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Short Communication

Bovine herpesvirus 4 infects differentiated neuronal cells in culture and establish persistent infection upon selection

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Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus with no clear disease association. Although BoHV-4 is not considered a neurotropic virus, it has been detected in peripheral and/or central nervous system tissues during persistent infection (Lopez *et al*, 1996, *Microb Pathogen* 21: 47–58; Yamamoto *et al*, 2000, *Arch Virol* 145: 2363–2370; Asano *et al*, 2003, *J Vet Med Sci* 65: 87–93). However, the direct interaction between BoHV-4 and neurons has not been studied so far. The authors investigated the interaction of BoHV-4 with N2a (neuroblastoma cell line) cells through the use of two recombinant viruses (BoHV-4/26A3*neo* and BoHV-4EGFPΔTK). Because of the unique biological characteristics of N2a cells, which differentiate in neuron-like cells producing dendrites, axon, and specific neuronal markers, the authors found that BoHV-4 infects differentiated N2a cells and a persistent infection can be established. BoHV-4 persistently infected N2a cells produce infectious viral particles, which do not interfere with cellular differentiation. *Journal of NeuroVirology* (2004) **10,** 123–130.

Keywords: bovine herpesvirus 4; cell culture model; neuron-like differentiated cells; persistent infection

Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus with no clear disease association (reviewed in Bartha *et al*, 1987; Thiry *et al*, 1989). Like other herpesviruses, it establishes persistent infections in its natural host (Dubuisson *et al*, 1989; Krogman and McAdaragh, 1982; Osorio and Reed, 1983) and in an experimental host, the rabbit (Osorio *et al*, 1982). Although BoHV-4 has been demonstrated in many tissues, accumulated evidences suggest that one site of persistence in both the natural and experimental host are cells of the monocyte/macrophage lineage (Dubuisson *et al*, 1989; Egyed and Bartha, 1998; Naeem *et al*, 1993; Osorio and Reed, 1983; Osorio *et al*, 1985).

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Cell lines persistently infected with gammaherpesviruses Epstein-Barr virus (EBV), herpesvirus saimiri (HVS), human herpesvirus 8 (HHV-8), and murine gammaherpesvirus-68 (MHV-68) have been established from cells isolated from infected hosts (Ceserman *et al*, 1995; Jung *et al*, 1999; Nilsson, 1979; Usherwood *et al*, 1996). This process has likely been greatly facilitated by the growth-transforming ability of these gammaherpesviruses (Flore *et al*, 1998; Jung *et al*, 1999; Miller, 1990; Moses *et al*, 1999). In contrast, no evidence for growth transformation has been obtained for BoHV-4. Each of the genes associated with transformation by other gammaherpesviruses is unique to each individual virus, and no homologous gene is found in other transforming gammaherpesviruses or BoHV-4 (Lomonte *et al*, 1996). In cell lines persistently infected with gammaherpesviruses, the infection is predominantly latent. In the vast majority of cells, viral gene expression is restricted to a specific subset of genes and the cells survive and replicate. In a small subset of cells, the virus is reactivated from latency, resulting in production of infectious virus and death of the cell (Kieff, 1996; Miller *et al*, 1997; Moses *et al*, 1999). In cell

lines persistently infected with gammaherpesviruses, the viral genome is maintained as a circular episome (Ceserman *et al*, 1995; Decker *et al*, 1996; Jung *et al*, 1999; Kieff, 1996; Usherwood *et al*, 1996). Origins of replication for the circular viral genomes, oriP, distinct from the origins of replication used during lytic viral replication, oriLyt, have been identified (Ballestas *et al*, 1999; Kung and Medveczky, 1996; Yates *et al*, 1984). A viral gene product is required for episomal maintenance of DNA containing oriP (Ballestas *et al*, 1999; Kung and Medveczky, 1996; Lupton and Levine, 1985; Yates *et al*, 1985).

BoHV-4 causes cytopathic effect (CPE) and replicates in a broad range of cell lines and primary cultures (Peterson and Goyal, 1988; Truman *et al*, 1986; Donofrio *et al*, 2002). In the present work, we have examined the interaction of BoHV-4 with undifferentiated and differentiated neuroblastoma cell line and although no CPE is noted, a persistent infection resulting in infectious virus production can be established by the use of a recombinant BoHV-4.

