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Immune Response to Bovine Herpes Herpesvirus Type 1 Infections: Virus-Specific Antibodies in Sera From Infected Animals

JAMES K. COLLINS,* ANN C. BUTCHER, AND CYNTHIA A. RIEGEL

Diagnostic Laboratory, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523

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The virus specificity of antibodies against bovine herpes virus type 1 was determined with a radioimmunoprecipitation assay and serum collected from natural and experimentally induced infections. By using sequentially collected sera, the development of antibodies to 4 to 5 viral glycoproteins and 11 to 12 nonglycosylated proteins was followed for the first 50 days after infection. The major and most consistent responses in experimentally and naturally infected animals were to four glycoproteins with molecular weights of 102,000, 96,000, 69,000, and 55,000, as well as to a major virion 115,000-molecular-weight nonglycosylated protein. The four glycoproteins were all coprecipitated by a neutralizing monoclonal antibody and were probably involved as target antigens in virus neutralization. Another antigenically unrelated glycoprotein with a molecular weight of 82,000 and a nonglycosylated protein with a molecular weight of 91,000 were also precipitated, but the immune response to these two proteins was transient. Reactivity to gp82 was only weakly detected in serum from naturally infected animals. Contact control animals which did not contract a bovine herpes virus type 1 infection but were exposed to infected animals with signs of severe illness had antibodies which recognized gp102, gp96, gp69 and gp55 as well as p115. These antibodies were present in low amounts and, in contrast to infected animals, did not increase between acute and convalescent sampling.

The specifity of the immune response necessary for resistance or recovery from bovine herpes virus type 1 (BHV-1) is not well defined. Although live virus vaccines have been prepared and shown to induce some level of protective immunity (9, 12, 17, 25), the protective functions and specificities are not known. Serum antibody titers to the virus do not assure that animals are protected; outbreaks occur in the presence of such titers (13, 16). Yet specific antibodies in bovine serum can be demonstrated to neutralize virus (6, 11), to stop viral spread to susceptible cells by antibody-dependent cellular cytotoxicity (1, 3, 24), and to carry out immune cytolysis of virus-infected cells (19). In some cases, the antibodies carrying out these functions have been determined to react with defined virus polypeptides (19). In addition to the humoral immune response, cell-mediated immune functions have been demonstrated to play a major role in recovery from BHV-1 infection (1, 7, 23). Cellular immunity may also be important in the prevention of recurrent infections, since immunosuppressive treatment can induce latent BHV-1 to cause disease (13, 16).

The present studies were carried out to analyze the polypeptide specificity of the humoral immune response to BHV-1. This specificity was determined by using serum and virus from an outbreak of BHV-1. Acute- and convalescent-phase serum samples were obtained from animals suffering from classic rhinotracheitis and from contact animals which were not clinically ill. Sequential serum samples taken from animals that were experimentally infected with this isolate were also analyzed. With serum obtained from such defined conditions, the deficits and fluctuations in the levels of virus-specific antibodies, as well as the consistency of recognition of several major viral polypeptides, could be identified.

* Corresponding author.

MATERIALS AND METHODS

Cells and virus. BHV-1 strain CSU 10902-82 was isolated from an outbreak of infectious bovine rhinotracheitis in Colorado. Virus was propagated in diploid bovine embryonic lung cells as described previously (6). The BHV-1 isolate was passaged once in bovine embryonic lung cells before labeling, purification, and use for experimental infections.

Radioactive labeling of infected cells. Second-passage bovine embryonic lung cells were infected with BHV-1 at a multiplicity of 1 to 5 50% tissue culture infective doses per cell in 25-cm^2 flasks. At 6 h postinfection, cells were washed three times with methionine-free minimal essential medium (MEM); 5 ml of MEM containing 25 μCi of [^{35}S]methionine per ml (1,096 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added per flask. For labeling with [^{3}H]glucosamine, medium was discarded at 6 h postinfection, and MEM containing 50 μCi of [^{3}H]glucosamine per ml (D-[6-^{3}H]glucosamine; ICN Pharmaceuticals Inc., Irvine, Calif.) was added. Labeling was carried out until 22 to 24 h postinfection, at which time the supernatant fluid was harvested for virus purification and the cells were lysed for radioimmunoprecipitation (RIP).

