VIROLOGY 206, 413–425 (1995)

The Role of the Major Tegument Protein VP8 of Bovine Herpesvirus-1 in Infection and Immunity[1]

S. VAN DRUNEN LITTEL-VAN DEN HURK,*,[2] S. GARZON,† J. V. VAN DEN HURK,* L. A. BABIUK,* AND P. TIJSSEN‡

*Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 0W0; †Université de Montreal, Montreal, Quebec, Canada, H3C 3J7; and ‡Institut Armand-Frappier, Laval-des Rapides, Quebec, Canada, H7N 4Z3

Received July 21, 1994; accepted October 25, 1994

The tegument of bovine herpesvirus-1 (BHV-1) carries an abundant protein of 96 kDa, termed VP8. Immunolabeling using VP8-specific antiserum and colloidal gold-labeled protein A as the electron-dense marker was used to identify VP8 in the virions and virus-infected cells. VP8 was confirmed to be a tegument protein that, like the herpes simplex virus-1 homologue VP13/14, contains O-linked carbohydrates. VP8 was found in the nucleus of virus-infected cells as early as 2 hr postinfection. Since VP8 is a gamma2 protein, this protein cannot be newly synthesized at this time and must be acquired from the inoculum. This supports the hypothesis that early during infection, VP8 has a function in modulation of alpha gene expression. Later during infection, VP8 was observed in the cytoplasm around nucleocapsids and in dense inclusions, which accumulated in the cisternae of the Golgi. In addition, *de novo*-synthesized VP8 continued to accumulate in the nucleus in dense areas and around nucleocapsids. In calves, VP8 stimulated T cell proliferation and antibody production, both after BHV-1 challenge and after immunization with purified VP8. These results suggest a role for VP8 in the induction of humoral and specifically cell-mediated immunity to BHV-1.

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) specifies more than 25 structural polypeptides (Pastoret *et al.,* 1980; Misra *et al.,* 1981). Three major glycoproteins, which constitute part of the virion envelope, have been extensively studied (van Drunen Littel-van den Hurk and Babiuk, 1986b). These glycoproteins, designated gI (gB), gIII (gC), and gIV (gD), are among the most prominent of the proteins recognized serologically by BHV-1-infected animals (Collins *et al.,* 1985; van Drunen Littel-van den Hurk and Babiuk, 1986a). Glycoprotein IV, but not gI and gIII, is also consistently recognized by CD_4^+ helper T lymphocytes from BHV-1 immune cattle (Hutchings *et al.,* 1990). A number of functions have been ascribed to these glycoproteins, including virus attachment to gI, gIII, and gIV (Liang *et al.,* 1991), penetration to gI and gIV (Hughes *et al.,* 1988; Fehler *et al.,* 1992), and cell fusion to gI and gIV (Fitzpatrick *et al.,* 1988, 1990; van Drunen Littel-van den Hurk *et al.,* 1992; Tikoo *et al.,* 1990). Any one of these glycoproteins, alone or in combination, induces protection against BHV-1 infection in cattle (Babiuk *et al.,* 1987; van Drunen Littel-van den Hurk *et al.,* 1990).

The proteins present in the tegument and nucleocapsid of BHV-1 have been poorly characterized. Tegument proteins have been defined as those that are neither totally released by nonionic detergent solubilization of the lipid envelope of mature intact virus particles, nor found associated with the nucleocapsids (Spear and Roizman, 1972). In herpes simplex virus (HSV), four tegument proteins have been identified and designated VP1/2, VP13/14, VP16, and VP22 (Spear and Roizman, 1972; Meredith *et al.,* 1991). Various functions have been assigned to tegument proteins, including protein kinase activity (Lemaster and Roizman, 1980), *trans*-acting stimulation of immediate-early gene transcription (Campbell *et al.,* 1984), inhibition of host cell peptide synthesis (Fenwick and Walker, 1978), and degradation of host cell mRNA (Schek and Bachenheimer, 1985). There is also evidence that VP13/14 and VP22 associate with the nuclear matrix during the virus replication cycle (Pinard *et al.,* 1987) and that they are able to bind HSV DNA (Blair and Honess, 1983).

One of the most abundant proteins in BHV-1 virions and virus-infected cells is a tegument protein of 96 kDa, designated VP8 (Misra *et al.,* 1981), VP7 (Pastoret *et al.,* 1980), 91K (Collins *et al.,* 1984), or 107K (Marshall *et al.,* 1986). The derived amino acid sequence of the VP8 gene has considerable homology with that of HSV-1 UL47, which codes for VP13/14 (McGeoch *et al.,* 1988; Carpenter and Misra, 1991; Laboissiere *et al.,* 1992). VP13/14 is phosphorylated and glycosylated (Meredith *et al.,* 1991). It has been shown to modulate alpha-transinducing factor-dependent induction of alpha genes (McKnight *et al.,* 1987; Zhang *et al.,* 1991). VP8 is also phosphorylated (Carpenter and Misra, 1991). However, the function of VP8

[1] Published as VIDO Journal Series No. 169.

[2] To whom correspondence and reprint requests should be addressed.

0042-6822/95 $6.00

and the biological relevance of its abundance is not understood. Preliminary evidence suggests that VP8 may be a target for BHV-1 specific immune responses in infected cattle (Collins *et al.,* 1985; van Drunen Littel-van den Hurk and Babiuk, 1986a; Hutchings *et al.,* 1990).

The purpose of the present study was to further elucidate the functions of VP8, both in the infected cell and in the host animal. To that end, we determined the localization of VP8 in the virus and virus-infected cells at the ultrastructural level, as well as identified the type of immune responses induced by VP8 in cattle.

MATERIALS AND METHODS

Cells and virus

Strain P8-2 of BHV-1 was propagated in Madin Darby bovine kidney (MDBK) cells grown in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Virus was purified on potassium tartrate gradients as described previously (van Drunen Littel-van den Hurk and Babiuk, 1985). Purified virus was treated with 1% nonidet P-40 (NP-40) or with 1% NP-40 and 1 *M* NaCl in TNE (0.1 *M* Tris–hydrochloride, 0.15 *M* NaCl, 0.001 *M* EDTA, pH 7.5). The detergent treated virus was centrifuged for 1 hr at 25,000 rpm in a SW 50.1 rotor (Beckman model L8-55, Beckman Instruments, Missisauga, ON, Canada). The supernatant was collected and the pellet was resuspended in TNE.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Affinity-purified VP8 or gradient-purified BHV-1 was suspended in electrophoresis sample buffer (0.0625 *M* Tris–HCl (pH 6.8), 1.25% sodium dodecyl sulphate (SDS), 12.5% glycerol, 0.15 *M* 2-mercaptoethanol, 0.00125% bromophenol blue) and boiled for 2 min. The viral proteins were separated on a 8.5% discontinuous polyacrylamide gel. For Western blotting the proteins were subsequently transferred to nitrocellulose in a mini-transblot cell (Hoefer Scientific, San Francisco, CA) at 100 V for 45 min in 25 m*M* Tris, 192 m*M* glycine (pH 8.3), 20% methanol. The nitrocellulose was then cut into 5-mm-wide strips and assayed for reactivity of BHV-1 hyperimmune or VP8-specific bovine sera by following the instructions supplied with the Bio-Rad immunoblot assay kit. BHV-1 hyperimmune sera were tested for reactivity with VP8, whereas VP8-specific sera were tested for reactivity with BHV-1 polypeptides.

Immunoprecipitation

The procedure for immunoprecipitation has been described in detail previously (van Drunen Littel-van den Hurk *et al.,* 1984). Briefly, BHV-1-infected and mock-infected MDBK cell lysates were incubated with bovine sera overnight at 4°, before addition of rabbit IgG anti-bovine Ig (Kirkegaard and Perry Laboratories, Gaithersburg, MD). After a 4 hr incubation at 4°, immune complexes were precipitated with Protein A CL-4B (Pharmacia, Baie d'Urfe, Quebec, Canada) and analyzed by SDS–PAGE.

Detection of carbohydrate

VP8 was purified by immunoadsorbent chromatography from BHV-1-infected MDBK cells as described previously (van Drunen Littel-van den Hurk and Babiuk, 1985). Subsequently, the purified VP8 was applied to nitrocellulose by SDS–PAGE and Western blotting. Carbohydrate was detected on VP8 using two approaches. First, carbohydrate on VP8 was oxidized with periodate followed by incorporation of biotin using biotin-hydrazide (Glycotrack kit, Oxford Biosystems Ltd., Rosedale, NY). Second, VP8 was incubated with a range of different biotinylated lectins (Sigma, Missisauga, ON, Canada; Boehringer-Mannheim, Laval, PQ, Canada). Biotinylated compounds were detected with streptavidin–alkaline phosphatase conjugate (Bio-Rad, Mississauga, ON, Canada).

