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Interference of Bovine Herpesvirus 1 (BHV-1) in Sorbitol-Induced Apoptosis

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Abstract In order to determine the ability of bovine herpesvirus type 1 (BHV-1) to suppress apoptosis, we examined the effects of BHV-1 infection on sorbitol-induced apoptosis on Madin–Darby bovine kidney (MDBK) cells. BHV-1 suppresses sorbitol-induced apoptosis in a manner similar to that of herpes simplex virus type 1 (HSV-1), indicating that BHV-1 has one or more anti-apoptotic genes. To elucidate the molecular mechanisms of apoptosis, expression of some genes encoding apoptosis-inhibiting and -promoting factors were analyzed on BHV-1 infected cells during the process of sorbitol-induced apoptosis. Our results revealed that the expression of bcl-2 and bcl-x_L decreased after 5 and 3 h p.i., respectively; while bax and procaspase-3 expression increased with respect to control as a function of p.i. times and at 7 h p.i. they were not observed. We further show that the expression of *p53* gene was also enhanced, suggesting that this apoptotic mechanism is p53 dependent. From these results, we propose that BHV-1 has one or more genes encoding apoptosis-inhibiting factors which interfere with the involvement of *bcl-2* gene family members and apoptotic pathway, depending upon caspase-3, triggered by sorbitol. J. Cell. Biochem. 89: 373–380, 2003.

Key words: BHV-1; sorbitol; apoptosis; p53; Bcl-2; Bax; Bcl-X_L

Bovine herpesvirus type 1 (BHV-1), a member of the Alphaherpesvirinae subfamily, is an economically important pathogen of cattle. BHV-1 is associated with a variety of clinical diseases including rhinotracheitis, infectious pustular vulvovaginitis, conjunctivitis, abortion, enteritis, encephalitis, and generalized systemic infections in the newborn [Gibbs and Rweyemamu, 1977; Kahrs, 1977; Ludwig, 1983; Wyler et al., 1989]. This virus, being able to modify the upper respiratory tract environment and cause immunomodulation, can be considered as one of the most responsible for the bovine respiratory disease complex [Yates, 1982]. The pathogenic profile of viral infection includes the development of an inflammatory response, the cytolytic action of natural killer cells, the antiviral activities of cytokines, and apoptosis.

Apoptosis or programmed cell death is an energy-dependent process of cell suicide in response to a variety of stimuli and is characterized by a number of distinct morphological features and biochemical processes including cell shrinkage and partial detachment from substratum, plasma membrane blebbing, chromatin condensation, and intra-nucleosomal cleavage and ultimately, cell fragmentation into apoptotic bodies which are phagocytosed without provoking an inflammatory response. The molecular mechanism of such changes is not completely clear. It is believed that an endogenous Ca^{2+} and Mg^{2+}-dependent endonuclease

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able to break double strand DNA at internucleosomal sites, is responsible for DNA fragments of oligonucleosomal size (180–200 bp) [Vaux et al., 1994].

During the last several years, apoptosis has become widely accepted as an important nonspecific antiviral host response. Premature death of an infected cell would have obvious deleterious consequences for the production of viral progeny, and, therefore, viruses and their gene products have been considered exquisitely useful tools in the molecular dissection of apoptotic pathways during infection [Razvi and Welsh, 1995].

The large DNA-containing viruses have proven to be a rich source of genes with anti-apoptotic activity. For example, it is well documented that wild-type herpes simplex virus 1 (HSV-1) blocks apoptosis induced by thermal [Leopardi and Roizman, 1996] or osmotic shock [Koyama and Miwa, 1997]. This has been considered to be an outcome of expression of a viral anti-apoptotic gene whose product inhibits one of the steps in the virus-induced signaling leading to apoptosis [Koyama and Miwa, 1997]. Although the molecular mechanism by which apoptosis is induced by sorbitol remains obscure, this method results to be very useful to detect anti-apoptotic activity of viruses [Koyama et al., 2000]. In any case, it seems that the number of hydroxyl groups present in the sorbitol molecule with respect to the high osmotic pressure plays a major role to induce apoptosis [Koyama et al., 2000].

