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Polypeptide Specificity of the Antibody Response after Primary and Recurrent Infection with Bovine Herpesvirus 1

SYLVIA VAN DRUNEN LITTEL-VAN DEN HURK AND LORNE A. BABIUK*

Department of Veterinary Microbiology, Western College of Veterinary Medicine, and the Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

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The polypeptide specificities and defense mechanisms of the humoral immune response to bovine herpesvirus 1 were analyzed. Sequential serum samples taken from cows which were experimentally infected with bovine herpesvirus 1 were tested for their reactivity with individual bovine herpesvirus 1 polypeptides by immunoprecipitation, immunoblotting, and enzyme-linked immunosorbent assay. All bovine immune sera reacted with each of the three major bovine herpesvirus 1 glycoproteins, GVP 6/11a/16, GVP 3/9, and GVP 11b, during primary as well as recurrent infection. Among these glycoproteins, GVP 6/11a/16 induced the earliest and most consistent immune response. The levels of antibody to GVP 3/9 and GVP 11b varied among the animals, and they were slightly lower than the level of antibody to GVP 6/11a/16. Antibodies to several nonglycosylated polypeptides and two additional glycoproteins were also detected with the immunoblot assay. However, these antibodies were usually apparent only during recurrent infection, whereas they were undetectable or low during primary infection. The antibodies in the sera from all animals mediated virus neutralization and destruction of virus-infected cells by two immune mechanisms, e.g., antibody- and complement-mediated lysis and antibody-dependent cell-mediated cytotoxicity.

Bovine herpesvirus type 1 (BHV-1) has been associated with a variety of clinical syndromes including rhinotracheitis, vulvovaginitis, abortions, conjuctivitis, encephalitis, and generalized systemic infections (14, 20). After recovery from primary infection, the virus remains in the host in a latent state. Reactivation of the virus can be provoked by certain endogenous or exogenous physical modifications in the animal or experimentally by treatment of the animals by glucocorticoids like dexamethasone (35).

Live, attenuated vaccines are extensively used to control BHV-1 infection in cattle and appear to induce some level of protective immunity (13, 19). However, they may not prevent the establishment of a latent infection by a virulent field strain. In addition, it has been shown that two BHV-1 vaccine strains can be reactivated by the use of dexamethasone (27), indicating that at least some BHV-1 vaccines can themselves establish a latent infection. Thus, subunit vaccines consisting of the immunogenic components of the virus or their immunoreactive portion should be considered as an alternative. A considerable amount of information is available regarding the immune mechanisms by which animals may recover from BHV-1 infection (31). Antibodies in sera from BHV-1-infected cows have been demonstrated to neutralize virus (2, 18) and to mediate immune lysis of virus-infected cells (2, 17, 32, 33). In addition to humoral immunity, cell-mediated immune responses have been shown to play a major role in the recovery from BHV-1 infection as well as in the prevention of recurrent infection (8, 31). However, little is known about the specificities and protective functions of the bovine cells and antibodies that are involved in these immune reactions or about the duration of the immune response after a primary or secondary infection.

For the preparation of a subunit vaccine, information regarding the polypeptides and mechanisms involved in the immune response against BHV-1 is needed. BHV-1 specifies more than 25 structural polypeptides (6, 23), of which 11 are supposedly glycosylated. By using monoclonal antibodies, four unique glycoproteins have been identified: GVP 6/11a/16, a complex of three glycoproteins with apparent molecular weights of 130,000, 74,000, and 55,000 (130K, 74K, and 55K proteins, respectively); GVP 7 (105K); GVP 9 (91K), which also occurs as a dimer GVP 3 (180K); and GVP 11b (71K) (24, 37; van Drunen Littel-van den Hurk and Babiuk, submitted for publication). Monoclonal antibodies against GVP 6/11a/16, GVP 3/9, and GVP 11b participated to different extents in virus neutralization and antibody- and complement-mediated (AbC′) cytolysis of virus-infected cells. In contrast, monoclonal antibodies against GVP 7 did not mediate neutralization or AbC′ lysis. In addition, purified GVP 3/9 and GVP 6/11a/16 stimulated the production of monospecific antibodies in rabbits, which neutralized viral infectivity and mediated immune lysis of virus-infected cells (38).

In the present study we analyzed the polypeptide specificities and defense mechanisms of the humoral response against BHV-1. Sequential serum samples were taken from cows experimentally infected with BHV-1 and tested for their reactivity against BHV-1 polypeptides. An early, strong, and consistent immune response was detected against GVP 6/11a/16. An equally strong, but slightly later and less consistent, response was directed against GVP 3/9 and GVP 11b. Several additional proteins and glycoproteins were recognized by the bovine antibodies. However, these reactions were weak, late, or inconsistent. The antibodies in the bovine sera mediated virus neutralization and destruction of virus-infected cells by two immune mechanisms, e.g., AbC′ lysis and antibody-dependent, cell-mediated cytotoxicity (ADCC).

MATERIALS AND METHODS

Cells and virus. Georgia bovine kidney (GBK) cells were cultured in Eagle minimal essential medium (GIBCO

* Corresponding author.

Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO). Strain P8-2 of BHV-1 was propagated in GBK cells and purified on potassium tartrate gradients as described previously (2, 23).

Animals. Healthy Hereford calves 6 months of age were exposed to an aerosol of BHV-1 strain 108 as described previously (5). All animals shed virus and exhibited clinical signs characteristic of BHV-1 infection. All animals recovered within 10 days of primary exposure to the virus. Approximately 4 months after primary infection, animals were either treated with dexamethasone (27) or exposed intranasally a second time to virulent BHV-1 virus.

Intracellular labeling. GBK cells were infected at a multiplicity of infection of 10. After adsorption for 1 h at 37°C, the virus was removed, and the monolayers were overlaid with methionine-free or glucose-free minimal essential medium containing 2% fetal bovine serum. At 6 h after infection, the cells were labeled with 50 μCi of L-[^{35}S]methionine or [^{3}H]glucosamine (Amersham Corp., Oakville, Ontario) per ml. At 24 h postinfection the cells were harvested and processed for gel electrophoresis or immunoprecipitation as described previously (37). The virus in the supernatant medium was concentrated by ultracentrifugation and purified on potassium tartrate gradients as described elsewhere (23).

Immunoprecipitation. The procedure for immunoprecipitation has been described in detail previously (37, 39).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 7.5% polyacrylamide discontinuous gels (21) as described previously (37, 39). Unless otherwise mentioned, electrophoresis was carried out under reducing conditions.