VP8-specific antibodies

A VP8-specific monoclonal antibody (1G4) was raised by immunization of mice with purified BHV-1, as described previously (Van Drunen Littel-van den Hurk *et al.,* 1984). VP8-specific polyclonal antibodies were raised by immunizing rabbits with VP8, purified by affinity chromatography on the VP8-specific monoclonal antibody column (Van Drunen Littel-van den Hurk and Babiuk, 1985), followed by preparative PAGE. The rabbits were immunized twice with 50 μg of pure VP8 in Freunds' adjuvant.

Immunofluorescence

The presence of VP8 in BHV-1-infected cells was determined at different times postinfection. MDBK cells were infected with BHV-1 at an m.o.i. of 10. At different times after infection, the cells were gently trypsinized and suspended in MEM with 10% FBS. Cytospin smears were prepared and fixed in methanol. They were incubated for 1 hr at 37° with a 1:20 dilution of VP8-specific rabbit serum or with a 1:100 dilution of a gIV-specific monoclonal antibody mixture (Hughes *et al.,* 1988). After two washes in PBS and double-distilled water, the cells were stained with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG (Boehringer-Mannheim, Laval, PQ, Canada) for 1 hr at 37° and washed again before being mounted in PBS–glycerol for examination.

Electron microscopy and protein A–gold (PAG) immunolabeling

For transmission electron microscopy, MDBK cells were infected with BHV-1 at an m.o.i. of 10. At 24 hr postinfection, the cell monolayers were fixed for 1 hr with 4% paraformaldehyde–1% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4, treated for 1 hr with 0.1 *M* NH_4Cl to quench free aldehyde groups, and washed with cacodylate buffer supplemented with 4.5% sucrose (w/v). The cells were scraped from the T flasks with a policeman, centrifuged at 800 *g* for 10 min at 4°, and embedded in 2% low-melting agarose (Seaplaque Agarose, FMC Corp., Rockland, MD). After gelification, the cell pellets were cut into small blocks (1 mm^3), dehydrated in graded ethanol, and embedded in Araldite 502 or Lowicryl K4M. Thin sections (70–90 nm) were mounted on 400-mesh nickel grids and processed for immunocytochemical labeling (Garzon *et al.,* 1982; Garzon and Bendayan, 1993). The grids were first floated for 5 min on a drop of TBS (0.05 *M* Tris–HCl, 0.15 *M* NaCl, pH 8.0), containing 0.05% Tween 20 (TBST), and then incubated for 60 min on a drop of a 200-fold dilution of VP8-specific rabbit serum in TBST. The grids were washed with TBST and then incubated for 60 min on a drop of PAG complex diluted in TBS. Protein A was labeled according to Bendayan (1984) with 10-nm colloidal gold particles by the method of Frens (1973). Finally, the sections were washed with TBS, rinsed in distilled water and counterstained with uranyl acetate and lead citrate. Microscopic examination was performed on an EM300 Philips microscope at 80 kV. The specificity of the labeling was demonstrated by using nonimmune sera, PAG complex alone, or mock-infected cells.

Experimental challenge and reactivation

Six-month-old Hereford calves were exposed to an aerosol of BHV-1 strain 108 as described previously (Bielefeldt-Ohman and Babiuk, 1985). Approximately 4 months after primary infection, animals were treated with dexamethasone to reactivate latent virus (Pastoret *et al.,* 1980).

Immunization

VP8 was purified on a monoclonal antibody column as described by van Drunen Littel-van den Hurk and Babiuk (1985). With this method, pure preparations of VP8 were obtained. Hereford calves were immunized with affinity-purified VP8, diluted to a concentration of 100 or 10 μg per dose and mixed with Avridine at a ratio of 1:1 as described previously (Babiuk *et al.,* 1987). The animals were injected intramuscularly and received two booster immunizations at 3-week intervals. The calves were bled at the times of immunization and 3 weeks after the last immunization for assessment of antibody responses. Blood was collected for isolation of peripheral blood mononuclear lymphocytes (PBML) and assessment of cell-mediated immunity before immunization and 7 days after each immunization.

Enzyme-linked immunosorbent assay (ELISA)

The antibody responses to VP8 were determined by ELISA, essentially as described previously (van Drunen Littel-van den Hurk *et al.,* 1984). Polystyrene microtiter plates (Immulon 2, Dynatech Laboratories Inc., Alexandria, VA) were coated with 0.05 μg of affinity-purified VP8 or with 0.5 μg of gradient-purified BHV-1 per well. Subsequently, the plates were incubated with serially diluted bovine sera, followed by horseradish peroxidase-conjugated rabbit anti-bovine IgG at a 1:5000 dilution (Kirkegaard and Perry Laboratories). ELISA titers were expressed as the reciprocal of the highest dilution resulting in a reading two standard deviations above the control value.

Virus neutralization assay

The neutralization titers of the VP8-specific bovine sera were determined, using 100 PFU of virus, as described previously (van Drunen Littel-van den Hurk *et al.,* 1984). The titers were expressed as the reciprocal of the serum dilution resulting in a 50% reduction of BHV-1 plaques relative to the virus control.

Isolation of PBML

Bovine blood was collected into citrate-dextran and centrifuged for 30 min at 1000 *g*. Buffy coat cells were aspirated, diluted with an equal volume of RPMI, and layered onto Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). After centrifugation for 40 min at 1000 *g,* the band of cells at the interface was collected. Viability, as assessed by trypan blue exclusion, was greater than 95%.

Blastogenesis assay

PBML were resuspended in RPMI-1640 medium containing 10% FBS (GIBCO) and added to triplicate wells of U-bottom 96-well microtiter plates (Corning, Richmond Hill, ON, Canada), to which VP8 or uv-irradiated BHV-1 had been added. Final culture conditions consisted of 10^6 PBML per ml in a volume of 200 μl per well. Optimum concentrations of antigen were 1 μg per ml of VP8 and 10^6 PFU per ml of BHV-1. Control cultures comprised cells cultured in RPMI alone. After 5 days of stimulation, 0.5 μCi of [*Methyl*-^{3}H]thymidine (TdR, 5.0 Ci/mmol; Amersham Canada Ltd., Oakville, ON, Canada) was added to all wells for the last 18 hr of culture. Incorporation of TdR into cellular DNA was assessed following harvesting of cells onto glass fiber filter mats (Skatron, Sterling, VA). ^{3}H incorporation

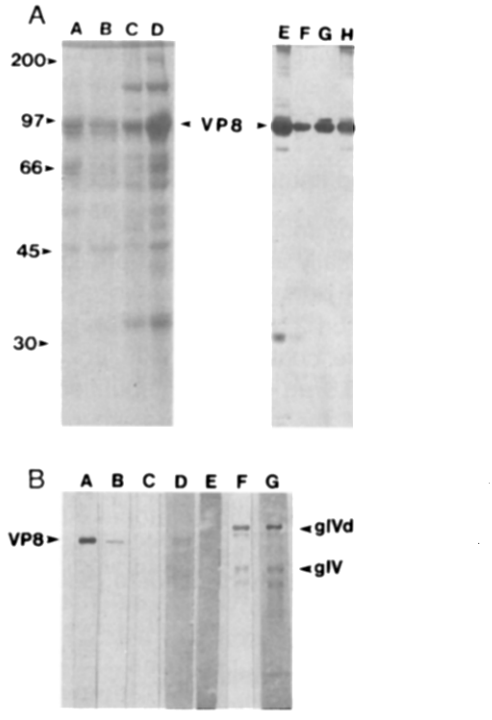

FIG. 1. (A) Localization of VP8 in BHV-1. Gradient-purified BHV-1 (lanes D and E), treated with 1% NP-40 (lanes A and F) or 1% NP-40 in 1 *M* NaCl (lane B and G) and separated by ultracentrifugation into supernatant (lanes A, B, F, and G) and pellet (lanes C and H). The viral polypeptides were identified by SDS–PAGE (8.5%) under reducing conditions and stained with Coomassie brilliant blue (lanes A, B, C, and D) or transferred to nitrocellulose and probed with VP8-specific monoclonal antibody 1G4 (lanes E, F, G, and H). The position of VP8 is indicated in the margin. The positions of molecular weight markers $\times 10^{-3}$ are shown in the left margin. (B) Carbohydrate detection. Affinity-purified VP8, probed with VP8-specific monoclonal antibody (lane A), probed with biotinylated *Dolichus biflorus* lectin (lane B), or oxidized with periodate and treated with biotin-hydrazide (lane D). Adenovirus hexon was probed with biotinylated *Dolichus biflorus* lectin (lane C) or oxidized with periodate and treated with biotin-hydrazide (lane E). Glycoprotein IV was probed with a gIV-specific monoclonal antibody mixture (lane F) or oxidized with periodate and treated with biotin-hydrazide (lane G).

was measured with a model 1701 liquid scintillation counter (Beckman Instruments, Richmond, BC, Canada). Results are expressed as a stimulation index (mean counts per minute with antigen/mean counts per minute without antigen).

