Susceptibility of sensory neurons to apoptosis following infection by bovine herpesvirus type 1

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Like other members of the alpha subfamily of herpesviruses, bovine herpesvirus type 1 (BHV-1) establishes latent infections in sensory neurons. BHV-1 induces apoptosis in lymphoid cells in vivo and in epithelial cell lines, but the ability of BHV-1 to induce apoptosis in sensory neurons remains unknown. In this report, the susceptibility of rabbit ganglionic neurons to infection by BHV-1 was examined in vitro and in vivo. Following infection of cultured neurons with BHV-1, hallmarks of apoptosis such as chromatin condensation, DNA fragmentation and membrane blebbing were detected. The appearance of these changes was preceded by active viral DNA replication as determined by in situ hybridization. When viral DNA replication was blocked by treatment of cultures with an inhibitor of eukaryotic DNA polymerases, apoptosis but not virus attachment to neurons or bICP0 gene expression was completely prevented. Taken together, these results demonstrate that sensory neurons are not intrinsically resistant to BHV-1-induced apoptosis and that viral DNA replication plays a role in triggering the apoptotic programme. Infection of rabbits with BHV-1 resulted in pathological changes in the trigeminal ganglia (TG) which included mononuclear cell infiltration and neuronophagia. Morphological evidence of apoptosis was not detected in neurons, even in cells with advanced cytopathology. Furthermore, whereas DNA fragmentation was common in infiltrating cells, it was very rare and sporadic in neurons. Therefore, mechanisms in the TG should exist to prevent neuronal apoptosis upon BHV-1 infection.

Introduction

Apoptosis, or programmed cell death, is a cell suicide programme characterized by chromatin condensation, DNA fragmentation, membrane asymmetry and membrane blebbing (Trump & Berezesky, 1998). Control of apoptosis by viruses may be critical to produce adequate levels of progeny virus, to spread virus in tissues or to facilitate virus persistence (reviewed by Shen & Shenk, 1995; Teodoro & Branton, 1997). Bovine herpesvirus type 1 (BHV-1), the aetiological agent of bovine infectious rhinotracheitis/vulvovaginitis (Snowdon, 1965), induces programmed cell death in bovine peripheral blood mononuclear cells (PMBCs) and CD4+ T cells in vivo, and in epithelial cells in vitro (Hannon et al., 1996, 1997, 1998; Esksra & Splitter, 1997; Winkler et al., 1999; Devireddy & Jones, 1999). The requirements for the induction of apoptosis seem to be cell-type specific. For example, both infectious and inactivated virus induce apoptosis in PMBCs, but only infectious BHV-1 is apoptotic in epithelial cells (Hannon et al., 1998; Devireddy & Jones, 1999).

Like herpes simplex virus 1 (HSV-1), the prototype member of the alpha subfamily of herpesviruses, BHV-1 establishes lifelong, non-productive latent infections in ganglionic sensory neurons (Homan & Easterday, 1980; Rock et al., 1987; for reviews see Rock, 1993, 1994; Jones, 1998). During the first days following infection by BHV-1 or by HSV-1, some neurons are productively infected (acute phase). However, other neurons are not permissive to virus replication and become latent infected. After the acute phase subsides, these cells continue to harbour viral DNA without producing infectious virus (latent phase). During latency, gene expression is restricted to a small region of the genome known as the latency-related (LR) (BHV-1) or latency-associated (HSV-1) region (Rock et al., 1987; Stevens et al., 1987). Expression of LR sequences in cycling cells resulted in cell cycle arrest (Schang et al., 1996) and in inhibition of apoptosis induced by sphingoid bases (Ciacci-Zanella et al., 1999). A role for the latency-associated region in neuronal survival has been proposed recently. Infection of rabbits with HSV-1 mutants which do not
express latency-associated transcripts (LATs) resulted in extensive neuronal apoptosis in the trigeminal ganglia (TG) (Perng et al., 2000). A significant loss of neurons was also observed after inoculation of mice with LAT null mutants. However, neuronal apoptosis was not detected in these animals (Thompson & Sawtell, 2001). These results open the question as to whether apoptosis or another neuronal death mechanism is the target for LATs.

