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INACTIVATED BOVINE HERPESVIRUS 1 INDUCES APOPTOTIC CELL DEATH OF MITOGEN-STIMULATED BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Bovine herpesvirus 1 (BHV-1) is able to inhibit the proliferation of bovine peripheral blood mononuclear cells. Here, we have demonstrated that live BHV-1 and, interestingly, inactivated BHV-1 can induce apoptosis of mitogen-stimulated bovine peripheral blood mononuclear cells in vitro.

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Bovine herpesvirus 1 (BHV-1) is a member of the Alphaherpesvirinae subfamily (24). In addition to causing initial respiratory infections (31), BHV-1 can predispose animals, presumably through immunodepression (3, 8), to secondary bacterial infections which lead to severe pneumonia and death (31). Virus-induced immunodepression has been reported for a large number of viral diseases, and various mechanisms have been described (18). After in vitro BHV-1 infection, the proliferation of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) and interleukin-2-dependent T lymphocytes is inhibited (6, 10). Similar observations have been reported with interleukin-2- and antigen-stimulated PBMCs (13). The mechanisms used by BHV-1 to inhibit the proliferative response to mitogen are poorly understood. Contradictory results have been obtained regarding the capacity of inactivated BHV-1 to induce this effect (6, 10, 13). Live or UVinactivated BHV-1 alters the proliferation of interleukin-2- and antigen-stimulated PBMCs (13). In contrast, only live BHV-1 is capable of inhibiting the proliferation of mitogen-stimulated PBMCs and interleukin-2-dependent T lymphocytes (6, 10). However, the inhibition by BHV-1 of interleukin- 2-dependent T-lymphocyte proliferation is associated neither with virus replication nor with expression of detectable levels of viral proteins but is caused by a virus-induced cell death resembling apoptosis under electron microscopy (10). Cell death can occur by two major distinct mechanisms, necrosis and apoptosis, which differ both morphologically and biochemically (7). Necrosis is considered to be a pathological reaction in response to lytic viral infections or toxic agents (7). In contrast, apoptosis is considered to be a physiological process which is part of the homeostatic regulation during normal tissue turnover (7), and studies have shown that it can be triggered by several viruses (reviewed in reference 21).

Because several studies demonstrated that BHV-1 inhibits the PBMC proliferative response to mitogen (6, 10, 13), we characterized in vitro the effects of BHV-1 on mitogen-stimulated PBMCs. The PBMCs were isolated on a Ficoll-Hypaque (Pharmacia, Roosendaal, The Netherlands) density gradient from the blood of steers serologically negative for BHV-1. The PBMCs were cultured in RPMI 1640 medium (Gibco, Gent, Belgium) supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 5 X 10-5 M 2-mercaptoethanol (Gibco) and stimulated either with 5 μg of concanavalin A (ConA) (Sigma, Bornem, Belgium) per ml or 0.5 μM ionomycin (Sigma) plus 10 nM phorbol 12,13-dibutyrate (Sigma) (IONO/PDB). BHV-1 Cooper (kindly provided by J. T. Van Oirschot, Lelystad, The Netherlands), BHV-1 8221, and the gammaherpesvirus BHV-4 V. Test strain (5) were used in this study. The viruses were multiplied on Madin Darby Bovine Kidney (MDBK) cells (American Type Culture Collection no. CCL22) and purified as described by Szilágyi et al. (28) with some minor modifications (17). In all of the experiments, the infections were performed with purified virus at a multiplicity of infection of 10 PFU per cell.

In order to determine if the inhibition of mitogen-stimulated PBMC proliferation by BHV-1 is associated with an increased cell death, we first investigated in vitro the effect of BHV-1 on cell viability (Table 1). Just before analysis, 5 μg of propidium iodide (Sigma) per ml was added to the culture medium, and the percentages of dead cells (propidium iodide-positive cells) were determined by flow cytometric analysis with a Becton Dickinson fluorescence-activated cell sorter (Facstar Plus) (Becton Dickinson, Erembodegem, Belgium).