Virus purification. Virus was purified as described previously (6; J. K. Collins, G. A. Bulla, C. A. Riegel, and A. C. Butcher, Vet. Microbiol., in press).

Virus serological tests. Virus neutralization (VN) was carried out by using an endpoint dilution procedure described by Carbrey (4). Enzyme-linked immunosorbent assay (ELISA) was carried out as described previously (Collins et al., in press), with an infected cell extract as the antigen.

RIP assay. All RIP assays were carried out with infected

cell lysates at 4°C. Radioactively labeled cells were lysed with lysing buffer which contained 1% Nonidet P-40 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), 0.5% sodium deoxycholate (Sigma Chemical Co., St. Louis, Mo.), and 0.1% sodium dodecyl sulfate (SDS) in 0.01 M Tris–0.15 M sodium chloride–1 mM EDTA (pH 7.2). After lysing (ca. 10^6 cells per ml), cells were frozen and thawed three times and then stored at −70°C. Preclearing of the lysate was carried out by mixing ca. 1×10^5 to 3×10^5 lysed cell equivalents with 0.05 ml of 10% (vol/vol) fixed *Staphylococcus aureus* as described previously (6). The mixture was incubated for 1 h and then centrifuged at 80,000 × *g* for 30 min. The supernatant fluid was drawn off, and 5 μl of bovine serum or 0.1 μl of ascitic fluid was added and allowed to react for 1 h. Fifty microliters of Cowan I strain (SaCI) was then added, followed by a second 1-h incubation. The SaCI was then washed three times with lysing buffer. After the final wash, the SaCI was boiled for 2 min with 0.05 ml of SDS-gel buffer and centrifuged; the supernatant was then collected for SDS-gel electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to previously described procedures (6, 14). All samples were dissolved by boiling in gel buffer containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol in 0.0625 M Tris (pH 6.8). Electrophoresis was carried out on slab gels (10 by 10 by 0.15 cm) stacked at 45 V for 1 h on a 3% stacking gel and run at 125 V for 2 1/2 to 3 h on a 7.5% resolving gel. Slab gels were dried and exposed to Kodak X-Omat AR film for 72 h. Silver staining was carried out with a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.).

Serum samples. Bovine serum samples were obtained from two sources: (i) acute and convalescent paired serum from Hereford yearling heifers and steers involved in a BHV-1 respiratory disease outbreak in a Colorado feedlot and (ii) sequential bleedings from 10 Holstein steers experimentally infected with the BHV-1 strain which was recovered from the disease outbreak. Animals involved in the field outbreak had been vaccinated with a modified live intramuscular BHV-1 vaccine 3 months before the outbreak; experimentally infected animals had no prior vaccination. Serum samples were collected in Vacutainer clot tubes, centrifuged, separated from the clot, heat inactivated at 56°C for 30 min, and stored at −20°C.

Experimental infections. Four-month-old sero-negative Holstein steers were obtained from a dairy with no history of animals with respiratory disease. On the date of transport to an isolation facility, the calves were inoculated intranasally with 2 ml of 2×10^8 50% tissue culture infective doses of BHV-1 virus per ml per nostril with a model no. 152 compressed air-powered DeVilbiss atomizer (DeVilbiss Co., Somerset, Pa.). To simulate feedlot conditions, ambient room temperature was kept at 90°F (ca. 32.2°C), and the animals were deprived of water for the first 24 h postinoculation. The animals were monitored daily by rectal temperature, clinical signs of respiratory distress, virus shedding, and by determining anti-BHV-1 titers. Virus shedding was monitored by inserting a swab 5 to 7 cm into the nostril and then transporting the swab in 1 ml of viral transport medium (6) to the laboratory and performing endpoint infectivity titrations. Clinical signs were monitored on a subjective scale of 1 to 4.