Several checkpoints exist in the pathways leading to sorbitol-induced apoptosis and many evidences indicate that a group of caspases, as well caspase-3 which has been found to be activated in several models of apoptosis [Porter and Janicke, 1999] are involved. A central role, as gene encoding apoptosis-promoting factors, is also attributed to wild-type p53 whose overexpression can result in rapid loss of cell viability, in a manner characteristic of apoptosis [Gottlieb and Oren, 1998].

p53-induced apoptosis can depend on the transcriptional regulation of apoptosis-related genes but can also occur independent of transactivation. Among the numerous p53-inducible gene products, several have well-defined role in apoptosis, such as Bax [Oltvai et al., 1993], however, none of them can be regarded as a single critical effector of p53 dependent cell death. In principle, activation of the apoptotic program also may rely on or, at least, be supported by the inhibition of anti-apoptotic mechanisms.

To get deeper insight into the molecular mechanisms involved in the sorbitol-induced apoptosis on Madin–Darby bovine kidney (MDBK) cells infected with BHV-1 at different times of post-infection, measurements of genomic DNA fragmentation, and evaluations of expression levels of p53, bax, bcl-2, bcl-x_L, and caspase-3 m-RNAs were carried out.

MATERIALS AND METHODS

Virus

The BHV-1 Cooper strain was kindly provided by Prof. Castrucci (University of Perugia, Italy). Virus stocks were routinely grown on MDBK cells (American Type Culture Collection, Manassas, Va, USA) and were also used for determination of virus titres. The virus was purified as described by Lyaku et al. [1992], resuspended in Dulbecco's modified Eagle's medium (DMEM) (Eurobio, Les Ulis Cedx B, France) supplemented with 2 mM L-glutamine (Bio-Whittaker, Rockland, ME, USA), and stored in liquid nitrogen until use.

Cells and Culture Conditions

MDBK cells were cultured in DMEM supplemented with 2 mM L-glutamine, 5% prescreened and heat-inactivated foetal calf serum (FCS) (Eurobio), 100 IU of penicillin, and 100 μg of streptomycin per ml (both antibiotics from Bio-Whittaker), in a 5% CO_2 incubator. This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus.

In the first series of experiments, MDBK cells (5×10^6 per flask) were seeded in 25-cm^2 culture flasks (Corning Costar Corp., Wiesbaden Germany) and after 24 h, the monolayers were incubated with different concentrations of sorbitol in the range of 0.1–1.2 M for 1 h at 37°C, briefly washed with DMEM, and then reincubated for an additional 3 h at 37°C in DMEM containing 2% FCS. Only the concentration of 1.0 M of sorbitol was selected to induce osmotic shock after the infection with BHV-1. The cells not treated with sorbitol, either mock infected with medium alone or infected with BHV-1, were considered controls.

Monolayers of MDBK cells were either mock infected with medium alone or infected with

BHV-1 Cooper at a multiplicity of infection of 10 TICD50/cell in DMEM without serum. Virus inoculum was allowed to proceed for 1 h at room temperature. After removal of the inoculum, the monolayers were overlaid with pre-warmed medium (DMEM containing 2 mM L-glutamine and 2% FCS) and incubated at 37°C in a 5% CO_2 incubator. At the end of various incubation times, one group of monolayers was incubated in medium containing 1 M sorbitol for 1 h at 37°C, rinsed once in DMEM, and then reincubated for an additional 2 h.

In some experiments, in order to investigate the effects of apoptosis on the multiplication of BHV-1, virus titre at different hours post-infection was evaluated. The yields of infectious virus were determined in MDBK cells infected with BHV-1 at a multiplicity of infection (moi) of 10 for 1 h both treated and not treated with 1 mM sorbitol at 37°C for 1 h. At different hours post-infection, from 0 to 48 h p.i., cell extracts, obtained by three cycles of freezing and thawing were evaluated by $TCID_{50}$ method.

EVALUATION OF APOPTOSIS

DNA Fragmentation Assay

Cells were washed twice with phosphate-buffered saline (PBS) and lysed by addition of a hypotonic solution (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl pH 7.5) [Herrmann et al., 1994]. After centrifugation for 5 min at 1,600*g*, the supernatant was collected and the extraction was repeated with the same lysis buffer. The supernatants were brought to 1% SDS and treated with RNase A (final concentration 5 μg/μl) for 2 h at 56°C followed by digestion with proteinase K (final concentration 2.5 μg/μl) at 45°C for at least 6 h. Before hydrolysis, a further extraction of DNA was performed with phenol-chloroform, followed by three successive ethanol precipitations in 2 M ammonium acetate.