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Fig. 1. *Electron micrographs of PBMCs stimulated with IONO/PDB, mock infected (A) or infected with BHV-1 (B and C) (multiplicity of infection of 10), and incubated for 48 h. (B) PBMC harboring cell shrinkage and condensed chromatin in several electron dense masses. (C) PBMC with nucleocapsids in the nuclei and in the cytoplasm (arrow) and with mature virus particles on the membrane (arrowhead). Bars, 1 μm.*

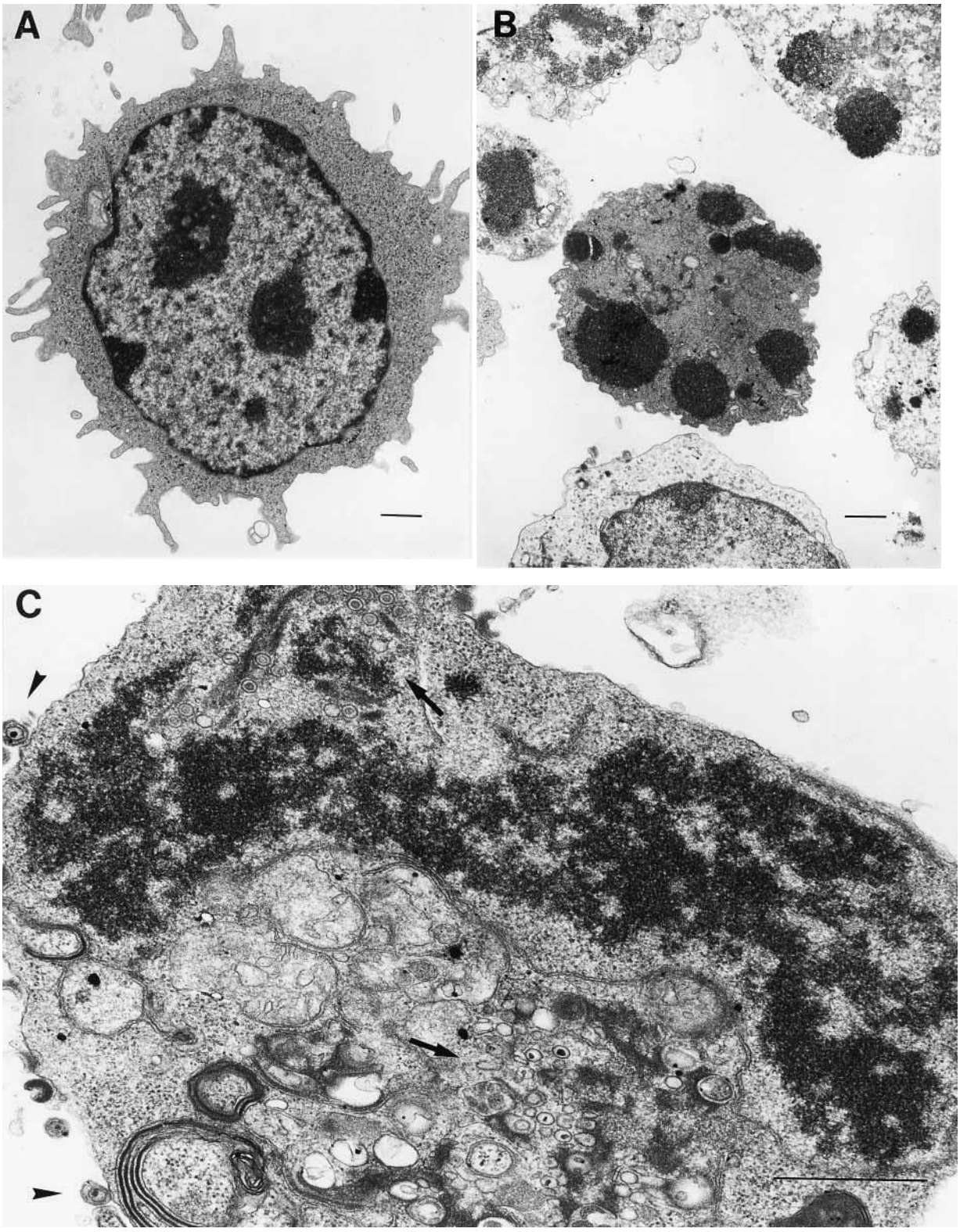

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Table 1. *Percentages of mortality of IONO/PDB-stimulated PBMCs mock infected or infected with BHV-1 or BHV-4a*

Incubation period (h)	% Mortality of infected IONO/PDB-stimulated PBMCs[b]			
	Mock infection	BHV-1 Cooper	BHV-1 8221	BHV-4 V. Test strain
2	9,4 ± 0,6	9,2 ± 0,5	9,4 ± 0,9	10,5 ± 0,9
24	12 ± 0,9	21,6 ± 1,4	20,4 ± 0,9	15 ± 0,6
48	14,5 ± 0,7	55,6 ± 0,9	49,6 ± 1,5	13,3 ± 1,2

[a] Other than for mock infection, virus was at a multiplicity of infection of 10.
[b] Each value represents the average ± standard deviation for triplicate cultures.

