A bovine macrophage cell line supports bovine herpesvirus-4 persistent infection

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Although bovine herpesvirus-4 (BHV-4), a gammaherpesvirus lacking a clear disease association, has been demonstrated in many tissues during persistent BHV-4 infection, a likely site of virus persistence is in cells of the monocyte/macrophage lineage. To establish an in vitro model of persistent infection potentially useful for examining the molecular mechanisms of BHV-4 persistence/latency, we infected the bovine macrophage cell line BOMAC. Following extensive cell death, surviving cells were found to be persistently infected, maintaining the viral genome over many passages and producing low levels of infectious virus. Although selection was unnecessary for the maintenance of the viral genome, cells persistently infected with recombinant BHV-4 containing a neomycin-resistance gene could be selected with geneticin, thus confirming that persistent BHV-4 infection was compatible with cell survival and replication. Furthermore, persistent BHV-4 infection caused no decrease in the growth rate of BOMAC cells. Sodium butyrate, which reactivates latent gammaherpesviruses in vitro, or dexamethasone, which reactivates latent BHV-4 in vitro, increased viral DNA by 10- to 15-fold in persistently infected BOMAC cells. This suggests that reactivation of latent BHV-4 by dexamethasone in vivo might involve direct action of dexamethasone on latently infected cells.

Bovine herpesvirus-4 (BHV-4) is a gammaherpesvirus with no clear disease association (reviewed by Bartha et al., 1987; Goyal & Naem, 1992; Thiry et al., 1989). Like other herpesviruses, it establishes persistent infections both in its natural host and in an experimental host, the rabbit (Castrucci et al., 1987b; Dubuisson et al., 1989; Krogman & McAdaragh, 1982; Osorio et al., 1982). Latent infections can be reactivated in vivo by dexamethasone treatment (Boerner et al., 1999; Castrucci et al., 1987a, b; Dubuisson et al., 1989; Krogman & McAdaragh, 1982). BHV-4 has been demonstrated in many tissues during persistent/latent infection by both PCR (Boerner et al., 1999; Egyed & Bartha, 1998) and in situ hybridization (Lopez et al., 1996) or by the recovery of virus after explant culture (Castrucci et al., 1987b; Dubuisson et al., 1989; Krogman & McAdaragh, 1982; Osorio & Reed, 1983; Osorio et al., 1985). Peripheral blood leukocytes and lymphoid organs are among the sites of persistent/latent BHV-4 infection most consistently detected (Boerner et al., 1999; Castrucci et al., 1987b; Dubuisson et al., 1989; Krogman & McAdaragh, 1982; Lopez et al., 1996; Osorio & Reed, 1983; Osorio et al., 1985). Identification of BHV-4 in non-T, non-B cells located in the marginal zone of the spleen of persistently/latently infected cattle and rabbits has implied that cells of the monocyte/macrophage lineage are a site of persistent/latent BHV-4 infection (Lopez et al., 1996; Osorio et al., 1985). Nothing is known about either viral genome maintenance or viral gene expression during persistent BHV-4 infection and the molecular mechanisms of reactivation are also unknown. An in vitro model for persistent BHV-4 infection would be useful for the study of these processes.

Cell lines that are persistently infected with the gammaherpesviruses Epstein–Barr virus, herpesvirus saimiri, human herpesvirus-8 and murine gammaherpesvirus-68 have been established from cells isolated from infected hosts (Cesarman et al., 1995; Jung et al., 1999; Nilsson, 1979; Usherwood et al., 1996). This process has been 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 by BHV-4 has been obtained. The BHV-4 genome contains none of the genes that are associated with transformation by other gammaherpesviruses (Lomonte et al., 1996). Therefore, it is unlikely that cell lines persistently infected with BHV-4 can be established from cells derived from infected cattle.

Cell lines that are persistently infected with and transformed by gammaherpesviruses show a predominantly latent virus infection. In the vast majority of cells, viral gene expression is restricted to a specific subset of genes and the cells survive and replicate. The viral genome is maintained as...
a circular episome (Cesarman et al., 1995; Decker et al., 1996; Jung et al., 1999; Kieff, 1996; Renne et al., 1996; Usherwood et al., 1996). In a small proportion of cells the virus spontaneously reactives from latency, resulting in the production of infectious virus and subsequent cell death (Kieff, 1996; Moses et al., 1999). Treatment with agents such as sodium butyrate or phorbol esters also leads to reactivation from latency (Kieff, 1996; Miller, 1990; Renne et al., 1996; Sun et al., 1999; Usherwood et al., 1996).