Western blot analysis. The Western blotting technique of Burnette (7) that was previously used (39) was slightly modified. Purified BHV-1 was suspended in electrophoresis sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 1.25% SDS, 12.5% glycerol, 0.15 M 2-mercaptoethanol, 0.00125% bromophenol blue) and fractionated by electrophoresis in reducing gels. Alternatively, the virus was suspended in 0.01 M Tris hydrochloride–0.15 M NaCl–0.001 M EDTA (pH 7.5)–1% Nonidet p-40 and left on ice for 15 min. Extracted proteins were separated from insoluble material by ultracentrifugation at 25,000 rpm for 2 h in an SB405 rotor at 4°C (model B60; International Equipment Co., Div. Damon Corp., Needham Heights, Mass.). The solubilized viral proteins were separated by electrophoresis in nonreducing gels. After electrophoresis, the polypeptides were transferred to nitrocellulose paper in a Bio-Rad transblot cell (Bio-Rad Laboratories, Mississauga, Ontario) at 30 V for 3 h. The electrode solution was 25 mM sodium phosphate buffer (pH 6.8) plus 0.01% SDS. Subsequently, the instructions for use of the Bio-Rad immunoblot assay kit were followed. Briefly, the nitrocellulose was incubated for 1 h in 0.02 M Tris hydrochloride–0.5 M NaCl, pH 7.5 (Tris-buffered saline), containing 3% gelatin. The nitrocellulose was then transferred to a 1:50 dilution of bovine sera in Tris-buffered saline containing 1% gelatin. After 3 h the nitrocellulose was washed for 30 min in three changes of Tris-buffered saline containing 0.05% Tween 20. Subsequently, the nitrocellulose was incubated for 1 h in a 1:1,000 dilution of affinity-purified, peroxidase-conjugated rabbit anti-bovine immunoglobulin G (IgG, Zymed Laboratories Inc., San Francisco, Calif.) in Tris-buffered saline containing 1% gelatin. The nitrocellulose was washed as described above and developed with the Bio-Rad development solution. After 15 to 30 min the reaction was stopped by transferring the nitrocellulose to dideoxy H_2O. All incubation steps were carried out at room temperature on a rocking platform.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed essentially as described previously (39). Antigens used for coating consisted of purified BHV-1 (5 μg/ml) or one of the BHV-1 polypeptides (0.25 μg/ml). Individual polypeptides were purified by affinity chromatography followed by preparative SDS-PAGE, as described in detail elsewhere (38, 39). Affinity-purified, peroxidase-conjugated rabbit anti-bovine IgG (Zymed) at a dilution of 1:3,000 was used as the detecting antibody.

Neutralization test. The neutralization titers of the bovine sera were determined as described previously (2). To determine complement-enhanced neutralization, guinea pig serum (1:40 final dilution) was added to the virus-antibody mixture. The titers were expressed as the reciprocal of the highest dilution of antibody which caused a 50% reduction of plaques relative to the virus control.

AbC′ cytolysis. The ability of the bovine sera to mediate complement-dependent cell lysis was determined essentially as described by Babiuk et al. (2). AbC′ lysis titers were expressed as the reciprocal of the highest dilution of antibody which caused more than 5% specific ^{51}Cr release.

ADCC assay. ADCC assays were performed in microtiter plates as described previously (33). The ratio of effector cells (polymorphonuclear cells) to target cells (BHV-1 infected, ^{51}Cr-labeled GBK cells) was 50:1. Controls consisted of BHV-1-infected GBK target cells plus anti-BHV-1 serum or target cells with polymorphonuclear cells in the absence of antibody.

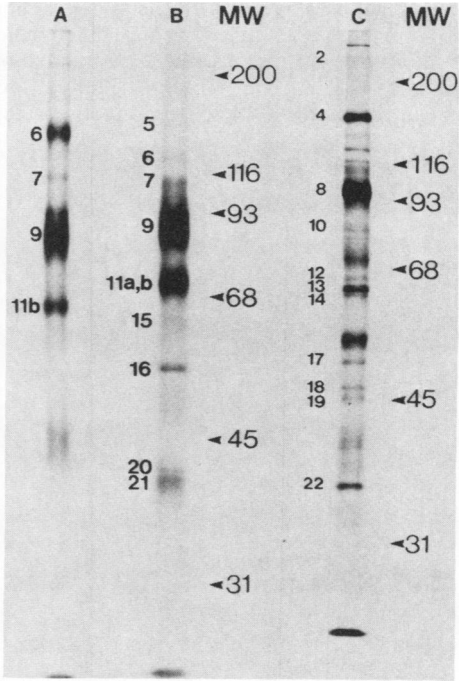

FIG. 1. Purified BHV-1 labeled with [^{3}H]glucosamine (lanes A and B) or L-[^{35}S]methionine (lane C). The structural polypeptides were identified by SDS-PAGE under nonreducing (lane A) or reducing (lanes B and C) conditions and numbered on the left. The positions of the molecular weight markers (×10^{3}) are shown in the right margin of the gels.

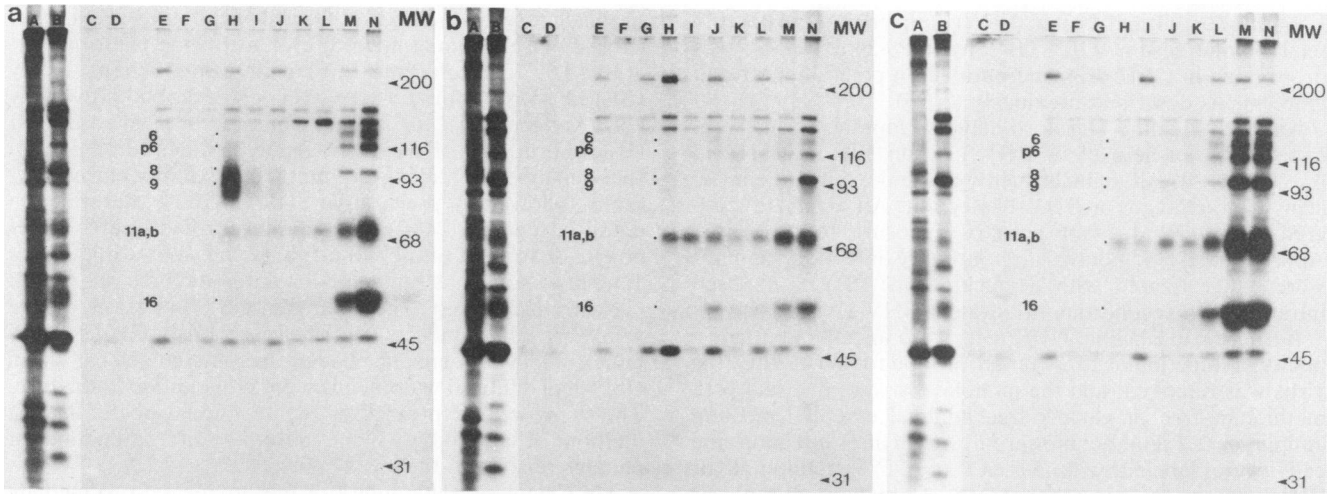

FIG. 2. Immunoprecipitation analysis of sequential sera from BHV-1-infected cows no. 61 (a), 62 (b), and 67 (c). L-[^{35}S]methionine-labeled polypeptides from mock-infected (lanes C and D) and BHV-1-infected (lanes E to N) cell lysates were precipitated with serum taken before infection (lanes E) or on day 5 (lanes F), 9 (lanes G), 14 (lanes H), 21 (lanes I), 29 (lanes J), 105 (lanes K), 118 (lanes L), 121 (lanes M), or 126 (lanes N) postinfection. Animals no. 61 and 67 were reactivated with dexamethasone, whereas no. 62 was challenged with virulent virus on day 118. Total mock-infected and BHV-1-infected cell lysates are shown in lanes A and B, respectively. The immunoprecipitates were analyzed by SDS-PAGE (7.5%). The viral proteins are numbered on the left, and the positions of the molecular weight markers ($\times 10^3$) are indicated in the right margin.