Pellets were dried for 30 min and resuspended in 200 μl Tris-EDTA pH 8.0. Aliquots of 20 μl containing 10 μg DNA were electrophoresed in 1.5% agarose gel with 0.5 × TBE running buffer (0.05 M Tris base, 0.05 M boric acid, and 1 M dissodium EDTA) for 4 h at 80 V. Gels were stained with 0.5 μg/ml ethidium bromide (Molecular Probes, Eugene, OR, USA) and photographed under UV transillumination with a Polaroid camera. Sizes of DNA fragments in the samples were compared with standard size fragments in a 1 kb DNA ladder (Invitrogen, Milan, Italy).

Morphological Analysis

At different times following infection or of incubation, 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 for morphological analysis following staining with acridine orange according to Mastino et al. [1997]. Briefly, a minimum of 600 cells, including those showing apoptotic characteristics, were counted using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation. The percentage of apoptotic cells was calculated as follows: % apoptosis = Total no. of cells with apoptotic nuclei/Total no. of cells counted × 100.

RNA Isolation and cDNA Preparation

The total RNA was isolated from BHV-1 infected and mock-infected MDBK cells, both pretreated or not treated with sorbitol, with Trizol LS reagent (Invitrogen) according to the manufacturer's instructions. The total RNA pellet was dissolved in 100 μl of nuclease-free water. RNA was reverse transcribed by using a random hexanucleotide primer (Boehringer, Mannheim, Germany) with Superscript II Reverse Transcriptase from Invitrogen according to the manufacturer's instructions. Briefly, 1 μg of total RNA was transcribed with reverse transcriptase at 37°C for 60 min with the final stage at 90°C for 5 min.

Semi-Quantitative RT-PCR Analysis

Primer pairs sequences were designed on the basis of published gene sequences as reported in Table I. One microliter of the RT reaction products was amplified by PCR in the presence of final concentration of 0.2 μM primers (sense and antisense mixture 1:1), 200 μM deoxynucleotide triphosphates (dNTPs) mixture (Sigma-Aldrich, Milan, Italy), and 0.5 U Taq DNA polymerase (Sigma). PCR reactions were conducted in a Stratagene RoboCycler Gradient 40 temperature cycler fitted with a Hot Top Assembly, using 500 μl thin-walled PCR tubes.

TABLE I. Primer Sequences Used in RT-PCR Analysis

Target gene	Oligonucleotide sequence
p53	5′ CCGAGGCCGGCTCTGAGTATACCACCATCC 3′ (+)
	5′ CTCATTCAGCTCCCGGAACATCTCGAAGCG 3′ (−)
Bax	5′ CTTTTGCTTCAGGGTTTCAT 3′ (+)
	5′ AAGTAAAAAGGGCCGCAAC 3′ (−)
Procaspase-3	5′ TTTGTTTGTGTGCTTCTGAGCC 3′ (+)
	5′ ATTCTGTTGCCACCTTTCGG 3′ (−)
bcl-2	5′ GTCGCTACCGTCGTGACTT 3′ (+)
	5′ CAGCCTCCGTTATCCTGGA 3′ (−)
bcl-X_L	5′ ATGGCAGCAGTAAAGCAAGCGC 3′ (+)
	5′ TTCTCCTGGTGGCAATGGCG 3′ (−)
β-actin	5′ GTGGGCCGCTCTAGGCACCAA 3′ (+)
	5′ CTCTTTGATGTCACGCACGATTTC 3′ (−)

The program for amplification consisted of 1 cycle at 95°C for 1 min, followed by 1 min of denaturation at 94°C, 1 min of annealing different temperature, and 2 min of extension at 72°C for 20 to 35 cycles in the linear range of the amplification cycle number, which was determined empirically in each case, and an additional incubation of 10 min at 72°C for final extension. PCR products were analyzed by electrophoresis on 1.5% ethidium bromide-containing agarose gels and visualized and quantitated under UV transillumination of apparatus BioRad Gel Doc 1000 (BioRad, Rome, Italy) with Quantity One program.