N2a cells become susceptible to BoHV-4 infection after differentiation

The mouse N2a neuroblastoma cell line (European Cell Culture Collection, ECACC no. 98141204) is classed as an adrenergic clone and expressing a low level of monoamine oxidase (MAO) activity (Nagatu *et al*, 1981). It is used in neurobiological studies to monitor initial morphological changes, owing to its ability to extend neuritis rapidly in response to serum withdrawal. Indeed, N2a cells have previously been shown to be a candidate model for neurobiological studies. Starting from that point, we analyzed the interaction between BoHV-4 and N2a cells and to determine whether N2a cells could support BoHV-4 infection. N2a cells were maintained as a monolayer using growth medium containing 90% Dulbecco's minimum essential medium (DMEM), 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin. Cells were subcultured to a fresh culture vessel when growth reached 70% to 90% confluence (i.e., every 3 to 5 days) and incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2.

First, N2a cells were differentiated by serum starvation. Cells were plated out in growth medium at a cell density of 10^6 cells per 75-cm^2 flasks, and incubated for 24 h to allow cell attachment and recovery. To induce differentiation, growth medium was carefully removed from cultureware so as to not disturb the attached cells and replaced with an equal volume of serum-free DMEM supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 10 μg/ml streptomycin (serum-free medium). Cultures were reincubated at 37°C in an atmosphere of 95% air/5% CO_2 for 48 h. Following differentiation, cells were exposed for 1 h in presence of 10% of FCS to 10

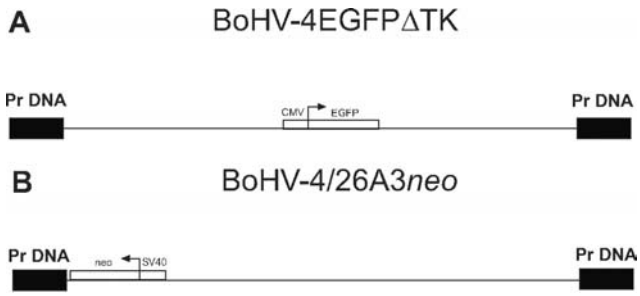

Figure 1 Schematic representation of recombinants BoHV-4 employed through the study (not on scale). (**A**) Diagram showing the structure of the recombinant BoHV-4EGFPΔTK, generated by homologous recombination with a CMV/EGFP expression cassette inserted into the TK locus of DN 599 BoHV-4 strain (Donofrio *et al*, 2002). Polyrepetitive DNA (Pr DNA) is indicated to the right and left end of the genome. (**B**) Diagram showing the structure of the recombinant BoHV-4/26A3*neo*, generated by homologous recombination. The fragment containing the neomycin-resistance gene (*neo*), driven by the SV40 promoter of large-T antigen (SV40), is located adjacent to the partial copy of the terminally repeated (prDNA) sequence at the right end of the *Eco*RI H fragment, and the 5′ end of the neomycin-resistance gene is oriented toward the BoHV-4 DNA (Donofrio *et al*, 2000).

multiplicity of infection (m.o.i) of a green recombinant BoHV-4 (BoHV-4EGFPΔTK) (Donofrio *et al*, 2002) (Figure 1A) obtained by insertion of an enhanced green fluorescent protein (EGFP) gene into the thymidine kinase (TK) locus of DN 599 BoHV-4

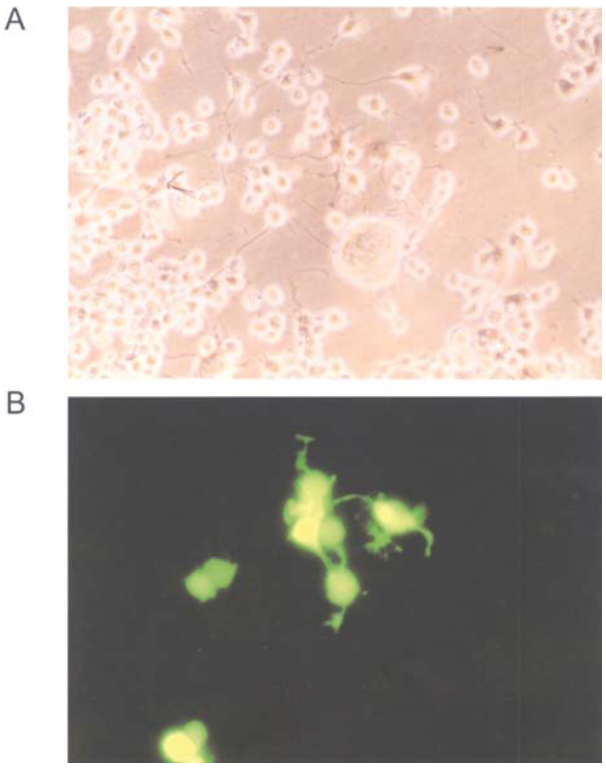

Figure 2 Culture of BoHV-4EGFPΔTK–infected differentiated N2a cells. (**A**) Phase-contrast microscope observation (40×). (**B**) Fluorescent microscope observation of neuron-like cells expressing EGFP (40×).

