INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), a member of the Alphaherpesvirinae (Roizman et al., 1992), causes infectious bovine rhinotracheitis, a disease of major economic concern (Yates, 1982). In addition to causing initial respiratory infections, BHV-1 can also predispose animals, presumably through immunosuppression (Bielefeldt Ohmann and Babiuk, 1985; Filion et al., 1983), to secondary bacterial infections that lead to severe pneumonia and death (Yates, 1982). Virus-induced immunosuppression has been reported in a large number of viral diseases, and various mechanisms, both direct and indirect, have been described (McChesney and Oldstone, 1987). The in vitro demonstration of virus-induced inhibition of leukocyte functions has focused on the interactions between the virus and the proliferating immune cells (McChesney and Oldstone, 1987). We have previously shown that BHV-1, even when inactivated, is able to induce apoptotic cell death in peripheral blood mononuclear cells (Hanon et al., 1996) or in bovine B lymphoma (BL-3) cells (Hanon et al., 1998a).

Apoptosis is considered to be a physiological process that is part of homeostatic regulation during normal tissue turnover. Apoptosis and necrosis differ both morphologically and biochemically (Martin et al., 1994; Duvall and Wyllie, 1986). When observed with an electron microscope, a cell undergoing apoptosis displays a distinct pattern of structural changes in the nucleus and in the cytoplasm including cell shrinkage, apoptotic body formation, and condensation of the chromatin into electron-dense masses (Martin et al., 1994; Duvall and Wyllie, 1986). Extensive damage of chromatin is associated with DNA cleavage into oligonucleosomal DNA fragments (Arends et al., 1990; Wyllie, 1980). The fragmentation of DNA is considered to be the key biochemical event of the apoptotic process (Martin et al., 1994; Duvall and Wyllie, 1986).

The mechanism by which BHV-1 induces apoptotic cell death is not yet understood. However, since BHV-1 viral particles inactivated by trioxsalen-UV light treatment are still able to induce apoptosis, the mechanism of induction could involve attachment, penetration, or decapsidation of BHV-1 (Hanon et al., 1996). Different BHV-1 envelope proteins mediate the initial interactions of BHV-1 viral particles with target cells (Tikoo et al., 1995). Glycoproteins B (gB) and gC have been shown to be involved in the attachment of BHV-1 to heparan sulfate proteoglycans on the cell surface (Tikoo et al., 1995; Okazaki et al., 1994; Liang et al., 1991; Byrne et al., 1995). In addition, gB, gD, and gH have been implicated in viral penetration (Liang et al., 1995; Fehler et al., 1992; Michelson et al., 1995; Schroder et al., 1997). We recently demonstrated that penetration of BHV-1 into target cells
is not required for the induction of apoptosis. Indeed, a glycoprotein H gene deleted BHV-1, which is deficient in viral penetration, still induces apoptosis in BL-3 cells (Hanon et al., 1998b). Therefore, one or more BHV-1 envelope glycoproteins could be involved in the activation of the apoptotic process. Among the different BHV-1 envelope glycoproteins, gD could be a potential candidate. This hypothesis is suggested by several studies demonstrating that the expression of high levels of gD is toxic for bovine cells (Fehler et al., 1992; Liang et al., 1995). In addition, it has been shown that gD of herpes simplex virus 1, another member of the Alphaherpesvirinae (Roizman et al., 1992), interacts with a member of the TNF/NGF receptor family (herpesvirus entry mediator, HVEM) (Montgomery et al., 1996). These receptors are implicated in a variety of cellular functions including proliferation, differentiation, or apoptosis (Baker and Reddy, 1996). Therefore, in order to further characterize the mechanism by which BHV-1 induces apoptosis, we investigated the involvement of gD in BHV-1-induced apoptosis.

