The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-γ

Camilo Raggo,1, 2 Monique Habermehl,2 Lorne A. Babiuk1, 2 and Philip Griebel2

Department of Veterinary Microbiology1 and Veterinary Infectious Disease Organization2, 120 Veterinary Road, University of Saskatchewan, Canada S7N 5E3

To study the biological relevance of using bovine herpesvirus-1 (BHV-1) as a vector for expressing cytokines, a BHV-1 virus that expressed bovine interferon-γ (IFN-γ) was constructed. This recombinant virus (BHV-1/IFNγ) was then used to infect the natural host in a respiratory disease model. In vitro characterization of the recombinant interferon-γ confirmed that the cytokine expressed in BHV-1-infected cells was biologically active. The in vivo effects of the recombinant IFN-γ were then analysed during a primary infection and after reactivation of a latent infection. During the primary infection, similar body temperature, clinical responses and virus shedding were observed for calves infected with either recombinant BHV-1/IFNγ or parental gC/NLacZ virus. An analysis of cellular and humoral responses did not reveal any significant immunomodulation by BHV-1/IFNγ during the primary infection. The stability and activity of recombinant IFN-γ was also analysed following the establishment of a latent infection. The presence of recombinant IFN-γ did not significantly alter virus shedding following reactivation. The isolation of reactivated BHV-1/IFNγ virus confirmed that a functional IFN-γ gene was retained during latency. Thus, herpesviruses may provide virus vectors that retain functional genes during latency and recrudescence.

Introduction

Bovine herpesvirus-1 (BHV-1) is a member of the subfamily Alphaherpesvirinae, genus Varicellovirus. Current interest in BHV-1 vaccine development has focused on the use of subunit vaccines, gene-deleted viruses and killed and live virus vectors (van Drunen Littel-van den Hurk et al., 1993; van Oirschot, 1996). The deletion mutants are used both to attenuate the virus and to serve as marker vaccines to distinguish vaccinated animals from naturally infected animals. These gene-deleted viruses can also be used as vectors for genes encoding an array of putative protective antigens or immunomodulatory molecules. The production of cytokines at the site of virus replication could have significant immunomodulation potential that could assist the host in controlling virus replication, preventing latency and possibly preventing secondary bacterial infections. The construction of a recombinant BHV-1 vector containing interferon-γ (IFN-γ) made it possible to investigate this novel vaccine approach in a primary respiratory infection model and following the reactivation from a latent infection.

IFN-γ has numerous immunological regulatory functions that may be beneficial in modulating virus diseases in cattle. These include Fc receptor up-regulation, activation of macrophages and polymorphonuclear phagocytic functions, up-regulation of major histocompatibility complex (MHC) class I and class II and inhibition of virus replication (Campos et al., 1989; Boehm et al., 1997). The production of IFN-γ by lymphocytes following BHV-1 infection plays an important role in the activation of non-MHC-restricted cytotoxicity (Campos et al., 1989). In addition, bovine IFN-γ (boIFN-γ) has been shown to up-regulate the expression of various immunespecific molecules, including MHC class II, Fc receptor and C3b receptor, and to influence a variety of effector functions (Bielefeldt Ohmann et al., 1987).

The capacity of IFN-γ to modulate virus infections when expressed in a recombinant virus vector has been investigated with several different viruses. A vaccinia virus (VV) vector expressing IFN-γ was shown to attenuate a typically lethal virus infection in an immunodeficient mouse model (Kohonen-Corish et al., 1990; Giavedoni et al., 1992). Recently, expression
of recombinant IFN-γ by a simian immunodeficiency virus (SIV) vector resulted in significantly lower virus loads in the blood of rhesus macaque monkeys when compared to mutant vector controls (Giavaldi et al., 1997).