In this study, the susceptibility of ganglionic neurons to infection by BHV-1 was examined. Infection of cultured ganglionic neurons with BHV-1 resulted in features of apoptosis such as DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. We found that BHV-1 induces neuronal apoptosis in a viral DNA replication-dependent manner. Infection of rabbits with BHV-1 induced mononuclear cell infiltration and neuronophagia in the TG. Apoptosis in the infected TG was mainly confined to infiltrating mononuclear cells, but no morphological evidence of apoptosis was detected in neurons. Furthermore, DNA cleavage in neurons was very limited and sporadic.

Methods

■ Virus and cells. The Cooper strain of BHV-1 was used in this study (National Veterinary Services Laboratories, Ames, Iowa, USA) (Jones et al., 1990). Viral stocks were prepared by infecting Madin–Darby bovine kidney cell (MDBK) monolayers at a m.o.i. of 0·1.

■ Nerve cell cultures. Sensory neurons from newborn rabbits were prepared as described previously (Jones et al., 1990). Briefly, dorsal root ganglia (DRG) were collected, incubated with 0·25% collagenase (Sigma) in Eagle’s essential medium (MEM, Sigma) for 3 h at 37 °C, and mechanically dissociated. Cells were washed twice with PBS (pH 7·3), resuspended in growth medium, and plated onto collagen-coated coverslips or chamber slides at a density of 5 × 10^3 to 10^4 neurons per well. The growth medium consisted of MEM containing 25 µg/ml gentamicin, 1·4 mM glutamine, 12 mg/ml glucose, 50 ng/ml nerve growth factor (Collaborative Research, Bedford, Mass., USA), and 10% foetal bovine serum. To eliminate non-neural cells, cultures were maintained in growth medium supplemented with 10 µM fluorodeoxyuridine (Sigma) and 10 µM cytosine arabinoside (Sigma) for 10 days. This treatment consistently yields cultures with more than 95% of cells being neurons as judged by cell morphology. Neuronal cultures were maintained at 37 °C with 5% CO2.

■ Ganglionic non-neural cell (NNC) cultures. DRG cell suspensions were obtained as described above and plated on plastic wells not covered with collagen. After 2 h incubation at 37 °C, 5% CO2, non-adherent cells were removed by gently pipetting the bottom of the well with fresh medium. Adherent cells were allowed to reach confluence, trypsinized, counted and split at a 1:3 ratio. This procedure eliminated virtually all neurons from the cultures.

■ Infection of cell cultures and virus titration. After antimitotic treatment, nerve cell cultures were washed three times with PBS and maintained for 2 days in growth medium. Neuron (5000 cells per well) or NNC cultures (25 000 cells per well) were infected with BHV-1 (m.o.i. 1:0) for 60 min at 37 °C. The cultures were rocked gently every 15 min to allow even spread of virus. After adsorption, the cultures were rinsed three times with PBS and once with growth medium, and refed with fresh growth medium. Virus was harvested at various days post-infection (p.i.) by three cycles of freeze–thawing the cells plus media. Viral titres were determined by standard plaque assays on MDBK cells. For each time-point, virus titres were the result of pooling three parallel cultures. For Fig. 1(a), the virus titres were normalized for the number of cells so that the given values are directly comparable.

■ Viral DNA replication block. To block viral DNA replication, neuronal cultures were treated with aphidicolin (APC, Sigma), a tetracyclic diterpenoid that binds the catalytic domain of herpesvirus DNA polymerase to form an inactive complex (Nishiyama et al., 1984). The drug was added to the growth medium at a final concentration of 25 µM, for 2 days (Delhon, 1990). Neurons were then infected (m.o.i. 1:0) and maintained in the presence of the drug until completion of the experiment.