Table 2. *Percentages of MDBK cells expressing viral proteins or 13-galactosidasea*

Method of detection	% of infected MDBK cells expressing viral protein or 13-galactosidase[b]				
	Mock infection	BHV-1 8221		BHV-1 Cooper	
		Live virus	Inactivated virus	Live virus	Inactivated virus
FACS–β -galactosidase assay	0.5 ± 0.1	93.2 ± 1.1	0.9 ± 0.2	ND[c]	ND
Anti-BHV-1 serum	0.5 ± 0.1	92.8 ± 0.9	2 ± 0.7	91.7 ± 1.3	1.28 ± 0.2
Anti-BICPO serum	0.7 ± 0.2	92.2 ± 1.2	2 ± 0.6	90.8 ± 1.5	1.1 ± 0.3
Anti-gC monoclonal antibody 1507	0.8 ± 0.3	92.4 ± 0.8	1.5 ± 0.3	90.1 ± 1.6	0.5 ± 0.2

[a] MDBK cells were mock infected or infected with live or inactivated BHV-1 (multiplicity of infection of 10). The activity of the 13-galactosidase enzyme and the expression of viral proteins were detected, respectively, 9 h after infection by the fluorescence-activated cell sorter (FACS)–13-galactosidase assay and 12 h after infection by indirect immunofluorescence staining.

[b] Each value represents the average ± standard deviation for triplicate cultures.

[c] ND, not determined.

Two hours after infection, we observed similar percentages of mortality in mock- and BHV-1-infected cell cultures ($P < 0.01$) (Table 1). However, 24 and 48 h after infection, the cell mortalities in BHV-1-infected cell cultures increased substantially and were significantly higher than those in mock-infected cell cultures ($P < 0.01$) (Table 1). Furthermore, the numbers of cells per milliliter were comparable 48 h after infection ($P < 0.01$). This indicated that the differences in mortalities between mock- and BHV-1-infected cell cultures were not due to cellular proliferation in mock-infected cell cultures. Stimulation with ConA gave results similar to those with IONO/PDB (data not shown). In contrast to BHV-1, PBMC cultures infected with BHV-4 did not show increased mortality compared with mock-infected cell cultures ($P < 0.01$) (Table 1). Furthermore, when a BHV-1 antiserum (obtained from a calf intranasally infected with BHV-1) was added to the culture medium of mock- and BHV-1-infected cell cultures, the percentage of mortality in BHV-1-infected PBMC cultures decreased from 44.2% ± 0.7% to 16.1% ± 1%.

BHV-1 specifically increased the percentage of dead cells in mitogen-stimulated PBMC cultures (Table 1). In order to gain insight into the death process induced by BHV-1, we studied the morphological characteristics of the BHV-1-infected cells by electron microscopy. Cell samples were treated as described by Bielefeldt Ohmann and Bloch (4) with a Zeiss 910 electron microscope. Forty-eight hours after infection, the PBMC cultures incubated with the BHV-1 Cooper strain contained half of the cells harboring cell shrinkage, membrane-bound apoptotic bodies, and distinctive condensation of the chromatin (Fig. 1B). These changes are typical

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morphological characteristics of cells undergoing apoptosis (7). The other half contained numerous viral nucleocapsids in their nuclei and some enveloped viral particles (Fig. 1C). Similar morphological observations were made when ConA or IONO/PDB was used to induce the cell proliferation (data not shown).

