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VIROLOGY 143, 104–118 (1985)

Effect of Tunicamycin and Monensin on Biosynthesis, Transport, and Maturation of Bovine Herpesvirus Type-1 Glycoproteins

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Received August 16, 1984; accepted January 10, 1985

The effect of tunicamycin and monensin on the biosynthesis, intracellular transport, and maturation of bovine herpesvirus type-1 (BHV-1) glycoproteins was examined. Tunicamycin completely inhibited the production of infectious virus particles and significantly reduced the incorporation of [^{3}H]glucosamine into viral glycoproteins. In the presence of monensin, reduced amounts of infectious virus particles were produced, which was mainly due to inhibition of virus release, rather than virus production. Monensin only slightly inhibited viral glycoprotein synthesis. The effects of these compounds on infectivity indicated that glycosylation is required for the production of infectious virus, though complete processing of the glycoproteins is not essential. In addition, egress of the virions from infected cells probably requires a functional Golgi complex. In the presence of tunicamycin or monensin various degrees of glycosylation of the major glycoproteins occurred, consequently their rates of migration differed from that of the normal glycoproteins. Tunicamycin completely blocked glycosylation of GVP 6/11a/16 and GVP 7. In contrast, GVP 3/9 and GVP 11b were partially glycosylated in the presence of tunicamycin. These results indicated that GVP 6/11a/16 and GVP 7 are N-linked glycoproteins, but GVP 3/9 and GVP 11b contain both N- and O-linked oligosaccharide side chains. Tunicamycin blocked the transport of all viral glycoproteins to the cell surface, suggesting that glycosylation is required for this process. In the presence of monensin, the viral glycoproteins were transported and expressed on the cell surface indicating that transport does not require complete processing of the glycoproteins and may occur via a Golgi-independent pathway. In addition, monensin-treated BHV-1 infected cells could act as target cells in an antibody-dependent cell cytotoxicity assay. Thus, complete glycosylation may not be essential for maintenance of antigenicity and participation in immune destruction.

INTRODUCTION

In the course of infection, herpesviruses specify several glycoproteins (Spear, 1975, 1976) that are located on the viral envelope and on the surface of the virus-infected cells (Roizman and Spear, 1971; Glorioso and Smith, 1977). As such, they play an important role during viral infection in a number of ways. Not only do they recognize receptor sites for the attachment and penetration of a virus into the cell (Manservigi *et al.*, 1977; Sarmiento *et al.*, 1979), they are also the antigens that determine the host's immune response to the virus. In that capacity they induce the production of neutralizing antibodies and they mediate interactions between the host's immune system and the virions or the virus-infected cells (Vestergaard and Norrild, 1979; Norrild *et al.*, 1979, 1980).

The most distinctive features of glycoproteins are the carbohydrate–peptide linkages which have been classified into two main types: N-glycosidic and O-glycosidic linkages (Sharon and Lis, 1981). The synthesis and processing of N-linked oligosaccharides are co- as well as post-translational events. First, a mannose-rich precursor oligosaccharide is transferred en bloc from a dolichol donor to an

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0042-6822/85 $3.00

asparagine residue in the nascent growing polypeptide (Waechter and Lennarz, 1976; Hubbard and Ivatt, 1981). Later during the glycosylation process, the oligosaccharide is modified by sequential removal and addition of carbohydrates (Berger *et al.*, 1982). In contrast to N-linked glycosylation, synthesis of O-linked oligosaccharides occurs by sequential addition of carbohydrates to the polypeptides, initiated by the transfer of *N*-acetylgalactosamine to a serine or threonine residue (Berger *et al.*, 1982). The latter steps involved in the processing of N-linked oligosaccharides and all or the greater part of O-linked glycosylation are thought to occur in the Golgi apparatus (Berger *et al.*, 1982). N-Linked oligosaccharides have been observed on most viral glycoproteins previously investigated (Ghosh, 1980), but recently, evidence has also been accumulating for the presence of O-linked oligosaccharides (Holmes *et al.*, 1981; Oloffson *et al.*, 1981; Rottier *et al.*, 1981; Shida and Dales, 1981; Johnson and Spear, 1982, 1983).

Bovine herpesvirus type-1 (BHV-1) specifies more than 25 structural polypeptides of which 11 are supposedly glycosylated (Misra *et al.*, 1981). Although there is considerable information available about immune destruction of BHV-1 infected cells (Babiuk *et al.*, 1975; Rouse *et al.*, 1976; Grewal *et al.*, 1977, 1980; Rouse and Babiuk, 1978), thus far very little was known about the glycoproteins that mediate immune lysis. In order to characterize the major BHV-1 glycoproteins with respect to their biochemical and immunological properties, we recently produced a panel of monoclonal antibodies against BHV-1 (van Drunen Littel-van den Hurk *et al.*, 1984). The availability of these monoclonal antibodies allowed us to identify four unique glycoproteins and their respective precursors: GVP 6/11a/16, a complex of three glycopeptides with apparent molecular weights of 130K, 74K, and 55K for which two precursors of 120K and approximately 105K were found; GVP 7 (105K) with one precursor (100K); GVP 9 (91K) which has two precursors of 72K and 64K and also occurs as a dimer, GVP 3 (180K); and GVP 11b (71K) with one precursor (63K) (van Drunen Littel-van den Hurk *et al.*, 1984; van Drunen Littel-van den Hurk and Babiuk, manuscript in preparation). These four unique glycoproteins were all expressed on the surface of the virus-infected cells and all, except GVP 7, participated in antibody and complement-mediated (AbC') cytolysis (Misra *et al.*, 1982; van Drunen Littel-van den Hurk *et al.*, 1984). In order to further characterize these four major BHV-1 glycoproteins, we used two compounds that interfere at different stages during glycosylation.