To determine whether APC affected cell numbers, neurons were grown in the presence or absence of 25 µM APC for 24 or 48 h and counted. To facilitate cell counting, a sterile needle was used to draw a grid on the surface of collagen-coated coverslips. Cell numbers per well were determined for four wells per condition and time-point in two parallel experiments. Individual values were analysed by one-way ANOVA.

■ In situ hybridization (ISH). To estimate the number of neurons actively replicating viral DNA, neuronal cultures grown on coverslips were infected with 1·0 TCID50 per cell, incubated for 12, 24, 36 or 48 h p.i., and fixed with 95% ethanol–glacial acetic acid (3:1) for 30 min at room temperature. ISH was performed essentially as described by Rock et al. (1992). RNA was eliminated by incubation with 100 µg/ml DNase-free RNase (Promega). Cultures were hybridized with 2·5 × 10^6 c.p.m. of 3H-labelled BHV-1 DNA fragments for 72 h at 45 °C. For determination of the percentage of neurons actively replicating viral DNA, cell counts for two cultures per time-point were performed. A total of 500 cells per culture were counted by randomly selecting fields of about 15 to 40 neurons each (magnification × 400). The results were expressed as the mean percentage of neurons positive for viral DNA replication from two cultures ± standard error of the mean. Cells were considered positive for viral DNA replication when they exhibited grains too numerous to count.

■ Electron microscopy. Neuronal cultures grown on chamber slides were infected with BHV-1 (m.o.i. 1:0) in the presence or absence of 25 µM aphidicolin. After 1, 24 or 48 h p.i., cells were fixed with 3% glutaraldehyde in 0·1 M sodium cacodylate pH 7·2, post-fixed in aqueous OsO4, dehydrated in a graded ethanol series and embedded in situ with Epon 12. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 100 apparatus.

■ TUNEL staining. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) technique detects endonucleolytically cleaved DNA by addition of labelled dUTP to DNA ends in situ. Neuronal cultures were infected with infectious or heat inactivated BHV-1 as described above (m.o.i. 1:0). At various times p.i., cells were washed in PBS, fixed with 4% buffered paraformaldehyde (pH 7·3) for 20 min, and permeabilized by incubation with proteinase K (20 µg/ml in PBS) for 15 min at room temperature. Cells were stained with an in situ apoptosis detection kit (Apop Tag, Intergen Co., NY, USA) following the manufacturer’s instructions. The percentage of TUNEL-positive cells was calculated as described for ISH. TG sections were processed as described above except sections were previously deparaffinized and rehydrated.

■ DAPI staining. For DNA staining, neuronal cultures were infected and fixed as described for the TUNEL technique. The cultures were incubated with 0·5% Triton X-100 for 10 min, washed with PBS (pH 7·3)
Induction of neuronal apoptosis by BHV-1

Fig. 1. BHV-1 replication kinetics in neuronal cultures. (a) Sensory neurons (○) or ganglionic NNC (●) were infected with BHV-1 (m.o.i. 1–0). At the indicated time-points, virus was harvested and the titres were determined by plaque assays on MDBK cells. Each value represents the average ± standard deviation of triplicate cultures. (b–d) Viral DNA replication in sensory neurons. Neuronal cultures were infected with BHV-1 as in (a) in the absence (b, d) or presence (c) of 25 µM APC. The cultures were fixed at various times p.i., processed for ISH and stained in a dark chamber with DAPI (2 µg/ml in absolute methanol; Sigma) for 2 min at room temperature. Coverslips were sequentially rinsed with absolute methanol and 70% ethanol, mounted with 20% glycerol in PBS and examined under UV light with a fluorescent microscope (Zeiss).

■ Animal inoculation. Seven-month-old New Zealand White rabbits were tranquillized with acepromazine and inoculated in the right and left conjunctival sacs and nostrils with, in total, 10^6 TCID_{50} of BHV-1. The volume of the inoculum was 10 µl per site. Animals (two per time point) were sacrificed at 0, 1, 2, 3, 4, 5 or 6 days p.i. and the TG processed for standard histology or the TUNEL technique.

