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 vivo, 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 & Naeem, 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). BHV-4 has been demonstrated in many tissues during persistent/latent infection by both PCR (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; 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 implicated 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 reactivates 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. Spliter (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 5 TCID50 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 TCID50 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 establish-
ment 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
antibody. The BOMAC cell line

3. In situ lysis gel electrophoresis and Southern

[Figure 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. (TCID50 per cell). Surviving cells were subjected to in situ lysis gel analysis (Gardella et al., 1984) (1 x 106 cells per lane) after the number of passages indicated. Viral DNA was detected by hybridization with the 3.7 kb BHV-4 EcoRI J fragment derived from the cloned 11 kb HindIII D fragment (pC1; van Santen & Chang, 1992) and labelled with 32P 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).]
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-

$\Delta$TK per cell were photographed at passage 4 using a standard fluorescein isothiocyanate filter-equipped microscope. No fluorescence was detected in uninfected BOMAC cells.

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$_{50}$ per cell and geneticin was used at 100, 200 or 400 $\mu$g/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$_{50}$ 26A3neo per cell and selected and maintained with 400 $\mu$g/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
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 the amount of viral DNA after dexamethasone treatment in vitro, consistent with reactivation, suggests that the reactivation of latent BHV-4 infection leading to virus shedding (following dexamethasone treatment in vitro) might be, at least partially, a result of direct action of dexamethasone on latently infected cells in addition to a relaxation of immunosurveillance due to the immunosuppressive effects of dexamethasone. This proposition is consistent with the observation that in the case of cattle that are latently infected with another bovine herpesvirus, BHV-1, viral nucleic acid (indicative of virus reactivation) is detectable in latently infected tissues by in situ hybridization within 6 h of dexamethasone treatment (Winkler et al., 2000).

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 subculturability 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.

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References


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