A BHV-1 vector expressing boIFN-γ (BHV-1/IFNγ) could potentially have beneficial immunomodulating effects. In this study, we evaluated the stability and biological activity of recombinant BHV-1/IFNγ during a primary infection of its natural host and following the reactivation of a latent herpesvirus infection.

Methods

■ Virus and cells. The Cooper strain of BHV-1, obtained from the National Veterinary Services Laboratories (Ames, IA, USA), was used as the prototype virus. A glycoprotein C (gC)-negative mutant expressing the LacZ gene (BHV-1/gC/gIII/LacZ) was previously produced and is designated gC/+LacZ (Liang et al., 1992). Field isolate of BHV-1 strain 108 was obtained from the Animal Diseases Research Institute (Alberta, Canada). All viruses were propagated in Madin–Darby bovine kidney (MDBK) cells that were obtained from the ATCC. Cells were maintained in minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) (Gibco/BRL).

■ Construction and characterization of recombinant virus. To construct a transfer vector with the boIFN-γ coding sequence, the boIFN-γ gene was isolated from plasmid pdTrpo/vCAAXP (Ciba Geigy) by an EcoRI/SspI digest followed by treatment with Klenow fragment to repair the protruding end (Cerretti et al., 1986). A BamH1 linker (dCCGG-ATCCG) was added for insertion into the BamH1 site of transfer vector pSD11SSIFNγ. DNA sequencing of the gC–boIFNγ junction confirmed the correct coding sequence. The cleaved product was predicted to retain the 143 aa sequence from the mature protein of boIFN-γ plus 6 aa from the gC fusion protein at the amino terminus (ARDPPEFM*QFF….) (Cerretti et al., 1986).

Parental BHV-1/gIII/LacZ (Liang et al., 1992) viral DNA was purified according to methods previously described (Raggo et al., 1996). To generate recombinant virus, MDBK cells were co-transfected with 1.5 μg pSD11SSIFNγ and 10 μg BHV-1/gIII/LacZ DNA by lipofection. Plasmid and viral DNA in 35 μl DH2O plus 40 μl lipofectin (Gibco/BRL) were added to MDBK cells. The next day, Opti-MEM (Gibco/BRL) supplemented with 2% FBS was added and cells were incubated for another day. Cells were then washed twice with MEM, trypsinized and resuspended into a 96-well plate. Wells expressing cytopathology were selected and 2% FBS was added and cells were incubated for 24 h. The supernatant was then collected and centrifuged at 75000 g for 1 h to remove active virus. Control wells were incubated with either 100 ng/ml of recombinant boIFN-γ or MEM. After a 24 h incubation, cells were collected from the wells by incubating adherent cells in PBS–BSA solution supplemented with 0.05% EDTA for 10 min. Cells were washed once and cell suspension of 0.05% EDTA for 10 min. Cells were washed once and cell suspensions were plated into a 96-well plate (5 x 10⁴ cells/well) and co-incubated for 30 min with lineage-specific monoclonal antibodies (MAbs); BoLa class II, a nonpolyorphic determinant (clone TH14B, IgG2a isotype); and monocyte-macrophage marker (clone DHH59B, IgG1 isotype) [Veterinary Medical Research and Development (VMRD), Pullman, WA, USA]. After two washes with PBS, cells were incubated for 30 min with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG1 or phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin (Becton Dickinson), then washed twice and fixed with 2% formaldehyde. Cells were stored in the dark at 4 °C until analysed. The intensity of fluorescence was evaluated using a FACScan (Becton Dickinson) and the Cell Quest program. Non-specific staining was determined by incubating cells with isotype-matched control MAbs and the FITC- and PE-conjugated secondary antibodies. To determine the percentage of positive staining, 5000 cells were analysed. For each sample forward- and side-scatter were used to restrict flow cytometric analyses to mononuclear cells.

Venous blood samples were collected in EDTA vacuum tubes (Becton Dickinson). Total and differential white blood cell counts were determined by Prairie Diagnostic Services (Saskatchewan, Canada).