■ Histology. The TG were fixed in 4% buffered paraformaldehyde (pH 7.4), dehydrated in a graded ethanol series, and embedded in paraffin. TG sections were deparaffinized, rehydrated, sectioned at 5 µm, and stained with haematoxylin & eosin (HE) or further processed for the TUNEL technique as described for neuronal cultures. Selected TG samples were embedded in Epon 12 resin, sectioned at 0.5–1 µm with an ultramicrotome, and stained with 1% Toluidine blue-Azur II.

■ RT–PCR analysis. To examine bICP0 and gC transcription, neuronal cultures were infected with BHV-1 at 1–0 TCID_{50} per cell in the presence or absence of APC. Twelve or 48 h.p.i. the cultures were washed twice with PBS and total RNA was extracted as described by Dyanov & Dzitoeva (1995). RT–PCR was performed by using a commercial kit (Access RT–PCR, Promega) following the manufacturer’s instructions. Primers and PCR conditions for amplification of bICP0 and gC were described previously (Hossain et al., 1995; Schang & Jones, 1997). Two µg of total RNA was incubated with 1 U of RNase-free DNase I (Gibco BRL) in the presence of 7 U of RNasin (Promega) for 30 min at 20 °C. The reaction was terminated by addition of EDTA at a final concentration of 2 mM and incubation for 10 min at 62 °C. Twenty µl of DNase-treated total RNA was mixed with 30 µl of a solution containing 1 x commercial reaction buffer, 200 µM dNTP mix, 1 µM of each primer, 1 mM MgSO_{4}, 5 U of AMV reverse transcriptase (omitted in the RT control), 5 U of Taq DNA polymerase, and 10% glycerol (omitted for gC). The reactions were covered with mineral oil and incubated 45 min at 48 °C (reverse transcription). The tubes were transferred to a thermocycler and hot started. Amplification was carried out for 40 cycles by denaturing 1 min at 95 °C, annealing 1 min at 65 °C and extending 2 min at 72 °C. For complete extension of the amplified products, a final incubation at 72 °C for 5 min was included. Amplified products were resolved by agarose gel electrophoresis. PCR primers for bICP0 were (sense) 5’ AGT CGA GGC GCA CGC GGG 3’ and (antisense) 5’ TGG GGG GCG CGG AAA CTG 3’. Primers for gC were (sense) 5’ GAG CAA AGC CCC GCC GAA GGA 3’ and (antisense) 5’ TAC GAA CAG CAG CAC GGG CGG 3’. The expected sizes of amplified products were 164 (bICP0) and 229 bp (gC).

Results

Replication of BHV-1 in neuronal cultures

To examine the kinetics of virus replication in sensory neurons and in ganglionic non-neuron cells (NNC), rabbit viral DNA with ^{3}H-labelled BHV-1 fragments. Representative fields of cultures 36 h.p.i. are shown (b, c). Arrowsheads in (b) illustrate neurons that were not considered to count towards the number of neurons actively replicating viral DNA because of the low number of grains. Note that label in (b) is almost exclusively concentrated in the nuclei of neurons. Counterstain: HE. Magnification: ×400. (d) Percentage of neurons replicating viral DNA as a function of time p.i.
primary ganglionic cultures were prepared as described in Methods. Neuronal (5000 cells per well) or NNC (25,000 cells per well) cultures were infected with BHV-1 (Cooper strain) at an m.o.i. of 1:0. At various times after infection, virus was harvested through three cycles of freeze–thawing the cells plus media and viral titres were determined by plaque assays. Fig. 1(a) shows the time-course of detection of viral progeny after normalizing the data for the number of cells. Maximal virus titres were about one-and-a-half logs higher in NNC than in neuronal cultures and were detected after 2 days p.i. in NNC cultures and 3 days p.i. in neuronal cultures. After day 2 p.i., virus production decreased abruptly in NNC, but remained relatively high in neuronal cultures. Between days 5–6, most neuron networks were detached from the substrate. Thus the production of progeny virus was delayed and showed lower maximal levels in ganglionic neurons than in ganglionic NNC.