RESULTS

BHV-1 structural proteins. The BHV-1 structural proteins were identified in [^{3}H]glucosamine- and L-[^{35}S]methionine-labeled, purified virus and given the designations proposed by Misra et al. (23) and modified by van Drunen Littel-van den Hurk and Babiuk (37; submitted) (Fig. 1). The viral glycoproteins, analyzed by SDS-PAGE under reducing conditions, are shown in Fig, 1, lane B. Those glycoproteins that have been identified and characterized by the use of monoclonal antibodies (24, 39; van Drunen Littel-van den Hurk and Babiuk, in preparation) have been given corresponding designations in a nonreducing gel (Fig. 1, lane A). Figure 1, lane C, demonstrates the nonglycosylated structural proteins, analyzed under reducing conditions. Only VP 8 has been identified by using monoclonal antibodies (39).

Polypeptide specificity of the antibody response to BHV-1. Nine cows were experimentally infected with BHV-1 strain 108. At approximately 4 months (118 days) after inoculation, dexamethasone was administered to five animals for 4 consecutive days to induce a recurrent infection (27), and the remaining four animals were rechallenged with virulent BHV-1. Sequential serum samples taken during primary infection, recovery, recrudescence, and reinfection were

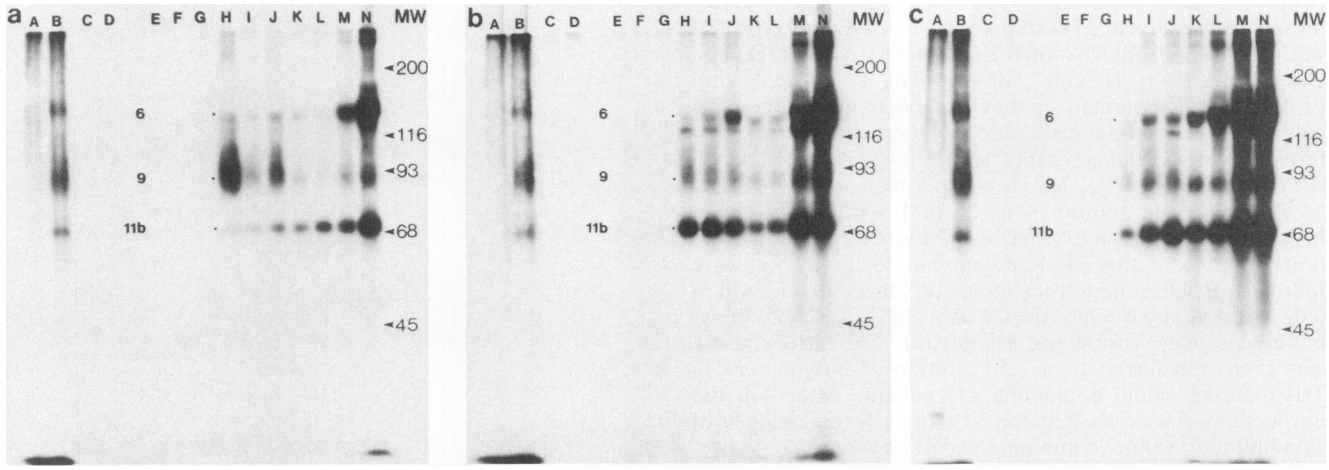

FIG. 3. Immunoprecipitation analysis of sequential sera from BHV-1-infected cows no. 61 (a), 62 (b), and 67 (c). [^{3}H]glucosamine-labeled polypeptides from mock-infected (lanes C and D) and BHV-1-infected (lanes E to N) cell lysates were precipitated with serum taken before infection (lanes E) or on day 5 (lanes F), 9 (lanes G), 14 (lanes H), 21 (lanes I), 29 (lanes J), 105 (lanes K), 118 (lanes L), 121 (lanes M), or 126 (lane N) postinfection. Animals no. 61 and 67 were reactivated with dexamethasone, whereas no. 62 was challenged with virulent virus on day 118. Total mock-infected and BHV-1-infected cell lysates are shown in lanes A and B, respectively. The immunoprecipitates were analyzed by SDS-PAGE (7.5%) under nonreducing conditions. The viral glycoproteins are numbered on the left, and the positions of the molecular weight markers ($\times 10^3$) are indicated in the right margin.

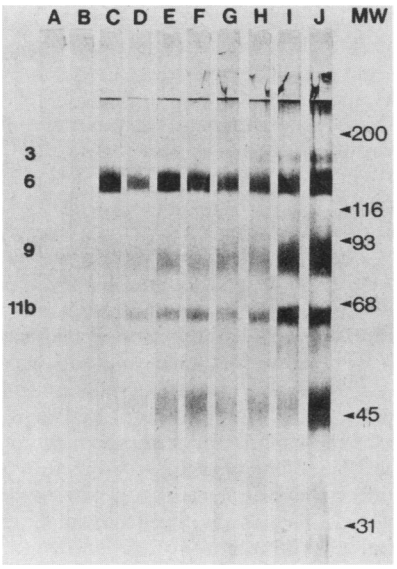

FIG. 4. Immunoblot analysis of sequential sera from BHV-1-infected cow no. 62. Nonidet P-40-extracted glycoproteins were fractionated by SDS-PAGE (7.5%) under nonreducing conditions and transferred to nitrocellulose, which was then incubated with preinfection serum (lane A) or serum taken on day 5 (lane B), 9 (lane C), 14 (lane D), 21 (lane E), 29 (lane F), 105 (lane G), 118 (lane H), 121 (lane I), or 126 (lane J) postinfection. Animal no. 62 was challenged with virulent virus on day 118. The viral glycoproteins are numbered on the left, and the positions of the molecular weight markers ($\times 10^3$) are indicated on the right.