BHV-4 causes cytopathic effect (CPE) and replicates in a variety of cell lines and primary cultures of bovine and various other animal species in culture (Lin et al., 1997; Peterson & Goyal, 1988; Truman et al., 1986). We recently examined the interaction of BHV-4 with a human rhabdomyosarcoma cell line, RD-4, and found that although no CPE was noted, some infectious virus was produced. Using recombinant BHV-4, we selected persistently infected RD-4 cells which expressed viral antigens and produced infectious virus at low levels but which continued to grow (Donofrio et al., 2000). To produce persistently infected cell lines that would be more similar to a persistent BHV-4 infection of cattle, we established persistent BHV-4 infection of a bovine macrophage cell line.

The M617 cell line (obtained at an unknown passage number from G. A. Splitter (School of Veterinary Medicine, University of Wisconsin-Madison, Madison, W.I., USA), through L. G. Adams (College Of Veterinary Medicine, Texas A & M University, College Station, TX, USA) is a bovine monocyte cell line originating spontaneously from peripheral blood mononuclear cells (Sager et al., 1999). BHV-4 (strain DN-599) infection of M617 cells at an m.o.i. of 2 TCID<sub>50</sub> per cell resulted in the death of all of the cells. The BOMAC cell line (obtained from J. R. Stabel, National Animal Disease Center, USDA-ARS, Ames, IA, USA) was established from peritoneal macrophages by transformation with SV40 DNA (Stabel & Stabel, 1995). BOMAC cells were used at passages 24–29 to establish a persistent BHV-4 infection. Uninfected BOMAC cells were shown to be free of BHV-4 genomes by in situ lysis gel electrophoresis followed by Southern blotting. BHV-4 (strain DN-599) infection of BOMAC cells at an m.o.i. of 5 or 50 TCID<sub>50</sub> per cell resulted in extensive CPE. However, a subpopulation of cells survived and could be passaged further. At every passage tested (over 20 passages), the viral genome was present in both circular and linear forms (Fig. 1). Therefore, the surviving cells are persistently infected. These surviving persistently infected cells continued to express SV40 T antigen (TAg) at a level similar to that in uninfected BOMAC cells, as indicated by indirect immunofluorescence using an anti-SV40 TAg monoclonal antibody (data not shown). The establishment of a persistent BHV-4 infection in the absence of selection contrasts with our results reported previously using the RD-4 human rhabdomyosarcoma cell line. The BHV-4 genome was rapidly lost from RD-4 cells unless the cells were infected with a recombinant BHV-4 virus expressing a drug-resistance gene and under drug selection (Donofrio et al., 2000).

As expected from the presence of linear viral DNA, which probably represents replicated, cleaved and packaged viral DNA, in surviving BOMAC cells, infectious BHV-4 was released from cells at all of the passages tested. Production of infectious virus indicated that viral genes of all kinetic classes were expressed. Therefore, we did not undertake the analysis of viral RNA expression. Immunostaining with a BHV-4-specific rabbit hyperimmune serum as described by Donofrio et al. (2000) resulted in extremely faint staining (data not shown). The amount of viral DNA and relative amounts of linear and circular viral DNA varied among samples of persistently infected cells at different passage numbers (Fig. 1) for unknown reasons. One possible explanation is that virus production is affected by growth conditions, such as the relative confluency of the cells or the concentration of available growth factors. Increased virus production would result firstly in the increase of linear viral DNA and secondly in the accumulation of more viral DNA, predominantly circular, after virus production had diminished. This possibility was not examined experimentally.

The extremely faint immunostaining of persistently infected BOMAC cells with anti-BHV-4 serum did not allow us to ascertain whether the culture was a carrier culture (only some of the cells are infected) or if all the cells were infected. In the case of a carrier culture, cell death might be an