Tunicamycin, by acting as an analog of UDP-*N*-acetylglucosamine, interferes with the formation of the *N*-acetylglucosamine pyrophosphoryldolichol intermediate and thereby completely inhibits the synthesis of N-linked oligosaccharide side chains (Schwartz and Datema, 1980). Consequently, all glycoprotein-containing enveloped viruses are affected by tunicamycin in such a way that it prevents maturation and production of infectious virus (Schwartz *et al.*, 1976; Morrison *et al.*, 1978; Nakamura and Compans, 1978; Stohrer and Huner, 1979; Pizer *et al.*, 1980). Monensin is a carboxylic ionophore that is able to equilibrate Na^+/K^+ levels with a selectivity for Na^+ (Pressman, 1976). Although it interferes with a variety of cellular functions (Tartakoff and Vasalli, 1977; Uchida *et al.*, 1980; Basu *et al.*, 1981; Wilcox *et al.*, 1982), one of its primary sites of action is the Golgi apparatus where it abolishes proton gradients that are crucial for Golgi functions (Pressman, 1976). Enveloped viruses are all affected by monensin in a similar manner, i.e., post-translational glycosylation (Chatterjee *et al.*, 1982; Johnson and Spear, 1982) and proteolytic processing (Johnson and Schlesinger, 1980; Chatterjee *et al.*, 1982), as well as protein secretion (Strous and Lodish, 1980; Payne and Kristensson, 1982) and transport of viral envelope proteins to the plasma membrane (Johnson and Schlesinger, 1980; Strous and Lodish, 1980; Alonso and Compans, 1981; Johnson and Spear, 1982; Payne and Kristensson, 1982) may be inhibited.

In the present study we used tunicamycin and monensin to obtain information about the contribution of the carbohydrate portion to biosynthesis and transport of the major BHV-1 glycoproteins, as well as maturation and infectivity of the BHV-1 virions. Glycosylation to some extent appeared to be required for proteolytic processing, transport, and expression of the viral glycoproteins on the cell surface. However, the glycosylation process did not have to be completed for these processes to occur. Similarly, partial but not complete processing of viral envelope glycoproteins seemed to be essential for nucleocapsid envelopment or virion infectivity. Finally, the use of these two compounds allowed us to conclude that GVP 6/11a/16 and probably GVP 7 contain only N-linked carbohydrates, whereas GVP 3/9 and GVP 11b have N- and O-linked oligosaccharides.

MATERIALS AND METHODS

Cells and virus. Georgia bovine kidney (GBK) cells were grown as monolayers in Eagle's minimal essential medium (MEM), (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (FBS) (Grand Island Biological Co.). Strain P8-2 of BHV-1 was propagated in GBK cells and quantitated by plaquing in microtiter plates with an antibody overlay as described previously (Rouse and Babiuk, 1974).

Potassium tartarate gradient analysis. Monolayers of GBK cells were infected with BHV-1 at a multiplicity of infection (m.o.i.) of 1. After adsorption for 1 hr at 37°, the virus was removed and the cells were overlaid with methionine-free MEM containing 2% FBS and tunicamycin or monensin, if used. Six hours later 25 μCi/ml of [^{35}S]methionine (Amersham, Oakville, Ontario) was added to the cells. At 34 hr postinfection, virus was purified from the infected cells (cell-associated virus) and the supernatant medium (extracellular virus). To release the intracellular virus the infected cells were sonicated for 15 sec at a setting of 7 on a Sonifier cell disrupter (Model W140D, Ultrasonics, Plainsview, N. Y.). After removal of the cell debris the virus was pelleted by centrifugation at 25,000 rpm for 2 hr in a SW41 rotor (Beckman Model L8-55). The virus pellet was resuspended in TNE (0.01 *M* Tris-hydrochloride, 0.15 *M* NaCl, 0.001 *M* EDTA, pH 7.5) by mild sonication and layered onto an 11-ml linear 20 to 50% potassium tartrate gradient in TNE. After centrifugation at 25,000 rpm for 1$\frac{1}{2}$ hr in a SW41 rotor at 4°, fractions were collected and assayed for radioactivity and infectivity. The virus-containing fractions were pooled, diluted with TNE, and centrifuged at 25,000 rpm for 2 hr in a SW41 rotor at 4° to collect the virus.

Preparation of radiolabeled cell lysates. Monolayers of GBK cells were infected with BHV-1 at an m.o.i. of 10. After adsorption for 1 hr at 37°, the virus was removed and the monolayers were overlaid with either methionine-free or glucose-free MEM containing 2% FBS. Monensin or tunicamycin was added at this time during infection. Six hours after infection, cells that were overlaid with methionine-free MEM were labeled with 25 μgCi/ml of L-[^{35}S]methionine, whereas cells, overlaid with glucose-free MEM, were labeled with 25 μCi/ml of [^{3}H]glucosamine (Amersham Corp., Oakville, Ontario). At 24 hr after infection the cells were harvested and washed with phosphate-buffered saline (PBS: 0.01 *M* Na_2HPO_4/NaH_2PO_4, 0.15 *M* NaCl, pH 7.4). For analysis by polyacrylamide gel electrophoresis (PAGE), the cells were resuspended in sample buffer (0.0625 *M* Tris-hydrochloride [pH 6.8], 1.25% sodium dodecyl sulfate (SDS), 12.5% glycerol, 0.15 *M* 2-mercaptoethanol, 0.00125% bromophenol blue) and boiled for 1 min. Alternatively, the cells were resuspended in modified RIPA buffer (0.02 *M* Tris-hydrochloride [pH 8.0], 0.15 *M* NaCl, 1% sodium deoxycholate (DOC), 1% Nonidet P40 (NP40)). After 15 min on ice, they were sonicated for 15 sec at a setting of 4 on a Sonifier cell disrupter (Model 140D, Ultrasonics). The cell debris was removed by centrifugation at 80,000 rpm for 15 min in a 30° A100 rotor (Beckman Airfuge) or at 25,000 rpm for 1

hr in a SB405 rotor at 4° (IEC Model B-60), and the supernatant was immediately used for immunoprecipitation.

Immunoprecipitation. The procedure for immunoprecipitation described previously (van Drunen Little-van den Hurk *et al.*, 1984) was slightly modified. Briefly, freshly prepared radiolabeled cell lysate in RIPA buffer was incubated overnight on ice with mouse ascites fluid. Rabbit IgG anti-mouse Ig (Cappel Laboratories, Cochranville, Pa.) was bound to protein A-Sepharose CL-4B beads (Pharmacia, Montreal, Quebec), and unbound antibody was removed by washing the beads three times in RIPA buffer. The beads, coated with rabbit IgG anti-mouse Ig, were then incubated for 3 hr on ice with the reaction mixture. The Sepharose beads were pelleted, washed four times with RIPA buffer containing 0.1% SDS, and resuspended in electrophoresis sample buffer. In order to dissociate antigen-antibody complexes, the samples were boiled for 5 min before electrophoresis.