RESULTS

RIP assay with bovine antiserum. Serum taken from animals after experimental infection with BHV-1 was used in RIP assays with [^{35}S]methionine-labeled polypeptides. The first polypeptides to be specifically recognized by bovine antibodies were identified by using serum taken at 11 days after infection (Fig. 1). The initial nonglycosylated polypeptides recognized had molecular weights of 115,000, 91,000, 50,000 to 52,000, 39,000 to 43,000, and 30,000 to 36,000 and were precipitated from a large number of [^{35}S]methionine-labeled polypeptides present in virus-infected cells (Fig. 1, lane M). The initial glycoproteins that were recognized included all of those found in [^{3}H]glucosamine-labeled infected cells (Fig. 1, lane G). These antigenic glycoproteins were identified by RIP assay with serum taken at 22 days after infection with [^{3}H]glucosamine-labeled infected cell lysate (Fig. 1, lane 22 [right side]). The molecular weights of these proteins were 102,000, 96,000, 82,000, 69,000, and 55,000. At 22 to 50 days postinfection, additional amounts of some polypeptides were precipitated, notably p115, p110, and gp55; the precipitation of others, notably p91 and gp82, waned (Table 1). A total of 15 to 16 specific polypeptides were identified that were recognized during the initial immune response to BHV-1 infection.

During the period from 8 to 50 days after infection, the antibody response was also followed by virus neutralization and by ELISA (Fig. 2). The serological responses closely followed the pattern of development of specific antibodies detected by RIP assay, with the earliest antibody titer demonstrated on day 8 by ELISA. There was a small amount of precipitation of two polypeptides in RIP assays done on days 3 and 8 after infection, but this was due to nonspecific binding of the polypeptides to the SaCI adsorbent (data not shown). The sera taken on day 3 lacked any anti-BHV-1 antibodies as shown by both the neutralization and the ELISA test. At the time that antibody titers and RIP activity increased, clinical signs and virus shedding decreased to normal levels (Fig. 2).

Antigenic polypeptides involved in virus neutralization. Two monoclonal antibodies, D9 and F2, which neutralized

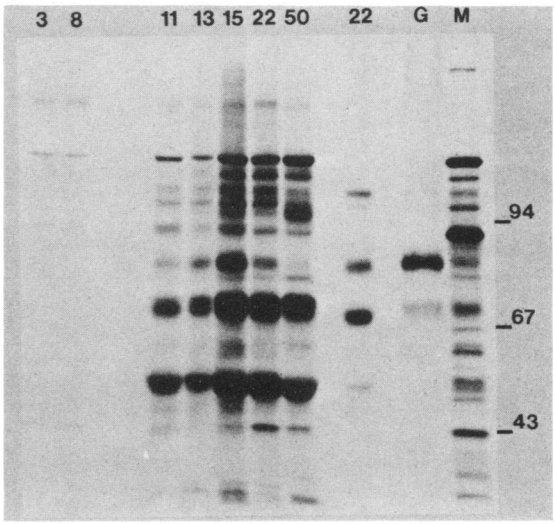

FIG. 1. BHV-1 polypeptides recognized after experimental infection. [^{35}S]methionine-labeled infected cell extracts were reacted with bovine serum taken at 3, 8, 11, 13, 15, 22, and 50 days after infection. One serum sample taken at day 22 (right side of figure) was used to precipitate proteins from a [^{3}H]glucosamine-labeled infected cell lysate. Lanes G and M, Proteins in the infected cell lysates without specific precipitation with serum.

BHV-1 (6) were used in a RIP assay and compared with the precipitating activity in bovine serum taken 22 days after infection. These are shown in lanes 1 to 3 respectively, of Fig. 3. D9 neutralizing antibody precipitated the glycoproteins gp102, gp96, gp69, and gp55, and F2 antibody precipitated gp82. All of these glycoproteins were also precipitated by bovine antiserum (Fig. 3, lane 3) and represent all of the major bands that were labeled with [^{3}H]glucosamine in virus-infected cells (Fig. 3, lane 5). All of these glycoproteins were present in intact BHV-1 as demonstrated with [^{35}S]methionine-labeled purified virus (Fig. 3, lane 4) and in infected cells (lane 6).