Statistical Analysis

The results are presented as mean ± SD of three independent 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). An error probability with $P < 0.05$ was selected as significant.

RESULTS

DNA Fragmentation

In preliminary studies, we established the effects of the dose and the time of incubation of sorbitol treatment in MDBK cells on induction of apoptosis. In these experiments, the criterion for induction of apoptosis was the fragmentation of chromosomal DNA resulting from random cleavage at intranucleosomal intervals evaluated by 1.5% agarose gel electrophoresis. Titrations of sorbitol in the range from 0.1 to 1.2 M, in MDBK cells incubated for 1 h at 37°C, indicated that concentrations from 0.6 to 1.2 M showed a major amount of fragmented chromosomal DNA than the ones treated at concentrations from 0.1 to 0.4 M sorbitol (data not shown). Furthermore, our results revealed that the time of incubation after sorbitol-treatment is a critical parameter; in fact, the typical ladder of apoptosis in agarose gel of cells treated with increasing concentrations of sorbitol appeared to have a major degree of DNA fragmentation after 2 h of incubation in fresh medium with respect to those incubated in fresh medium for less time (data not shown).

The effectiveness of BHV-1 infection in blocking apoptosis induced by sorbitol is shown in Figure 1. MDBK cells infected with BHV-1 and

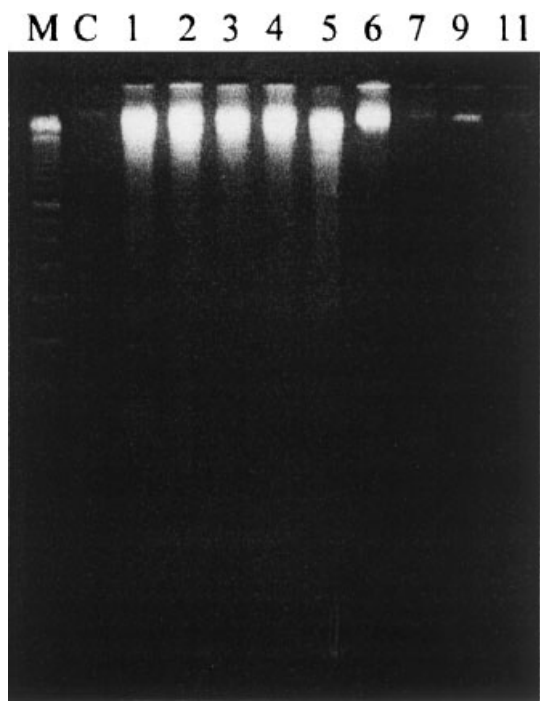

Fig. 1. Fragmentation of chromosomal DNA in the bovine herpesvirus 1 (BHV-1)-infected Madin–Darby bovine kidney (MDBK) cells during sorbitol treatment. At the indicated intervals, BHV-1-infected MDBK cells were treated with 1.0 M sorbitol for 60 min, followed by incubation in the reagent-free medium for 120 min. **Lane M** corresponds to molecular weight markers. One representative experiment of three is shown.

treated with sorbitol from 1 h upto 5 h p.i. showed a degree of DNA fragmentation similar to that of the non-infected cells treated with sorbitol alone. While the DNA fragmentation disappeared when the sorbitol treatment took place at 7 h p.i. At this time, the sorbitol was no more able to induce the formation of nucleosomal oligomers.

Results from morphological analysis confirmed DNA laddering result. As shown in Figure 2, a high percentage of cells, showing the apoptotic feature in the morphology, was observed in those treated with sorbitol immediately after virus infection. The percentage of apoptotic cells decreased upto a low value after 7-h post-infection. From this time point, the values are very similar to those of untreated-sorbitol infected cells in agreement with the results of DNA fragmentation analysis by agarose gel electrophoresis.

These results show that BHV-1 inhibits or slows down the cellular osmotic shock induced by sorbitol. Furthermore, the infection with BHV-1 alone did not induce apoptosis, at least upto 24 h p.i. whereas lysates from cells infected for 48 h showed extensive DNA fragmentation (Fig. 3).