RESULTS

The effect of BHV-1 gD null mutant on BL-3 cells

To investigate the involvement of gD in BHV-1-induced apoptosis, we used a BHV-1 gD null mutant, which offers the opportunity to test whether the presence of gD in BHV-1 viral particles is required for the induction of apoptosis in target cells. For this purpose, the BHV-1 gD null mutant was first multiplied on normal Madin Darby bovine kidney (MDBK) cells to generate a virus stock in which virions do not contain gD in the viral envelope (BHV-1 gD−/−) and, consequently, are no longer infectious. A second virus stock in which virions contain gD in the viral envelope but do not genetically encode gD (BHV-1 gD+/−) was generated after multiplication of the BHV-1 gD null mutant on gD-expressing MDBK cells. As control, a wt BHV-1 virus stock was also generated on normal MDBK cells. We first investigated whether BHV-1 gD−/− can induce DNA fragmentation in BL-3 cells. For this assay, BL-3 cells were cultivated in the presence or in the absence of the BHV-1 gD+/−, BHV-1 gD−/+, and wt BHV-1 virus stocks (concentration of 1000 physical viral particles per BL-3 cell) for 48 h. The cells were then harvested and further processed to detect the occurrence of DNA fragmentation by in situ DNA fragment labeling. Figure 1A shows that very low percentages (9.5%) of apoptosis were obtained in cultures incubated with virions devoid of gD (BHV-1 gD−/−). These percentages were similar to those obtained in mock-infected cultures (8.4%). In contrast, wt BHV-1 and BHV-1 gD+/+ induced high levels of apoptosis (70.8 and 76.9%, respectively) (Fig. 1A). Since infection by herpesviruses might lead to DNA cleavage, the occurrence of apoptosis as measured by the TUNEL assay must be confirmed by agarose gel electrophoresis in which a specific DNA ladder pattern resulting from internucleosomal cleavage is observable. The DNA isolated from BL-3 cells incubated with wt BHV-1 and BHV-1 gD−/− clearly showed the apoptosis-specific internucleosomal laddering (Fig. 2). In contrast, background levels of DNA laddering were observed in the DNA isolated from BL-3 cells that were mock-infected or incubated with BHV-1 gD−/− (Fig. 2). These observations clearly demonstrate that the deletion of gD affects the ability of BHV-1 to induce apoptosis in target cells. The inability of BHV-1 gD−/− to induce apoptosis could not be due to a deficiency in viral penetration since we previously demonstrated that the deletion of gH that also causes a deficiency in viral penetration did not affect the induction of apoptosis (Hanon et al., 1998b).

To confirm that the induction of apoptosis in BL-3 cells is due to BHV-1 viral particles harboring gD in the viral envelope, we investigated the effect of anti-gD monoclonal antibodies (Mabs) on the ability of wt BHV-1, BHV-1 gD+/+, and BHV-1 gD−/− to induce apoptosis in BL-3 cells. For this assay, virus stocks were preincubated with a mix of anti-gD Mabs (ascites diluted at 1/1000) for 20 min at 37°C and then incubated with BL-3 cells as described above. As shown in Fig. 1A, the incubation of wt BHV-1 and BHV-1 gD−/+ with anti-gD Mabs drastically reduced the percentage of apoptotic BL-3 cells. We also compare anti-gD, anti-gB, and anti-gC Mabs for their effect on the ability of wt BHV-1 to induce apoptosis in target cells (Fig. 1B). Flow cytometric analysis showed that all Mabs directed against gD (3C1, 10C2, 2C8, 3E7, 9D6, 4C1), even 10,000-fold diluted, strongly reduced the percentage of apoptosis in BHV-1-infected cultures. In contrast, the anti-gC (1507) and anti-gB (5106, 4807) Mabs slightly reduced the level of apoptosis when used at a dilution of 1/10,000 and only the 5106 Mab (anti-gB) had an inhibitory effect at a dilution of 1/1000 (Fig. 1B). These results indicate that, in contrast to anti-gB or anti-gC Mabs, anti-gD Mabs can strongly inhibit the ability of BHV-1 to induce apoptosis in target cells.