**Viral DNA replication in cultured neurons**

To determine the number of neurons actively replicating viral DNA, neurons were grown on coverslips for 12 days and then infected with BHV-1 (m.o.i. 1:0). At various times p.i., neurons were processed for ISH and probed for viral DNA with $^3$H-labelled BHV-1 DNA fragments as described in Methods. Under these conditions, label was observed almost exclusively in the nuclei of infected neurons and ranged from
BHV-1 infection induces apoptosis in cultured neurons

Cleavage of DNA into nucleosomal fragments is characteristic of apoptosis. To determine whether BHV-1 infection is associated with DNA fragmentation in neurons, neuronal cultures were infected with BHV-1 (m.o.i. 1-0) and processed for TUNEL, a technique that detects free DNA ends with condensed chromatin. Original magnification: ×400.

Fig. 3. BHV-1 infection induces chromatin condensation in sensory neurons. Neuronal cultures were infected (b) or mock-infected (a) with BHV-1 (m.o.i. 1-0). At 48 h p.i., the slides were stained with DAPI and examined under UV light. The arrowheads in (b) point to clusters of nuclei with condensed chromatin. Sporadic grains compatible with background signal levels to dense accumulations of grains (Fig. 1b). Almost 50% of neurons were involved in active viral DNA replication by 36 hours p.i. (500 cells per time-point in two parallel experiments) (Fig. 1d). Taken together these results are consistent with a delayed virus infectious cycle in neurons relative to NNC (Nichol et al., 1996).

Inhibition of viral DNA replication prevents neuronal apoptosis

To investigate whether viral DNA replication is required for the induction of neuronal apoptosis by BHV-1, neuronal cultures were infected with virus in the presence of aphidicolin (APC), an inhibitor of viral as well as certain cellular DNA polymerases (Nishiyama et al., 1984; Delhon, 1990; Marheineke & Hyrien, 2001). Because inhibition of cellular DNA polymerases by APC could be toxic for neurons, cell counts were performed on neuronal cultures maintained in the absence or presence of APC for 24 or 48 h as described in Methods. No differences in cell counts were found between APC-treated and untreated neuronal cultures at both time-points (p > 0.005).

Ten-day-old neuronal cultures were treated with APC for 2 days before virus infection (m.o.i. 1-0) and maintained in the presence of the drug throughout the length of the experiment. Decreasing low levels of infectious virus could be recovered from culture supernatants for the first 2 days p.i., but not on day 3 or beyond, suggesting that detected virus represents inoculum virus and not progeny virus (not shown). At various times p.i. in the presence of APC, neuronal cultures were processed for the detection of viral DNA by ISH and scored as positive or negative for ongoing viral DNA replication as described above. No positive neurons were observed after 24, 36, 48 or 72 h p.i. (Fig. 1c). When parallel cultures were stained with the TUNEL technique, or DAPI, no TUNEL-positive cells or cells with condensed chromatin were detected (Fig. 2b, d; not shown). Transmission electron microscopy of APC-treated neurons infected with BHV-1 demonstrated that virus was able to attach to neurons in the presence of the inhibitor (Fig. 4D). These results indicate that viral DNA replication plays a role in the induction of neuronal apoptosis by BHV-1 in vitro.