![Fig. 1. In situ lysis gel electrophoresis and Southern blot analysis of viral DNA in BHV-4-persistently infected BOMAC cells. Cells were infected at the indicated m.o.i. (TCID<sub>50</sub> per cell). Surviving cells were subjected to in situ lysis gel analysis (Gardella et al., 1984) (1 x 10<sup>6</sup> cells per lane) after the number of passages indicated. Viral DNA was detected by hybridization with the 3–7 kb BHV-4.EcoRI I fragment derived from the cloned 11 kb HindIII D fragment (pC1; van Santen & Chang, 1992) and labelled with P by random primer extension (RadPrime kit, BRL). Arrowheads indicate circular (C) and linear (L) viral genomes. Linear viral DNA was detectable in all samples on the original autoradiograph. No hybridization to samples from uninfected BOMAC cells was observed (data not shown).](image-url)
obligatory outcome of infection, but the culture would be maintained by multiplication of the uninfected cells. Infection of this culture would then be maintained by a new infection of cells by virus that had been produced by the infected cells. Alternatively, the presence of virus in all cells would suggest that BHV-4 infection is compatible with the survival and replication of BOMAC cells. The recombinant virus BHV-4EGFPΔTK, which contains an enhanced green fluorescent protein (EGFP) gene linked to the human cytomegalovirus immediate early promoter inserted into the BHV-4 thymidine kinase gene, was used to demonstrate that persistently infected BOMAC cells, initially infected with 50 TCID<sub>50</sub> per cell, were unlikely to be a carrier culture. BOMAC cells were infected with 25 TCID<sub>50</sub> BHV-4EGFPΔTK per cell. Observation of cells by fluorescent microscopy indicated that all surviving cells expressed EGFP and were, therefore, infected (Fig. 2). Fluorescence of some cells was quite faint: subjective observation suggested that the intensity of fluorescence decreased with the increasing passage number of the cell line. Others have also observed a decrease in the intensity of fluorescence of cells expressing EGFP over time, presumably due to the toxicity of EGFP resulting in the selection of cells expressing less EGFP (Felts et al., 2000). In our case, the decrease could be due to the selection of cells with either fewer viral genomes per cell or less efficient gene expression from the viral genomes.

These data suggested that our persistently infected BOMAC cells did not contain the number of uninfected cells required to maintain a carrier culture. To determine whether a carrier culture could be established or if infection would spread to all cells, BOMAC cells were infected with 0.25 TCID<sub>50</sub> BHV-4EGFPΔTK per cell and observed by fluorescence microscopy. Initially, not all cells were infected, but by 28 days post-infection (p.i.) (after 5 passages) virus infection had spread to include all cells (data not shown). This is consistent with our observation that persistently infected cells produce infectious virus. Our results do not rule out the possibility that a carrier culture could be established if cells were initially infected at a much lower m.o.i., but do support the conclusion that persistently infected BOMAC cells established by infection at 5 or 50 TCID<sub>50</sub> per cell are not carrier cultures.

To confirm that infected BOMAC cells could survive and replicate, we infected cells with the recombinant BHV-4 26A3neo (Donofrio et al., 2000), which carries the neomycin-resistance gene, and selected infected cells with geneticin. Four different protocols, differing in the m.o.i. of the initial infection, time of application of selection and the concentration of geneticin, were used successfully to establish geneticin-resistant, persistently infected BOMAC cells. The initial m.o.i. was 5 or 50 TCID<sub>50</sub> per cell and geneticin was used at 100, 200 or 400 µg/ml. Geneticin was usually added 1 day p.i. However, delaying geneticin selection until 66 days p.i. (after 15 passages) also resulted in selection of geneticin-resistant cells. In all cases, colonies developed from cells surviving both the virus infection and the drug selection. These colonies were cultivated into cell lines that could be passaged in the presence of the selective drug. These cells contained the BHV-4 genome, as demonstrated both by in situ lysis gel analysis and by PCR (data not shown). Therefore, the presence of the BHV-4 genome is compatible with both the survival and the replication of BOMAC cells. These cells also produced infectious BHV-4. Immunostaining with a BHV-4-specific rabbit hyperimmune serum resulted in extremely faint staining (data not shown). The ability to select geneticin-resistant cells 15 passages after infection with recombinant BHV-4 is further evidence that persistent infection can be established in the absence of drug selection, as would be the case following the natural infection of cattle with wild-type virus.

BOMAC cells that were persistently infected with BHV-4 (strain DN-599 or 26A3neo) grew in a similar way to uninfected BOMAC cells for over 20 passages. To more accurately assess the effect of persistent BHV-4 infection on cell growth, the rate of growth of BOMAC cells selected for persistent BHV-4 infection (cells infected with 50 TCID<sub>50</sub> 26A3neo per cell and selected and maintained with 400 µg/ml geneticin) was compared to that of uninfected BOMAC cells over a 4 day period in the absence of geneticin using an MTT assay, as described previously (Donofrio et al., 2000). Results (not shown) indicated that the persistently infected cells grew at a rate identical to that observed for uninfected cells. This is consistent with our observations that selection is not necessary to maintain persistent infection. Again, this result contrasts with persistently infected RD-4 cells, which grew more slowly than uninfected cells (Donofrio et al., 2000).