Variability of immune response to BHV-1. In addition to sera collected sequentially from one experimentally infected animal, nine other animals were also infected, and serum was collected at 3 (acute phase) and 15 (convalescent phase) days after infection. Virus neutralization titers for these animals are shown in Table 2. RIP assays with these sera were analyzed for variability in the precipitation of viral polypeptides and are shown in Fig. 4. All nine animals showed very similar RIP patterns. No animal had any reactivity to BHV-1 polypeptides at 3 days after infection, but by 15 days, all had responded. This correlated well with the recovery from infection shown by the decline in clinical signs (Fig. 2). Each animal precipitated at least seven major polypeptides and several minor polypeptides. Five of the seven major bands were the glycoproteins gp102, gp96, gp82, gp69, and gp55, which were identified earlier by the monoclonal antibodies. In addition, the nonglycosylated polypeptides p115, p94, p91, p69, p50-52, p30, and p10-15 were precipitated. Besides small quantitative differences, the only significant variation observed was in the precipitation of p110, which was absent from the convalescent responses of animals 1, 2, and 4.

TABLE 1. Polypeptides of BHV-1

No. and mol wt ($\times 10^3$) of viral polypeptides[a]		Recognized by bovine antibodies on day after infection[b]		
Purified virus	Infected cell lysate	1–8	11–15	22–50
	~200			
145	p145	±		
115	p115 (M)	±	+	++
	p110		+	++
102	gp102		+	++
97	gp96			+
	p94		++	++
88	p91 (M)	±	+++	+
81	gp82 (M)		+++	+
77	p79			
70	p70			
	gp69 (M)		++	+++
65	p66			
62.5	p61.5		+	++
61	p60			
59				
57	p57			
	gp55 (M)		+	++
	p52			
49	p50			
47				
41.5	p43			
	p40		+	++
	p39			
36	p36			
33				
31	p30	±	+	++
	p10-15			

[a] p, Polypeptide; gp, glycoprotein; M, major virus band. Polypeptides on purified virus (n = 19) were determined by silver staining polyacrylamide gels; polypeptides in infected cell lysate (n = 25) were determined by [^{35}S]methionine labeling.

[b] ±, Nonspecific precipitation; +, ++, and +++, relative amounts of polypeptide.

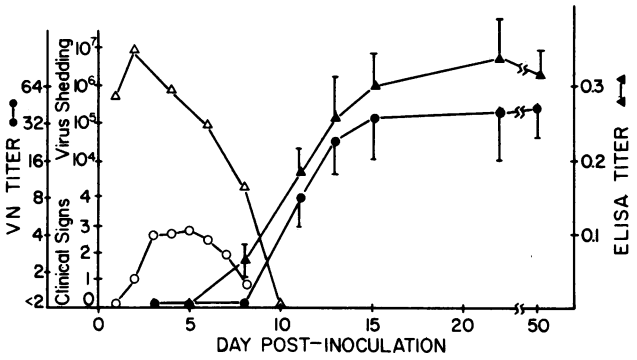

FIG. 2. Serological response to infection with BHV-1. After infection, virus shedding (△) and clinical signs (○) were monitored. Antibody response was measured by neutralization (●) and by ELISA (▲).

RIP assays with sera from natural infections. Sera were collected from feedlot animals that had become infected under natural conditions, and these were analyzed for neutralization titers and by RIP assay. Two groups of animals were identified, those who were acutely ill (no. 10 to 15;

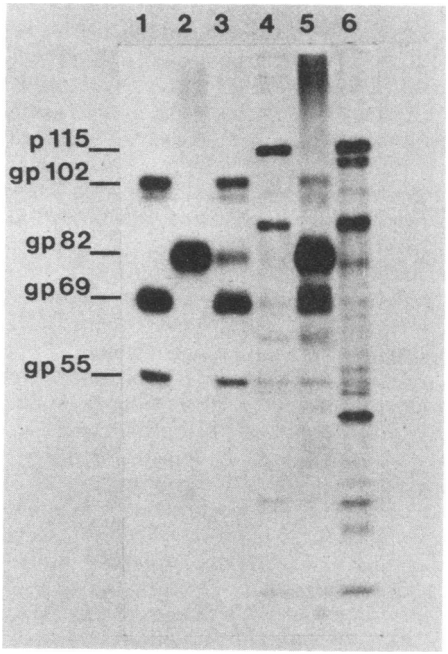

FIG. 3. RIP of [^{35}S]methionine-labeled and [^{3}H]glucosamine-labeled BHV-1-infected cell lysates. Lanes 1 to 3 show RIP patterns with [^{3}H]glucosamine-labeled polypeptides. Lanes: 1, monoclonal antibody D9; 2, monoclonal antibody F2; 3, bovine antiserum taken at 50 days postinfection; 4, [^{35}S]methionine-labeled purified BHV-1; 5, [^{3}H]glucosamine-labeled BHV-1-infected cell lysate; and 6, [^{35}S]methionine-labeled BHV-1-infected cell lysate.