examined for the presence of BHV-1-specific antibodies by using an immunoprecipitation assay. No major differences were observed between animals in which virus was reactivated and those rechallenged with virus. Some BHV-1 glycoproteins, e.g., GVP 3/9, are not readily labeled with L-[^{35}S]methionine (Fig. 1). Therefore. BHV-1-infected, L-[^{35}S]methionine- and [^{3}H]glucosamine-labeled cell lysates were used as antigen preparations. To differentiate between GVP 6/11a/16 and GVP 11b, [^{3}H]glucosamine-labeled immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions. Figures 2 and 3 show the humoral immune response of three representative cows, nos. 61 (Fig. 2a and 3a), 62 (Fig. 2b and 3b), and 67 (Fig. 2c and 3c). During primary infection (Fig. 2 and 3, lanes F to J), the main antibody response of the animals was directed against GVP 6/11a/16 (130K/74K/55K), pGVP 6 (120K), and GVP 11b (71K), whereas a weak antibody response was found against VP 8 (96K). Cow no. 61, and to a lesser extent cows no. 62 and 67, also developed antibodies against GVP 9 (91K). After recovery (Fig. 2 and 3, lanes K and L), the antibody levels against these seven polypeptides decreased, although they never became completely negative. During recurrent infection (Fig. 2 and 3, lanes M and N), the antibody levels against all polypeptides increased dramatically. Although cow no. 61 appeared to have the strongest immune response against GVP 9 during primary infection, the levels of antibody against GVP 9 were similar to those in cows no. 62 and 67 during recurrent infection.

Time course of the antibody response to BHV-1. An immunoblot assay was used to study the time of appearance of the antibodies against the various polypeptides in the sera of the BHV-1-infected animals. There are several reasons for choosing this technique for this purpose. First, the immunoblot assay appeared to be more sensitive than the immunoprecipitation assay. Second, differences in methionine content or extent of glycosylation or both between the various BHV-1 polypeptides pose limitations to a quantitative interpretation of the imunoprecipitation assay, but do not affect the interpretation of the immunoblot assay. Purified virus or Nonidet P-40-extracted viral glycoproteins were fractionated by SDS-PAGE. Again, to differentiate between GVP 6/11a/16 and GVP 11b, the Nonidet P-40-extracted glycoproteins were electrophoresed under nonreducing conditions. The separated polypeptides were electrophoretically transferred to nitrocellulose, which was then incubated with the different bovine sera. The humoral immune response of one representative cow (no. 62) is shown in Fig. 4 and 5. A strong antibody response against the major viral glycoproteins, especially GVP 6/11a/16, was observed during primary as well as recurrent infection. In contrast, the response against the viral nonglycosylated polypeptides and the minor glycoproteins was weak during primary infection (Fig. 4 and 5, lanes B to F), but became stronger after recurrent infection (Fig. 4 and 5, lanes I and J). Specifically, the GVP 6/11a/16 complex was recognized by the bovine antibodies between day 5 and day 9 postinfection. The antibodies reacted with GVP 3/9 between day 9 and day 14 postinfection. Similarly, they recognized GVP 11b between day 9 and day 14 after infection. Additional polypeptides, which were weakly or inconsistently recognized during primary infection, included VP 4 (151K) on day 5 and VP 8, VP 12 (66K), and VP 13 (64K) between day 5 and day 9. The levels of the antibodies against the glycoproteins and VP 8 rose during primary infection. After recovery (Fig. 4 and 5, lanes G and H), they decreased slightly, to increase dramatically again after recurrent infection. In contrast, the antibody levels

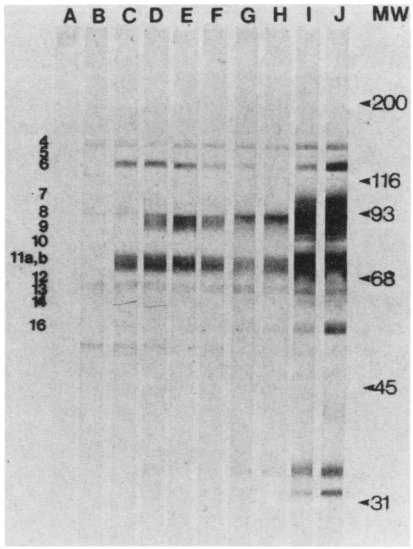

FIG. 5. Immunoblot analysis of sequential sera from BHV-1-infected cow no. 62. Purified virus was fractionated by SDS-PAGE (7.5%) and transferred to nitrocellulose, which was then incubated with preinfection serum (lane A) or serum taken on day 5 (lane B), 9 (lane C), 14 (lane D), 21 (lane E), 29 (lane F), 105 (lane G), 118 (lane H), 121 (lane I), or 126 (lane J) postinfection. Animal no. 62 was challenged with virulent virus on day 118. The viral polypeptides are numbered on the left, and the positions of the molecular weight markers ($\times 10^3$) are indicated on the right.

against the remaining nonglycosylated polypeptides did not increase until after recrudesence or reinfection occurred. Several additional polypeptides were recognized by the bovine antibodies during recurrent infection, notably GVP 5 (138K), GVP 7 (105K), VP 10 (79K), and VP 14 (62K). The low-molecular weight bands may either correspond to GVP 20 (40K), GVP 21 (38K), VP 22 (35K), and VP 23 (32K) or represent breakdown products. The results summarized in Table 1 suggest that the antibody response during primary infection is primarily directed against the major glycoproteins, particularly against the GVP 6/11a/16 complex.

Variability of the antibody response to BHV-1. Although the immunoprecipitation and immunoblot assays did not show a significant variation in specificity of the antibody response of the different cows, some quantitative differences were observed. To evaluate the extent of these differences, the variability of the antibody response was examined in an ELISA. BHV-1 or one of the major glycoproteins was used as the antigen. Serum samples were taken from nine cows at different times during primary BHV-1 infection, recovery, and recurrent infection. The ELISA titers of the sequential sera of four representative cows, nos. 61, 62, 65, and 67, are shown in Fig. 6. The titers against BHV-1 were approximately 10-fold higher than those against the individual glycoproteins. The ELISA titers against GVP 6/11a/16 were fourfold higher than those against GVP 3/9, which, in turn, were twofold higher than those against GVP 11b. The titers against BHV-1 and GVP 6/11a/16 reached peak levels at 14 days postinfection, whereas those against GVP 9 and GVP 11b did not reach their peak until 14 to 21 and 21 to 29 days postinfection, respectively. After a gradual decrease during recovery, the ELISA titers against all antigens rose dramatically after dexamethasone treatment or reinfection, reaching 5- to 10-fold higher levels than those achieved after the primary infection. The patterns and the levels of the antibody titers were very similar in all nine animals. The only significant variation observed was in the relative antibody levels against GVP 6/11a/16, GVP 3/9, and GVP 11b. For example, animal no. 62 had higher antibody titers against GVP 6/11a/16 than the other three cows. However, the antibody titers against GVP 3/9 were highest in animals no. 61 and 62, whereas the titers against GVP 11b were highest in animal no. 65.