strain to monitor the establishment of infection. Cells were successfully infected, as indicated by EGFP expression, which was detectable as soon as 48 h post infection (p.i.). Infected N2a cells appeared very well differentiated, exhibiting the distinct neuronal morphology where EGFP expression could be observed (Figure 2). Because we previously have shown the interaction of BoHV-4 with murine embryonic stem cells (ESCs) (Donofrio *et al*, 2003), we next tested the possibility of BoHV-4 to infect undifferentiated N2a cells. N2a cells were exposed to 10 m.o.i. of BHV-4EGFPΔTK in presence of 10% of FCF to avoid differentiation and infection was monitored under fluorescent microscope by EGFP expression starting from 48 h p.i. for a period of 3 days. Undifferentiated N2a cells did not show EGFP expression (Figure 3A, B), and no CPE was apparent, even at m.o.i. as high as 50 and even after passage of the cells (Figure 3C, D). We then concluded that BoHV-4 infects differentiated N2a cells and the infection is predominantly nonpermissive and noncytopathic. The reason of the resistance of undifferentiated cells is not clear. A first explanation may be the change of the intracellular microenvironment, transcription factors expression and activation, and cell-cycle progression during N2a cells differentiation, which would allow the switching on or off the viral human cytomegalovirus promoter used to drive the transcription of EGFP expression cassette accomodated inside of the TK locus of the BoHV-4 genome. A second explanation could be the lack of expression of a cell-specific receptor for BoHV-4 on the surface of undifferentiated N2a cells. To exclude one of the possibilities, we transfected differentiated and undifferentiated N2a cells with pEGFP-C1 (Clontech), a plasmid carrying the same EGFP expression cassette used to build the green recombinant BoHV-4 (Donofrio *et al*, 2002). Both differentiated and undifferentiated N2a cells showed EGFP expression (data not shown), giving evidence for the lack of expression of a cell-specific receptor for BoHV-4 on the surface of undifferentiated N2a cells.

N2a cells support BoHV-4 persistent infection

Based on results described thus far, the BoHV-4 infection of differentiated N2a cells is predominantly nonpermissive and noncytopathic. Because the extent of

