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THE INVOLVEMENT OF OXIDATIVE STRESS IN BOVINE HERPESVIRUS TYPE 4-MEDIATED APOPTOSIS

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1. ABSTRACT

Bovine herpesvirus type 4 (BHV-4) belongs to the gamma-2-herpesviruses of the Gammaherpesvirinae subfamily. BHV-4 has a worldwide distribution and has been isolated in a variety of clinical diseases as well as from healthy cattle. In this report we demonstrate that BHV-4 induces apoptosis in MDBK cells. In the early phases of apoptosis, cells show an increase in the intracellular level of reactive oxygen species, which is indicative of oxidative stress. This precedes DNA fragmentation, a hallmark typical of apoptosis. Cells were protected from apoptosis only by certain antioxidants (butylated hydroxyanisole and ebselen), whereas N-acetylcysteine turned out to be ineffective. Antioxidants that protected cells from apoptosis prevented oxidative stress but failed to block virus growth. These observations suggest that oxidative stress may be a crucial event in the sequence leading to apoptotic cell death but apoptosis is not required for the multiplication of BHV-4.

2. INTRODUCTION

Apoptosis, or programmed cell death, is an active process of cellular suicide consisting of a predictable sequence of cellular or morphological events leading to nuclear DNA cleavage and chromatin condensation (1, 2). Interest in this mode of cell death has risen due to discoveries that apoptosis plays a role not only in normal development but also in a number of disease processes. Apoptotic cell death pathways are induced by a variety of stimuli (e.g. different chemical insults, DNA damage, radiation, growth factor deprivation, cytotoxic T-lymphocytes or virus infections), many of these signals are known to also induce oxidative stress (3). Evidence regarding the role of reactive oxygen species (ROS) in the replication and pathogenesis of several viruses including human immunodeficiency virus has led to an increased interest in the role of oxidative stress in other virus infections (4-8). The killing of infected cells by apoptosis is one of the mechanisms of host defence against virus infections. Thus, it is not surprising that numerous viruses have developed strategies to prevent apoptosis (9-11) through different mechanisms. The strategies include modulation of the Bcl-2/Bax pathway (12) early in the apoptotic programme or interference with caspases (13), a group of proteases known to be part of the death effector mechanism of apoptosis. The discovery of a selenoprotein homologue to the antioxidant enzyme glutathione peroxidase in a poxvirus indicates that certain viruses may have acquired cellular mechanisms aimed at counteracting intracellulary-generated oxidants (14).

Bovine herpesvirus type 4 (BHV-4) is a member of the Gammaherpesvirinae subfamily of herpesviruses. It belongs to the gamma-2-herpesviruses on the basis of its thymidine kinase activity. Unlike other herpesviruses, BHV-4, originally isolated by Bartha et al. (15), is characterized by a wide host range both *in vivo* and *in vitro*.

Bovine herpesvirus type 4 shows genomic similarity to other gammaherpesvirinae, such as herpesvirus Saimiri (HSV) and human herpesvirus 8 (HHV-8), the etiological agent of Kaposi's sarcoma in

humans (16-21). In fact, it has been demonstrated that BHV-4 carries a gene, which encodes an antiapoptotic Bcl-2 homologue strictly related to that encoded by HHV-8 (22). However, it is difficult to study HHV-8 that cannot yet be grown efficiently in cell culture.

Moreover, recently, Sciortino et al. (23) have demonstrated that BHV-4 could simultaneously carry genes able to block and induce apoptosis. Such evidence on the proapoptotic/antiapoptotic role of BHV-4 prompted us to investigate the role of ROS in BHV-4 infection as an experimental model for gamma-2-herpesvirus infection.

3. METHODS

3.1. Reagents

Butylated hydroxyanisole (BHA), N-acetylcisteine (NAC) and ebselen were purchased from Sigma Company (Milan, Italy). BHA, NAC and ebselen were dissolved in ethanol, distilled water and DMSO, respectively, prior to dilution in cell culture medium. All other chemicals were of the highest purity that is commercially available.

3.2. Cells and viruses

Madin Darby Bovine Kidney (MDBK) cells (American Type Culture Collection CCL22) were maintained in complete medium (CM) consisting of Dulbecco Minimal Essential Medium (DMEM) containing 10% foetal calf serum (w/o FCS after virus infection), 2mM L-glutamine, 1% Non Essential Aminoacid, 100 U/ml penicillin, and 100 microg/ml streptomycin, at 37 °C in a CO_2 atmosphere.