Attachment of BHV-1 gD null mutant to BL-3 cells

Attachment of BHV-1 to target cells has been shown to be necessary for induction of apoptosis (Hanon et al., 1998b). The deletion of gD does not affect the ability of BHV-1 to bind to MDBK cells (Fehler et al., 1992). Nevertheless, this observation has not been confirmed with other cell lines, including BL-3 cells. Therefore, we next investigated whether the inability of BHV-1 gD−/− to induce apoptosis is due to a defect in viral attachment. For this purpose, BL-3 cells were incubated in the presence or in the absence of the wt BHV-1, BHV-1 gD−/+, and BHV-1 gD−/− virus stocks (concentration of 1000 physical viral particles per BL-3
cell) for 1 h at 37°C. The cells were then processed to detect attached viral particles by indirect immunofluorescence and analyzed by flow cytometry. Figure 3 shows that wt BHV-1, BHV-1 gD−/+, and BHV-1 gD−/− bound to comparable number of BL-3 cells (26.1, 28.1, and 21.2%, respectively). As expected, culture of BL-3 cells incubated in the absence of virus only showed 0.4% positive cells. This experiment indicates that the absence of gD in the viral envelope does not affect the ability of BHV-1 to bind to BL-3 cells. Consequently, the inability of BHV-1 gD−/− to induce apoptosis is not due to a defect in viral attachment.

The effect of purified gD on BL-3 cells

To further investigate the role of gD in BHV-1-induced apoptosis, we determined the effect of affinity-purified gD on BL-3 cells. For this purpose, BL-3 cells were incubated in the presence or in the absence of purified gD (0.05, 0.5, or 5 μg/ml) for 48 h, harvested, and then processed to detect the occurrence of DNA fragmentation by in situ DNA fragment labeling. Flow cytometric analysis showed that incubation of BL-3 cells with even 5 μg/ml of purified gD did not increase the background level of apoptosis, indicating that gD alone is not sufficient to activate the apoptotic process in BL-3 cells (Fig. 1).
4). Affinity-purified gD could require another virion component to induce apoptosis. In this case, affinity-purified gD should be able to inhibit the ability of BHV-1 viral particles to induce apoptosis. Therefore, we next investigated the effect of affinity-purified gD on BHV-1-induced apoptosis. For this purpose, BL-3 cells were incubated in the presence or in the absence of purified gD (0.05, 0.5, or 5 μg/ml) at 37°C for 1 h. The cells were then incubated with BHV-1 (multiplicity of infection (m.o.i.) of 10) for 48 h. After being harvested, the cells were further processed to detect the occurrence of DNA fragmentation as described above. The analysis showed 49.9% of BL-3 cells incubated with BHV-1 were apoptotic (Fig. 4). In contrast, the presence of affinity-purified gD (0.05, 0.5, or 5 μg/ml, respectively) reduced drastically the ability of BHV-1 to induce apoptosis in cultures of BL-3 cells (6.3, 9.4, and 22.6%, respectively) (Fig. 4). Therefore, although binding of affinity-purified gD to BL-3 cells does not lead to induction of apoptosis, it inhibits the ability of BHV-1 viral particles to induce apoptosis.

**DISCUSSION**

The mechanism(s) by which BHV-1 induces apoptosis is not yet understood. However, it has been recently demonstrated that attachment but not penetration of BHV-1 is necessary to induce apoptosis into target cells (Hanon et al., 1998b), suggesting that one or more BHV-1 envelope proteins could be involved in the activation of the apoptotic process. In this study, we provide evidence for the direct or indirect involvement of gD in the mechanism by which BHV-1 induces apoptosis. This finding is based on the following observations. Although BHV-1 virions devoid of gD (BHV-1 gD−/−) still bind to BL-3 cells (Fig. 3), they are no longer able to induce apoptosis in BL-3 cells (Figs. 1A and 2). In contrast, virions that contain gD in the viral envelope but do not genetically encode gD (BHV-1 gD−/+ ) induce a level of apoptosis comparable to that produced by wt BHV-1 (Figs. 1A and 2). In addition, Mabs directed against gD, but not against gB or gC, strongly reduced the high levels of apoptosis induced by BHV-1 (Fig. 1). This observation confirms that the induction of apoptosis is directly due to BHV-1 viral particles harboring gD in the viral envelope. Interestingly, binding of affinity-purified gD to BL-3 cells inhibited the ability of wt BHV-1 viral particles to induce apoptosis in BL-3 cells (Fig. 4). Altogether, these results demonstrate that the presence of gD in the viral envelope is required for BHV-1 viral particles to induce apoptosis in target cells.