RESULTS

VP8 is a glycosylated tegument protein

Treatment with nonionic detergent has been used as a means to solubilize BHV-1 polypeptides. Treatment of purified virions with NP-40 resulted in the release of a portion of VP8 into the detergent fraction. Further treatment of the pellet fraction with 1% NP-40 and 1 *M* NaCl caused more VP8 to dissociate from the nucleocapsid fraction, although a portion of VP8 still remained associated with the pellet (Fig. 1A). The association of VP8 with envelope as well as nucleocapsid supports the hypothesis that VP8 is a tegument protein (Bolton *et al.*, 1983; Marshall *et al.*, 1986). Since VP8 is homologous to VP13/14 of HSV-1, we investigated whether VP8 is glycosylated. The predicted amino acid sequence of VP8 has no N-linked glycosylation sites (Carpenter and Misra, 1991). In addition, the presence of tunicamycin during virus infection does not have an effect on the electrophoretic mobility of VP8 (van Drunen Littel-van den Hurk and Babiuk, 1985), confirming that VP8 does not contain any N-linked oligosaccharides. Previous studies have shown that it is not possible to label VP8 with [^{3}H]-glucosamine. However, carbohydrate was detected on VP8 following periodate oxidation (Fig. 1B). Out of a range of different lectins with specificities for different carbohydrate groups, only *Dolichus biflorus* lectin [specific for N-acetyl galactosamine (GalNAc)-α-3GalNAc] bound to VP8 (Fig. 1B). This indicates that VP8 contains O-linked carbohydrate. No carbohydrate was detected on the hexon protein of hemorrhagic enteritis virus, an unglycosylated structural protein, whereas carbohydrate was observed on BHV-1 gIV.

Intracellular distribution of VP8

In order to determine the localization of VP8 during the course of an infection, MDBK cells were infected with BHV-1 and VP8 was identified by indirect immunofluorescence (Fig. 2). Before infection, no staining was observed. As early as 2 hr postinfection, VP8 was identified in the nucleus of the infected cell (Fig. 2B). The intensity of the nuclear staining increased until 6 hr after infection, indicating transport of input VP8 into the nucleus (Fig. 2D). Small amounts of VP8 were found in the cytoplasm from about 6 to 10 hr after infection (Figs. 2D and 2E). The amount of VP8 continued to increase both in the nucleus and in the cytoplasm until 14 hr after infection, which is probably due to *de novo* synthesis (Fig. 2F) (Carpenter and Misra, 1991). In contrast, the early glycoprotein gIV was observed in the cytoplasm from 4 hr postinfection (Fig. 2G). Later during infection, increasing amounts of gIV accumulated in the cytoplasm and on the plasma membranes of the infected cells (Fig. 2H and 2I). Glycoprotein IV was never observed in the nucleus. From 14 until 24 hr postinfection, the intensity of the VP8 and gIV staining did not change.

Ultrastructural localization of VP8 in infected cells

BHV-1-infected MDBK cells were incubated with VP8-specific rabbit serum, followed by protein A–gold complex (Fig. 3A). Gold labeling was observed in the nucleus over dense areas surrounding the nucleocapsids, in the cytoplasm over dense structures, and in

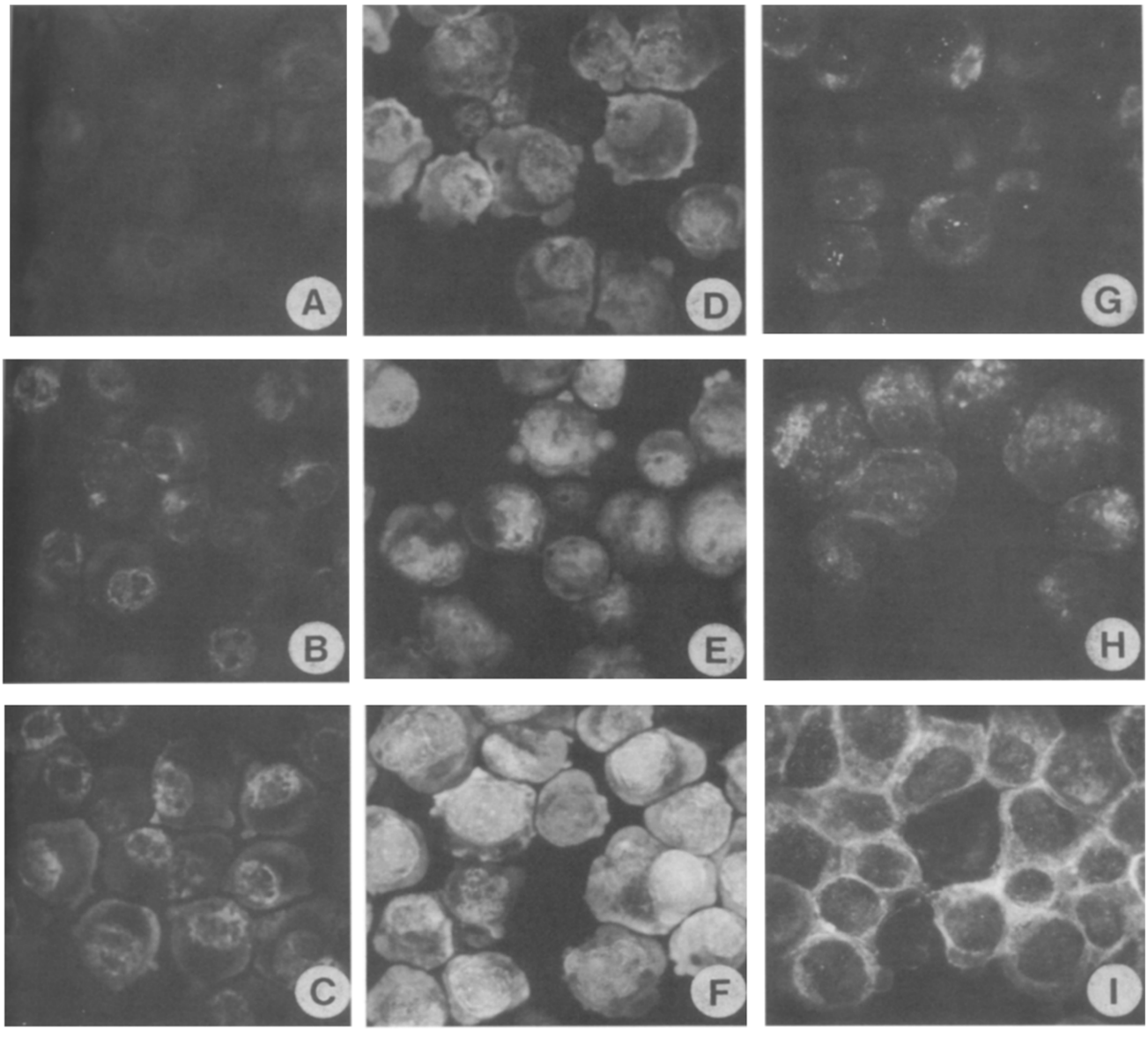

FIG. 2. Time course of VP8 expression in BHV-1-infected MDBK cells. The localization of VP8 was determined by indirect immunofluorescence with a specific polyclonal rabbit serum at time points 0 hr (before infection) (A), 2 hr (B), 4 hr (C), 6 hr (D), 10 hr (E), and 14 hr (F) after BHV-1 infection. A gIV-specific monoclonal antibody mixture was used as a control at time points 4 hr (G), 8 hr (H) and 14 hr (I). Magnification, ×175.

the Golgi area. VP8 was not present on the cell surface, which confirms that VP8 is not expressed on the plasma membrane. However, it was found along the cytoplasmic membranes over budding virus particles. Virus particles released from infected cells are shown in Figs 3B–D. Figure 3B shows the typical morphology of BHV-1 virions at different section levels. Intact and partially disrupted virions were gold-labeled in between the nucleocapsid and the envelope, again suggesting a tegument localization of VP8. However, some of the gold particles appeared associated with the surface of the virion (Figs. 3C and 3D).