BHV-4/FI, deriving from a bovine affected by postpartum metritis, isolated, characterized and kindly provided by Prof. C. Buonavoglia (University of Bari, Italy), was used in all the experiments.

3.3. Virus infection

MDBK cells seeded in 25 cm^2 culture flasks at a density of 10^6 cells per flask were infected with the BHV-4/FI strain in a small volume of culture medium without FCS for 1 h at 37 °C. After 72 h of infection, cell extracts, obtained by three cycles of freezing and thawing, were pooled, collected, and stored in aliquots at –80 °C. The virus-containing cell-extract stocks were successively tested by the $TCID_{50}$ method 7 days after infection. For experimental infections, 24 h after cultures had been split, cells grown to approximately 80% confluence were inoculated in CM with BHV-4 at different multiplicity of infection (m.o.i.) ratios and incubated at 37 °C in 5% CO_2. Control cultures were inoculated with equivalent quantities of medium only. At 1h p.i. medium containing virus inoculum was removed from the cultures to which fresh CM or CM associated with BHA, NAC or Ebselen was then added for the remaining culture time. Uninfected and BHV-4-infected cells were grown at 37 °C in CM.

3.4. DNA fragmentation

The fragmentation of cellular DNA was evaluated by FACS analysis according to Cossarizza et al. (24). At different times following infection, adherent cells, removed from the culture substrate by treatment with trypsin-EDTA solution, were again mixed with cells previously collected by centrifugation in supernatant from the same flask and resuspended at an adequate concentration in PBS. Thus, the entire cell population of the culture was reconstituted and processed for further analysis. Cells, collected as previously described, were fixed by a solution consisting of 9 ml of 80% ethanol and 1 ml of cells suspension, and seeded in 15 ml polypropylene tubes (Falcon) agitated continuously and stored at –20 °C for 4 h. Cells were then washed twice with PBS in 15 ml polypropylene tubes (Falcon). The cell pellet was immediately resuspended gently at room temperature in 1 ml of hypotonic solution consisting of 50 μg/ml propidium iodide, 0.1 % sodium citrate, and 1% Triton X-100 in distilled water. The tubes were placed at 22-24 °C for 30 minutes in the dark. Flow cytometry analysis was subsequently performed using a FACscan flow cytometer (DAKO Partek). A correct threshold value was experimentally selected to exclude the majority of cell debris. At least 5000 events were acquired for each sample.

3.5. Determination of cell viability by MTT

Cell viability was assayed by means of the MTT (Sigma) assay. The principle of this method is that MTT, a soluble tetrazolium salt, is converted to insoluble formazan by active mitochondrial dehydrogenases of living cells. Such conversion from yellowish soluble tetrazolium to purple formazan can be assayed spectrofluorimetrically. MTT was added to cultured cells, treated according to the above-mentioned protocols. After 4 hours of incubation, the medium was removed and replaced with DMSO to solubilize the MTT formazan crystals. The spectrophotometer absorbance at 570 nm was determined. Data are presented as a percentage of the control, and results are the mean ± SD of three experiments performed in duplicate.

3.6. Measurements of the cellular redox state

Free radical production was measured by incubation of the MDBK in the presence of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Irvine CA, USA), (25). DCFH-DA is a stable, non-fluorescent molecule that readily crosses the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH), which is rapidly oxidized in the presence of peroxides to the highly fluorescent 2',7'-dichlorofluorescin (DCF). Cells ($2x10^6$/ml) were loaded with 1 μM DCFH-DA for 30 min at 37 °C, in a medium whose composition was in mM: NaCl 138; KCl 2,7; CaCl2 1,2; $MgCl_2$ 1,2; phosphate buffer saline (PBS) 10, glucose 10, pH 7,4 (standard medium). After the loading period, cells were washed twice before the experiment was performed. The fluorescence was recorded at 495 nm Ex and 530 nm Em in a SPEX Fluoromax spectrophotofluorimeter.

3.7. Statistical analysis

The results are presented as the mean ± S.D. of three experiments. One-way ANOVA with Turkey's post test was performed using GraphPad InStat Version 3.00 for Windows 95 (GraphPad Software, San Diego California USA).