TABLE 2. Virus neutralization titers

Serum source no.	Titer[a]	
	Acute phase	Convalescent phase
Experimentally infected		
1	<2	32
2	<2	128
3	<2	32
4	<2	16
5	<2	32
6	<2	128
7	<2	64
8	<2	64
9	<2	64
Field infection		
10	16	32
11	64	128
12	8	128
13	16	128
14	256	128
15	4	128
Field contact		
16	32	16
17	4	8
18	16	8
19	32	32
20	4	8
21	16	16

[a] Titers were determined by an all-or-none endpoint dilution assay with 100 50% tissue culture infective doses.

Table 2 and Fig. 5) and contact control animals which did not become sick (no. 16 to 21, Table 2; no. 16 to 18, Fig. 5). Sick animals demonstrated severe respiratory signs including labored breathing, elevated temperature, nasal and ocular exudates, and depression; of ca. 100 animals that showed clinical signs, 15 died. Serum sampling was carried out on two occasions, at the time acute clinical illness was first evident and 14 days later. The RIP assay data for the sick animals are shown in Fig. 5; it was evident that much greater variability was present in comparison to the amount of precipitation obtained with sera from controlled infections. Most of this variability was due to differences in sampling times. The exact time course of the infections was not precisely known, as shown by animal 14 (Table 2; Fig. 5), which had already developed specific antibodies at the acute-phase sampling time.

The polypeptides recognized by each naturally infected animal were similar to those polypeptides recognized under experimental conditions, with the glycoproteins constituting the main polypeptides precipitated (Fig. 5). Dramatic increases in the amounts of the precipitation of polypeptides were found in sick animals 11 to 13 and animal 15 for gp102, gp96, gp69, and gp55 and also for the nonglycosylated proteins p115, p110, and p91. Some of these glycoproteins (gp102 and gp96) were also precipitated to a very small extent by the acute-phase samples taken from these animals. One of these animals, no. 10, showed no increase in polypeptide precipitation. A major difference was observed between these convalesent-phase samples and those taken from the experimentally infected animals in the precipitation of gp82. The convalescent sera from these field cases (Fig. 5, no. 10 to 15) showed much less reactivity to gp82 than did the convalescent sera from the experimental animals (Fig. 4).

Figure 5 also shows the acute and convalescent pattern found by RIP assay with sera from contact animals which did not demonstrate clinical illness (no. 16 to 18). It was apparent that little or no increase in precipitation activity or antibody titers was evident between acute and convalescent sera. Animals 19 to 21 were similar in their RIP patterns to animals 16 to 18 (data not shown). In these field contact animals, both acute- and convalescent-phase sera exhibited RIP patterns almost identical to the RIP patterns found by using acute-phase sera from animals which later experienced clinical disease.

DISCUSSION

The polypeptides which carry the major antigenic determinants on BHV-1 have been identified by using sera from both natural and experimentally induced infections. The most striking feature of the reactivities of these antisera was the consistent precipitation of all viral glycoproteins that were identified by glucosamine labeling. These included at least two antigenic groups of glycoproteins. The first group was characterized by four glycoproteins (gp102, gp96, gp69, and gp55) which were coprecipitated by monoclonal antibody D9. Preliminary work suggests that these glycoproteins are related to each other by a precursor-product biosynthetic pathway (unpublished data). The second antigenic glycoprotein was gp82, recognized by monoclonal antibody F2. All of these glycoproteins were recognized early in the immune response to BHV-1 infection.