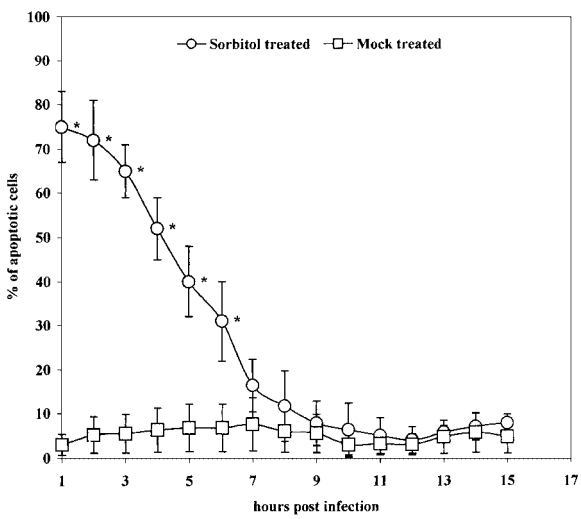

Fig. 2. Percentage of apoptotic cells in MDBK monolayers infected (□) with BHV-1 Cooper strain (moi 10) or mock-infected (O) and treated with 1.0 M sorbitol at different hours p.i. followed by incubation in the reagent-free medium for 120 min. Apoptosis was evaluated using fluorescence microscopy by calculating the percentage of cells showing nuclear morphology of apoptosis after staining with acridine orange. Results are expressed as mean ± SD of three independent experiments. $^{*}P < 0.05$.

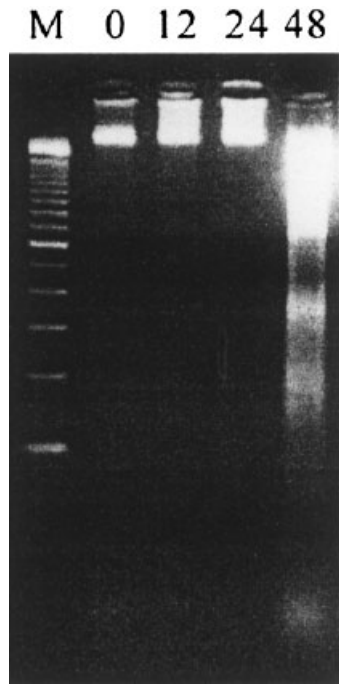

Fig. 3. Fragmentation of chromosomal DNA in the BHV-1-infected MDBK cells. MDBK cells were infected with BHV-1 at an moi of 10. At the indicated time, the infected cells were harvested and fragmented DNA was extracted. **Lane M** corresponds to molecular weight markers. The results represent one of three separate experiments, which gave similar results.

Expression of Sorbitol-Induced Apoptosis-Related Genes in MDBK Cells

To identify the genes involved in sorbitol-induced apoptosis in MDBK cells, we investigated the mRNA expression of some genes encoding apoptosis-promoting and -inhibiting factors by a semi-quantitative RT-PCR method (Fig. 4). After 1 h of incubation with 1 M sorbitol and 2 h of incubation in fresh medium, from MDBK monolayers extraction of RNA was performed. Expression of *p53*, *bax*, and *procaspase-3* genes resulted to be augmented with respect to those of control cells, and precisely the mRNAs of treated cells were upregulated about twofold, as determined by Quantity One program of Gel doc (BioRad).

In contrast, no differences in bcl-2 and bcl-x_L expression between sorbitol-treated or -untreated MDBK cells were observed. As an internal control, a β-actin DNA band was distributed at similar levels in both samples.

Expression of Sorbitol-Induced Apoptosis-Related Genes in BHV-1-Infected MDBK Cells

To identify the genes involved in sorbitol-induced apoptosis in MDBK cells infected with