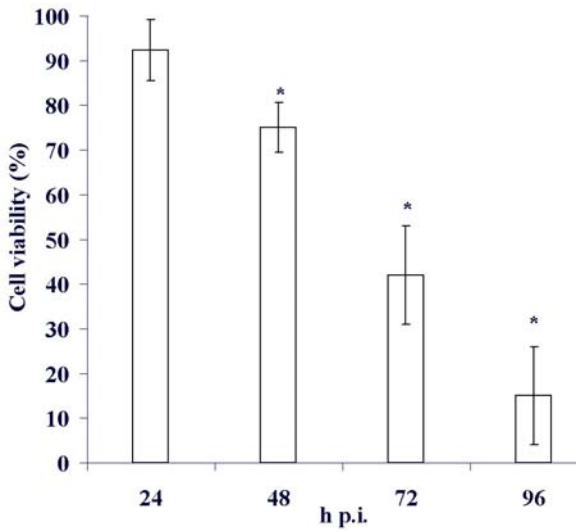

Figure 1. BHV-4/FI induces cell death in MDBK cells. MDBK cells in 96-well plates were either mock-infected or infected at m.o.i. 10. Viable, adherent cells were stained with MTT at 24 h, 48 h, 72 h or 96 h post-infection and the absorbance assayed as described in the Methods section. Data are presented as a percentage of the control, and results are expressed as the mean ± SD of three experiments performed in duplicate.*=P< 0.05 versus control

4. RESULTS

Infection of MDBK cells with the BHV-4/FI strain resulted in cell death in a time-dependent manner. In this cell line, the cytopathic effect appeared at 48 hours post infection and reached a massive effect at 72 – 96 hours p.i. (Figure 1).

The BHV-4/FI-induced cell death occurred via apoptosis, as detected by subgenomic DNA ('sub-Gl peak') analysed by PI staining of the cell nuclei and FACS analysis (Figure 2). Results showed an increase in the percentage of apoptotic cells following BHV-4 infection, when compared with control cells, which were uninfected.

Figure 3 summarizes the results of three independent experiments in which apoptosis induced by BHV-4/FI at various multiplicity of infection and different times post infection (p.i.) was evaluated by propidium iodide assay. Low levels of apoptosis were detected at 24 h p.i., while at 48 h p.i. cultures infected at all m.o.i. tested showed a high percentage of characteristic apoptotic cells in a dose-dependent fashion. Induction of apoptosis was invariably detected following infection and was dependent on the dose of virus inoculum and on the length of time of culture following infection (Figure 3).

Figure 4 shows the effect of selected antioxidants (BHA, Ebselen and NAC) on BHV-4/FI induced apoptosis at different hours p.i.. BHV-4/FI-induced apoptosis was significantly inhibited by the antioxidants BHA and Ebselen in a time-dependent manner, while *N*-acetylcysteine (NAC), another well known antioxidant, did not prevent BHV-4/FI-induced apoptosis (Fig 4). Maximal inhibition was seen at 100 microM BHA, whereas concentrations higher than 200 microM were slightly toxic to the cells (Fig 5). Ebselen at 5 microM inhibited BHV-4/FI-induced apoptosis but at higher concentrations the compound itself was toxic to MDBK cells (Figure 5).

In fact, we found that, after 24 h of incubation, both Butylated hydroxyanisole (BHA) and N-acetyl cysteine, did not modify, significantly, the cells' viability. Ebselen, a mimic of cellular enzyme glutathione peroxidase (26), showed a toxic effect on uninfected cells (Figure 5).

The induction of apoptosis was associated with significant levels of viral production as assayed by detecting plaque formation after 7 days p.i. (Fig.6). Additionally, BHA did not significantly inhibit the production of infectious virus from BHV-4/FI-infected cells as determined by titrations of cell culture supernatants (Figure 6).

To analyze whether BHV-4/FI-induced apoptosis was accompanied by increased production of ROS, we loaded MDBK cells with DCFH-DA. This dye is trapped intracellularly after deacetylation, and DCF green fluorescence was analyzed spectrophotofluorimetrically (Figure 7). The analysis showed that, at 24-36 h post-infection, BHV-4/FI induced a massive increase in cellular oxidative stress. BHA and Ebselen completely prevented the increase in intracellular oxidative stress, whereas *N*-acetylcysteine (NAC) did not prevent BHV-4/FI-induced ROS production.