Glycoprotein D could participate either directly or indirectly in BHV-1-induced apoptosis. Interestingly, Mauri et al. (1998) recently demonstrated that HVEM, which is a cellular receptor for HSV-1 gD, can bind two cellular ligands, secreted lymphotoxin α (LTα) and LIGHT, a new member of the TNF superfamily. LIGHT has been recently identified as a strong inducer of the apoptotic process in lymphoid cells (Zhai et al., 1998). Therefore, the interaction of BHV-1 gD with a bovine receptor similar to HVEM could be responsible for the induction of apoptosis. Nevertheless, binding of BHV-1 gD to BL-3 cells
appeared to be not sufficient for activation of the apoptotic process (Fig. 4). Several hypotheses could explain this observation. (i) BHV-1 gD may require an association with the viral envelope to mediate its biological function. In this context, it is interesting to note that LIGHT, which is the natural ligand of HVEM, is a membrane-bound cytokine (Mauri et al., 1998). (ii) Activation of apoptosis in target cells might be due to another virion envelope protein(s) whose expression or function is linked to the presence of BHV-1 gD on the viral envelope. One or several other viral glycoproteins could play a role but not the gE, gI, gG, and gH glycoproteins since BHV-1 deleted for these glycoproteins still induced apoptosis in target cells (Hanon et al., 1998b). (iii) Finally, we cannot exclude the possibility that the purification procedure partially affected specific biological domains of BHV-1 gD, inhibiting its ability to induce apoptosis.

The direct or indirect involvement of gD in BHV-1-induced apoptosis may help to better elucidate the pathogenesis of BHV-1. Following BHV-1 infection, the high susceptibility of infected animals to secondary bacterial infection is associated with immunosuppression (Bielefeldt Ohmann and Babiuk, 1985; Filion et al., 1983). BHV-1 has been shown to induce apoptosis in T lymphocytes, B lymphocytes, and monocytes/macrophages in vitro (Hanon et al., 1998a). This suggests that a wide range of immune cells could undergo BHV-1-induced apoptosis in vivo, which in turn could severely affect different immune cell functions. In addition, Renjifo et al. (1998) already observed that, in contrast to wild-type virus, BHV-1 gD null mutant does not affect the ability of bovine macrophages to process and present antigens to lymphocytes in vitro. Therefore, the deletion or subtle amino acid substitutions in gD could eliminate the ability of BHV-1 to induce immunosuppression in vivo. Moreover, the observation that Mabs directed against BHV-1 gD strongly inhibit the ability of BHV-1 to induce apoptosis in target cells (Fig. 1) supports the use of gD as a subunit vaccine (van Drunen Littel-van den Hurk et al., 1993). Immunization experiments demonstrated that purified gD protects more efficiently animals from disease than purified gC or gB (van Drunen Littel-van den Hurk et al., 1993). The higher protection conferred by gD is presumably due to the induction of an efficient immune response, which can limit the multiplication of BHV-1 in infected animals. Anti-gD antibodies produced in immunized animals could also protect immune cells from BHV-1-induced apoptosis and therefore allow the induction of a faster and stronger immune response against BHV-1.

In conclusion, the results presented in this study provide evidence for the direct or indirect involvement of gD in the mechanism by which BHV-1 induces apoptosis. This finding could have important implications in the development of new, more effective, and safer vaccines and also help to better elucidate the pathogenesis of BHV-1.