Analysis of polypeptides by polyacrylamide gel electrophoresis. High resolution SDS-PAGE was performed by the discontinuous method of Laemmli (1970). The polypeptides of the radiolabeled cell lysates and immunoprecipitates were separated in 7.5% SDS-polyacrylamide slab gels with 4.5% stacking gels. Unless otherwise mentioned, electrophoresis was performed under reducing conditions. Samples containing ^{35}S were analyzed by autoradiography of the gels on 3M X-ray film (Picker, Saskatoon, Sask.). Gels containing ^{3}H-labeled samples were treated with En^{3}Hance (New England Nuclear Corp., Lachine, Quebec) for fluorography on preflashed film. Prior to drying, the gels were stained with Coomassie blue to visualize the molecular weight markers (BioRad Laboratories, Mississauga, Ontario) that were electrophoresed in parallel with the samples.

Cell surface immunofluorescence. Infected GBK cells were removed from monolayers by mild trypsinization and 1×10^6 cells were resuspended in 250 μl of bovine anti-BHV-1 antiserum. After reaction for 45 min at 4° the cells were washed three times in cold Hanks' balanced salt solution (HBSS) and reacted a second time against a 1:16 dilution of fluorescein-labeled rabbit anti-bovine IgG antiserum (Cappel Laboratories). After reaction for 45 min at 4° the cells were washed three times in cold HBSS and finally resuspended in 10% glycerol-PBS, mounted on glass slides, and observed with the aid of a fluorescence microscope.

Antibody-dependent cell cytotoxicity (ADCC) assay. ADCC assays were performed in microtiter plates as described previously (Rouse *et al.*, 1976). The effector (polymorphonuclear cells, PMNs) to target (BHV-1 infected ^{51}Cr-labeled GBK cells) cell ratio was 50:1. Controls consisted of BHV-1 infected GBK target cells plus anti-BHV-1 serum, or target cells with PMNs in the absence of antibody. Treatment of the BHV-1 infected target cells with tunicamycin or monensin was initiated immediately after adsorption of the virus.

RESULTS

Production of infectious virus. In order to determine what effect inhibition of glycosylation had on infectious virus production, BHV-1 infected GBK cells were treated with increasing concentrations of tunicamycin or monensin immediately after infection. As Table 1 illustrates, both tunicamycin and monensin decreased the yields of infectious BHV-1, but over a different concentration range and to different extents.

Concentrations as low as 0.001 μg/ml of tunicamycin were sufficient to cause a 80% decrease in the production of infectious BHV-1. As tunicamycin concentrations increased, the quantity of infectious virus decreased even more dramatically, such that at 5 μg/ml infectious virus production was negligible (Table 1).

Monensin also decreased total infectious virus production, but to a much lower extent (data not shown). Since monensin is a compound that affects the transport and secretion mechanisms of the cell we assessed whether the decrease in infectious virus reflected a reduction in virus

TABLE 1

EFFECT OF TUNICAMYCIN AND MONENSIN ON PRODUCTION OF INFECTIOUS BHV-1

Tunicamycin[a]		Monensin[a]		
			Virus titer	
Concentration (μg/ml)	Virus titer total	Concentration (μM)	Intracellular	Extracellular
0	3×10^8 (100)[b]	0	2.3×10^7 (100)[b]	2.5×10^7 (100)[b]
0.001	6×10^7 (20)	0.01	2.1×10^7 (91)	1.1×10^7 (44)
0.01	5×10^7 (17)	0.05	1.7×10^7 (74)	2.7×10^6 (11)
0.1	1.5×10^5 (0.5)	0.1	2.9×10^6 (13)	1.2×10^4 (0.04)
1	4×10^2 (<0.001)	0.5	8×10^4 (0.3)	1.5×10^3 (<0.001)
5	<10 (<0.001)	5	3.9×10^5 (1.7)	3.3×10^4 (0.1)

[a] GBK cells were infected with BHV-1 at an m.o.i. of 1. Tunicamycin or monensin was added to the cells immediately after virus adsorption and maintained until the cells were harvested at 48 hr postinfection.

[b] Virus yields were determined by plaquing on GBK cells with an antibody overlay. Cell-associated virus was released by sonication of the cells. The number in parentheses represent the virus titer expressed as a percentage of the control value for untreated cells.

release or actual virus production. Table 1 demonstrates that a monensin concentration of 0.05 μM reduced the extracellular virus yield to 44% of the control value, whereas the amount of cell-associated virus was still 91% of the control value. This same trend continued, with increasing concentrations of monensin having a greater effect on virus release rather than on actual production of virus within the cells.

Assembly and release of BHV-1. Table 1 clearly demonstrated that tunicamycin as well as monensin inhibited the production of infectious BHV-1 in a dose-dependent fashion. In addition, it appeared that no infectious virus, whether cell-associated or extracellular, was produced in the presence of high enough concentrations of tunicamycin, whereas some infectious virus was produced, even at very high concentrations of monensin. In order to obtain further information regarding the assembly and release of the virions, BHV-1 was labeled with L-[^{35}S]methionine and analyzed on 20 to 50% potassium tartrate gradients. Gradients of extracellular and cell-associated virus from untreated GBK cells showed two peaks of radioactivity. The denser peak represents complete particles and corresponds to the peak of infectivity, whereas the lighter peak consists principally of incomplete particles that lack DNA (Misra *et al.*, 1981) (Fig. 1A).

Virus assembly was dramatically affected by the presence of tunicamycin in the cells during infection (Figs. 1B, C). At 1 μg/ml tunicamycin no extracellular virus was detected and the amount of cell-associated virus was reduced to approximately 35% of the control value. In addition, a shift in position of the peak of cell-associated virus was observed. This could possibly reflect an altered size, density, or conformation of the virions. No infectivity was detectable in this peak (Fig. 1C).

Monensin, however, had a much less dramatic effect on virus assembly (Figs. 1D, E). At 0.2 μM as well as 20 μM monensin, the quantity of cell-associated virus was only reduced to between 70 and 80% of the control value. Although the slightly reduced peak of cell-associated virus showed a shift in position in the gradient, similar to that observed after tunicamycin treatment, this shift was not as pronounced. Accordingly a reasonable amount of infectivity was detected in this peak (Fig. 1E). In agreement with the above observation on the production of