5. DISCUSSION

Induction of apoptosis represents a method by which viruses can induce host cell death while limiting inflammatory and other immune responses. A number of studies, in fact, have demonstrated that viruses belonging to a variety of families, particulary those characterized by persistence, should harbor, in their genomes, genes that are able to counteract apoptotic mechanisms (22, 27-33). BHV-4 as well as other gamma-2-herpesviruses have been shown to posses such antiapoptotic machinery (34-39). Results reported in the present study confirm, according to the results of Sciortino et al. (23), that BHV-4 is able to induce apoptosis in permissive cells lines and, therefore, BHV-4 encodes for anti-apoptotic as well as for pro-apoptotic genes.

Moreover, in this study, we report that BHV-4 induces oxidative stress 24 -36 h p.i. of MDBK cells and use of selected antioxidants, which interfere with oxidative stress, is able to prevent BHV-4 induced apoptosis.

Since the protection from cell death was not due to inhibition of virus replication or formation of infectious progeny, our results indicate that cell death and replication of BHV-4 can be dissociated. This is in contrast with Levine et al. (40) where the antiapoptotic protein Bcl-2 was shown to inhibit Sindbis virus-induced host mortality by inhibiting virus replication rather than by effect on apoptosis.

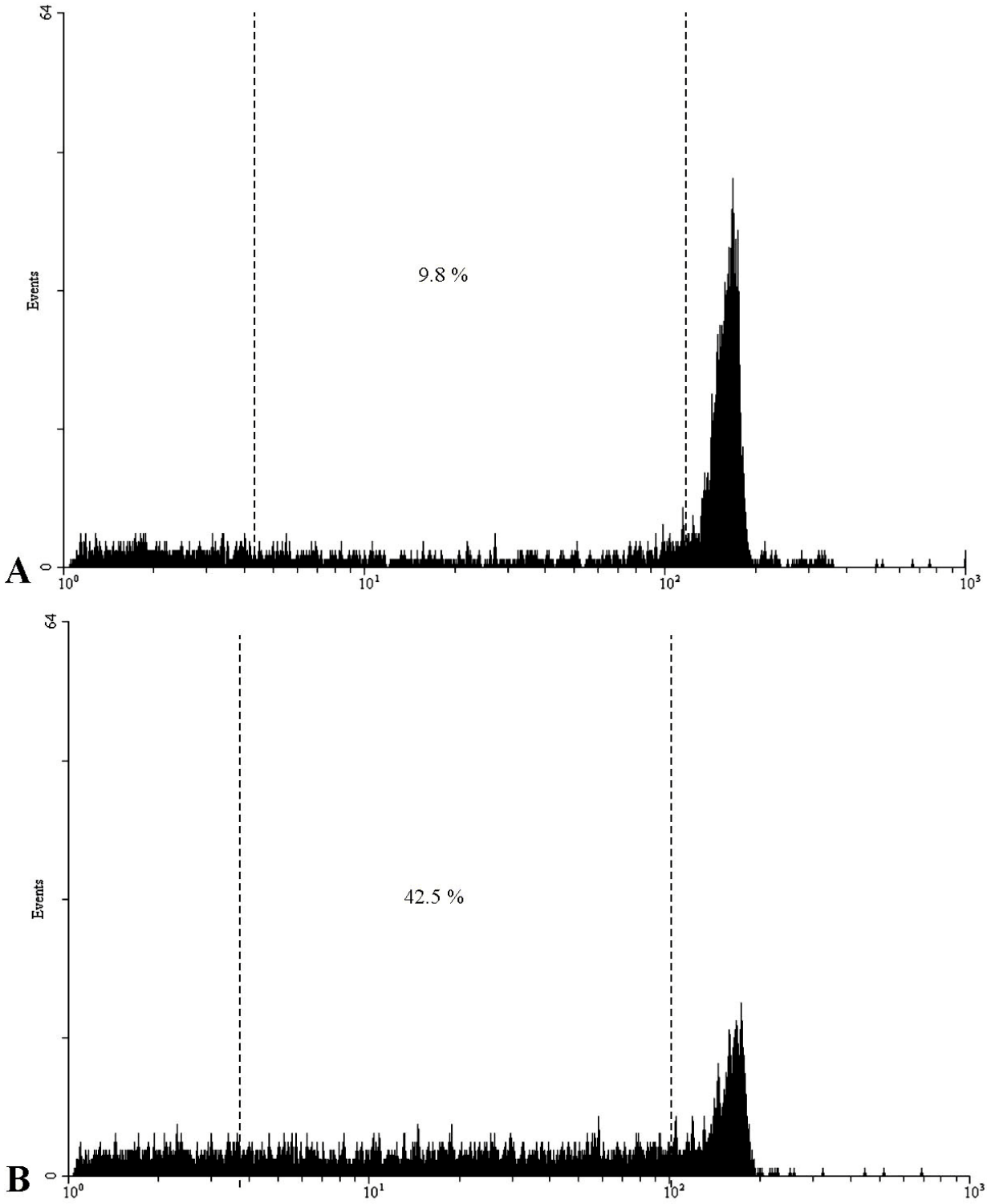