Two nonglycosylated proteins (p115 and p91) were also uniformly precipitated in RIP assays by bovine antiserum. These two proteins were both major constituents of the virus as measured by methionine labeling. The antibody response to p115 increased after experimental infection (up to 50 days postinfection), whereas the response to p91 was strong only immediately after infection (on day 15). The response to p69 was similar to the response to gp82 in that it was also short lived and trailed off by 50 days after infection. The significance of this type of response was unclear, except that it may have indicated that these antigenic polypeptides provided a less potent immunogenic stimulus. This was supported by the finding that convalescent-phase sera from field infections showed a reduced precipitation of gp82, reflecting that the sampling time may have missed the maximal gp82 response.

Besides the variability of reactivity to p91 and gp82, the immune responses were remarkably similar among the different categories of infection and exposure. All of the animals which were subjected to controlled infections and who had not had any prior exposure to the virus responded in nearly identical fashion as shown by RIP assay. Approximately 15 to 16 viral polypeptides were recognized by 15 days after infection. The response to the field infection was more heterogeneous; the variability, however, was not due to different polypeptides recognized but to the amount of reactivity to the same 15 to 16 polypeptides. The most consistent response to the natural infections in the field was the recognition of p115 and the gp102, gp96, gp69, and gp55 group of glycoproteins. Similar to the clinically ill animals, most of the contact animals already had some specific antibodies to these glycoproteins at the time of acute sampling. This was most likely due to prior vaccination. All of the contact animals were in the same feedlot pen with sick animals for ca. 2 months. Given this information, it could be concluded that the presence or absence of these antibodies alone could not be correlated with protective immunity.

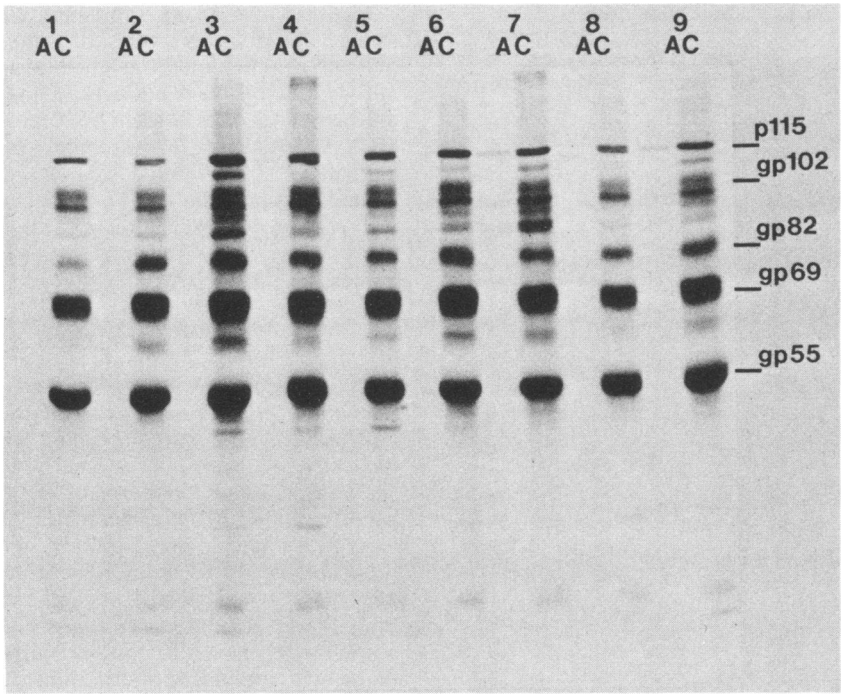

FIG. 4. RIP obtained with acute (A)- and convalescent (C)-phase serum from nine experimentally infected animals with [^{35}S]methionine-labeled BHV-1-infected cell lysate.

RIP activity in the bovine sera correlated well with antibodies detected by ELISA and VN. When maximal RIP activity was observed, the VN titers were also elevated. VN titers reached levels similar to those found by others carrying out experimental infections (12) or after vaccination with modified live viruses (9, 17, 25). However, it was not possible to correlate which polypeptides were recognized in the ELISA or in the VN tests because the RIP assay detected activity to so many polypeptides.