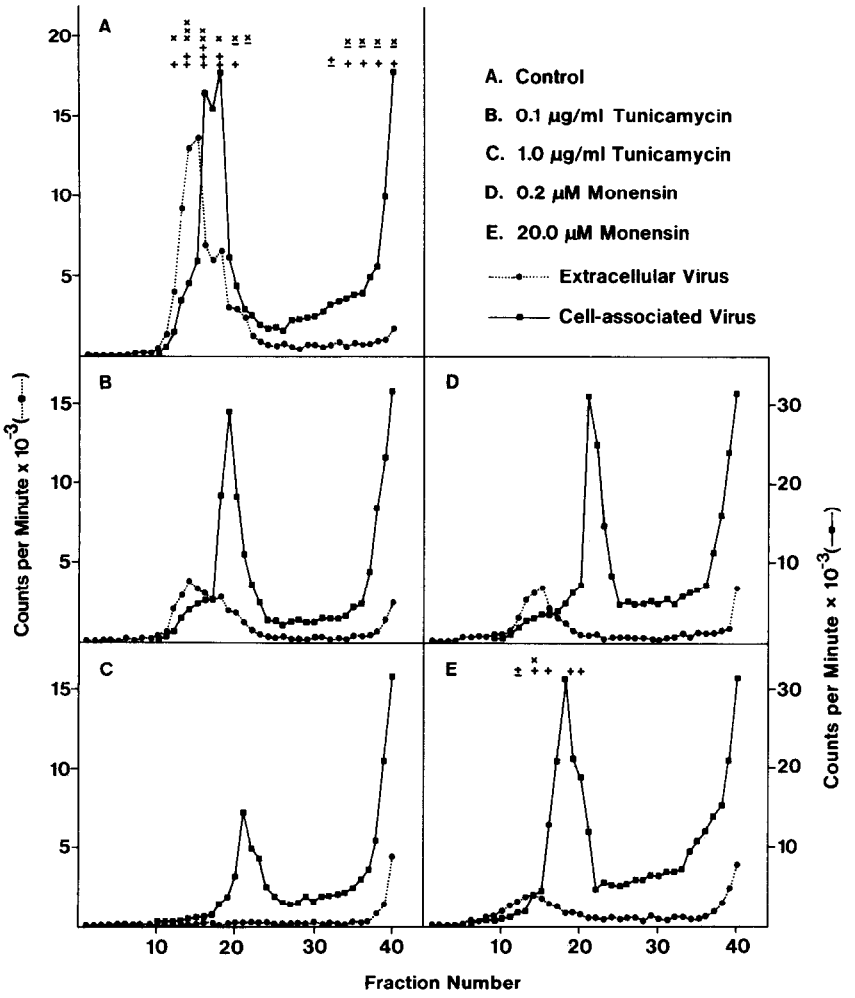

FIG. 1. Analysis of BHV-1 on potassium tartrate gradients. Immediately after virus adsorption, GBK cells were treated with tunicamycin (0.1 and 1.0 μg/ml), monensin (0.2 and 20.0 μ*M*), or left untreated (control). The cells were labeled with L-[^{35}S]methionine 6 hr postinfection. At 24 hr postinfection the virus was collected from the infected cells (cell-associated virus) and the supernatant medium (extracellular virus), and analyzed on 20 to 50% potassium tartrate gradients. The gradients were fractionated from the bottom of the tubes. The infectivity of individual extracellular (X) and cell-associated (+) virus fractions in A, C, and E is indicated at the top of each fraction. XXX and +++: >1000 pfu/ml; XX and ++: 100-1000 pfu/ml; X and +: 10-100 pfu/ml; *X* and +: 1-10 pfu/ml.

infectious virus, virus release was drastically reduced in the presence of 0.2 μ*M*, as well as 20 μ*M* monensin. Only 10 to 15%, as compared to the control value, was released (Fig. 1E). This virus had very low infectivity.

Viral protein synthesis and glycosylation. In order to determine the effect of these glycosylation inhibitors on viral polypeptide synthesis and glycosylation, mock-infected, and BHV-1 infected GBK cells were treated with increasing concentrations of tunicamycin or monensin, immediately after adsorption. Six hours later the cells were labeled with L-[^{35}S]methionine or [^{3}H]glucosamine. At 24 hr postinfection the cells were harvested and the incorporation of L-[^{35}S]methionine and

[^{3}H]glucosamine into viral polypeptides was analyzed by SDS-PAGE. At the three lower concentrations of tunicamycin no effect on the incorporation of L-[^{35}S]-methionine into viral or cellular polypeptides was observed. Only at 1 and 10 μg/ml of tunicamycin was viral and cellular protein synthesis reduced (data not shown). The incorporation of [^{3}H]glucosamine into all individual viral polypeptides was significantly reduced in the presence of increasing concentrations of tunicamycin (Fig. 2). Since glycosylation of cellular polypeptides was inhibited to the same extent (data not shown), the synthesis of cellular and viral glycoproteins probably involves the same or similar dolichol-mediated carbohydrate transfer. In contrast, monensin had little effect on protein synthesis or glycosylation of any of the viral or cellular glycoproteins (Fig. 2).

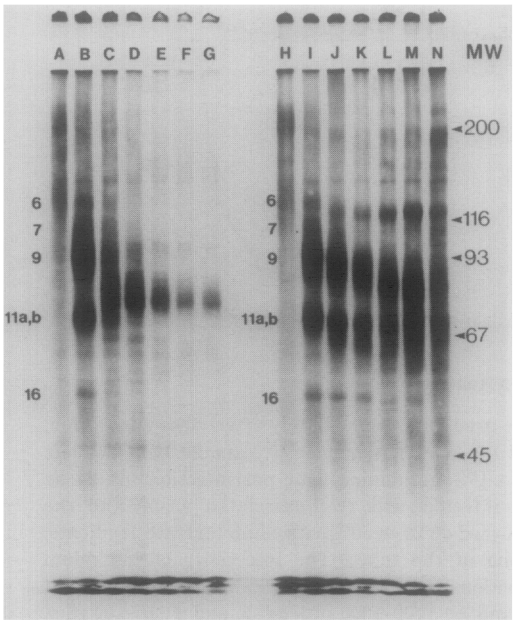

FIG. 2. PAGE (7.5%) analysis of [^{3}H]glucosamine-labeled polypeptides from mock-infected (lanes A and H) and BHV-1 infected (lanes B to G and I to N) cells. Immediately after virus adsorption, the cells were treated with 0.02 (lane C), 0.1 (lane D), 0.5 (lane E), 2.5 (lane F), and 10.0 (lane G) μg/ml tunicamycin, 0.02 (lane J), 0.1 (lane K), 0.5 (lane L), 2.5 (lane M), and 10.0 (lane N) μ*M* monensin, or left untreated (lanes A, B, H, and I). The major viral glycoproteins are numbered in the left margin. Molecular weight markers $\times 10^{-3}$ are shown in the right margin.