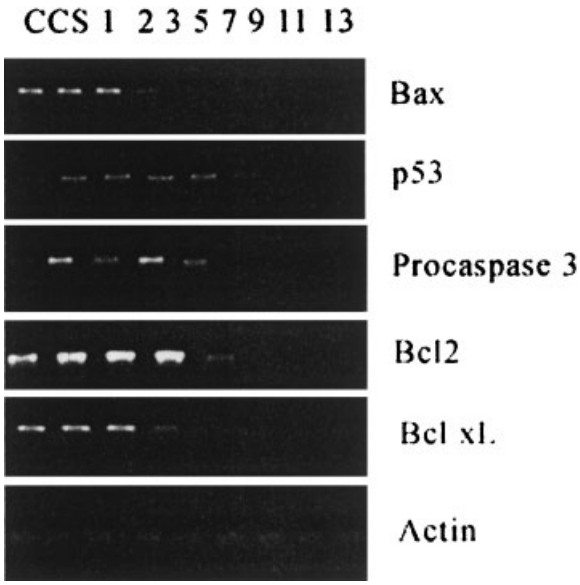

Fig. 4. Effect of sorbitol on the steady state levels of p53, bax, procaspase 3, bcl 2, bcl X_L mRNA in MDBK cells at different times of BHV-1 infection. Cultures were incubated either with culture medium (C, control) or 1.0 M sorbitol for 1 h (CS), or treated with sorbitol at the indicated intervals of BHV-1 infection (1, 3, 5, 7, 9, 11, and 13 h p.i.). The cultures were subjected to RNA extraction and RT-PCR amplification. Reaction products were run on 1.5% agarose gel and β-actin was the positive transcription control. This figure is representative of three identical experiments, which gave similar results.

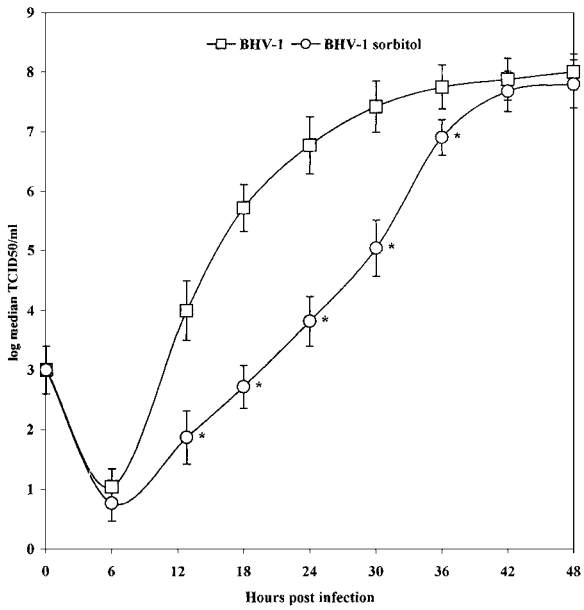

Fig. 5. One step growth curve of BHV-1 in sorbitol treated (O) or mock treated (□) cells. MDBK cells, preincubated in medium containing 1 M sorbitol for 1 h at 37°C, were infected with BHV-1 Cooper strain at a multiplicity of infection of 10, at the indicated intervals the amounts of progeny virus were determined by $TCID_{50}$ method. Results are expressed as mean ± SD of three independent experiments.

BHV-1, we investigated the kinetics of mRNA expression of some genes encoding apoptosis-promoting and -inhibiting factors by a semi-quantitative RT-PCR (Fig. 4).

Expression of the *p53* gene was augmented in infected cells upto 5 h p.i., then decreased remarkably, and did not recover by 9 h p.i. Expression of the *bax* and *procaspase-3* genes increased showing a peak after 3 h of p.i., and then decreased until complete disappearance at 7 h p.i. Expression of bcl-2 and bcl-x_L did not change remarkably with respect to control cells until 5 and 3 h p.i., respectively, when they were barely detectable. β-actin DNA band was distributed at similar levels in all samples.

Multiplication of BHV-1 in Apoptotic Cells

To understand the role of the anti-apototic activity in the life cycle of the virus, the effect of apoptosis on the multiplication of BHV-1 was examined. Figure 5 shows one-step growth curve of the virus in sorbitol-treated and untreated cells. In treated cells, the virus could grow but with a much longer eclipse period. From our results, it could be postulated that virus–cell molecule interactions during the attachment or penetration processes were not affected by pre-treatment with 1 M sorbitol. Instead, a remarkable difference is seen in the interval time from 12 to 30 h, when the multiplication of BHV-1 in the sorbitol-pretreated cells resulted reduced with respect to the control. These results indicate that the multiplication of BHV-1 is sensitive to apoptotic process but not completely abolished.