TABLE 1. BHV-1 polypeptides recognized by bovine sera[a]

Viral polypeptide[b]	Mol wt ($\times 10^3$) (7.5% gel)	Primary infection[c]	Recurrent infection[d]
GVP 1	>330		
VP 2	263		
GVP 3	180		
VP 4	151	±	+
GVP 5	138	±	+
GVP 6	130	+ + +	+ + +
GVP 7	105		+
VP 8	96	±	+ +
GVP 9	91	+ +	+ + +
VP 10	79		+
GVP 11a	74	+ + +	+ + +
GVP 11b	71	+ +	+ + +
VP 12	66	±	+
VP 13	64	±	+
VP 14	62		+
GVP 15	56		
GVP 16	55	±	+ + +
VP 17	50		
VP 18	46		
VP 19	44		
GVP 20	40		(+)
GVP 21	38		(+)
VP 22	35		(+)
VP 23	32		(+)

[a] Determined by an immunoblot assay: ±, +, + +, + + +, relative amount of antibody; (+), tentative assignment.

[b] Identified by Misra et al. (23) and van Drunen Littel-van den Hurk et al. (39); VP, virion polypeptide; GVP, glycosylated virion polypeptide.

[c] Days 0 to 29 postinfection.

[d] Induced by dexamethasone on days 118 to 122 postinfection (cows no. 61 and 67) or rechallenged with virulent virus on day 118 (cows no. 62 and 65). Symbols are as for primary infection.

Biological importance of the antibody response to BHV-1. Cows which were experimentally infected with BHV-1 responded by producing antibodies against a number of BHV-1-specific polypeptides. In an attempt to assess the possible involvement of these antibodies in recovery and prevention of reinfection, the ability of the antibodies to neutralize viral infectivity and to mediate destruction of virus-infected cells was investigated. The antibodies from all nine cows were able to neutralize viral infectivity. As shown in Fig. 7A and B for representative cows no. 61, 62, and 67, the neutralization titers reached peak levels on day 14 postinfection. After a decline during recovery, the titers rose dramatically during recrudescence or reinfection. The neutralization titers in the presence of complement (Fig. 7B) were approximately 15-fold higher than those in the absence of complement (Fig. 7A) during primary infection, whereas there was only a 5-fold difference during recurrent infection. The antibodies from the same animals did participate in immune-mediated destruction of BHV-1-infected cells. The highest levels of AbC′ lysis were found on day 14 (cow no. 61) or 21 (cows no. 62 and 67) postinfection (Fig. 7C), whereas the peak of ADCC was reached on day 14 (cow no. 61), 21 (cow no. 62), or 29 (cow no. 67) postinfection (Fig. 7D). After recovery, the titers decreased, to rise again after recurrent infection. However, in contrast to antibody and neutralization titers, the AbC′ lysis and ADCC titers were not higher than those observed after primary infection.

DISCUSSION

The different responses of the bovine immune system to a BHV-1 infection, which may result in virus neutralization or cell lysis, have been studied extensively (2, 17, 31). However, the role of the individual polypeptides in inducing protective immunity has not been investigated. The virus-specific polypeptides that are synthesized during a BHV-1 infection have recently been identified (6, 23, 39), thus allowing a thorough investigation of the kinetics of immune responses to each polypeptide as well as to determine which ones might be most valuable in the recovery phase. In the present study, we investigated which viral polypeptides are recognized by antibodies to BHV-1 during primary and recurrent infection and which biological functions are mediated by these antibodies.

The most interesting observation of this study was the strong antibody response of all BHV-1-infected cows against the major viral glycoproteins GVP 6/11a/16, GVP 3/9, and GVP 11b during primary infection. GVP 6/11a/16 in particular induced an early and very consistent immune response. Antibodies against GVP 3/9 and GVP 11b appeared later after infection, and the levels varied among immune sera from different animals. The antibody levels against all three glycoproteins decreased after recovery, but rose dramatically again during reinfection or after reactivation of latent