Transcription from the bICP0 gene is not prevented in APC-treated neurons

Using RT–PCR, we observed that gC, a late virus gene, is never transcribed at detectable levels in APC-treated infected neurons (Fig. 5). However, it is theoretically possible that virus genes expressed before the initiation of viral DNA replication are transcribed in APC-treated neurons and, therefore, could participate in or be responsible for the induction of apoptosis. This is especially significant for genes like bICP0, the BHV-1 homologue of HSV-1 ICP0 (Wirth et al., 1989, 1992). The bICP0 gene is toxic when expressed in mammalian cells in the absence of other viral proteins (Ciacci-Zanella et al., 1999;
Inman et al., 2001). Using RT–PCR, we explored the possibility that the dual kinetics bICP0 gene is expressed under circumstances where viral DNA replication is pharmacologically prevented. Following infection with BHV-1 (m.o.i. 1:0), total RNA from APC-treated or untreated neurons was extracted, reverse transcribed and PCR amplified. No DNA amplification resulted from RNA extracted from uninfected cultures (Fig. 5, lane 4) or from infected cultures when the RT step was omitted (Fig. 5, lane 2). An amplified DNA fragment of the expected size was obtained from infected cultures at 12 h p.i. in the absence of APC (Fig. 5, lane 3). Transcription from the bICP0 gene was consistently detected in APC-treated neuronal cultures after 12 but not 48 h p.i. (Fig. 5, lanes 5 and 6), supporting the idea that virus is internalized by neurons in the presence of APC. These results indicate that transcription from the bICP0 gene is not prevented in APC-treated neurons.
Neuronal apoptosis is not a common event in BHV-1-infected TG

To determine whether infection by BHV-1 is associated with apoptosis in ganglionic neurons in vivo, rabbits were infected with $10^5$ TCID$_{50}$ of virus as described in Methods. The animals were sacrificed at various days p.i. and the TG collected and processed for standard histology. Pathological changes were detected starting on day 2 p.i. and consisted of mononuclear cells, mainly lymphocytes and macrophages, infiltrating individual neurons. On days 3 to 6 p.i., the number of mononuclear cells increased, forming multiple distinctive inflammatory foci which involved several neurons (Fig. 6a). In these foci, some mononuclear cells exhibited hallmarks of apoptosis such as chromatin condensation and nuclear fragmentation (Fig. 6b). Additional TG sections processed for the TUNEL technique (four sections per time-point, two per animal, one per TG) confirmed the occurrence of apoptosis in BHV-1-infected TG. Mononuclear infiltrating cells, mostly but not exclusively around neurons, were positive for DNA fragmentation starting on day 2 p.i. The number of TUNEL-positive cells increased with the time after infection as the inflammatory reaction in the TG increased in magnitude. A positive signal for TUNEL was observed in both apparently intact and fragmented cells (Fig. 6g, h).

Neuronophagia within inflammatory foci was common between 3 and 6 days p.i. in all the TG examined (Fig. 6c–e). The first signs of neuronophagia consisted of infiltrating immune cells transversing the satellite cell glial barrier and making direct contact with the neuron cell bodies (Fig. 6c). This was followed by progressive recession of the neuronal cytoplasm until the entire neuron was eventually replaced by infiltrating cells (Fig. 6d, e). In spite of these dramatic changes, no signs of chromatin condensation, nuclear fragmentation, or apoptotic body formation were associated with these neurons. We were unable to detect TUNEL-positive neurons in mock-infected rabbits (Fig. 6f), BHV-1-infected rabbits on days 1, 2, 3 or 5 p.i., or in infected animals when incubation with TdT was omitted. Even neurons associated with infiltrating immune cells or being phagocytosed failed to show a TUNEL-positive signal. We detected one TUNEL-positive neuron on day 4 p.i., and five TUNEL-positive neurons in one of two rabbits on day 6 p.i. TUNEL-positive neurons were associated with incipient rather large inflammatory foci and showed a preserved morphology (Fig. 6g, h). Taken together, these results indicate that apoptosis in BHV-1-infected rabbit TG is mainly confined to mononuclear inflammatory cells.

Discussion

For herpesviruses such as BHV-1, control of virus-induced apoptosis may play a role in the balance between productive vs latent infection at the site of virus persistence. In this report, we demonstrate that the infection of cultured rabbit ganglionic neurons with BHV-1 induces changes associated with apoptotic cell death such as DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage (Figs 2 and 3). In support of these observations, we have recently found that infection of neurons with BHV-1 induces loss of plasma membrane asymmetry (unpublished results), which is an early marker of apoptosis (Martin et al., 1995). Early ultrastructural changes induced by virus infection included chromatin rearrangement and loss of the normal nucleolar architecture (Fig. 4A–C). Whether the ultrastructural changes are part of the virus-induced cytopathic effect (Delhon, 1990) or they represent specific early morphological features of apoptosis in neurons remains to be determined.