The electrophoretic profiles of the [^{35}S]methionine-labeled viral polypeptides, synthesized in the presence and absence of tunicamycin or monensin, were similar. In contrast, the patterns of the [^{3}H]-glucosamine-labeled viral glycopeptides, showed that the major BHV-1 glycoproteins GVP 6 (130K), GVP 7 (105K), GVP 9 (91K), GVP 11a (74K), GVP 11b (71K), and GVP 16 (55K) were absent in cells treated with tunicamycin or monensin, whereas new bands with higher electrophoretic mobilities appeared (Fig. 2).

Identity of the (glyco)proteins, appearing in the presence of tunicamycin and monensin. Monoclonal antibodies, which were previously prepared in this laboratory, were used to establish the identity of the faster migrating bands that appeared in the presence of tunicamycin and monensin. Mock-infected and BHV-1 infected cells were treated with two different concentrations of tunicamycin and monensin and labeled with L-[^{35}S]methionine or [^{3}H]glucosamine. Cell lysates were prepared and precipitated with monoclonal antibodies specific for GVP 6/11a/16 (1E11), GVP 7 (3D9T), GVP 3/9 (1D6), and GVP 11b (3D9S). The precipitates were analyzed by SDS-PAGE. Since under reducing conditions, which are normally used, the majority of GVP 6 is dissociated into GVP 11a and GVP 16, GVP 6/11a/16 precipitates were also analyzed under nonreducing conditions.

Figure 3 illustrates that monoclonal antibody 1E11 precipitated the glycopeptides GVP 6 (130K), pGVP 6 (120K), GVP 11a (74K), and GVP 16 (55K) from untreated virus-infected cells, labeled with L-[^{35}S]methinine or [^{3}H]glucosamine. However, in cells treated with tunicamycin, only one polypeptide (105K) was precipitated which may correspond to the nonglycosylated precursor form of GVP 6, ppGVP 6 (van Drunen Littel-van den Hurk, and Babiuk, manuscript in preparation). This polypeptide was neither glycosylated, nor cleaved, suggesting that GVP 6/11a/16 contains only N-linked oli-

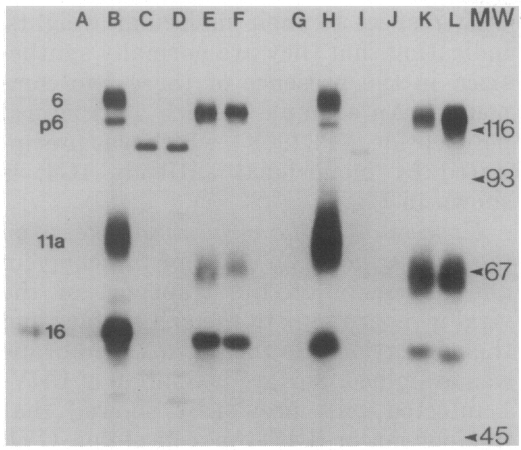

FIG. 3. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled (lanes A to F) or [^{3}H]glucosamine-labeled (lanes G to L) polypeptides from mock-infected (lanes A and G) and BHV-1 infected (lanes B to F and H to L) cells. Cell lysates were precipiated with monoclonal antibody 1E11. Immediately after virus adsorption, the cells were treated with 0.1 (lanes C and I) and 2.5 (lanes D and J) μg/ml tunicamycin, 0.1 (lanes E and K) and 2.5 (lanes F and L) μM monensin, or left untreated (lanes A, B, G, and H). The precipitated polypeptides are shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

gosaccharides and that glycosylation is required for cleavage of GVP 6 into GVP 11a and GVP 16. In contrast, three glycopeptides with apparent molecular weights of 125K, 69K, and 53K were precipitated from monensin-treated L-[^{35}S]methionine- and [^{3}H]glucosamine-labeled cells. These glycopeptides appeared to correspond to incompletely processed forms of GVP 6, GVP 11a, and GVP 16 which indicates that monensin inhibits further processing of these glycoproteins. Complete glycosylation was not needed for cleavage of GVP 6 into GVP 11a and GVP 16. Analysis of the GVP 6/11a/16 precipitates under nonreducing conditions confirmed that the faster migrating bands observed in tunicamycin and monensin treated cells are the non- or partially glycosylated counterparts of GVP 6 (Fig. 4).

Monoclonal antibody 3D9T precipitated GVP 7 (105K) and pGVP 7 (100K) from untreated, virus-infected cells (Fig. 5). No polypeptides were precipitated from infected cells treated with tunicamycin. One glycopeptide with an apparent molecular weight of 102K was precipitated from monensin-treated cells. This glycopeptide appeared to be an incompletely glycosylated form of GVP 7.

Figure 6 demonstrates that GVP 9 (91K) and pGVP 9 (72K) were precipitated by monoclonal antibody 1D6 from untreated, virus-infected cells, labeled with L-[^{35}S]-methionine and [^{3}H]glucosamine. After treatment of the cells with tunicamycin, two polypeptides with apparent molecular weights of 81K and 64K were precipitated. The 81K polypeptide was glycosylated, indicating that GVP 9 contains both N- and O-linked oligosaccharides. The 64K polypeptide may correspond to a nonglycosylated precursor form of GVP 9, ppGVP 9 (van Drunen Littel-van den Hurk and Babiuk, manuscript in preparation). From cells, treated with monensin, two glyco-

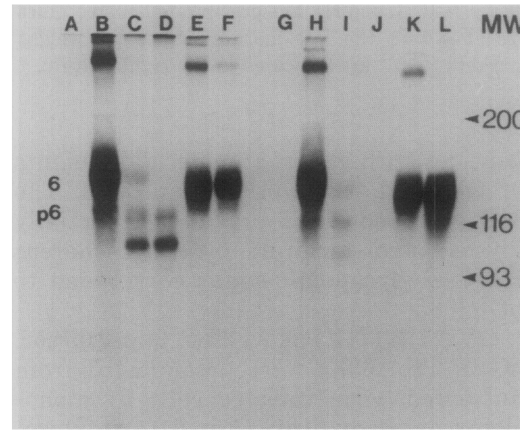

FIG. 4. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled (lanes A to F) or [^{3}H]glucosamine-labeled (lanes G to L) polypeptides from mock-infected (lanes A and G) and BHV-1 infected (lanes B to F and H to L) cells. Electrophoresis was performed under nonreducing conditions. Cell lysates were precipitated with monoclonal antibody 1E11. Immediately after virus adsorption, the cells were treated with 0.1 (lane C and I) and 2.5 (lanes D and J) μg/ml tunicamycin, 0.1 (lanes E and K) and 2.5 (lanes F and L) μM monensin, or left untreated (lanes A, B, G, and H). The precipitated polypeptides are shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