MATERIALS AND METHODS

Cells and viruses

Bovine lymphoma B cells (American type culture collection CRL 8037) were cultured in Optimem medium.
As described by Fehler et al. (1992), the BHV-1 gD null mutant (BHV-1/80-221) in which the ORF encoding gD is replaced by a lacZ cassette was propagated on gD-expressing MDBK cells (clone BUIV3-7; m.o.i. of 10) to generate a virus stock in which virions contain gD in the viral envelope but do not genetically encode gD (BHV-1 gD+/−). A second virus stock in which virions do not contain gD and, consequently, are no longer infectious (BHV-1 gD−/−) was generated after multiplication of the BHV-1 gD null mutant on normal MDBK cells (clone Bu100; kindly provided by W. Lawrence and L. Bello, Philadelphia, PA; m.o.i. of 10). Wild-type BHV-1 (Cooper strain; kindly provided by Dr. J. T. van Oirschot, Lelystad, The Netherlands) was propagated on MDBK cells (m.o.i. of 10). After multiplication of the viruses, the culture media were clarified by centrifugation at 1500 g for 20 min at 4°C and the viruses were pelleted by ultracentrifugation at 26,000 g for 2 h at 4°C. Viral pellets were then resuspended in OptiMem medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin and stored at −70°C until use.

**Viral particle count**

Virus suspensions were ultracentrifuged at 100,000 g for 1 h, resuspended in water, and then mixed with an equal volume of a 2% phosphotungstic acid (Sigma, Bornem, Belgium) and a suspension of latex beads (1.6 × 10^11 particles/ml) (Laborimpex, Brussels, Belgium). A droplet of this suspension was placed on an electron microscope grid (Laborimpex) and after 5 min the excess suspension was removed and the particles were counted under the electron microscope (Zeiss 910).

**Monoclonal antibodies and affinity-purified gD**

Monoclonal antibodies (3C1, 10C2, 2C8, 3E7, 9D6, 4C1) directed against different antigenic domains of gD (Hughes et al., 1988) were used in this study. The Mabs directed against gB (5106, 4807) and gC (1507) were kindly provided by Dr. G. Letchworth (University of Wisconsin–Madison). Full-length gD was affinity-purified from BHV-1-infected cells as previously described (Li et al., 1995).

**Detection of BHV-1 viral particles attached on BL-3 cells**

BL-3 cells were first fixed in PBS containing 0.5% paraformaldehyde for 15 min at 20°C. The cells were then washed with PBS containing 10% FCS and incubated with BHV-1 viral particles at 37°C. One hour later, the cells were washed with PBS containing 10% FCS and fixed again in PBS containing 0.5% paraformaldehyde for 15 min at 20°C. To label the attached BHV-1 viral particles, BL-3 cells were incubated for 30 min at 20°C in PBS containing the appropriate predetermined concentration of anti-gB Mab (5106). The cells were then washed with PBS containing 5% FCS and further incubated in PBS containing the PE-conjugated F(ab')2 goat anti-mouse IgG (H+L chains) (Dako) suspension at 20°C for 30 min. After an additional wash with PBS containing 5% FCS, the cells were resuspended in PBS and analyzed by flow cytometry for orange fluorescence.

**Detection of apoptosis**

Detection of DNA fragmentation was carried out either by the TUNEL procedure or by agarose gel electrophoresis. For the TUNEL procedure, cells were washed with PBS containing 10% FCS and fixed in PBS containing 2% paraformaldehyde. The TUNEL reaction was carried out as described in the apoptosis detection kit (in situ cell death detection kit, fluorescein; Boehringer Mannheim, Mannheim, Germany). Samples were analyzed by flow cytometry for green fluorescence.

For DNA gel electrophoresis, DNA extraction was carried out as described per the instructions of a commercial kit (DNAzol reagent, Gibco). Briefly, cells were lysed in DNAzol reagent. Each DNA sample was electrophoresed on a 1.5% agarose gel. The DNA was visualized under UV light after being stained with ethidium bromide.

**Flow cytometry**

Flow cytometric analyses were performed using a Becton Dickinson fluorescence-activated cell sorter (Facstar Plus), equipped with an argon laser (excitation lines at 488 nm). Debris were excluded from the analysis by the conventional scatter gating method. Ten thousand events per sample were collected in a list mode, stored, and analyzed by the Consort 32 system (Becton Dickinson).

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