BHV-1 causes apoptosis in a number of cell systems (Devireddy & Jones, 1999; Eskra & Splitter, 1997; Hannon et al., 1996, 1997, 1998; Winkler et al., 1999). Requirements for induction seem to be cell-type specific. For example, both infectious and inactivated BHV-1 are able to induce apoptosis in mitogen-stimulated PMBCs (Hannon et al., 1996) or activated CD4$^+$ cells (Eskra & Splitter, 1997). Attachment of virus particles is sufficient for induction in PMBCs (Hannon et al., 1998). Whereas infectious BHV-1 induced apoptosis in epithelial cell lines, inactivated virus was unable to efficiently induce apoptosis (Devireddy & Jones, 1999). The fact that virus attachment to the cell membrane per se is not followed by apoptosis in cultured neurons was indicated by ultrastructural examination of APC-treated cultures inoculated with BHV-1 (Fig. 4D).
Fig. 6. Pathological changes associated with BHV-1 infection in rabbit TG. (a) Infiltrating mononuclear cells forming neuron-associated inflammatory foci (4 days p.i.). HE, x 400. (b–e) Semi-thin sections of resin-embedded tissue stained with Toluidine blue-Azur II. Various stages of chromatin condensation in infiltrating cells are indicated by arrowheads in (b) (4 days p.i.). In (c), two infiltrating cells (large arrowheads) traversed the glial cell barrier (small arrowheads) and took direct contact with the neuron cell body. In (d), about half of the neuron cell body (arrowhead) was replaced by mononuclear cells. Most of the neuron cell body (arrowheads) was replaced by invading infiltrating cells in (e). (c–e) 6 days p.i. Original magnification: x 1000. (f–h) Detection of DNA fragmentation by the TUNEL technique (6 days p.i.). (f) Mock-infected rabbit. (g) A TUNEL-
In cultured neurons infected with BHV-1, we found that 0, 12 and 34% of neurons exhibited a TUNEL-positive signal at 12, 24 and 48 h p.i., respectively. These values represent roughly half of the neurons supporting viral DNA replication at the corresponding time-points (Fig. 1d), suggesting that the apoptotic programme is triggered after viral DNA replication is initiated. The observation that not all neurons supporting viral DNA replication exhibit a TUNEL-positive signal could reflect the fact that a critical stage in viral DNA replication (one that is not discerned by ISH) should be achieved before apoptosis is triggered in neurons. Another possibility is that template amplification above a critical threshold is required for the induction of apoptosis.

It was previously shown that rabit neuronal cultures can be maintained for several weeks in the presence of APC without any obvious secondary toxic effects (Delhon, 1990). APC inhibits eukaryotic DNA polymerases, including viral polymerases, thus blocking DNA replication at an early stage (Marheineke & Hyrien, 2001). In this report, BHV-1-induced apoptosis was completely prevented when viral DNA replication was blocked with APC (Fig. 2b, d). Although at this time we cannot rule out the possibility that other factors associated with virus infection are involved in the induction of apoptosis, the fact that treatment with APC did not preclude virus attachment to the cell surface (Fig. 4D), nor viral gene expression (Fig. 5), supports the idea that viral DNA replication plays an important role in BHV-1-induced neuronal apoptosis in vitro.