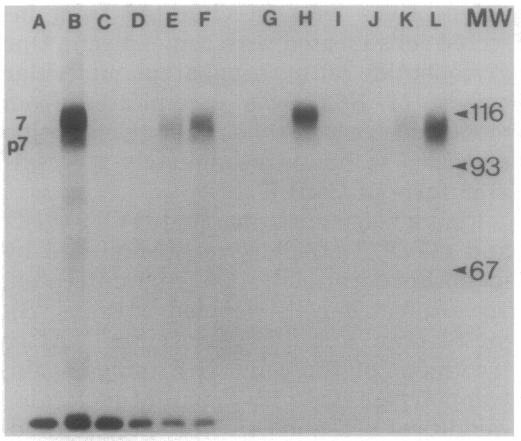

FIG. 5. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled (lanes A to F) or [^{3}H]glucosamine-labeled (lanes G to L) polypeptides from mock-infected (lanes A and G) and BHV-1 infected (lanes B to F and H to L) cells. Cell lysates were precipitated with monoclonal antibody 3D9T. Immediately after virus adsorption, the cells were treated with 0.1 (lanes C and I) and 2.5 (lanes D and J) μg/ml tunicamycin, 0.1 (lanes E and K) and 2.5 (lanes F and L) μM monensin, or left untreated (lanes A, B, G, and H). The precipitated polypeptides are shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

peptides with apparent molecular weights of 86K and 72K were precipitated. The 86K glycopeptide is probably a partially glycosylated form of GVP 9, whereas the 72K glycopeptide may correspond to pGVP 9.

GVP 11b (71K) as well as its precursor, pGVP 11b (63K) were precipitated from untreated, virus-infected cells by monoclonal antibody 3D9S (Fig. 7). One glycopeptide with an apparent molecular weight of 65K was precipitated from tunicamycin-treated cells, suggesting that GVP 11b contains N-linked as well as O-linked oligosaccharides. From monensin-treated cells one glycopeptide with an apparent molecular weight of 68K was precipitated, which probably is an incompletely processed form of GVP 11b.

In contrast to the glycoproteins, whose molecular weights had changed due to incomplete processing in the presence of tunicamycin or monensin, the nonglycosylated polypeptides were not affected with respect to their molecular weights, indicating that they are normally synthesized in the presence of these two compounds. An example of such a structural polypeptide, VP8 (96K), which was precipitated by monoclonal antibody 1G4, is shown in Fig. 8.

Expression of the viral glycoproteins on the cell surface. The effect of tunicamycin and monensin on the transport of the viral glycoproteins to the cell surface and their insertion into the plasma membrane was examined. Surface iodination of BHV-1 infected cells previously showed that the four major BHV-1 glycoproteins, GVP 6/11a/16, GVP 3/9, GVP 7, and GVP 11b were all expressed on the surface of the virus-infected cell (Misra *et al.*, 1982; van Drunen Littel-van den Hurk *et al.*, 1984). Cell surface immunofluorescence confirmed these observations by demonstrating that the viral glycoproteins were expressed on the surface of untreated, virus-infected cells (data not shown). Treatment of BHV-infected cells with 5 μM monensin

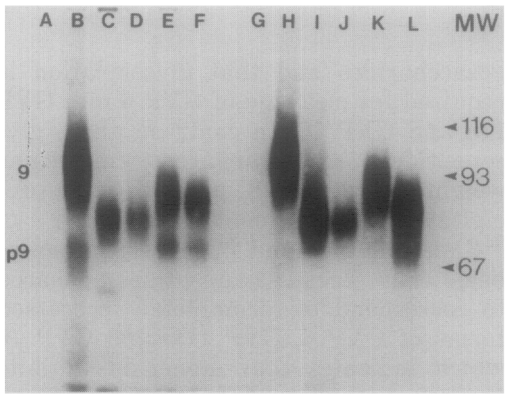

FIG. 6. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled (lanes A to F) or [^{3}H]glucosamine-labeled (lanes G to L) polypeptides from mock-infected (lanes A and G) and BHV-1 infected (lanes B to F and H to L) cells. Cell lysates were precipitated with monoclonal antibody 1D6. Immediately after virus adsorption, the cells were treated with 0.1 (lanes C and I) and 2.5 (lanes D and J) μg/ml tunicamycin, 0.1 (lanes E and K) and 2.5 (lanes F and L) μM monensin, or left untreated (lanes A, B, G, and H). The precipitated polypeptides are shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

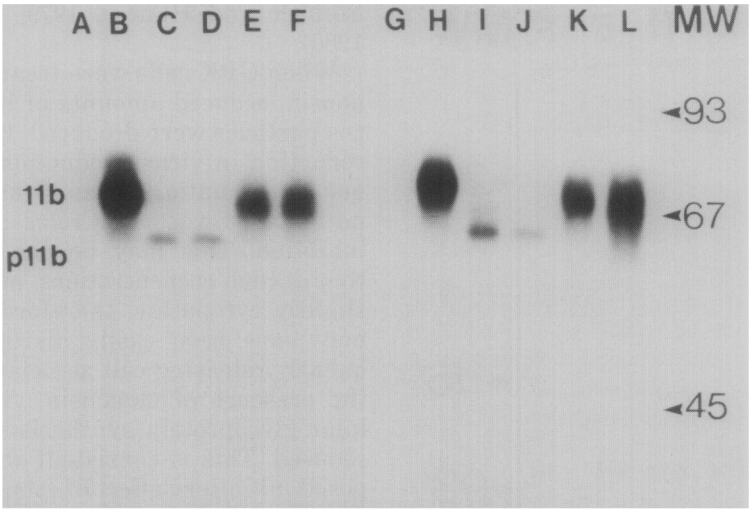

FIG. 7. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled (lanes A to F) or [^{3}H]glucosamine-labeled (lanes G to L) polypeptides from mock-infected (lane A and G) and BHV-1 infected (lanes B to F and H to L) cells. Cell lysates were precipitated with monoclonal antibody 3D9S. Immediately after virus adsorption, the cells were treated with 0.1 (lanes C and I) and 2.5 (lanes D and J) μg/ml tunicamycin, 0.1 (lanes E and K) and 2.5 (lanes F and L) μM monensin, or left untreated (lanes A, B, G, and H). The precipitated polypeptides are shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

did not affect surface expression. In contrast, when the cells were infected with BHV-1 in the presence of 1 μg/ml of tunicamycin, no surface immunofluorescence could be detected, suggesting inhibition of expression of any of the viral glycoproteins on the cell surface.