Fig. 2. *Percentages of cells undergoing DNA fragmentation in cultures of PBMCs stimulated with IONO/PDB, mock infected or infected with BHV-1 (multiplicity of infection of 10), and incubated at different times. Each value represents the average ± standard deviation for triplicate cultures.*

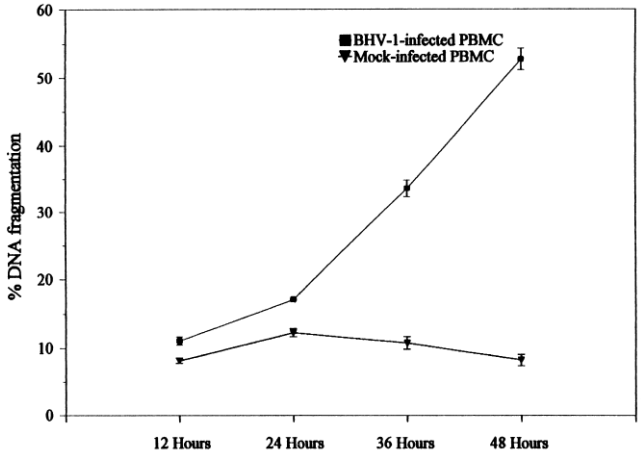
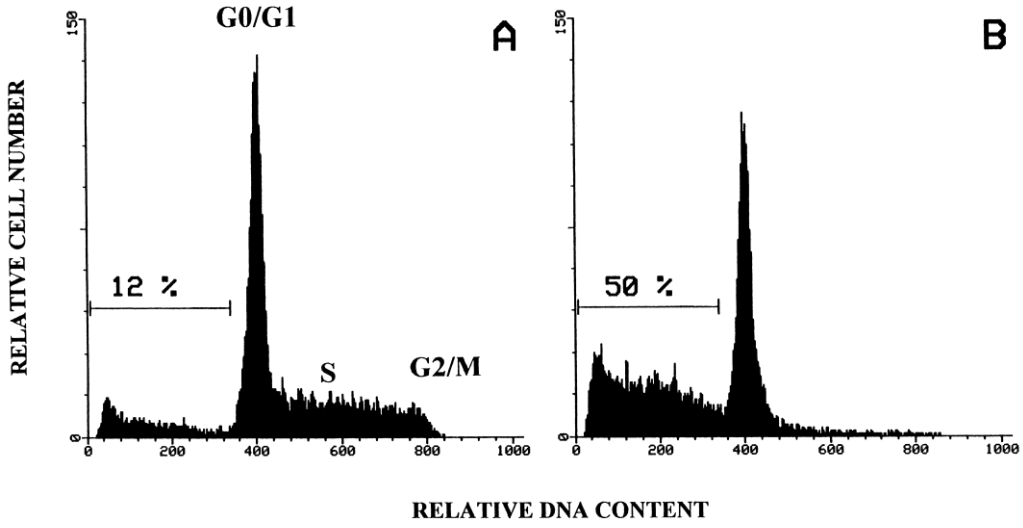


Fig. 3. *DNA content analysis of PBMCs stimulated with IONO/PDB, mock infected (A) or infected with BHV-1 (B) (multiplicity of infection of 10), and incubated for 48 h. Results for the various growth phases (G0/G1, S, and G2/M) are shown.*



In order to confirm that some cells in BHV-1-infected PBMC cultures undergo apoptosis, we analyzed these cultures for cellular DNA fragmentation, which is considered to be the key biochemical event of apoptosis (1, 7, 30). The DNA fragmentation was first detected by 3' end labelling with fluorescein isothiocyanate-dUTP (26) with a commercial kit (in situ cell death detection kit, fluorescein ; Boehringer Mannheim, Mannheim, Germany). Flow cytometric