Because quantification of transcription was not attempted here, differences in the level of bICP0 transcription between APC-treated and untreated neurons remain unknown. Previous work with HSV-1-infected primary neurons has shown that inhibition of viral DNA replication results in a dramatic decrease in the expression of all classes of viral genes, including the ICP0 gene (Nichol et al., 1996). Such dependence of viral gene expression on viral DNA replication has also been observed during infection of the mouse TG with thymidine kinase-negative HSV-1 mutants (Kosz-Vnenchak et al., 1990, 1993). bICP0 is toxic for cycling cells when expressed in the absence of any other viral gene (Ciacchi-Zanella et al., 1999), but does not directly induce apoptosis (Inman et al., 2001). The observation that neither DNA cleavage nor chromatin condensation was detected in APC-treated neurons over a 3 day period (Fig. 2; not shown), and that cell numbers were not decreased by APC, suggest that the level of bICP0 expression is not apoptotic or toxic in neurons under the effect of APC. It remains to be determined whether expression of bICP0 is apoptotic or toxic in transiently transfected neurons.

It is tempting to speculate about the role of viral DNA replication in the induction of apoptosis in neurons. Viral DNA replication could induce apoptosis through at least two mechanisms which are not mutually exclusive. One mechanism is that viral DNA replication per se triggers the apoptotic programme. The other mechanism is that viral DNA replication is required for maximal expression of viral genes, which in turn triggers the apoptotic programme. This could partially explain why we observed less TUNEL-positive neurons than neurons actively replicating viral DNA at the corresponding time-points. The idea of viral DNA replication as an apoptosis trigger in neurons is consistent with previous works showing that unscheduled DNA replication or activation of proliferation genes in post-mitotic neurons leads to apoptosis instead of cell division (Freeman et al., 1994; Lee et al., 1994; reviewed by Ross, 1996). In this context, it is interesting that expression of cell cycle progression markers such as cyclins D1, E and A, is induced in TG neurons following acute infection by BHV-1 (Schang et al., 1996; Winkler et al., 2000).

TG from acutely infected rabbits but not from mock-infected animals showed pathological changes which increased in magnitude as a function of the time p.i. These changes included mononuclear cell infiltration, apoptosis and neuromophagia. Features of apoptosis such as chromatid condensation and DNA fragmentation were observed in mononuclear cells surrounding neurons (Fig. 6). In contrast, morphological hallmarks of apoptosis were absent in TG neurons. Furthermore, whereas neurons exhibiting pathological changes were observed in all the TG between 3 and 6 days p.i., TUNEL-positive neurons were exceedingly rare and sporadic, indicating that neuronal apoptosis is not a common event in BHV-1-infected TG. This low level of in vivo neuronal apoptosis in the TG is coincident with those observed in rabbit or mouse injected with wild-type HSV-1 (Perng et al., 2000; Thompson & Sawtell, 2001; Ahmed et al., 2002), or in swine infected with pseudorabies virus (PRV) (Aleman et al., 2001). As in PRV-infected TG, TUNEL-positive neurons in BHV-1-infected TG exhibited a well preserved morphology and were associated with moderate inflammatory reactions. Because virus detection was not attempted in this work, we do not know whether TUNEL-positive TG neurons (and mononuclear infiltrating cells) are virus-infected cells. These cells could represent virus-infected cells, cells under the effect of cytokines released in the ganglion during virus infection, or both (Allsopp & Fazakerley, 2000; Chao et al., 1995; Sei et al., 1995; Shimeld et al., 1999). In swine infected with PRV, the sporadic TUNEL-positive TG neurons were also positive for PRV antigens, suggesting that virus infection induced the apoptotic programme (Aleman et al., 2001).

The results presented here indicate that ganglionic neurons are not intrinsically resistant to virus-induced apoptosis. However, neuronal apoptosis was very rare in the infected TG

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(h) A TUNEL-positive, fragmented infiltrating cell in direct apposition to a TUNEL-negative neuron (small arrowhead). The large arrowhead points to a TUNEL-positive neuron. Counterstain: methyl green. Magnification: ×400 (f, g); ×1000 (h).
as reported for other two alphaherpesviruses. Therefore, mechanisms in the TG should exist to prevent neuronal apoptosis upon virus infection. If one function of the LR gene is to ensure neuronal survival and given that viral DNA replication is a major inducer of apoptosis, it will be of great interest to elucidate how and when the LR gene exerts its protective role.

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Induction of neuronal apoptosis by BHV-1


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