DISCUSSION

In the present study, we have investigated the expression of some representative genes encoding apoptosis-promoting and -inhibiting factors during the course of sorbitol-induced apoptosis in MDBK cells infected with BHV-1. The premise underlying this study is that the ability of virus to block sorbitol-induced apoptosis indicates that the viral gene product(s) acts on the cellular products involved in the signaling pathway leading to apoptosis. In fact, the suppression of the DNA fragmentation in MDBK cells infected with BHV-1 and then treated with sorbitol indicates that viral function(s) requires expression of a BHV-1 anti-apoptotic gene(s).

The lytic cycle of BHV-1 infection exhibits, as in other herpesviruses, three temporal phases termed immediate-early, early, and late [Wirth et al., 1989]. Following interaction of the virus with host cell receptors, there is a very rapid penetration and initiation of viral replication with evidence of the viral antigen presence on the surface of infected cells within 3–4 h post-infection and the beginning of complete assembly and extracellular spread by 7 and 8 h, respectively [Ludwig and Letchworth, 1987].

Interestingly, our results show that the degree of chromosomal DNA fragmentation is absent when the cells were treated with sorbitol after 5 h p.i. This can be considered to be an outcome of expression of a BHV-1 anti-apoptotic gene/s and also as an early event in the viral replication cycle.

Several studies have shown that a variety of viruses have acquired or evolved a formidable battery of anti-apoptotic genes, which play a central role of apoptosis in the host antiviral response [Gillet and Brun, 1996].

Osmotic shock by exposure of cells to high concentrations of sorbitol has been demonstrated to induce apoptosis by altering the structure of the plasma membrane such that membrane-embedded proteins cluster and activate their signaling pathways [Rosette and Karin, 1996]. It has already been reported that the exposure of cells to hyper-osmolar media causes a marked increase in complex protein kinase activity, whose mechanism is yet an object of study. However, it is known that the osmotic shock induced by sorbitol activates the stress-activated protein kinases c-Jun N-terminal kinases (JNKs) and p38 protein kinase as well as the extracellular signal-regulated kinases (ERKs). Furthermore, it stimulates the accumulation of cytoplasmic cytochrome c and the cleavage of procaspase-3 [Bilney and Murray, 1998].

Many cysteine protease enzymes known as caspases are very important in the apoptotic pathway and proteolytically activated caspase-3 has been found to be necessary to trigger apoptosis by cleavage of several substrates, among which DNA fragmentation factor has been demonstrated to play a fundamental role [Wong and Choi, 1997].

We hypothesize that the anti-apoptotic effect of BHV-1 is likely mediated through regulation of genes involved in apoptosis and/or cell survival at the transcriptional level, and it might interfere in the pathway triggered by the sorbitol. Nevertheless, the explanation of this effect could of course also turn upon a signal-mediated control of post-transcriptional steps in gene expression, that is to say that a specific signal could be transduced into an alteration in the physiological state of the recipient cell. Furthermore, it is also possible that the viral gene products blocking apoptosis might interact either with the elements of the apoptosis induced-signaling pathway or with the factors for the cellular elements that normally block the same pathway. In any case, in our experimental conditions, the mechanism clearly results to be p53-dependent. p53 is one of the representative apoptosis-promoting factors and, as already mentioned in the results, the levels of expression of this gene appears to be affected during the course of anti-apoptotic effect of BHV-1 in our model of sorbitol-induced apoptosis, as well those of bax and procaspase-3 mRNAs. All these factors revealed an increased expression with respect to that of control during the first 5 h p.i. Moreover, at the same time, also the expression of bcl-2 and bcl-x_L mRNAs (apoptosis-inhibiting factors) appears to be modified. In fact, these expression levels clearly decreased after 5 and 3 h p.i., respectively.

It is widely accepted that the bcl-2 family is involved in the inhibition of apoptosis [Adams and Cory, 1998], so this result showing the absence of expression at 7 h p.i. of apoptosis-inhibiting and apoptosis-promoting factors indicates the connection to the anti-apoptotic effect of BHV-1.

In conclusion, our results show that the expression of some members of bcl-2 family in response to sorbitol-induced apoptosis is regulated by p53. A pro-apoptotic member, bax, was upregulated, whereas anti-apoptotic members, bcl-2 and bcl-x_L, were repressed in response to p53-induction, and that befitted well with a common principle according to which pro-apoptotic factors are upregulated and anti-apoptotic factors are repressed.