Figure 2. Induction of apoptosis by BHV-4 infection in MDBK cells, detected by flow cytometry. DNA fragmentation in mock (A) or BHV-4/FI (B) infected MDBK cells (m.o.i. 10) at 48 h post-infection was analyzed by PI staining of nuclei followed by FACS analysis. The lines indicate the boundaries among the peaks of hypodiploid nuclei and presumably of debris, which were arbitrarily set on untreated samples and maintained for all other samples. The numbers in cytograms represent the percentages of hypodiploid nuclei, based on a total number of nuclei from which debris was excluded. One representative experiment out of three is shown.

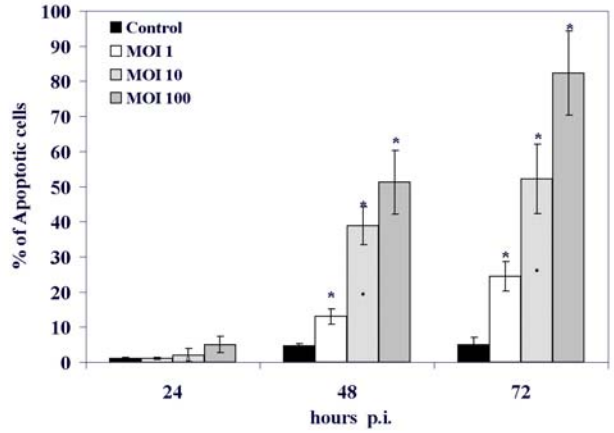

Figure 3. Kinetic and dose dependency of BHV-4/FI-induced apoptosis in MDBK cells. Apoptosis induced by BHV-4/FI at indicated m.o.i. was evaluated by flow cytometry after 24, 48 and 72 hours post-infection. Results are expressed as the mean ± SD of three experiments performed in duplicate. *=P< 0.05 versus control.

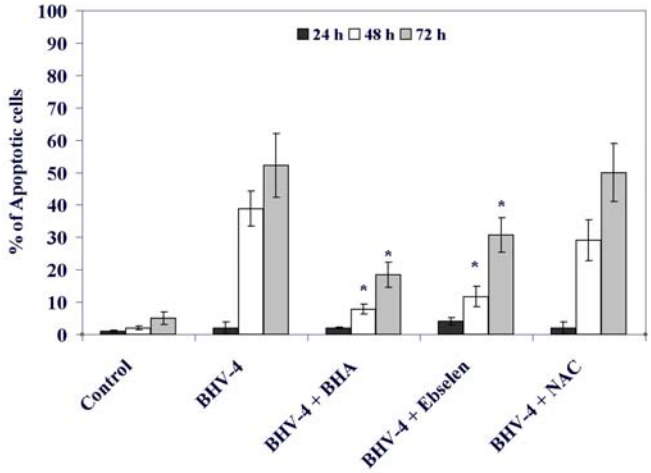

Figure 4. Effect of selected antioxidants on BHV-4/FI-induced apoptosis. Cells were infected with BHV-4/FI and incubated in the presence or absence of BHA (100 microM), Ebselen (5 microM) or NAC (100 microM). Apoptotic nuclei were quantified by FACS analysis of PI-stained nuclei at 24, 48 and 72 hours p.i. as described in the Methods section. Results are expressed as the mean ± SD of three experiments performed in duplicate. *=P< 0.05 versus BHV-4/FI-infected cells