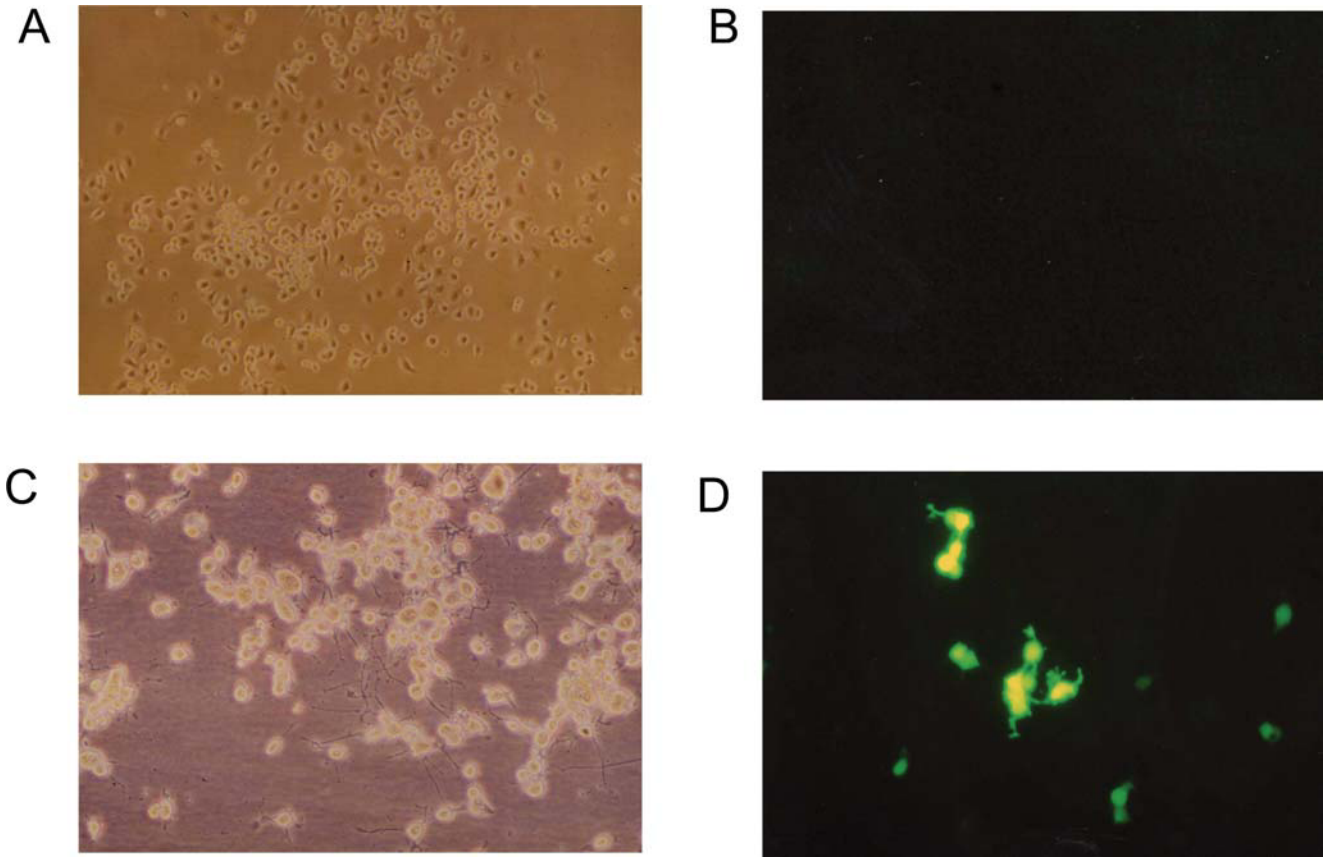

Figure 3 Resistance of undifferentiated N2a cells to BoHV-4EGFPΔTK infection. (**A**) Phase-contrast microscope observation of BoHV-4EGFPΔTK–infected undifferentiated N2a cells and (**B**) corresponding fluorescent microscope observation, with no detectable EGFP fluorescence, demonstrating lack of infection. (**C**) Phase-contrast microscope observation of a control culture of BoHV-4EGFPΔTK–infected differentiated N2a cells and (**D**) corresponding fluorescent microscope observation, where EGFP expression is well detectable.

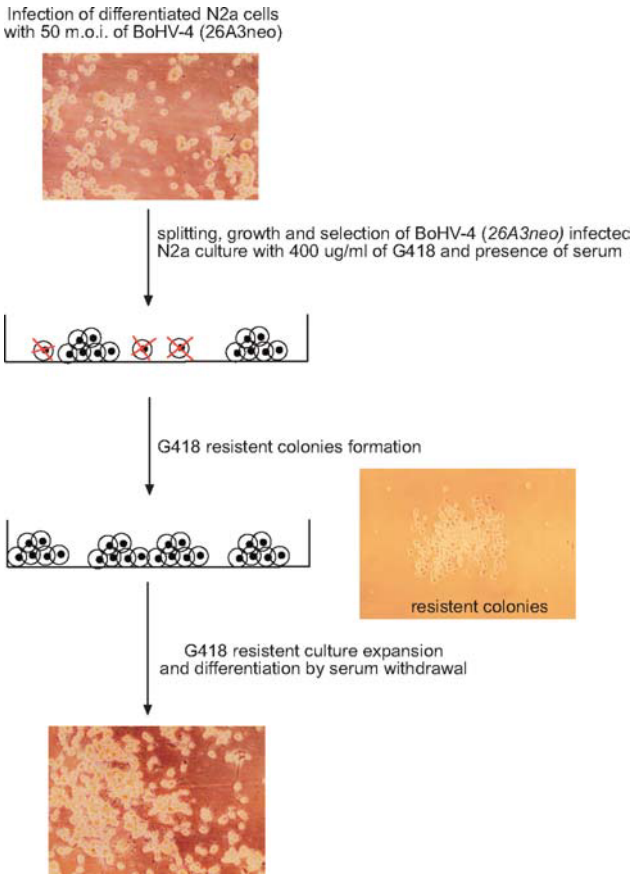

Figure 4 Schematic explanation of the strategy used to obtain a homogeneous population of BoHV-4 persistently infected N2a cells.