In the nucleus, the immunogold label appeared to be concentrated in dense areas surrounding a large number of nucleocapsids (Fig. 4A). High concentrations of VP8-specific label were also found along the nuclear membrane, suggesting transport of VP8 through the membrane (Fig. 4B). In the cytoplasm, the VP8-specific label was primarily localized over dense material, which accumulates progressively in the cisternae of the Golgi (Fig. 5A). Numerous nucleocapsids or enveloped virus particles were included in these heavily labeled structures (Figs. 5B and 5C). Enveloped virus particles were also observed in Golgi-derived cytoplasmic vesicles. Intracytoplasmic dense bodies, which have also been described for HCMV, were strongly labeled, suggesting that they may consist of excess VP8 (Fig. 5C). The passage or accumulation of VP8 in the Golgi was confirmed by the colocalization of VP8 (VP8-specific serum and protein A–gold, 5 mm)

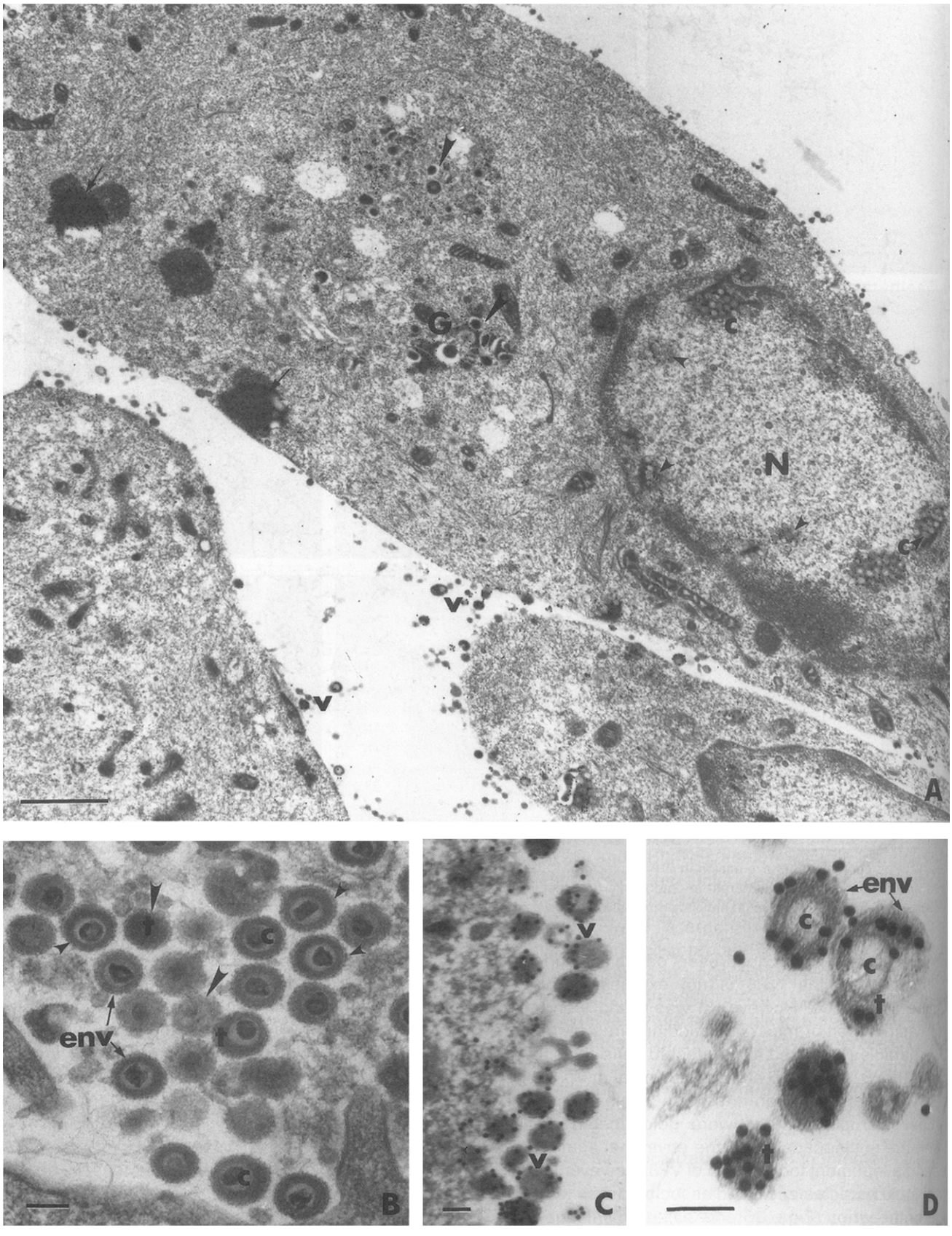

FIG. 3. Postembedding immunolocalization of VP8 in BHV-1-infected cells and virions. VP8 was detected with VP8-specific rabbit serum and protein A–gold (10 nm). (A) VP8-specific gold label was localized in the nucleus (N) in dense areas around nucleocapsids (c), in the cytoplasm over dense structures (arrow), in the Golgi (G) area (arrowhead), and along the cytoplasmic membrane over the budding virus particles (v). The

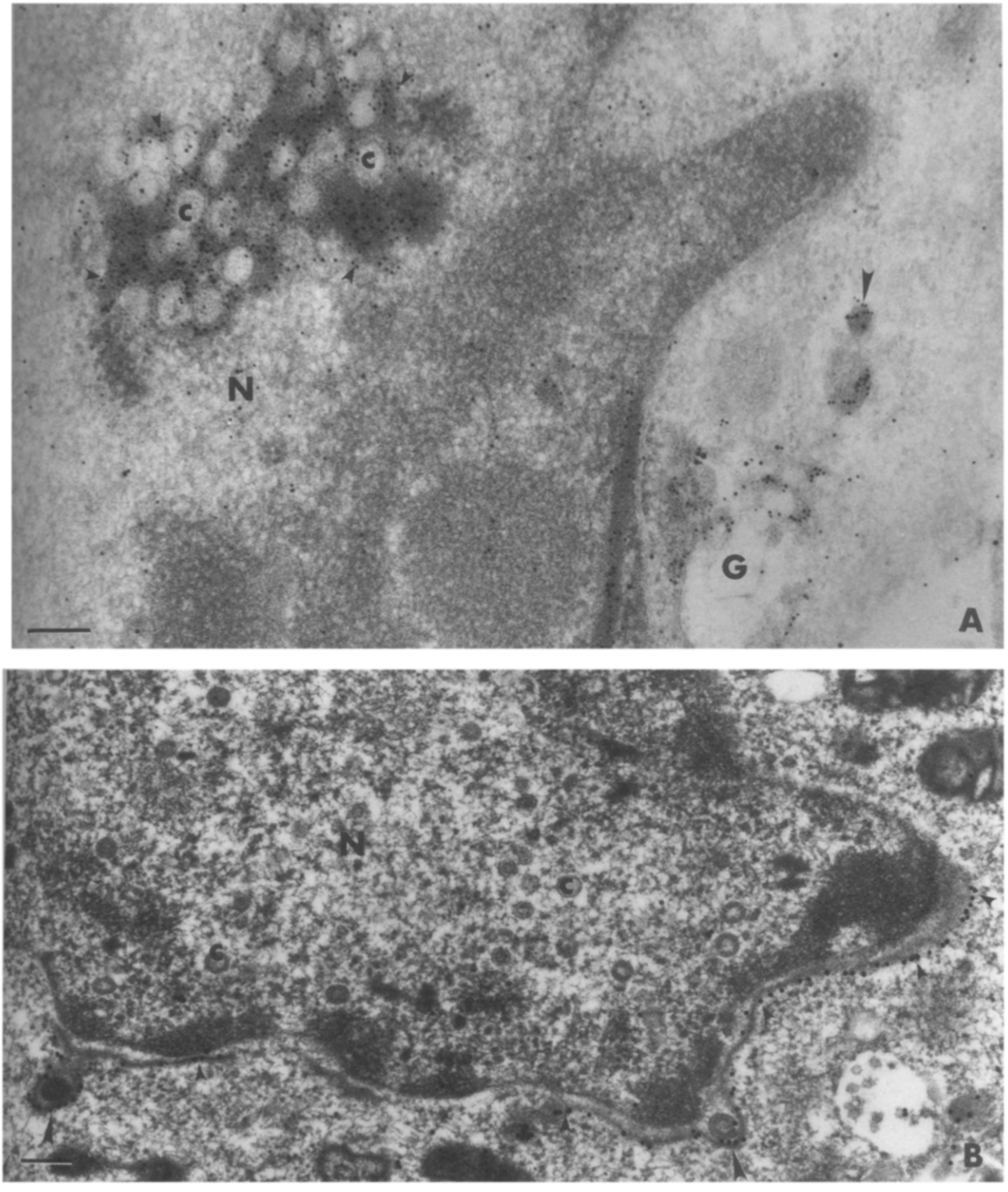

FIG. 4. Nuclear immunolocalization of VP8 in BHV-1 infected cells. (**A**) The nuclear dense material surrounding the nucleocapsids was heavily labeled (short arrowhead). (**B**) VP8-specific gold label was also present on the nuclear membrane (short arrowheads) around budding of nucleocapsids (long arrowheads). The bar represents 0.2 μm.