Antibody-dependent cell cytotoxicity. To further confirm that tunicamycin prevented surface expression of viral glycoproteins and to see whether the glycoproteins expressed on the cell surface in the presence of monensin could be recognized and participate in immune-mediated destruction of BHV-1 infected cells, we included these cells as targets in an ADCC assay. The presence of increasing concentrations of tunicamycin in the cells during the infection caused a reduction of specific ^{51}Cr release of 27 to 98%, which corresponded to the decrease and finally lack of expression of any of the glycoproteins on the surface of the infected cells (Fig. 9). In contrast, no reduction of specific ^{51}Cr release was observed after treatment of BHV-1 infected cells with increasing concentrations of monensin (Fig. 9). These results support the previous results which showed that tunicamycin inhibited cell surface expression of BHV-1 antigens whereas monensin did not.

DISCUSSION

GBK cells failed to produce infectious virus particles when treated with tunicamycin. No extracellular and only small amounts of cell-associated, noninfectious virus were detected. The virus produced under these circumstances was considered defective, since it was not infectious and it had a lower density in potassium tartrate gradients than infectious virus. At increasing concentrations of tunicamycin, viral as well as cellular glycoprotein synthesis was specifically and increasingly inhibited. This is consistent with the known inhibitory effect of tunicamycin on N-linked glycosylation in eukaryotic cells (Schwartz and Datema, 1980). The effect of tunicamycin on the glycosylation of BHV-1 proteins suggested that, like all other enveloped viruses (Ghosh, 1980),

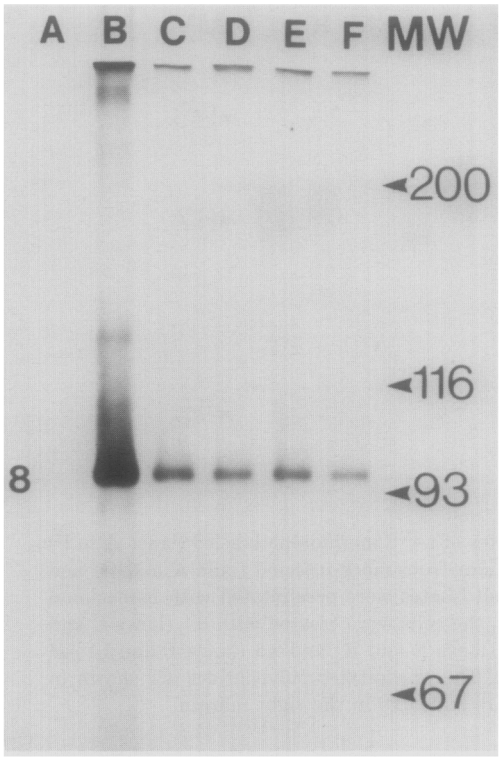

FIG. 8. PAGE (7.5%) analysis of immunoprecipitates of L-[^{35}S]methionine-labeled polypeptides from mock-infected (lane A) and BHV-1 infected (lanes B to F) cells. Cell lysates were precipitated with monoclonal antibody 1G4. Immediately after virus adsorption, the cells were treated with 0.1 and 2.5 μg/ml tunicamycin (lanes C and D), 0.1 and 2.5 μM monensin (lanes E and F), or left untreated (lanes A and B). The precipitated polypeptide is shown in the left margin. Molecular weight markers $\times 10^{-3}$ are indicated in the right margin.

BHV-1 uses cellular enzymes for glycosylation and that BHV-1 proteins produced in the presence of tunicamycin essentially lack N-linked oligosaccharide side chains. This may explain the lower density of the noninfectious virus particles produced by tunicamycin-treated cells. The influence of tunicamycin on infectivity indicated that glycosylation of the viral proteins is required for the production of infectious virus, a phenomenon previously reported for several other enveloped viruses (Schwartz *et al.*, 1976; Morrison *et al.*, 1978; Nakamura and Compans, 1978; Stohrer and Hunter, 1979; Pizer *et al.*, 1980).

When GBK cells were treated with monensin, reduced amounts of infectious virus particles were produced. However, the reduction in virus production per se was not as dramatic as was virus release. In no instance was virus release completely inhibited. This may be due to the fact that at high cocnentrations, monensin was slightly cytopathic, therefore, some cells may have been easily disrupted, consequently releasing cell-associated virus. In the presence of monensin, viral and cellular glycoprotein synthesis was slightly reduced. This is consistent with the supposed inhibitory effect of monensin on the Golgi complex (Pressman, 1976) where all or the greater part of processing of N-linked oligosaccharides and synthesis of O-linked oligosaccharides is thought to occur (Berger *et al.*, 1982). This suggested that BHV-1 proteins synthesized in the presence of monensin lack fully processed N-linked and/or O-linked carbohydrates and many explain the somewhat lower density of the cell-associated virus particles, produced by monensin-treated cells. It also indicated that complete processing

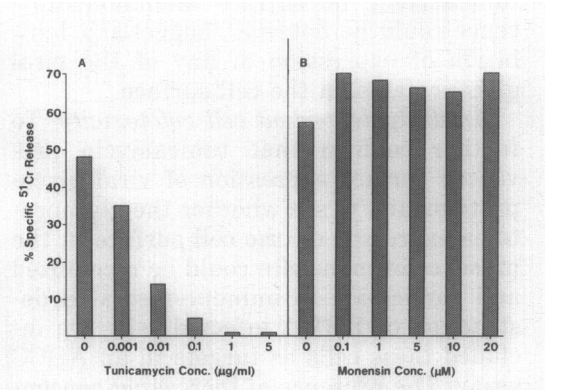

FIG. 9. Antibody-dependent cell cytotoxicity. GBK cells were infected with BHV-1 at an m.o.i. of 1. Various concentrations of tunicamycin or monensin were added to the cells immediately after virus adsorption and maintained until the cells were harvested at 24 hr postinfection. The effector (PMN) to target cells were harvested at 24 hr postinfection. The effector (PMN) to target cell ratio was 50:1. Each assay was done in triplicate. Nonspecific release in each case was less than 12%.

of the glycoproteins is not needed for infectivity, since a considerable fraction of the virions, associated with monensin-treated cells, was still infectious, even though their glycoproteins were not fully processed. Similarly results were reported for influenza virus (Alonso and Compans, 1981) and HSV-1 (Campadelli-Fiume *et al.*, 1982; Johnson and Spear, 1982). The observation that monensin mainly affected release of BHV-1 virions supports the proposition that the Golgi apparatus is involved in egress of herpesvirions from the cells (Johnson and Spear, 1982).