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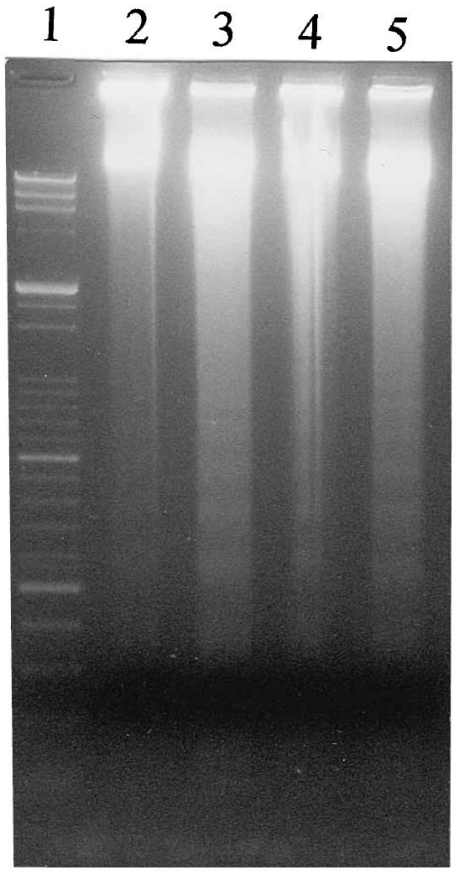
analysis showed that 12 h after infection, the BHV-1-infected ConA- and IONO/ PDB-stimulated PBMC cultures contained small numbers of cells undergoing DNA fragmentation. Furthermore, there was a significant increase in the percentages of cells with DNA fragmentation 24, 36, and 48 h after infection ($P < 0.01$) (Fig. 2). The mock-infected cell cultures always exhibited background levels of DNA fragmentation (Fig. 2). The accumulation of sub-G0/G1 phase cells, as shown in the DNA content analysis performed as described by Telford et al. (29), confirmed the occurrence of DNA fragmentation in BHV-1-infected cultures (Fig. 3B). The mock-infected cultures showed the typical pattern of DNA content with G0/G1, G2/M, and S phase cells (Fig. 3A). The DNA fragmentation was also demonstrated by agarose gel electrophoresis as described by Jeurissen et al. (14), in which the apoptosis-specific internucleosomal laddering in DNA from BHV-1-infected PBMCs was clearly observed (Fig. 4, lane 3).

Fig. 4. *Agarose gel electrophoresis of DNA extracted from IONO/PDB-stimulated PBMCs mock infected (lane 2), infected with live BHV-1 (lane 3), mock infected (in the presence of trioxsalen) (lane 4), or infected with inactivated BHV-1 (by trioxsalen-UV light treatment) (lane 5). Lane 1 corresponds to molecular weight markers.*



Taken together, these data (Table 1 and Fig. 1 to 4) indicate that there is an accumulation of cells with morphological and biochemical characteristics of apoptotic cell death in PBMC cultures incubated with BHV-1. These results also indicate that BHV-1 replication occurred in at least a fraction of the cells of the BHV-1-infected cultures. To determine whether BHV-1 gene expression is required for the induction of apoptosis, we investigated the capacity of inactivated BHV-1 to induce DNA fragmentation in PBMC cultures.

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For this assay, the BHV-1 8221 (which carries the *Escherichia coli* 13-galactosidase gene under the control of the mouse cytomegalovirus IE gene promoter/enhancer) and Cooper strains were inactivated by a photochemical treatment (11, 22) which involved an incubation of the virus with 1 μg of 4,5',8- trimethylpsoralen (trioxsalen) (Sigma) per ml and an exposure to UV light (peak wavelength, 302 nm) (model UVM-57; UPV Inc., San Gabriel, Calif.). Viral plaque assays (data not shown), determination of 13-galactosidase expression as described by Nolan et al. (19), and viral protein expression experiments were performed to confirm the inactivation of viral gene expression (Table 2).

IONO/PDB-stimulated PBMCs were mock infected (in the presence of trioxsalen, which was either irradiated or not irradiated) or infected with live (in the presence of trioxsalen) or inactivated (by trioxsalen-UV light treatment) virus. Forty-eight hours later, the PBMCs were fixed and end labelled in situ with fluorescein isothiocyanate-dUTP-fluorescein to detect the fragmentation of DNA. The flow cytometric analysis showed that the BHV-1 Cooper and 8221 strains induced DNA fragmentation in 53.3% ± 1.3% and 43.3% ± 1.1% of the PBMCs, respectively (each value reported represents the average ± standard deviation for triplicate cultures). These values were significantly higher than those of the controls (in the absence of the viruses) ($P < 0.01$), which were 11.3% ± 0.9% and 11.1% ± 1%, respectively. The inactivation of the BHV-1 Cooper and 8221 strains only slightly decreased the percentages of DNA fragmentation (48.5% ± 0.7% and 33.9% ± 0.7%, respectively). The occurrence of internucleosomal DNA cleavage in PBMCs incubated with inactivated BHV-1 was also demonstrated by agarose gel electrophoresis (Fig. 4, lane 5).