---

bar represents 1 μm. (**B**) Ultrastructural morphology, typical for BHV-1 shown at the different section levels of the particle from tangential (long arrowhead) to transversal (short arrowhead) plane. (**C**) and (**D**) VP8-specific label, localized over the tegument (t) of the virus (v) in between the nucleocapsid (c) and the envelope (env). The bar represents 0.1 μm.

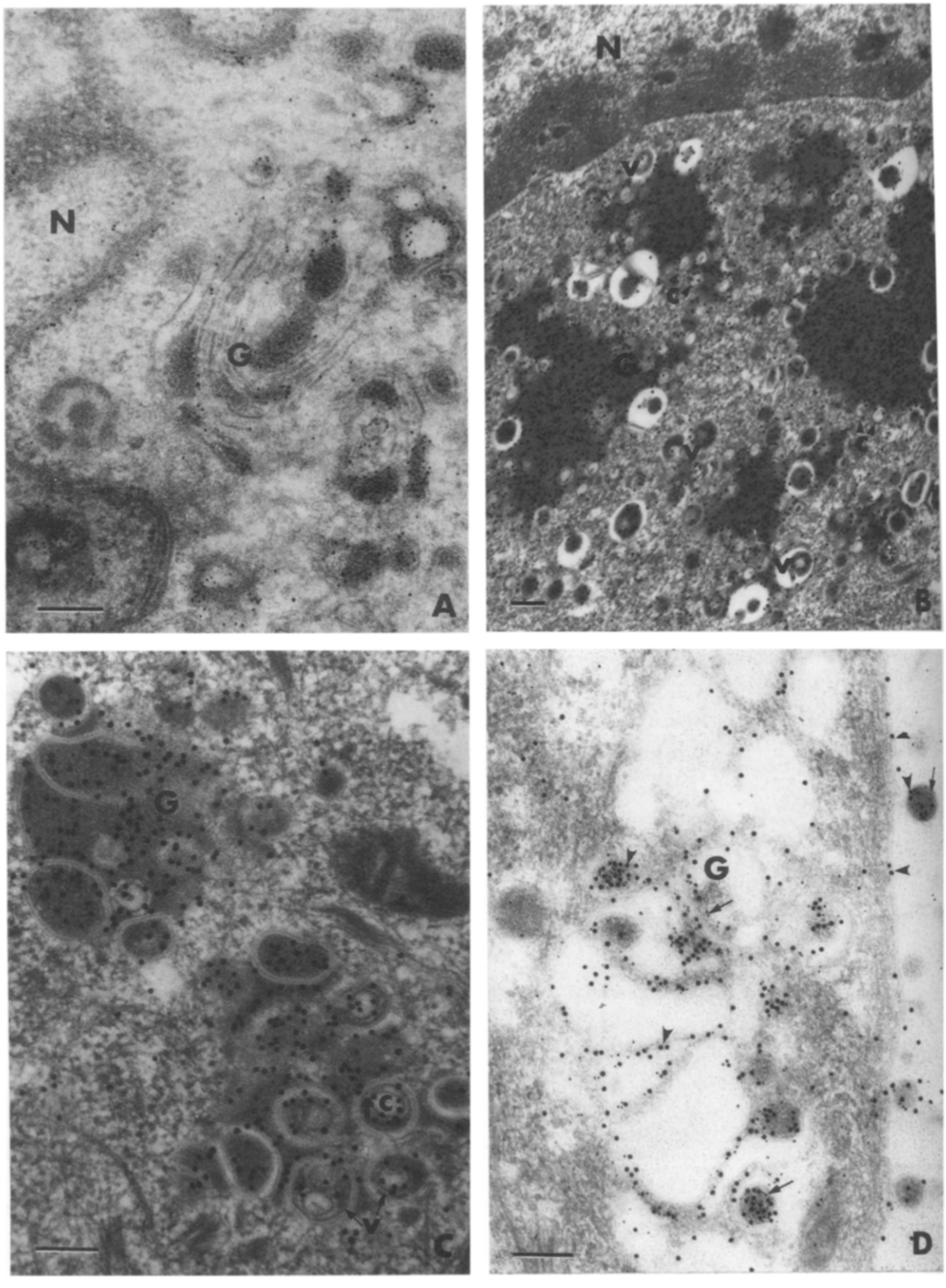

FIG. 5. Intracyplasmic immunolocalization of VP8 in BHV-1-infected cells. (**A**) VP8-specific gold label was most prevalent in association with dense material, which accumulates progressively in the cisternae of the Golgi (G). (**B**) and (**C**) Numerous nucleocapsids or enveloped viral particles were included in the heavily labeled structures. (**D**) The passage of VP8 in the Golgi (G) was confirmed by the colocalization of VP8 (VP8-specific rabbit serum and protein A–gold, 5 nm, arrows) and galactose (*Ricinus communis agglutinin*-gold, 15 nm, arrowheads) in the Golgi area and over virus particles transported through the Golgi or budding on the cytoplasmic membrane. The bar represents 0.2 μm.

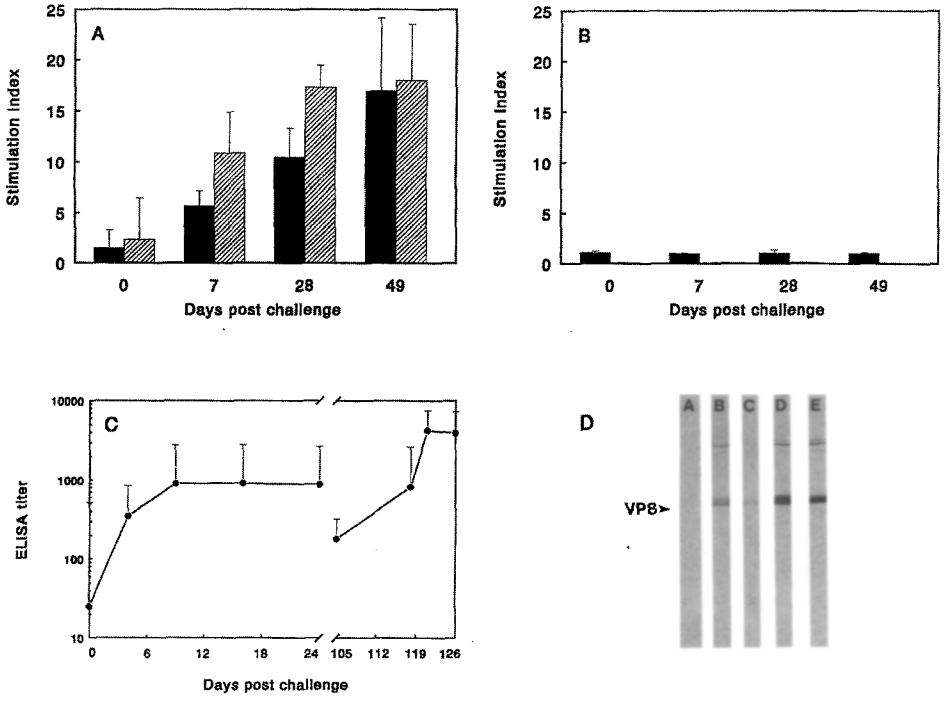

FIG. 6. VP8-specific immune responses of calves challenged with BHV-1 (A) Proliferative response to VP8 (■) and BHV-1 (▨) in BHV-1-challenged animals. (B) Proliferative response to VP8 (■) and BHV-1 (▨) in control animals. (C) VP8-specific ELISA titers, expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the control value. Error bars show the standard deviation of the mean of 10 animals. (D) VP8-specific reaction on a Western blot on Days 0 (lane A), 24 (lane B), 105 (lane C), and 126 (lane D) after challenge. Reactivation occurred on Day 105. Purified VP8 was transferred from a 8.5% polyacrylamide gel to nitrocellulose, which was then probed with the different bovine sera. The reaction of a VP8-specific monoclonal antibody (lane E) is shown on the right.

and galactose (*Ricinus communis* agglutinin–gold, 15 mm) in the Golgi area and over virus particles transported through the Golgi or budding through the cytoplasmic membranes (Fig. 5D). No immunogold label was observed in mock-infected cells or in BHV-1-infected cells treated with nonimmune serum or PAG complex alone.