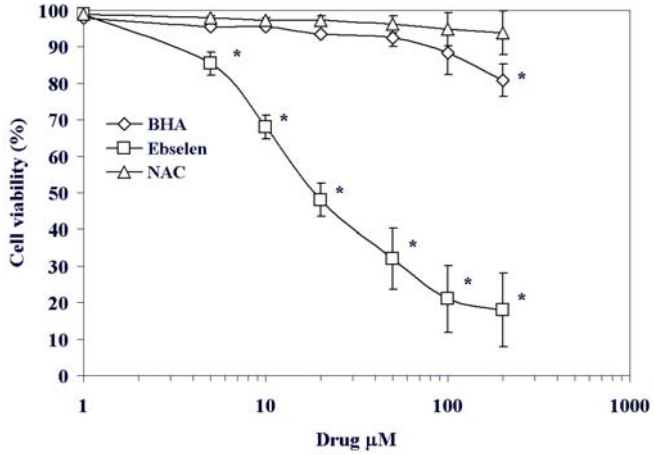

Figure 5. Effect of selected antioxidants (BHA, Ebselen and NAC) in uninfected MDBK cells on cell viability. Viable, adherent cells were stained with MTT at 24 h of incubation and the absorbance assayed as described in the Methods section. Data are presented as a percentage of the control, and results are expressed as the mean ± SD of three experiments performed in duplicate. *=P< 0.05 versus control

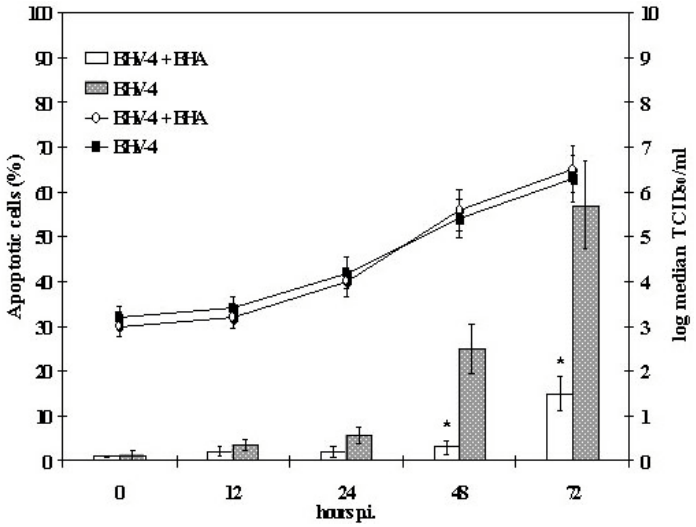

Figure 6. Time course of virus production and apoptosis in MDBK cells infected with BHV-4/FI (m.o.i. 10) treated or untreated with BHA (100 microM). Bars represent the percentage of apoptotic cells (axis on the left) while lines represent the virus titre (axis on the right). Results are expressed as the mean ± SD of three experiments performed in duplicate.*=P<0.05 versus untreated BHV-4/FI-infected cells.