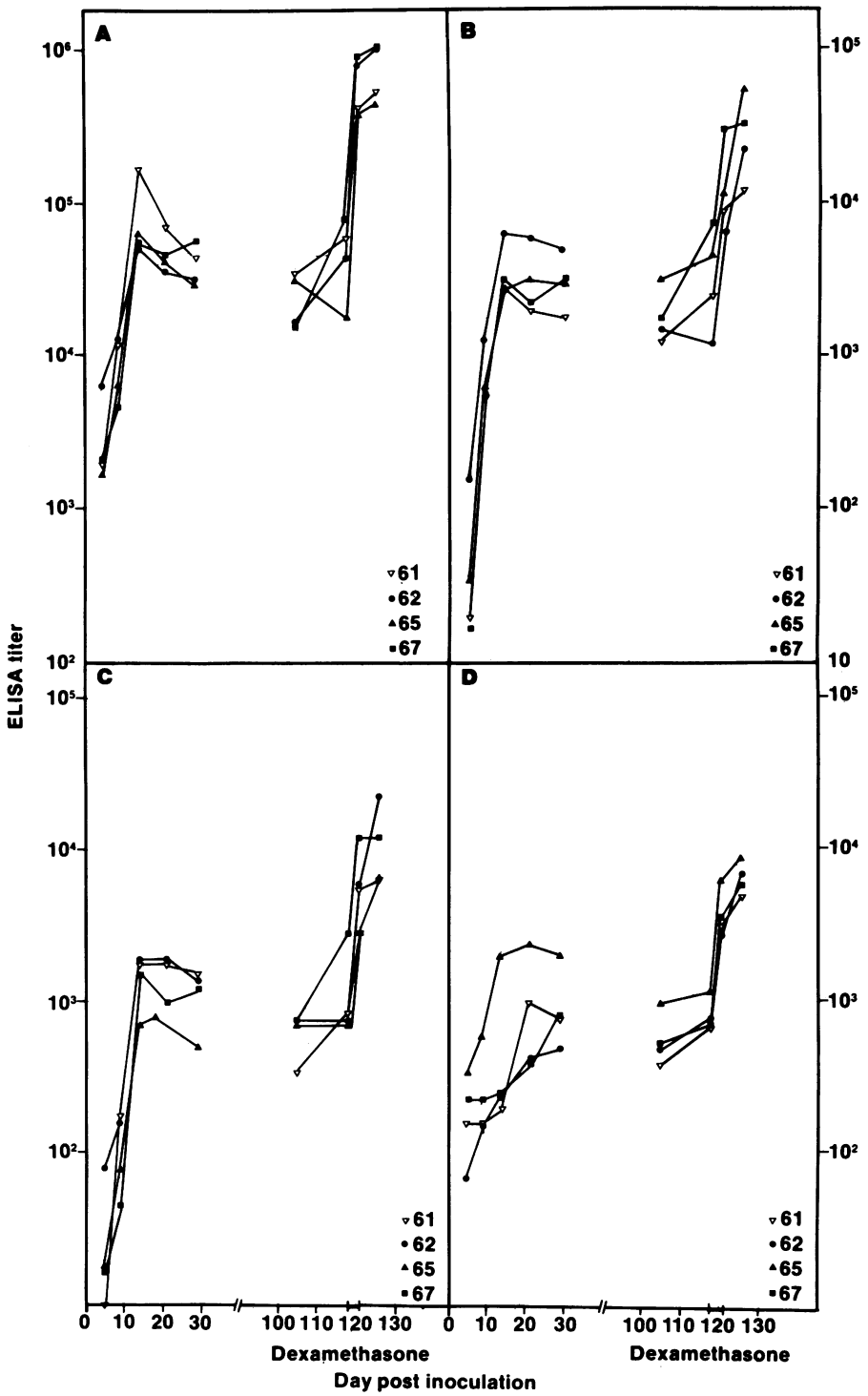

FIG. 6. Serological response of cows no. 61 (▽), 62 (●), 65 (▲), and 67 (■) to infection with BHV-1. The animals were experimentally infected on day 0. Animals no. 61 and 67 were reactivated with dexamethasone, whereas no. 62 and 65 were rechallenged with virulent virus on day 118. The specificity of the antibody response was determined by an ELISA with BHV-1 (A), GVP 6/11a/16 (B), GVP 3/9 (C), or GVP 11b (D) as the antigen.

virus with dexamethasone. These observations are consistent with similar studies on the immune response to herpes simplex virus type 1 (HSV-1) (11, 15, 41), in which the major reactivity in HSV-1 immune sera was found to be directed against a glycoprotein with an apparent molecular weight of ~130,000. However, the identity of this 130K glycoprotein does not appear to be firmly established. Whereas Gilman et al. (15) named this glycoprotein gC, Eberle and Mou (11) defined it as gB. In addition to antibodies to gB, Eberle and Mou (11) also detected antibodies to gC and gD in almost all

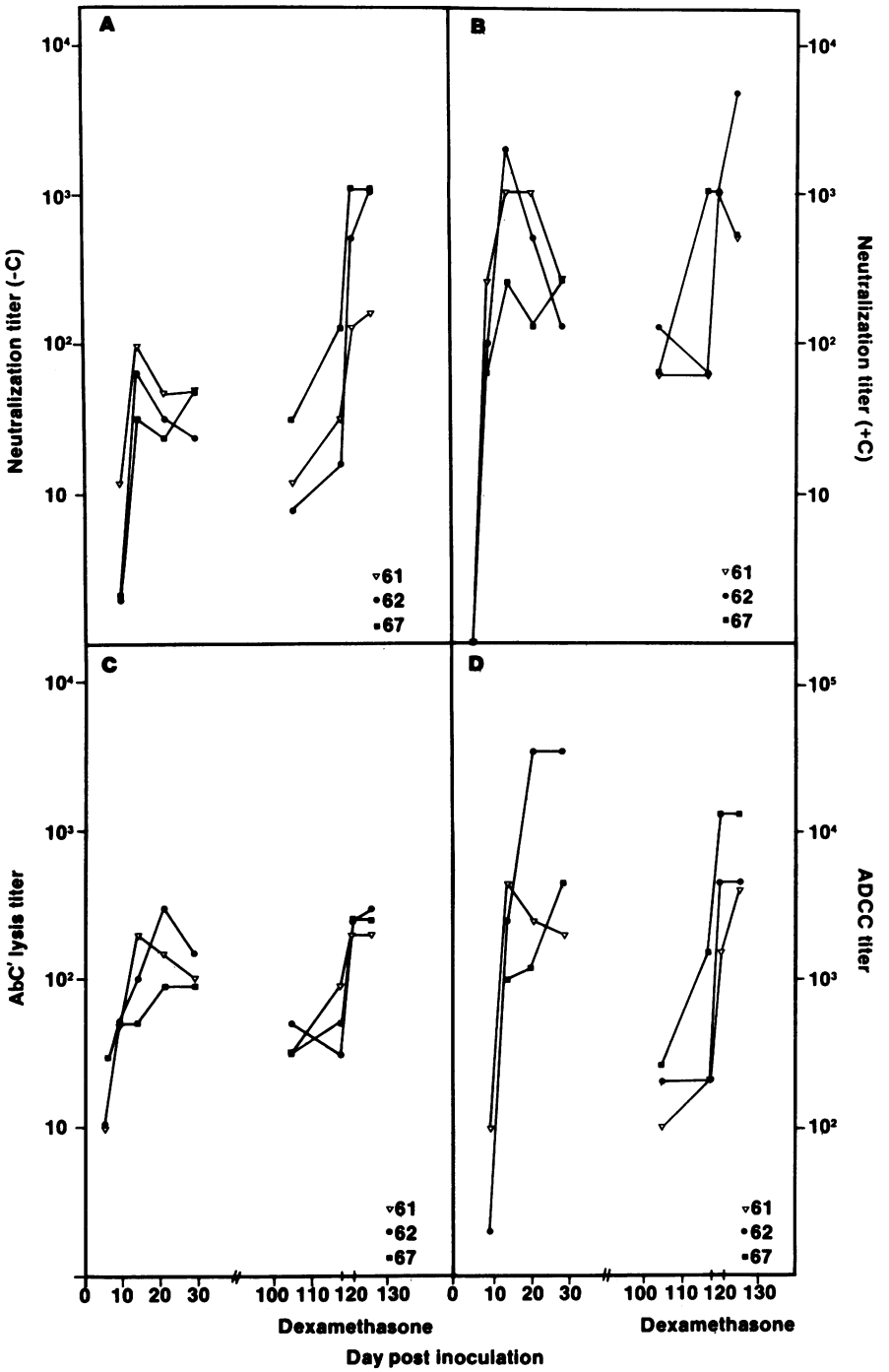

FIG. 7. Activity of sera from BHV-1-infected cows no. 61 (▽), 62 (●), and 67 (■) in neutralization, AbC' lysis, and ADCC. Neutralization titers were determined in the absence (A) and presence (B) of guinea pig serum as a source of complement and expressed as the reciprocal of that dilution which caused a 50% reduction of plaques relative to the virus control. AbC' lysis (C) and ADCC (D) titers were expressed as the reciprocal of the highest dilution causing more than 5% specific ^{51}Cr release. Animals no. 61 and 67 were reactivated with dexamethasone, whereas no. 62 was rechallenged with virulent virus on day 118.