VP8-specific immune responses

To examine the presence of VP8-specific cellular and humoral immunity, calves were challenged with BHV-1, and the virus was reactivated 4 months later. When PBMC from BHV-1-challenged animals were cultured in the presence of VP8 or BHV-1, antigen-specific proliferation was enhanced 7 days after challenge. The proliferative responses continued to increase until 49 days after challenge (Fig. 6A). Antigen-specific proliferation was not observed in control animals (Fig. 6B). VP8-specific antibodies were detected by ELISA as early as 4 days after challenge. The levels of VP8-specific antibody continued to rise until 8 days postinfection. Following dexamethasone treatment on Day 105, the meanwhile reduced VP8-specific antibody titers increased again (Fig. 6C). The specificity of the immune response was confirmed by Western blotting (Fig. 6D).

Immunogenicity of VP8

Since VP8, which is the most abundant protein in the virion, was recognized by PBMC and antibodies of BHV-1 hyperimmune animals, its immunogenicity in cattle was assessed. Calves were immunized with 100 or 10 μg of affinity-purified VP8 (Fig. 7A). After two immunizations with 100 μg of VP8, serum neutralizing antibody levels were enhanced, with a further increase following the third immunization. In contrast, with 10 μg of VP8 two doses were needed, before neutralizing antibody titers started to increase (Fig. 7B). To determine whether T lymphocytes were stimulated by immunization of calves with VP8, the proliferative response of PBMC, cultured in the presence of VP8 or BHV-1, was measured *in vitro*. Animals immunized with 100 μg of VP8 showed an enhanced proliferative response as early as 7 days after the first immunization. This response continued to rise until after the last immunization (Fig. 7C). However, three doses of 10 μg of VP8 were needed for an appreciable increase in proliferation to occur (Fig. 7D). The immune response was VP8-specific as shown by ELISA (Fig. 7E). In contrast, none of the animals reacted with gI, gII (not shown), gIII, or gIV (Fig. 7F) in an ELISA. In addition, BHV-1 hyperimmune sera recognized the major glycoproteins gI, gIII, and gIV, as well as several other BHV-1 proteins,

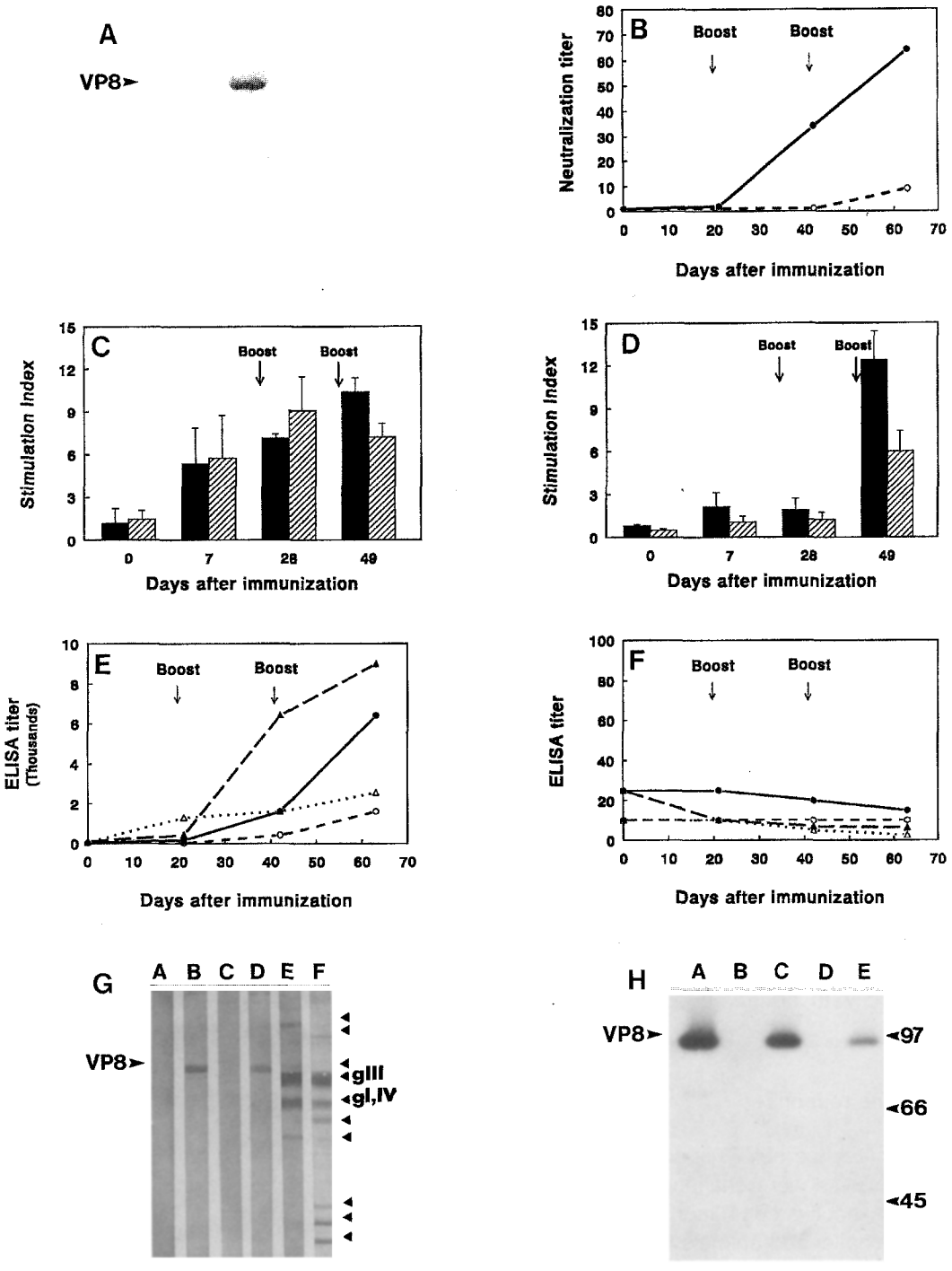

FIG. 7. Kinetics of the immune response to VP8. (A) Affinity-purified VP8, identified by SDS–PAGE (8.5%) under reducing conditions and stained with Coomassie brilliant blue. The position of VP8 is indicated in the left margin of the gel. (B) Virus neutralizing antibody titers were determined in sera of calves immunized with 100 μg (●) or 10 μg (○) of VP8. (C) Lymphoproliferative response was measured in calves immunized with 100 μg of VP8 against 1 μg/ml of VP8 (■) or 10^6 PFU/ml of uv-irradiated BHV-1 (▨) and expressed as a stimulation index (mean counts per minute with antigen/mean counts per minute without antigen). (D) Lymphoproliferative response was measured in calves immunized with 10 μg of VP8 against 1 μg/ml of VP8 (■) or 10^6 PFU/ml of uv-irradiated BHV-1 (▨) and expressed as a stimulation index (mean counts per minute with antigen/mean counts per minute without antigen). (E)) Antibody titers were determined by ELISA in sera of calves immunized with 100 μg of VP8 against VP8 (●) and BHV-1 (▲) and in sera of calves immunized with 10 μg of VP8 against VP8 (○) and BHV-1 (△). (F) ELISA titers were determined in sera of calves immunized with 100 μg of VP8 against gIII (●) and gIV (▲) and in sera of calves immunized with 10 μg of VP8 against gIII (○) and gIV (△). (G) Specificity of the immune response of animals before (lane A) and after (lane B) immunization with 100 μg of VP8, before (lane C) and after (lane D) immunization with 10 μg of VP8 or after immunization with BHV-1 (lanes E and F). Purified BHV-1 was disrupted in electrophoresis sample buffer, separated by electrophoresis and transferred to nitrocellulose, which was then probed with the different bovine sera. The positions of VP8, gI, gIII, and gIV are indicated in the margins. (H) Immunoprecipitation of [^{35}S]methionine-labeled polypeptides from BHV-1-infected cells

whereas the sera from the VP8-immunized animals only recognized VP8 in Western blot and immunoprecipitation assays, further proving that they were not accidentally exposed to BHV-1 or other viral proteins during the immunization period (Figs. 7G and 7H).