As described previously, the caspase-3 is involved in sorbitol-induced apoptosis [Teramachi and Izawa, 2000], which correlates well with our results showing that sorbitol can upregulate expression of *procaspase-3* gene. Similar results have also been observed in the okadaic acid-induced apoptosis [Wan-Cheng et al., 2001]. In addition, it is highly plausible that the BHV-1 potential, to protect MDBK cells from sorbitol-induced apoptosis, requires the expression of

some viral gene/s, which interferes with this process. Further work is required to elucidate the mechanism/s of this inhibition and to characterize the viral gene/s involved.

REFERENCES

Adams JM, Cory S. 1998. The bcl-2 protein family: Arbiters of cell survival. Science 281:1322–1326.

Bilney AJ, Murray AW. 1998. Pro- and anti-apoptotic effects of K^+ in HeLa cells. FEBS Lett 424:221–224.

Gibbs EPJ, Rweyemamu M. 1977. Bovine herpes viruses. Part I. Bovine herpesvirus 1. Vet Bull 47:317–343.

Gillet G, Brun G. 1996. Viral inhibition of apoptosis. Trends Microbiol 4:312–317.

Gottlieb TM, Oren M. 1998. p53 and apoptosis. Semin Cancer Biol 8:359–368.

Herrmann M, Lorenz HM, Voll R, Grunke M, Woith W, Kalden JR. 1994. A rapid and simple method for the isolation of apoptotic DNA fragments. Nucleic Acids Res 22:5506–5507.

Kahrs RF. 1977. Infectious bovine rhinotracheitis: A review and update. J Am Vet Med Assoc 171:1055–1064.

Koyama AH, Miwa Y. 1997. Suppression of apoptotic DNA fragmentation in herpes simplex virus type 1-infected cellr. J Virol 71:2567–2571.

Koyama AH, Arakawa T, Adachi A. 2000. Characterization of apoptosis induced by sorbitol: A unique system for the detection of antiapoptotic activities of viruses. Microbes Infect 2:599–606.

Leopardi R, Roizman B. 1996. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. Proc Natl Acad Sci USA 93:9583–9587.

Ludwig H. 1983. Bovine herpesviruses. In: Roizman B, editor. The herpesviruses. Vol. II. New York: Plenum Press. pp. 135–214.

Ludwig GV, Letchworth GJ. 1987. Temporal control of bovine herpesvirus 1 glycoprotein synthesis. J Virol 61: 3292–3298.

Lyaku JRS, Nettleton PF, Marsden HS. 1992. A comparison of serological relationships among ruminant alphaherpesviruses by ELISA. Arch Virol 124:333–341.

Mastino A, Sciortino MT, Medici MA, Perri D, Ammendolia MG, Grelli S, Amici C, Pernice A. 1997. Herpes simplex virus 2 causes apoptotic infection in monocytoid cells. Cell Death Differ 4:629–638.

Oltvai ZN, Milliman CL, Korsmeyer SJ. 1993. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609–619.

Porter AG, Janicke RU. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6(2):99–104.

Razvi ES, Welsh RM. 1995. Apoptosis in viral infections. Adv Virus Res 45:1–60.

Rosette C, Karin M. 1996. Ultraviolet light and osmotic stress: Activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 274:1194–1197.

Teramachi K, Izawa M. 2000. Rapid induction of apoptosis in human gastric cell lines by sorbitol. Apoptosis 5:181–187.

Vaux DL, Haecker G, Strasser A. 1994. An evolutionary perspective on apoptosis. Int Rev Cytol 68:251–305.

Wan-Cheng Li D, Xiang H, Mao YW, Wang J, Fass U, Zhang XY, Xu C. 2001. Caspase-3 is actively involved in okadaic acid-induced lens epithelial cell apoptosis. Exp Cell Res 266:279–291.

Wirth UV, Gunkel K, Engels M, Schwyzer M. 1989. Spatial and temporal distribution of bovine herpesvirus 1 transcripts. J Virol 63:4882–4889.

Wong B, Choi Y. 1997. Pathways leading to cell death in T cells. Curr Opin Immunol 9:358–364.

Wyler R, Engels M, Schwyzer M. 1989. Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1). In: Whittmann G, editor. Herpesvirus diseases of cattle, horses, and pigs. Developments in veterinary virology. Boston: Kluwer Academic Publishers. pp. 1–172.

Yates WDG. 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.