virus replication, if any, must be extremely low, we felt it might be difficult to distinguish between input virions and new infectious virus. Therefore, we did not determine whether the N2a cells produced infectious virus, but selected for infected cells by a recombinant BoHV-4 (BoHV-4/26A3*neo*) containing the neomycin-resistance gene (*neo*) inserted near the junction of unique and prDNA (Donofrio *et al*, 2000) (Figure 1B). Differentiated N2a cells were infected with 50 m.o.i. of BoHV-4/26A3*neo* and after infection, inactivation of extracellular virus was carried out by low-pH treatment (Mettenleiter, 1989). Briefly, the medium was removed and the plates were washed once with phosphate-buffered saline (PBS), than incubated for 2 min with either PBS (control plates) or a buffer (pH 3) containing 40 mM citric acid, 10 mM KCl, and 135 mM NaCl. This procedure completely inactivated and eliminated nonpenetrated infectious particles. One day p.i., cells were split with presence of serum and 400 μg/ml geneticin (G418) was added. Although N2a cells are sensitive to 300 μg/ml of G418, a higher dose was used in an attempt to avoid the selection of spontaneously resistant cells. Ten days p.i., colonies began to appear and by 14 days p.i., the colonies were macroscopic (Figure 4). Colonies were trypsinized, pooled, and allowed to continue to grow in the presence of G418. Starting from fourth passage of G418-resistent N2a cells, cells were analyzed for viral genome presence and virus production. Viral-infected cells were lysed overnight in TEL (10 mM TrisHCl, pH 7.5, 1 mM EDTA) buffer containing 0.5% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K per ml at 37°C. Nucleic acids were extracted by treatment with phenol-chloroform and precipitated with ethanol (Maniatis *et al*, 1989). Treatment with 100 μg/ml of RNase A (Sigma) was performed for 1 h, after which the DNA was extracted with phenol and precipitated with ethanol again. The samples were kept at −20°C. One-microgram sample of DNA was amplified over 30 cycles, each cycle consisting of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and chain elongation with 1 U of Taq polymerase (Boehringer-Diagnostics) at 72°C for 2 min. Polymerase chain reaction (PCR) amplification was performed in a final volume of 50 μl of 10 mM Tris-hydrochloride, pH 8.3, containing 0.2 mM deoxinucleoside triphosphate, 3 mM $MgCl_2$, 50 mM KCl, and 0.25 μM of each primer. In the first cycle, the samples were denatured at 94°C for 5 min, and in the last cycle, the extension step was increased to 7 min. The primers used for amplification are listed in Table 1 and were selected from the published sequence of the BoHV-4 genome. The PCR product was electrophoresed in 1% agarose gel and visualized after ethidium bromide staining. PCR allowed us to detect BoHV-4 DNA in G418-resistent N2a cells and the integrity of the viral genome was confirmed through the generation of three different amplicons positioned in the central (TK and IE2) and extreme right hand side (*neo*) of the BoHV-4/26A3*neo* genome (Figure 5A and Table 1) (Zimmerman *et al*, 2001). The presence of viral DNA in G418-resistent N2a cells (Figure 5B) suggested

Table 1 Nucleotide sequence of the primers employed to show the integrity of BoHV-426A3*neo* genome by PCR

	Gene-specific primers			
Target gene	*Forward primer*	*Reverse primer*	*Size of amplicon*	*GeneBank accession number*
neo	(a) tcccccggggatgattgaacaagatggatt	(b) tcccccgggtcagaagaactcgtcaagaag	2538 bp	U02434
TK locus	(c) cgaattctagtctaaagtcatcctc	(d) cgaattccattggcttcatcccaca	854 bp	S49773
IE2	(e) gctagccaccatgaaggggattatttatccc	(f) ggatccttaccctcgtcttgttatttcccccaa	813 bp	L01099

Note. The reported nucleotide position on BoHV-4 genome is referred to the published long unique region sequences (GeneBank accession numbers) of BoHV-4 genome.