We examined the effects of dexamethasone, which reactivates latent BHV-4 infection in vivo (Boerner et al., 1999; Castrucci et al., 1987a, b; Dubuisson et al., 1989; Krogman & McAdaragh, 1982), and sodium butyrate, which reactivates latent infections of other gammaherpesviruses in vitro (Kieff, 1996; Miller, 1990; Sun et al., 1999), on BHV-4 replication in persistently infected BOMAC cells. BOMAC cells that were selected for BHV-4 persistent infection with
gp geneticin and treated with either dexamethasone or sodium butyrate exhibited a 10- to 15-fold increase of viral DNA, implying activation of virus replication (Fig. 3). The increase of butyrate exhibited a 10- to 15-fold increase of viral DNA, geneticin and treated with either dexamethasone or sodium butyrate or dexamethasone was added to the medium of subconfluent BOMAC cells (in 6-well plates) that were selected with geneticin for persistent BHV-4 infection (cells infected with 50 TCID₅₀ 26A3neo per cell and selected and maintained with 400 µg/ml geneticin). Total DNA was prepared 2 days later by treatment with SDS and proteinase K followed by phenol extraction and digested with HindIII. The relative amount of viral DNA in 5 µg total DNA from treated and untreated cells was determined by Southern blot analysis using a digoxigenin-labelled PCR fragment that detected a 4-7 kb BHV-4 fragment.

![Table](image)

**Fig. 3.** Induction of viral DNA replication by sodium butyrate and dexamethasone treatment. Either sodium butyrate or dexamethasone was added to the medium of subconfluent BOMAC cells (in 6-well plates) that were selected with geneticin for persistent BHV-4 infection (cells infected with 50 TCID₅₀ 26A3neo per cell and selected and maintained with 400 µg/ml geneticin). Total DNA was prepared 2 days later by treatment with SDS and proteinase K followed by phenol extraction and digested with HindIII. The relative amount of viral DNA in 5 µg total DNA from treated and untreated cells was determined by Southern blot analysis using a digoxigenin-labelled PCR fragment that detected a 4-7 kb BHV-4 fragment.

Although persistent BHV-4 infection can be established in both RD-4 and BOMAC cell lines, the infections differ in several important ways: (1) BHV-4 causes no CPE in RD-4 cells, but does cause cell death of the majority of BOMAC cells and persists in surviving cells; (2) in BOMAC cells, persistent infection can be established in the absence of drug selection but BHV-4 does not persist in RD-4 cells in the absence of the drug selection for a recombinant virus; (3) viral antigen expression is more easily detected by a polyclonal rabbit hyperimmune serum in persistently infected RD-4 cells than in persistently infected BOMAC cells; (4) persistently infected, RD-4 cells grow more slowly than uninfected cells, whereas persistent infection of BOMAC cells has no effect on cell growth; and (5) most importantly, persistent BHV-4 infection of a bovine macrophage cell line is possibly relevant to the persistence of BHV-4 in cattle.

Lin et al. (1999) recently reported persistent infection of bovine arterial endothelial (BAE) cell cultures with BHV-4. They observed CPE at approximately passage 35 in several separate BAE cell cultures and isolated BHV-4 from these cultures. They concluded that the cultures were persistently infected when isolated from the animal and that continuous subculturing activated replication of the virus. An alternative explanation is that infection of the BAE cultures, which are highly susceptible to BHV-4 infection (Lin et al., 1997), occurred by contamination during passaging. Our persistently infected BOMAC cells differ significantly from those of Lin et al. (1997). The ‘persistently infected’ BAE cultures were completely killed by the virus, whereas our persistently infected BOMAC cells continue to grow at the same rate as uninfected cells.

This is Auburn University College of Veterinary Medicine publication number 2579. We are thankful to Dr J. R. Stabel for providing BOMAC cells and to Dr L. G. Adams for providing M617 cells. We gratefully acknowledge the technical assistance of Ms K. Sparks and Mr J. B. Carr.

G. Donofrio and V. L. van Santen contributed equally to this work.

References


Received 10 October 2000; Accepted 11 January 2001