DISCUSSION

VP8 is one of the most abundant proteins in BHV-1-infected cells and virions. However, little is known about its structural and functional properties. In this study we determined the ultrastructural localization of VP8 in the virions and virus-infected cells, as well as the interactions between VP8 and the host's immune system.

In the virions, VP8 was found in the tegument. It was partially extracted with nonionic detergent and partially with buffer of neutral pH and high ionic strength, indicating that this protein is associated with capsid and tegument proteins by ionic interactions. VP8 appeared less strongly associated with the nucleocapsid than its HSV homologue VP13/14, which was not released at all by nonionic detergent treatment (Meredith *et al.*, 1991). The localization of VP8 in the tegument was confirmed by electron microscopy.

In the virus-infected cells, VP8 was found in the nucleus as early as 2 hr postinfection, suggesting that this gamma2 protein is not newly synthesized within the cells, but acquired from the inoculum. The phenomenon of uptake and subsequent detectable nuclear localization of a viral protein before virus replication has been reported for human cytomegalovirus (HCMV) pp65, one of the viral tegument proteins (Grefte *et al.*, 1992). One reason for the abundance of VP8 in the tegument may be this early transport to the nucleus and the need for sufficient quantities of this protein to carry out its function, which, in analogy to the role of the HSV homologue VP13/14 (McKnight *et al.*, 1987; Zhang *et al.*, 1991), may be modulation of alpha gene expression. Later during infection, VP8 was also present in the cytoplasm and more VP8 accumulated in the nucleus. This indicates that *de novo*-synthesized VP8 is also transported into the nucleus late during the infectious cycle. It is likely that at this time VP8 has another function. In the nucleus, VP8 was primarily found in dense inclusions and surrounding nucleocapsids which may indicate tegument formation. If VP8 is indeed complexed with the nucleocapsids in the nucleus, it may also function in the translocation of nucleocapsids to the cytoplasm, where large quantities of VP8 were found in dense inclusions and around nucleocapsids, while they move through the Golgi. In the cytoplasm, as well as in the nucleus, VP8 was present in abundance, indicating the importance of sufficient quantities of VP8 in the tegument.

In cattle, VP8 was recognized following challenge with BHV-1, both by T cells and by antibodies, indicating the potential of VP8 to act as an immunogen during infection. This was confirmed by the ability of purified VP8 to induce low levels of neutralizing antibodies and T cell activation in cattle. Although the VP8-specific neutralizing antibody titers were lower than those induced by glycoproteins gIII (gC) and gIV (gD), they were similar to those induced by gI (gB) (Babiuk *et al.*, 1987; van Drunen Littel-van den Hurk *et al.*, 1990). In addition, VP8 induced a stronger cell-mediated immune response than gI or gIII (Hutchings *et al.*, 1990). The ability of VP8 to induce antibodies in cattle that neutralize virus infection agrees with a previous report by Collins *et al.* (1984), who generated a VP8-specific neutralizing monoclonal antibody. These data suggest that VP8 may be partially exposed on the virion. Neutralization by VP8-specific antibodies probably occurs postattachment, perhaps during uncoating of the virion. Another potential mechanism of neutralization by VP8-specific antibodies could involve inhibition of VP8-induced modulation of alpha gene expression.

The sequence of VP8 has been shown to be homologous to those of VP13/14 of HSV (Carpenter and Misra, 1991; Davison and Scott, 1986) and gp10 of EHV (Whittaker *et al.*, 1991). In addition, we have shown that VP8, like VP13/14 and gp10, contains O-linked carbohydrates, which is apparently acquired during transport of tegumented nucleocapsids through the Golgi. Although the role of this type of modification to virus structural proteins is not clear, carbohydrate residues appear to be common on proteins involved in transcriptional control (Jackson and Tijan, 1988). BHV-1 VP8 also resembles HCMV pp65 in many ways. These proteins are both transported to the nucleus early after infection and are both phosphorylated (Meredith *et al.*, 1991; Whittaker *et al.*, 1991). Furthermore, like VP8, pp65 has been reported to be a major target of the cell-mediated immune response (Forman *et al.*, 1985) during natural infection with HCMV. The pp65 protein also causes a humoral immune response in animals as well as in humans, albeit not as strongly as some of the other HCMV antigens (Gibson and Irmiere, 1984).

In this study we demonstrated expression and localization of VP8 in the virions and virus-infected cells. VP8 clearly has a function in the nucleus early after infection, which may account for its abundance in the virion, as well as late during infection, which may ex-

with VP8-specific monoclonal antibody 1G4 (lane A) or bovine sera before (lane B) and after (lane C) immunization with 100 μg of VP8 or before (lane D) and after (lane E) immunization with 10 μg of VP8. The immunoprecipitates were analyzed by electrophoresis under reducing conditions. The position of VP8 is indicated in the left margin. The positions of molecular weight markers $\times 10^{-3}$ are shown in the right margin.

plain the abundant *de novo* synthesis in the infected cells. In addition, VP8 was shown to be a good immunogen in cattle, specifically for the induction of cell-mediated immunity.

ACKNOWLEDGMENTS

The authors are grateful to Tammy Wolfe and Henry Strykowski for technical assistance, the animal support staff at VIDO for care and handling of the animals, and Sandra Calver for preparing this manuscript. Financial support was provided by the Natural Science and Engineering Council and the Medical Research Council of Canada.

REFERENCES

BABIUK, L. A., L'ITALIEN, J., VAN DRUNEN LITTEL-VAN DEN HURK, S., ZAMB, T. J., LAWMAN, M. P. J., HUGHES, G., and GIFFORD, G. A. (1987). Protection of cattle from bovine herpesvirus type-1 (BHV-1) infection by immunization with individual glycoproteins. *Virology* **159,** 57–66.

BENDAYAN, M. (1984). Protein A-gold electron microscopic immunocytochemistry: Methods, applications and limitations. *J. Electron Microsc. Tech.* **1,** 243–248.

BIELEFELDT-OHMAN, H., and BABIUK, L. A. (1985). Viral-bacterial pneumonia in calves: Effect of bovine herpesvirus-1 on immunologic functions. *J. Infect. Dis.* **151,** 937–947.

BLAIR, E. D., and HONESS, R. W. (1983). DNA-binding proteins specified by herpesvirus saimiri. *J. Gen. Virol.* **64,** 2697–2715.

BOLTON, D. C., ZEE, Y. E., and ARDANS, A. A. (1983). Identification of envelope and nucleocapsid proteins of infectious bovine rhinotracheitis virus by SDS–polyacrylamide gel electrophoresis. *Vet. Microbiol.* **8,** 57–68.

CAMPBELL, M. E. M., PALFREYMAN, J. W., and PRESTON, C. M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J. Molec. Biol.* **180,** 1–19.

CARPENTER, D. E., and MISRA, V. (1991). The most abundant protein in bovine herpesvirus-1 virions is a homologue of herpes simplex virus type 1 UL47. *J. Gen. Virol.* **72,** 3077–3084.

COLLINS, J. K., BUTCHER, A. C., and RIEGEL, C. A. (1984). Neutralizing determinants defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus-1. *J. Virol.* **52,** 403–409.

COLLINS, J. K., BUTCHER, A. C., and RIEGEL, C. A. (1985). Immune response to bovine herpesvirus type-1 infections: Virus-specific antibodies in sera from infected animals. *J. Clin. Microbiol.* **21,** 546–552.

DAVISON, A. J., and SCOTT, J. E. (1986). The complete DNA sequence of varicella zoster virus. *J. Ser. Virol.* **67,** 1759–1816.

FEHLER, F., HERRMANN, J. M., SAALMULLER, A., METTENLEITER, T. C., and KEIL, G. M. (1992). Glycoprotein IV of bovine herpesvirus 1-expressing cell line complements and rescues a conditionally lethal viral mutant. *J. Virol.* **66,** 831–839.

FENWICK, M. L., and WALKER, M. J. (1978). Suppression of the synthesis of cellular macromolecules by herpes simplex virus. *J. Gen. Virol.* **41,** 37–51.