Using monoclonal antibodies, specific for the major BHV-1 glycoproteins, we have shown that the non- or partially glycosylated polypeptides of lower molecular weight, found in tunicamycin- and monensin-treated cells, were antigenically related to glycoproteins GVP 6/11a/16, GVP 7, GVP 3/9 or GVP 11b. In tunicamycin-treated cells a nonglycosylated counterpart of GVP 6 was found which may be the precursor polypeptide, pp GVP 6 (van Drunen Littel-van den Hurk and Babiuk, manuscript in preparation). This indicated that GVP 6/11a/16 contains only N-linked carbohydrate side chains. Although the majority of GVP 6 is normally cleaved into GVP 11a and GVP 16, this nonglycosylated polypeptide, present in tunicamycin-treated cells, was not cleaved. Apparently, glycosylation is required for the proper conformation and/or location of the polypeptide, needed for cleavage. No nonglycosylated counterpart of GVP 7 was detected in tunicamycin-treated cells. There may be several reasons for this observation. It has been reported that nonglycosylated polypeptides are sometimes difficult to detect, because (i) complete translation of the glycoprotein mRNA may depend on cotranslational addition of N-linked oligosaccharide side chains, (ii) nonglycosylated polypeptides may be very susceptible to degradation by host cell proteases, or (iii) the nonglycosylated polypeptide may be aggregated and thus not solubilized (Schwartz *et al.*, 1976; Leavitt *et al.*, 1977; Diggelman, 1979; Gibson *et al.*, *1979; Pizer et al.*, 1980; Stallcup and Fields, 1981). Whichever reason is correct, it suggests that GVP 7 may be an N-linked glycoprotein. One partially glycosylated counterpart of GVP 9 was found in tunicamycin-treated cells, suggesting that GVP 3/9 contains N-linked, as well as O-linked carbohydrate side chains. The intensity of this partially glycosylated polypeptide indicated that a considerable fraction of the oligosaccharides in GVP 9 is O-glycosidically linked. In addition, small amounts of a nonglycosylated polypeptide was detected. This may be the precursor polypeptide, pp GVP 9 (van Drunen Littel-van den Hurk and Babiuk manuscript in preparation). Similarly, one partially glycosylated counterpart of GVP 11b was present in tunicamycin treated cells, indicating that GVP 11b also contains N-linked and O-linked oligosaccharide side chains.

In the presence of monensin, an increase in electrophoretic mobility of all four BHV-1 glycoproteins was observed, indicating that processing of N-linked oligosaccharides and/or synthesis of O-linked oligosaccharides was inhibited. Monensin has been shown to block the processing of Sindbis virus (Johnson and Schlesinger, 1980) and HSV-1 (Johnson and Spear, 1982; Wenske *et al.*, 1982) glycoproteins at the high-mannose stage. However, the glycoproteins observed in monensin-treated, BHV-1 infected cells did not correspond to the normal precursors of these four BHV-1 glycoproteins, suggesting that partial processing beyond the high-mannose stage occurred. In order to determine whether this difference was due to the virus or the cell system used, BHV-1 as well as HSV-1 were grown in the presence of monensin in three different cell lines. These experiments (data not shown) demonstrated that the effect of monensin on BHV-1 or HSV-1 glycoproteins was independent of the cell line used. In addition, whereas the BHV-1 glycoproteins were processed beyond the high-mannose stage in the presence of monensin, the HSV-1 glycoproteins were blocked at the high-mannose stage, which is in agreement with previously mentioned observations

(Johnson and Spear, 1982; Wenske *et al.*, 1982). Thus, the difference in the effect of monensin on BHV-1 and HSV-1 glycoprotein appears to be a reflection of the virus rather than of the cell line.

Tunicamycin blocked the transport of all BHV glycoproteins to the cell surface. This is consistent with previous reports on a number of other enveloped viruses (Nakamura and Compans, 1978; Norrild and Pederson, 1982; Payne and Kristensson, 1982; Peak *et al.*, 1982) and clearly suggests that glycosylation is required for this process. In contrast to data reported for HSV-1 (Johnson and Spear, 1982), BHV-1 glycoproteins were transported to and expressed on the cell surface in the presence of monensin. The reasons for this discrepancy are not fully understood, but they could be related to both the virus and the cell systems used. Such discrepancies are not uncommon, since many reports on the effect of monensin on the transport of glycoproteins to the cell surface have been controversial. It is often shown that monensin does prevent transport (Johnson and Schlesinger, 1980; Alonso and Compans, 1981; Johnson and Spear, 1982; Payne and Kristensson, 1982), but sometimes no effect is observed (Alonso and Compans, 1981; Chatterjee *et al.*, 1982). One communication described the blockage of transport of both VSV and influenza virus glycoproteins to the surface of baby hamster kidney (BHK-1) cells, whereas influenza hemagglutinin, but not VSV G protein was expressed on the surface of Madin-Darby canine kidney (MDCK) cells (Alonso and Compans, 1981). These results indicate that both the virus and the cell may influence the final result. The observation that in the presence of monensin BHV-1 glycoproteins were expressed on the cell surface, whereas HSV-1 glycoproteins are not (Johnson and Spear, 1982), may be due to the above mentioned difference in degree of glycosylation, which appears to be a reflection of the virus. Furthermore, BHV-1 glycoproteins may be transported by vesicles, like influenza hemagglutinin, whose formation is not dependent upon the Golgi complex and thus insensitive to monensin.

BHV-1 infected cells, grown in the presence of tunicamycin, could not act as target cells in the ADCC assay, as one would expect since no glycoproteins were expressed on their surface. Similar results were reported for HSV-1 infected cells treated with tunicamycin (Norrild and Pederson, 1982). In contrast, monensin did not affect the efficiency of lysis in the ADCC assay, even though the glycoproteins, synthesized in the presence of this compound were incompletely glycosylated. Thus, the antibodies recognized the BHV-1 glycoproteins, produced in the presence of monensin, and killed the virus-infected cells by ADCC, suggesting that alterations in glycosylation induced by this compound did not affect the antigenicity of the glycoproteins. Consequently, although glycosylation may be required for the proper folding and conformation of the glycoproteins, the complete carbohydrate portion may not be needed and may not play a major role in antigenicity and immunogenicity of BHV-1 glycoproteins.

ACKNOWLEDGMENTS

We thank Elaine Gibbons, Terry Beskorwayne, and Jim Gilchrist for their technical assistance. This work was supported by grants from the Medical Research Council, Farming for the Future, and Canada Department of Agriculture.

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