HSV-1 immune sera they examined. The levels of antibody against these two glycoproteins showed a variation among sera similar to those against GVP 3/9 and GVP 11b. The observation that the major reactivity of the bovine immune sera was directed against the viral glycoproteins was not surprising, since herpesvirus glycoproteins are expressed on the surface of the virion (26, 34) and the plasma membrane of the host cell (16, 24, 30, 39) and thus are prime candidates for interactions with the host's immune system.

Antibodies, induced after infection with BHV-1, mediated virus neutralization and immune lysis of BHV-1-infected cells, supporting their role in the recovery process. Interestingly, the neutralization titers peaked at approximately 14 days postinfection, whereas the AbC' lysis and ADCC titers

reached their highest levels around 21 days after infection. This observation may indicate a relationship between virus neutralization and the presence of antibodies to GVP 6/11a/16, which appeared early during infection. In contrast, antibodies to GVP 3/9 and GVP 11b may have a more important function in lysis of virus-infected cells. This hypothesis is supported by earlier studies, which showed that monoclonal and monospecific polyclonal antibodies directed against GVP 6/11a/16 neutralized virus more efficiently, whereas antibodies to GVP 3/9 and GVP 11b were more efficient in mediating lysis of infected cells (24, 38, 39).

Most viruses have only one protein or glycoprotein that is important for virus attachment and interactions with the immune system of the host. The data presented here indicate an important role for all the major viral glycoproteins in the humoral immune response to BHV-1 infection. This is in agreement with previous studies that demonstrated the involvement of monoclonal and monospecific polyclonal antibodies against the major glycoproteins in the response to BHV-1 (38, 39). In this respect, the behavior of BHV-1 is very similar to that of other herpesviruses, particularly HSV, which also elicit a functionally important immune response to a number of glycoproteins. Several studies have shown that each of the HSV glycoproteins gB, gC, gD, and gE is involved in the humoral immune response (4, 10, 25, 28, 29, 40). Recently, a definite role for gB and gC in the cell-mediated response was also established (1, 12). In addition, HSV glycoprotein gD (22), as well as monoclonal antibodies against several of the HSV glycoproteins, induced protection in mice (3, 9). Consequently, not one but several herpesvirus glycoproteins appear to be relevant to the interactions between virus and host. Several reasons may be postulated as to why the antibody response of the BHV-1-infected animals against GVP 6/11a/16 was more consistent and thus seemed to be more important than that against GVP 3/9, GVP 11b, or any of the other, minor glycoproteins. First, GVP 6/11a/16 is synthesized early in the infectious cycle. However, so are GVP 7 and GVP 11b (23, 24; van Drunen Littel-van den Hurk and Babiuk, in preparation). Alternatively (and more likely), the size of this glycoprotein complex, as well as its quantity and perhaps its position on the virion envelope and the plasma membrane of the host cell, may influence its capacity to induce a strong immune response.

Although the reactivity of the bovine sera was primarily directed at the three major BHV-1 glycoproteins, the antibodies also recognized a number of nonglycosylated polypeptides and two additional minor glycoproteins, GVP 5 and GVP 7. However, the immune response against these polypeptides was weak or absent during primary infection. Only during recurrent infection was a significant increase in antibody levels observed. Of these polypeptides, only GVP 5 and GVP 7 have been detected on the surface of the virion envelope and the plasma membrane of the virus-infected cell (39), implying that the remaining polypeptides are confined to internal rather than surface locations in the virion and infected cells. This explains why, during initial exposure to the virus, the bovine immune system is not likely to respond to these polypeptides or responds weakly. However, after repeated exposure to the virus, which occurs during recurrent infections, the host may respond to these antigens due to destruction of infected cells and subsequent release of these polypeptides. The reason why GVP 5 and GVP 7 appear to be only weakly immunogenic is presently unclear, but it may be related to size, quantity, or location of these glycoproteins.

Three BHV-1 glycoproteins were identified as major immunogens in the bovine system by using three different techniques. Very few polypeptides were recognized in the immunoprecipitation assay, which may be due to incomplete solubilization of the viral polypeptides or suboptimal antigen-antibody concentrations. In the immunoblot assay a much larger number of polypeptides was recognized by the antibodies in the bovine sera, even though the antigens were presented in a denatured form. Although this denatured state of the antigens may account for those polypeptides that were not recognized, it is more likely that a number of polypeptides are of lesser importance as immunogens and therefore are not (or very weakly) detected by antibodies in the bovine sera. With the ELISA it was possible to determine the relative antibody titers to the individual BHV-1 polypeptides, but only if these were available in purified form. Thus, depending on whether visualization or quantitation is desired, the immunoblot assay or ELISA, respectively, is the one that should be used for studies designed to analyze the kinetics of immune responses to BHV-1 antigens.

In summary, all bovine immune sera reacted with each of the three major BHV-1 glycoproteins, GVP 6/11a/16, GVP 3/9, and GVP 11b, during primary as well as recurrent infection. Among these three glycoproteins, GVP 6/11a/16 induced the earliest and most consistent immune response. Antibodies to several nonglycosylated polypeptides and to two additional glycoproteins were also detected with the immunoblot assay, which was the most sensitive and useful method. However, these antibodies were usually only apparent after recurrent infection.

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LITERATURE CITED

1. **Abendroth-Bishop, G., S. D. Marlin, S. A. Schwartz, and J. C. Glorioso.** 1984. Human natural killer cell recognition of herpes simplex virus type-1 glycoproteins: specificity analysis with the use of monoclonal antibodies and antigenic variants. J. Immunol. **133**:2206–2214.
2. **Babiuk, L. A., R. C. Wardley, and B. T. Rouse.** 1975. Defense mechanisms against bovine herpesvirus: relationship of virus host cell events to susceptibility to antibody complement lysis. Infect. Immun. **12**:958–963.
3. **Balachandran, N., S. Bacchetti, and W. E. Rawls.** 1982. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun. **37**:1132–1137.
4. **Balachandran, N., D. Harnish, W. E. Rawls, and S. Bacchetti.** 1982. Glycoproteins of herpes simplex virus type 2 as defined by monoclonal antibodies. J. Virol. **44**:344–355.
5. **Bielefeldt Ohmann, H., and L. A. Babiuk.** 1985. Viral-bacterial pneumonia in calves: effect of bovine herpesvirus-1 on immunologic functions. J. Infect. Dis. **151**:937–947.
6. **Bolton, D. C., Y. E. Zee, and A. A. Ardans.** 1983. Identification of envelope and nucleocapsid proteins of infectious bovine rhinotracheitis virus by SDS-polyacrylamide gel electrophoresis. Vet. Microbiol. **8**:57–68.
7. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
8. **Davies, D. H., and L. E. Carmichael.** 1973. Role of cell-mediated immunity in the recovery of cattle from primary and recurrent