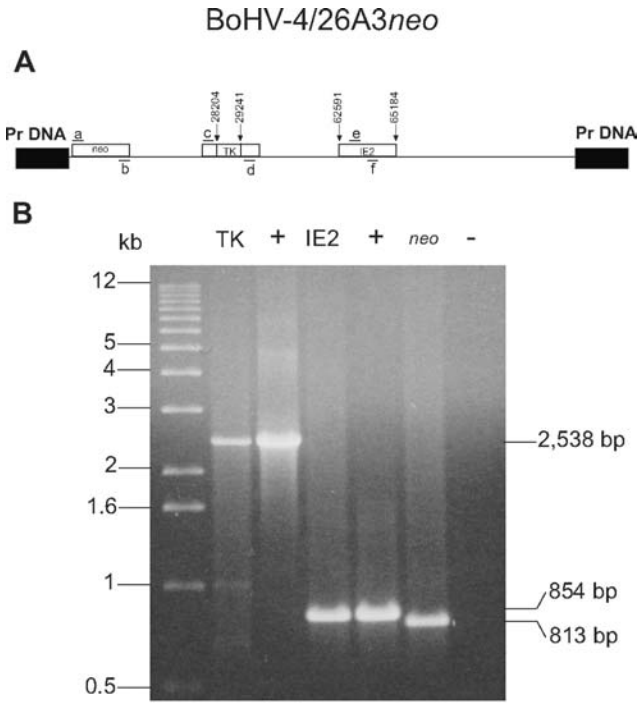

Figure 5 Assessment of BoHV-4/26A3*neo* genome presence and integrity by PCR in persistently infected differentiate N2a cells. (**A**) Diagram showing target genes position (clear boxes) employed to assess the presence and integrity of BoHV-4 genome by PCR in persistently infected differentiated N2a cells. Underlined a, b, c, d, e, and f indicates primer positioning on the target gene and numbers indicates nucleotide position in the BoHV-4 sequence (Table 1). Polyrepetitive DNA (Pr DNA) is indicated to the right and left end of the genome and corresponds to the black boxes. (**B**) PCR products were generated from BoHV-426A3*neo* persistently infected differentiated N2a cells DNA, with indicated primer pairs listed in Table 1. For each amplicons (TK, IE2, and *neo*), a positive control was introduced (+) using wild-type (w. t.) BoHV-4 DN 599–infected cells DNA. The loss of product for the *neo* gene of the w. t. BoHV-4 DN 599–infected cells DNA (−) is clearly due to the lack of the target sequence in the w. t. BoHV-4 DN 599 genome, which can be well considered as a negative-control sample in the PCR reaction. Molecular sizes are shown on the left in kilobase, with amplicons size on the right in base pairs.

that these cells could produce infectious virus. To determine whether G418-resistent N2a cells were producing infectious BoHV-4, medium recovered fromG418-resistent N2a cells was inoculated onto susceptible BAE-7372 cell for 30 min. Bovine arterial endothelial cell line (BAE-7372) (obtained from Dr. Stefano Grolli, Veterinary Biochemistry Institute, Parma University, Italy) was used, due to the high sensitivity towards BoHV-4 and other bovine herpesviruses (Lin *et al*, 1997). Cells were grown at 37°C in MEM (Gibco-BRL, Paisley, UK) supplemented with 10% heat-inactivated FCS, 100 IU/ml of penicillin, and 10 μg/ml of streptomycin, in a humidified atmosphere containing 95% air/5% CO_2.

Because the medium contained 400 μg/ml geneticin, it was removed after 30 min and replaced with fresh medium. Plaques typical of BoHV-4 were visible after 5 days. Immunohistochemical staining

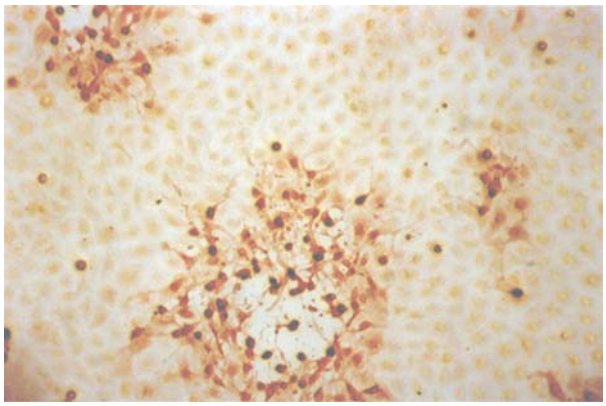

Figure 6 Phase-contrast microscope image of immunostained BoHV-4 plaque, originated from BoHV-426A3*neo* particles coming from BoHV-426A3*neo* persistently infected differentiated N2a cells. Staining control was assessed using only the secondary antibody (data not shown).

was performed to confirm the presence of BoHV-4 (Figure 6) and the plaque numbers allowed us to quantify the amounts of virus produced.