In some viral models, the expression of viral proteins has been shown to be necessary for the induction of apoptosis (12, 16, 27). In contrast, in human immunodeficiency virus-infected patients, the interaction of the soluble human immunodeficiency virus gp120 envelope protein with the CD4 molecules is thought to induce a defective signal transduction that leads to the apoptosis of the T helper population (2, 15, 25). T-cell receptor-mediated activation of apoptosis has been described for T lymphocytes and thymocytes (9, 20). In addition, antibodies that bind to the cell surface APO-1 and Fas proteins mediate apoptosis (9). The mechanism by which BHV-1 induces apoptotic cell death is not yet understood. However, since the inactivated BHV-1 viral particles have the capacity to induce apoptosis in PBMC cultures, it could be postulated that the mechanism of induction involves (i) virus-cell molecule interactions during the attachment or penetration processes or (ii) the effect of viral structural protein(s) released into the cell. To date, we are investigating the role played by the BHV-1 gC, gI, gE and gG structural glycoproteins in the activation of the apoptotic process. We have already observed that the recombinant BHV-1 virus with the gene coding for gC deleted (kindly provided by F. A. M. Rijsewijk, Lelystad, The Netherlands) (23) still induces DNA fragmentation, and, therefore, it seems most likely that the gC glycoprotein is not involved in the induction of apoptosis (data not shown).

In conclusion, we demonstrated that live or inactivated BHV-1 is capable of inducing apoptosis, in mitogen-stimulated PBMC cultures. This might have important implications for the biology and pathogenesis of the virus in vivo. Furthermore, this experimental model provides the opportunity to characterize the BHV-1 viral proteins involved in the activation of the apoptotic process.

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References

1. Arends, M. J., R. G. Morris, and A. H. Wyllie. 1990. Apoptosis: the role of the endonuclease. Am. J. Pathol. 136:593–608.
2. Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R.-P. Sekaly, and T. Helman Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176:1099–1106.
3. Bielefeldt Ohmann, H., and L. A. Babiuk. 1985. Viral-bacterial pneumonia in calves: effect of bovine herpesvirus-1 on immunologic functions. J. Infect. Dis. 151:937–947.
4. Bielefeldt Ohmann, H., and B. Bloch. 1982. Electron microscopic studies of bovine viral diarrhea virus in tissues of diseased calves and in cell cultures. Arch. Virol. 71:57–74.
5. Bublot, M., P. Lomonte, A.-S. Lequarre, J.-C. Albrecht, J. Nicholas, B. Fleckenstein, P.-P. Pastoret, and E. Thiry. 1992. Genetic relationships between bovine herpesvirus 4 and the gammaherpesviruses Epstein-Barr virus and herpesvirus saimiri. Virology 190:654–665.
6. Carter, J. J., A. D. Weinberg, A. Pollard, R. Reeves, J. A. Magnuson, and N. S. Magnuson. 1989. Inhibition of T-lymphocyte mitogenic responses and effects on cell functions by bovine herpesvirus 1. J. Virol. 63:1525–1530.
7. Duvall, E., and A. H. Wyllie. 1986. Death and the cell. Immunol. Today 7:115–119.
8. Filion, L. G., R. L. McGuire, and L. A. Babiuk. 1983. Nonspecific suppressive effect of bovine herpesvirus type 1 on bovine leukocyte functions. Infect. Immun. 42:106–112.
9. Green, D. G., and D. W. Scott. 1994. Activation-induced apoptosis in lymphocytes. Curr. Opin. Immunol. 6:476–487.
10. Griebel, P. J., H. Bielefeldt Ohmann, M. J. P. Lawman, and L. A. Babiuk. 1990. The interaction between bovine herpesvirus type 1 and activated bovine T lymphocytes. J. Gen. Virol. 71:369–377.
11. Hanson, C. V., J. L. Riggs, and E. H. Lennette. 1978. Photochemical inactivation of DNA and RNA viruses by psoralen derivatives. J. Gen. Virol. 40:345–358.
12. Hinshaw, V. S., C. W. Olsen, N. Dybdahl-Sissoko, and D. Evans. 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. 68:3667–3673.
13. Hutchings, D. L., M. Campos, L. Qualtiere, and L. A. Babiuk. 1990. Inhibition of antigen-induced and interleukin-2-induced proliferation of bovine peripheral blood leukocytes by inactivated bovine herpesvirus 1. J. Virol. 64:4146–4151.
14. Jeurissen, S. H. M., F. Wagenaar, J. M. A. Pol, A. J. van der Eb, and M. H. M. Noteborn. 1992. Chicken anemia virus causes apoptosis of thymocytes after in vivo infection and of cell lines after in vitro infection. J. Virol. 66:7383–7388.
15. Laurent-Crawford, A. G., E. Coccia, B. Krust, and A. G. Hovanessian. 1995. Membrane-expressed HIV envelope glycoprotein heterodimer is a powerful inducer of cell death in uninfected CD4+ target cells. Res. Virol. 146:5–17.
16. Laurent-Crawford, A. G., B. Krust, S. Muller, Y. Rivière, M. A. Rey-Cuillé, J. M. Béchet, L. Montagnier, and A. G. Hovanessian. 1991. The cytopathic effect of HIV is associated with apoptosis. Virology 185:829–839.