FITZPATRICK, D. R., ZAMB, T. J., PARKER, M. D., VAN DRUNEN LITTEL-VAN DEN HURK, S., BABIUK, L. A., and LAWMAN, M. J. P. (1988). Expression of bovine herpesvirus-1 glycoproteins gI and gIII in transfected murine cells. *J. Virol.* **62,** 4239–4248.

FITZPATRICK, D. R., ZAMB, T. J. and BABIUK, L. A. (1990). Expression of bovine herpesvirus-1 glycoprotein gI in transfected bovine cells induces spontaneous cell fusion. *J. Gen. Virol.* **71,** 1215–1219.

FORMAN, S. J., ZAIA, J. A., CLARK, C. L., WRIGHT, C. L., MILLS, B. J., POTTATHIL, R., RACKLIN, M. T., GALLAGHER, M. T., WELTE, K., and BLUME, K. G. (1985). A 64,000 dalton matrix protein of human cytomegalovirus induces *in vitro* immune responses similar to those of whole viral antigen. *J. Immunol.* **134,** 3391–3395.

FRENS, G. (1973). Controlled nucleation for the regulation of the particle size in monodisperse gold solutions. *Nat. Phys. Sci.* **241,** 20–22.

GARZON, S., BENDAYAN, M., and KURSTAK, E. (1982). Ultra-structural localization of viral antigens using the protein A-gold technique. *J. Virol. Methods* **5,** 67–73.

GARZON, S., and BENDAYAN, M. (1993). Colloidal gold probe: An overview of its application in viral cytochemistry. *In* "Immuno-gold Electron Microscopy in Virus Diagnosis and Research" (A. D. Hyatt and B. T. Eaton, Eds.), pp. 137–176. CRC Press, Boca Raton.

GIBSON, W., and IRMIERE, A. (1984). Selection of particles and proteins for use as human cytomegalovirus subunit vaccines. *In* "CMV: Pathogenesis and Prevention of Human Infection" (S. A. Plotkin, S. Michelson, J. S. Pagano, and F. Rapp, Eds.), pp. 305–324. Alan R. Liss, Inc., New York.

GREFTE, J. M. M., VAN DER GUN, B. T. F., SCHMOLKE, S., VAN DER GIESSEN, M., VAN SON, W. J., PLACHTER, B., JAHN, G., and DE THE, T. H. (1992). The lower matrix protein pp65 is the principal viral antigen present in peripheral blood leukocytes during an active cytomegalovirus infection. *J. Gen. Virol.* **73,** 2923–2932.

HUGHES, G., BABIUK, L. A., and VAN DRUNEN LITTEL-VAN DEN HURK, S. (1988). Functional and topographical analyses of epitopes on bovine herpesvirus-1 glycoprotein IV. *Arch. Virol.* **103,** 47–60.

HUTCHINGS, D. L., VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1990). Lymphocyte proliferative responses to separated bovine herpesvirus-1 proteins in immune cattle. *J. Virol.* **64,** 5114–5122.

JACKSON, S. P., and TIJAN, R. (1988). O-glycosylation of eukaryotic transcription factors: Implication for mechanisms of transcriptional regulation. *Cell* **55,** 125–133.

LABOISSIERE, S., TRUDEL, M., and SIMARD, C. (1992). Characterization and transcript mapping of a bovine herpesvirus type-1 gene encoding a polypeptide homologous to the herpes simplex virus type 1 major tegument proteins VP13/14. *J. Gen. Virol.* **73,** 2941–2947.

LEMASTER, S., and ROIZMAN, B. (1980). Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. *J. Virol.* **35,** 798–811.

LIANG, X., BABIUK, L. A., VAN DRUNEN LITTEL-VAN DEN HURK, S., FITZPATRICK, D. R., and ZAMB, T. J. (1991). Bovine herpesvirus-1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. *J. Virol.* **65,** 1124–1132.

MARSHALL, R. L., RODRIGUEZ, L. L., and LETCHWORTH, G. J., III (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus-1) by biochemical and immunological methods. *J. Virol.* **57,** 745–753.

MCGEOCH, D. J., DALRYMPLE, M. A., DAVISON, A. J., DOLAN, A., FRAME, M. C., MCNAB, D., PERRY, L. J., SCOTT, J. E., and TAYLOR, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69,** 1531–1574.

MCKNIGHT, J. L. C., PELLETT, P. E., JENKINS, F. J., and ROIZMAN, B. (1987). Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate alpha-transducing factor dependent activation of alpha genes. *J. Virol.* **61,** 992–1001.

MEREDITH, D. M., LINDSAY, J. A., HALLIBURTON, I. W., and WHITTAKER, G. R. (1991). Post-translational modification of the tegument proteins (VP13 and VP14) of herpes simplex virus type 1 by glycosylation and phosphorylation. *J. Gen. Virol.* **72,** 2771–2775.

MISRA, V., BLUMENTHAL, R. M., and BABIUK, L. A. (1981). Proteins specified by bovine herpesvirus-1 (infectious bovine rhinotracheitis virus). *J. Virol.* **40,** 367–378.

PINARD, M.-F., SIMARD, R., and BIBOR-HARDY, V. (1987). DNA-binding proteins of herpes simplex virus type 1-infected BHK cell nuclear matrices. *J. Gen. Virol.* **68,** 727–735.

SCHEK, N., and BACHENHEIMER, S. L. (1985). Identification and mapping of two polypeptides encoded within the herpes simplex virus type 1 thymidine kinase gene sequences. *J. Virol.* **38,** 593–605.

SPEAR, P. G., and ROIZMAN, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. *J. Virol.* **9,** 143–159.

TIKOO, S. K., FITZPATRICK, D. R., BABIUK, L. A., and ZAMB, T. J. (1990). Molecular cloning, sequencing and expression of functional bovine herpesvirus-1 glycoprotein IV in transfected bovine cells. *J. Virol.* **64,** 5132–5142.

TIKOO, S. K., ZAMB, T. J., and BABIUK, L. A. (1993). Analysis of bovine herpesvirus-1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia virus. *J. Virol.* **67,** 2103–2109.

VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1985). Antigenic and immunogenic characteristics of bovine herpesvirus type-1 glycoproteins GVP 3/9 and GVP 6/11a/16, purified by affinity chromatography. *Virology* **144,** 204–215.

VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1986a). Polypeptide specificity of the antibody response after primary and recurrent infection with bovine herpesvirus-1. *J. Clin. Microbiol.* **23,** 274–282.

VAN DRUNEN LITTEL-VAN DEN HURK, S., and BABIUK, L. A. (1986b). Synthesis and processing of bovine herpesvirus-1 glycoproteins. *J. Virol.* **59,** 401–410.

VAN DRUNEN LITTEL-VAN DEN HURK, S., VAN DEN HURK, J. V., GILCHRIST, J. V., MISRA, V., and BABIUK, L. A. (1984). Interactions of monoclonal antibodies and bovine herpesvirus type-1 (BHV-1) glycoproteins: Characterization of their biochemical and immunological properties. *Virology* **135,** 466–479.

VAN DRUNEN LITTEL-VAN DEN HURK, S., GIFFORD, G. A., and BABIUK, L. A. (1990). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine* **8,** 358–368.

VAN DRUNEN LITTEL-VAN DEN HURK, S., PARKER, M. D., FITZPATRICK, D. R., VAN DEN HURK, J. V., CAMPOS, M., BABIUK, L. A., and ZAMB, T. (1992). Structural, functional and immunological characterizaton of bovine herpesvirus-1 glycoprotein gI expressed by recombinant baculovirus. *Virology* **190,** 378–392.

VAN DRUNEN LITTEL-VAN DEN HURK, S., PARKER, M. D., MASSIE, B., VAN DEN HURK, J. V., HARLAND, R., BABIUK, L. A., and ZAMB, T. J. (1993). Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. *Vaccine* **11,** 25–35.

WHITTAKER, G. R., RIGGIO, M. P., HALLIBURTON, I. W., KILLINGTON, R. A., ALLEN, G. P., and MEREDITH, D. M. (1991). Antigenic and protein sequence homology between VP13/14, a herpes simplex virus type 1 tegument protein, and gp10, a glycoprotein of equine herpesvirus 1 and 4. *J. Virol.* **65,** 2320–2326.

ZHANG, Y., SIRKO, D. A., and MCKNIGHT, L. C. (1991). Role of herpes simplex virus type 1 UL46 and UL47 in alphaTIF-mediated transcriptional induction: characterization of three viral deletion mutants. *J. Virol.* **65,** 829–841.