Cells were washed three times with PBS and fixed with 4% paraformaldehyde 10 to 15 min at 37°C, then washed two times with PBS containing 0.1% bovine serum albumin (BSA) and incubated 5 min at 20°C with the same solution. Cells were incubated 5 min at 20°C with PBS containing 0.3% Triton-X100, washed 2 to 3 times with PBS, and incubated 10 min at 37°C with 0.15% H_2O_2 in PBS. Rabbit anti–BoHV-4 hyperimmune serum, diluted 1/500 in PBS, was incubated with the cells 2 h at 37°C and cells were washed 3 times with PBS. Secondary antibody (peroxidase-conjugated anti-rabbit immunoglobulin G [IgG]; Sigma), diluted 1/500 in PBS, was incubated with cells 1 h at 37°C. Cells were washed 3 times with PBS. Bound secondary antibody was detected by development in 250 μg/ml diaminobenzidine (DAB) (Sigma), 0.015% H_2O_2, 50 mM Tris, pH 7.4, for approximately 10 min at 20°C. Development was stopped by washing with 4°C PBS.

Virus was produced by persistently infected N2a cells, through all passages tested (20 passages), in a dose of 0.1 plaque-forming units (p.f.u.)/cell. We concluded that complete viral particles had been assembled in infected N2a cells. This result shows a persistent infection.

BoHV-4 persistent infection does not interfere with N2a cells differentiation

Persistently infected N2a cells were induced to differentiate by serum withdrawal, which restricted cell division and produced morphological alterations characterized by the elaboration of axonal like processes. Persistently infected differentiated N2a cultures were washed in Tris-buffered saline (TBS; 50 mM Tris-HCl and 150 mM NaCl, pH 7.4) before fixation with

ice-cold 90% (*v*/*v*) methanol in TBS at −20°C for 20 min. Cells were viewed using an inverted light microscope at 200× magnification. The total number of cells and axon-like processes, which were defined as cellular extensions greater than a cell body diameter in length (Keilbaugh *et al*, 1991), were recorded. Five random fields were examined from each well, giving a total cell count of at least 200 cells per well.

Measurements of axon-like processes number did not show significant statistical differences as compared to differentiated uninfected N2a control cells (Figure 7).

Viruses are confined to use the metabolic and biosynthetic pathways of the cells they infect. These pathways vary not only between cell types but also within any given cell lineage with stage of differentiation and between activated and resting cells. There are many well-documented examples of viruses that replicate in specific cell type and at specific stage in cell growth, differentiation, or activation. Examples would be reactivation of herpesvirus from latency by host-cell differentiation (Dutko and Oldstone, 1981); initiation of papillomavirus replication on differentiation of keratinocytes (Dollad *et al*, 1993); and replication of minute virus in testicular cells on differentiation (Guetta *et al*, 1986). One key mechanism mediating these effects is the regulation of the viral gene expression by host factors. Specific enhancers and transcriptional activators have been shown to be important and to vary between cell types and stages of cellular differentiation (Wirth *et al*, 1992).

Although BoHV-4 is not considered a neurotropic virus, it has been detected in peripheral and/or central nervous system tissues during persistent infection (Lopez *et al*, 1996; Yamamoto *et al*, 2000; Asano *et al*, 2003). However, not a clear association has been shown so far among the presence of the virus in the neural tissue, viral replication, and persistent infection of the neuronal cells. Therefore, an *in vitro* model for that purpose would be very useful. N2a cells are a relatively unexplored neuronal cell culture system for the evaluation of the viral infection