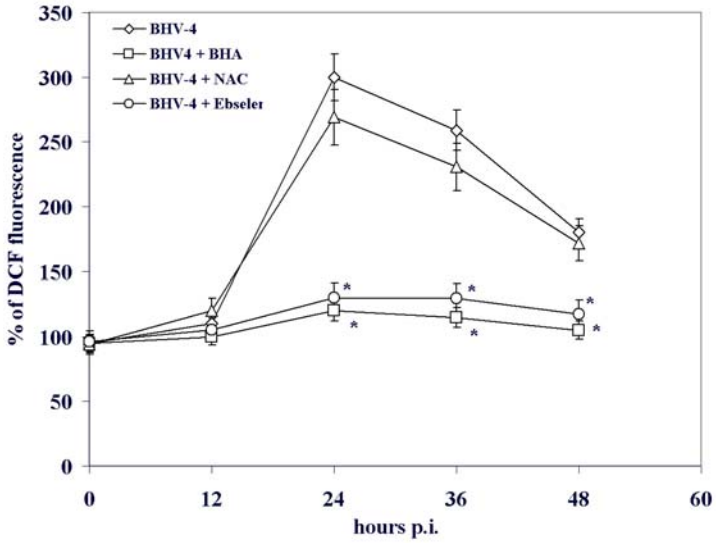

Figure 7. Intracellular production of ROS is an early event in BHV-4-induced apoptosis. Mock or BHV-4/FI infected MDBK cells were incubated in medium in the presence or absence of BHA (100 microM), Ebselen (5 microM) or NAC (100 microM). The intracellular redox state was measured by quantification of DCF fluorescence by spectrophotofluorimetry as described in the Methods. Data are presented as a percentage of the control (100%) and results are the mean ± SD of three experiments performed in duplicate. *=P< 0.05 versus BHV-4/FI-infected cells.

Oxidative stress has been suggested to be a mediator of apoptosis induced by a variety of triggers, including virus infections (3, 41, 7). This was frequently shown by the addition of ROS or by the antiapoptotic effect of certain antioxidants. In our experiments, only BHA (42) protects cell from BHV-4-induced apoptosis. Other antioxidants, which are known to inhibit cell death in other cells, e.g. NAC (24) failed to protect them. Ebselen, protected cells from BHV-4-induced apoptosis but was toxic to MDBK cells. Differences in the effects of chemically diverse antioxidants have previously been observed in other cell types. NAC in low millimolar concentration was shown to induce apoptosis in the uman CEM-T-cell line (43), or dithiocarbamates such as PDTC were reported to trigger apoptosis in thymocytes (44). Moreover, only NAC and PDTC inhibited influenza virus (H3N2 subtype)-induced cell death in J774.1 macrophages (45), whereas other antioxidants failed to protect the cells. However, neither NAC nor PDTC protected MDCK cells from apoptosis induced by influenza virus of the H5N9 subtype (46). NAC is also able to provide a strong inhibitory effect on EIV-induced apoptosis (47).

Our data show that antioxidants that failed to protect against BHV-4-induced cell death also failed to change the intracellular redox status as indicated by DCF fluorescence. This suggests that the protective effect of BHA was due to the direct antioxidant action rather than to interference with other pathways.

Butylated hydroxytoluene (BHT, an antioxidant homologue to BHA) was reported to reduce the infectivity of Newcastle disease virus *in vitro* and *in vivo* (48), probably by a direct effect on the lipid-containing virus. In contrast, BHA did not interact directly with BHV-4, since preincubation of the virus with BHA for 1 h at 37 °C did not reduce virus infectivity (data not shown).

Oxidative stress is defined as any imbalance between oxidants and antioxidants in favour of the oxidants (49). The change in DCF fluorescence, which is indicative of oxidative stress, may thus be due to increased production of ROS, to a decreased capacity of antioxidant defence mechanims, or to both.

The inhibition of cellular protein synthesis observed during infection with viruses may also contribute to oxidative stress. Thus, it seems conceivable that the overall inhibition of cellular protein synthesis may reduce the steady level of antioxidant enzymes, such as superoxide dismutases or glutathione peroxidases. Moreover, additional mechanisms are known to contribute to a decreased antioxidant capacity, e.g. the extrusion of cytosolic glutathione (50, 51) or the down regulation of the transcription of the antioxidant enzyme manganese superoxide dismutase (52).

Although our data proved that BHV-4 increases cellular oxidative stress, it cannot elucidate the exact mechanisms responsible for the increased level of ROS in BHV-4-infected cells. This issue is currently under investigation in our laboratory.

In conclusion, our results demonstrate a virus inoculum-dependent and time-dependent induction of apoptosis in cells productively infected by BHV-4 *in vitro*. A ROS-dependent pathway mediates this induction of apoptosis. Antioxidants prevented BHV-4-induced apoptosis but did not affect BHV-4 replication. These results suggest that oxidative stress may be a crucial event in the sequence leading to apoptotic cell death and confirm that apoptosis is not essential in BHV-4 productive infection.

Finally, the availability of the experimental model of apoptotic BHV-4 infection *in vitro* could also be useful for future identification of viral genes involved in the control of apoptosis by other gamma-2-herpesviruses for which productive infection *in vitro* represents a serious problem.

6. ACKNOWLEDGMENTS

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