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Status : Postprint (Author's version)

17. Lyaku, J. R. S., P. F. Nettleton, and H. S. Marsden. 1992. A comparison of serological relationships among ruminant alphaherpesviruses by ELISA. Arch. Virol. 124:333–341.

18. McChesney, M. B., and M. B. A. Oldstone. 1987. Viruses perturb lymphocyte functions: selected principles characterizing virus-induced immunosuppression. Annu. Rev. Immunol. 5:279–304.

19. Nolan, G. P., S. Fiering, J.-F. Nicolas, and L. A. Herzenberg. 1988. Fluorescence-activated cell analysis and sorting of viable mammalian cells based on -D-galactosidase activity after transduction of *Escherichia coli lacZ*. Proc. Natl. Acad. Sci. USA 85:2603–2607.

20. Radvanyi, L. G., G. B. Mills, and R. G. Miller. 1993. Religation of the T cell receptor after primary activation of mature T cells inhibits proliferation and induces apoptotic cell death. J. Immunol. 150:5704–5715.

21. Razvi, E. S., and R. M. Welsh. 1995. Apoptosis in viral infections. Adv. Virus Res. 45:1–60.

22. Redfield, D. C., D. D. Richman, M. N. Oxman, and L. H. Kronenberg. 1981. Psoralen inactivation of influenza and herpes simplex viruses and of virus-infected cells. Infect. Immun. 32:1216–1226.

23. Rijsewijk, F. A. M., M. J. Kaashoek, G. Keil, H. A. Paal, R. C. Ruuls, F. A. C. Van Engelenburg, E. Thiry, P.-P. Pastoret, and J. T. Van Oirschot. 1995. In vitro and in vivo role of the non essential glycoproteins gC, gE, gI and gG of bovine herpesvirus 1, p. 27. In Abstracts of the Symposium on IBR and other ruminant herpesvirus infections. European Society for Veterinary Virology, Sart-Tilman (Liege), Belgium.

24. Roizman, B., R. C. Desrosiers, B. Fleckenstein, C. Lopez, A. C. Minson, and M. J. Studdert. 1992. The family of Herpesviridae: an update. Arch. Virol. 123:425–449.

25. Schwartz, O., M. Alizon, J.-M. Heard, and O. Danos. 1994. Impairment of T cell receptor-dependent stimulation in CD4+ lymphocytes after contact with membrane-bound HIV-1 envelope glycoprotein. Virology 198:360–365.

26. Sgonc, R., G. Boeck, H. Dietrich, J. Gruber, H. Recheis, and G. Wick. 1994. Simultaneous determination of cell surface antigens and apoptosis. Trends Genet. 10:41–42.

27. Strand, M. R., and L. L. Pech. 1995. Microplitis demolitor polydnavirus induces apoptosis of a specific hemocyte morphotype in *Pseudoplusia includens*. J. Gen. Virol. 76:283–291.

28. Szilágyi, J. F., and C. Cunningham. 1991. Identification and characterization of a novel non-infectious herpes simplex virus-related particle. J. Gen. Virol. 72:661–668.

29. Telford, W. G., L. E. King, and P. J. Fraker. 1991. Evaluation of glucocorticoid-induced DNA fragmentation in mouse thymocytes by flow cytometry. Cell. Prolif. 24:447–459.

30. Wyllie, A. H. 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (London) 284:555–556.

31. Yates, W. D. G. 1982. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. Can. J. Comp. Med. 46:225–263.