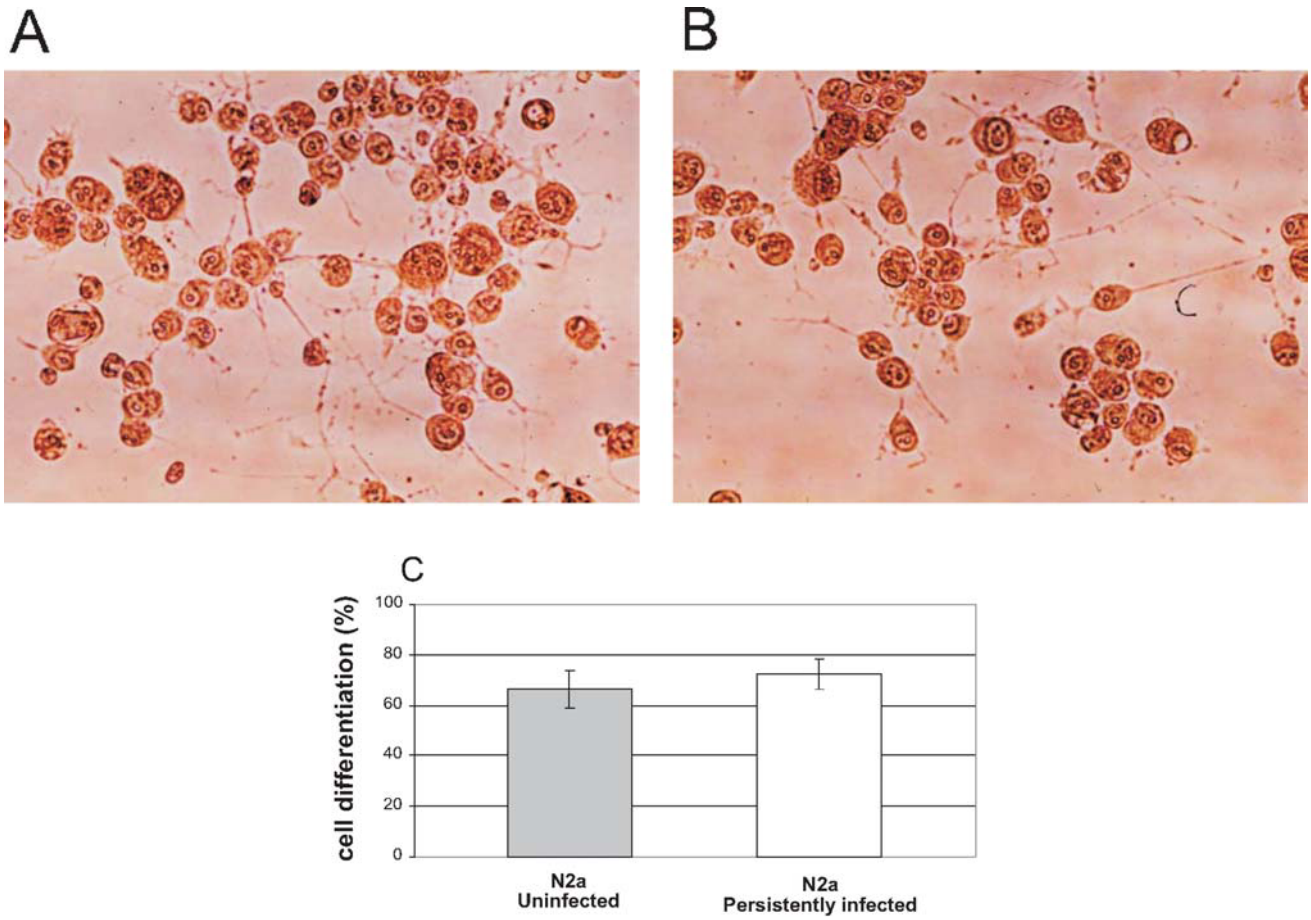

Figure 7 Effect of BoHV-4 persistent infection on N2a differentiation. Phase-contrast microscope image of differentiated persistently infected (**A**) uninfected (**B**) N2a cells. Note that no reduction in number of axon-like processes can be observed if compared with uninfected control cells. (**C**) Quantification of persistently infected N2a cells differentiation. Cells were fixed, then viewed on an inverted light microscope; cells were scored as positive when they had processes larger than 1 cell diameter. Data are expressed as mean ± standard error of the percentage of cells bearing processes in five randomly chosen photographic fields. No significant statistical difference can be detected with respect to control cells.

and replication. These cells are a neuroblastoma cell line derived from murine neuroblastoma (Nagatsu *et al*, 1981). N2a differentiate reversibly into neuron-like cells upon serum withdrawal. During differentiation, these cells develop dendrites and axons and express a variety of neuronal cell marker characteristic of central nervous system neurons (Tan *et al*, 2002). Following differentiation, they restrict their dividing phenotype, which can be reverted upon splitting the culture in the presence of serum. Because of these unique biological characteristics, differentiated N2a cells appear to be a promising cell system for evaluating the interaction of BoHV-4 and neurons as well as BoHV-4 vector-directed gene delivery to neurons and molecular mechanisms involved on neuronal persistent infection.

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