There are most likely many important antigenic determinants involved in immunity to BHV-1. The antigenically related group of gp102, gp96, gp69, and gp55 glycoproteins, as well as gp82, probably play a role in neutralization, as evidenced by the neutralizing activity of monoclonal anti-

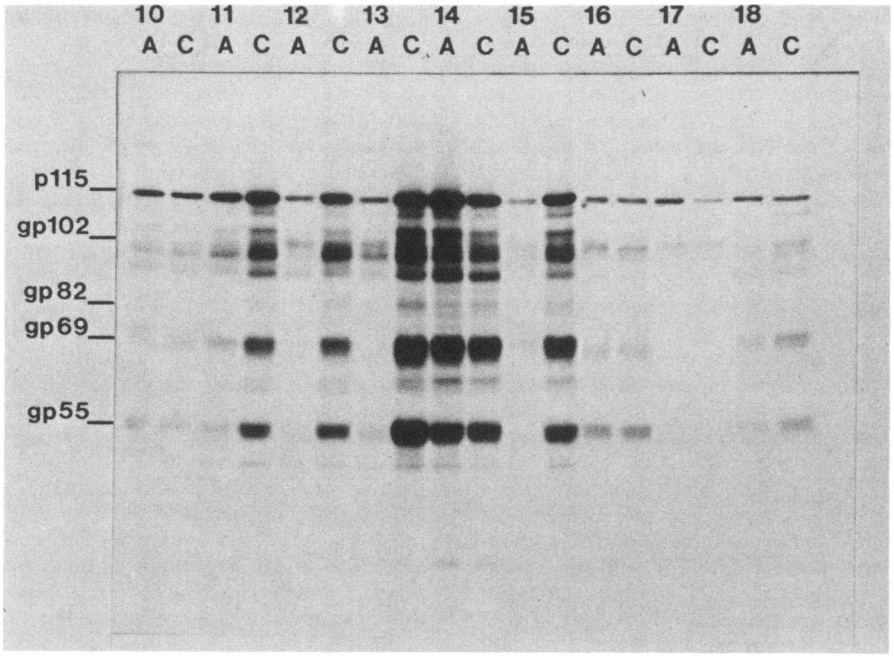

FIG. 5. RIP obtained with acute (A)- and convalescent (C)-phase serum from six naturally infected clinically ill animals (no. 10 to 15) and three contact clinically normal animals (no. 16 to 18).

bodies D9 and F2, respectively (6). A monoclonal antibody which recognizes a 71.5-kd glycoprotein can carry out immune cytolysis of BHV-1-infected cells (19). Bovine antibody with neutralizing activity and activity in immune cytolysis has an important role in limiting the spread of virus infections (3, 19). Thus, neutralizing epitopes and epitopes that are not involved in neutralization and yet reside on infected cell membranes may play critical roles in immunity. The nature of these serologically defined antigenic polypeptides, as well as determinants involved in cell-mediated immune functions (23), remain to be elucidated with BHV-1.

The large number of BHV-1 polypeptides makes the analysis of immunological reactivities complex. The polypeptides that are defined by RIP assays are only a small percentage of those virus-specific polypeptides that are found inside infected cells, and it is unclear whether all of these polypeptides might be shown to be antigenic with other assays. In addition, the results of RIP assays vary, depending on the technical details of the procedure. The present studies and others (18) have relied upon the binding of bovine immune complexes to protein A, a technique which has been shown to efficiently detect bovine immunoglobulin G_2 but to detect other bovine immunoglobulin classes with much less efficiency (10). Other technical details which could also affect the results include the metabolic labeling method, the virus strain used, and the method of antibody induction. With the large number of polypeptides and the concomitant technical considerations, various analyses of BHV-1 polypeptides and antigens have demonstrated apparently different results. Bolton et al. have found 33 BHV-1 polypeptides, several of which were envelope proteins based on their absence from deenveloped particles (2). Misra et al. (18) demonstrated 25 to 33 polypeptides depending on polyacrylamide gel technique, with 11 of these being glycoproteins, 9 of which were in the same size range as described for the glycoproteins found here. By comparison with other herpesviruses, particularly herpes simplex virus, most or all of the BHV-1 glycoproteins are likely to be involved in the immune response (20, 26). Some of the herpes simplex virus glycoproteins are able to induce significant protective antibodies (21, 15); others are involved with both the humoral immune response and act as antigens in cell-mediated responses (5, 8, 20). To determine what BHV-1 polypeptides are involved in the various immune-mediated functions, further comparisons will be necessary to clarify the nature and variability of BHV-1 antigenic polypeptides.

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