infections with infectious bovine rhinotracheitis virus. Infect. Immun. **8**:510–518.
9. **Dix, R. D., L. Pereira, and J. R. Baringer.** 1981. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurological disease. Infect. Immun. **34**:192–199.
10. **Eberle, R., and R. J. Courtney.** 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein regions of herpes simplex virus type 1. J. Virol. **35**:902–917.
11. **Eberle, R., and S.-W. Mou.** 1983. Relative titers of antibodies to individual polypeptide antigens of herpes simplex virus type-1 in human sera. J. Infect. Dis. **148**:436–444.
12. **Eberle, R., R. G. Russell, and B. T. Rouse.** 1981. Cell-mediated immunity to herpes simplex virus: recognition of type-specific and type-common surface antigens by cytotoxic T cell populations. Infect. Immun. **34**:795–803.
13. **Gerber, J. D., A. E. Marron, and C. J. Kucera.** 1978. Local and systemic cellular and antibody immune responses of cattle to infectious bovine rhinotracheitis virus vaccine administered intranasally or intramuscularly. Am. J. Vet. Res. **39**:753–760.
14. **Gibbs, E. P. J., and M. M. Rweyemamu.** 1977. Bovine herpesviruses. I. Bovine herpesvirus-1. Vet. Bull. (London) **47**:317–343.
15. **Gilman, S. C., J. J. Docherty, and W. E. Rawls.** 1981. Antibody responses in humans to individual proteins of herpes simplex viruses. Infect. Immun. **34**:880–887.
16. **Glorioso, J. C., and J. W. Smith.** 1977. Immune interactions with cells infected with herpes simplex virus: antibodies to radioiodinated surface antigens. J. Immunol. **1**:114–121.
17. **Grewal, A. S., B. T. Rouse, and L. A. Babiuk.** 1977. Mechanisms of resistance of different cell types in mediating antibody-dependent cell mediated cytotoxicity. Infect. Immun. **15**:698–703.
18. **House, J. A., and J. A. Baker.** 1971. Bovine herpesvirus IBR-IPV. The antibody virus neutralization reaction. Cornell Vet. **61**:320–335.
19. **Jericho, K. W. F., and L. A. Babiuk.** 1983. The effect of dose, route and virulence of bovine herpesvirus-1 vaccine on experimental respiratory disease in cattle. Can. J. Comp. Med. **47**:133–139.
20. **Kahrs, R. F.** 1977. Infectious bovine rhinotracheitis: a review and update. J. Am. Vet. Med. Assoc. **171**:1055–1064.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Long, D., T. J. Madara, M. Ponce de Leon, G. H. Cohen, P. C. Montgomery, and R. J. Eisenberg.** 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. Infect. Immun. **37**:761–764.
23. **Misra, V., R. M. Blumenthal, and L. A. Babiuk.** 1981. Proteins specified by bovine herpesvirus type 1 (infectious bovine rhinotracheitis virus). J. Virol. **40**:367–378.
24. **Misra, V., J. E. Gilchrist, G. Weinmaster, L. Qualtiere, S. van den Hurk, and L. A. Babiuk.** 1982. Herpesvirus induced "early" glycoprotein: characterization and possible role in immune cytolysis. J. Virol. **32**:1046–1054.
25. **Norrild, B., S. L. Shore, and A. J. Nahmias.** 1979. Herpes simplex glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immune cytolysis and their correlation with previously identified glycopeptides. J. Virol. **32**:741–748.
26. **Olshevsky, U., and Y. Becker.** 1972. Surface glycoproteins in the envelope of herpes simplex virus. Virology **50**:277–279.
27. **Pastoret, P. P., L. A. Babiuk, V. Misra, and P. Griebel.** 1980. Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. Infect. Immun. **29**:483–488.
28. **Pereira, L., T. Klassen, and J. R. Baringer.** 1980. Type-common and type-specific monoclonal antibody to herpes simplex virus type 1. Infect. Immun. **29**:724–732.
29. **Powell, K., A. Buchan, C. Sim, and D. Watson.** 1974. Type-specific protein in herpes simplex virus reacts with neutralizing antibody. Nature (London) **249**:360–361.
30. **Roizman, B., and P. Spear.** 1971. Herpesvirus antigens on cell membranes detected by centrifugation of membrane-antibody complexes. Science **171**:298–300.
31. **Rouse, B. T., and L. A. Babiuk.** 1978. Mechanisms of recovery from herpesvirus infections. Can. J. Comp. Med. **42**:414–427.
32. **Rouse, B. T., R. C. Wardley, and L. A. Babiuk.** 1975. The role of antibody-dependent cytotoxicity in recovery from herpesvirus infections. Cell. Immunol. **22**:182–190.
33. **Rouse, B. T., R. C. Wardley, and L. A. Babiuk.** 1976. Antibody-dependent, cell-mediated cytotoxicity in cows: comparison of effector cell activity against heterologous erythrocytes and herpesvirus-infected bovine target cells. Infect. Immun. **13**: 1433–1441.
34. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. **29**:1149–1158.
35. **Sheffy, B. E., and D. H. Davies.** 1972. Reactivation of a bovine herpesvirus after corticosteroid treatment. Proc. Soc. Exp. Biol. Med. **140**:974–976.
36. **Spear, P. G.** 1975. Glycoproteins specified by herpes simplex virus type-1: their synthesis, processing and antigenic relatedness to HSV-2 glycoproteins. Int. Agency Res. Cancer Sci. Publ. **11**:49–60.
37. **van Drunen Littel-van den Hurk, S., and L. A. Babiuk.** 1985. Effect of tunicamycin and monensin on biosynthesis, transport and maturation of bovine herpesvirus type-1 (BHV-1) glycoproteins. Virology **143**:104–118.
38. **van Drunen Littel-van den Hurk, S., and L. A. Babiuk.** 1985. Antigenic and immunogenic characteristics of bovine herpesvirus type-1 (BHV-1) glycoproteins GVP 3/9 and GVP 6/11a/16, purified by immunoadsorbent chromatography. Virology **144**:204–215.
39. **van Drunen Littel-van den Hurk, S., J. V. van den Hurk, J. E. Gilchrist, V. Misra, and L. A. Babiuk.** 1984. Interactions of monoclonal antibodies and bovine herpesvirus type-1 (BHV-1) glycoproteins: characterization of their biochemical and immunological properties. Virology **135**:466–479.
40. **Vestergaard, B. F., and B. Norrild.** 1979. Crossed immunoelectrophoretic analysis and viral neutralizing activity of five monospecific antisera against five different herpes simplex virus glycoproteins. Int. Agency Res. Cancer Sci. Publ. **24**:225–234.
41. **Zweerink, H. J., and L. W. Stanton.** 1981. Immune response to herpes simplex virus infections: virus-specific antibodies in sera from patients with recurrent facial infections. Infect. Immun. **31**:624–630.