For flow cytometric analysis, leukocytes were incubated with MAbs specific for the following bovine leukocyte surface markers: CD3 (clone MM1A), CD4 (ILA12), CD8 (clone CACT80C), IgM (clone PIG45A), B-cell (BAQ44A) and granulocytes/monocytes (DHH59B). Leukocytes were also dual-labelled for the MHC class II molecule (TH14B) and CD4 (ILA12). Antibodies were obtained from VMRD. The IL12A (CD4) monoclonal antibody was a generous gift from J. Ellis (Western College of Veterinary Medicine, Saskatoon, Canada). Labelling by MAbs was evaluated using a FACScan (Becton Dickinson) and the Lysis II program.

■ Cattle challenge model. All animals were cared for under the guidelines provided by the Canadian Council on Animal Care (1993). BHV-1 sero-negative 6-month-old Holstein calves were randomly divided into three groups. At day 0, animals were intranasally challenged with 4 x 10⁶ p.f.u./ml of either wild-type Cooper strain, gC–/LacZ– mutant or recombinant BHV-1/IFNγ aerosol using a Devilbiss nebulizer model 65. Approximately 3 x 10⁶ p.f.u. of virus was administered by aerosol during a 5 min period. Each experimental group was housed separately to prevent cross-infection by different virus strains. Animals were monitored daily for 10 days by a veterinarian who assessed clinical responses. Thirty-five days after the primary infection, animals were treated for 5 days with 0.1 mg/kg dexamethasone to reactivate latent virus infections (Homan & Easterday, 1983). Clinical responses were monitored and nasal swabs were taken to detect virus shedding for 10 days after dexamethasone treatment.

Briefly, calves were euthanized and both lungs were removed. MEM (1 l) was then poured into the trachea and the lung lobes were gently massaged. The lungs were next inverted to collect the MEM solution that contained alveolar macrophages. This procedure was repeated three times. The collected medium was pooled and centrifuged at 1500 r.p.m. in a Beckman GH-3.7 rotor for 10 min. The cell pellet was resuspended and washed twice with MEM before viable cells were counted. Alveolar macrophages were incubated in 6-well plates at 15 x 10⁶ cells per well in MEM containing 5% FBS.

To detect IFN-γ-induced MHC class II up-regulation, 3 ml of isolated alveolar macrophages (4 x 10⁶ cells/ml) were incubated with 2 ml of supernatant from MDBK cells infected with either wild-type Cooper virus, gC–/LacZ– or BHV-1/IFNγ. Virus supernatant was centrifuged at 75000 g for 2 h to remove active virus. Control wells were incubated with either 100 ng/ml of recombinant boIFN-γ or MEM. After a 24 h incubation, cells were collected from the wells by incubating adherent cells in PBS–BSA solution supplemented with 0.05% EDTA for 10 min. Cells were washed once and cell suspensions were placed into a 96-well plate (5 x 10⁷ cells/well) and co-incubated for 30 min with lineage-specific monoclonal antibodies (MAbs); BoLa class II, a nonpolyorphic determinant (clone TH14B, IgG2a isotype); and monocyte-macrophage marker (clone DHH59B, IgG1 isotype) [Veterinary Medical Research and Development (VMRD), Pullman, WA, USA]. After two washes with PBS, cells were incubated for 30 min with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG1 or phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin (Becton Dickinson), then washed twice and fixed with 2% formaldehyde. Cells were stored in the dark at 4 °C until analysed. The intensity of fluorescence was evaluated using a FACScan (Becton Dickinson) and the Cell Quest program. Non-specific staining was determined by incubating cells with isotype-matched control MAbs and the FITC- and PE-conjugated secondary antibodies. To determine the percentage of positive staining, 5000 cells were analysed. For each sample forward- and side-scatter were used to restrict flow cytometric analyses to mononuclear cells.

Venous blood samples were collected in EDTA vacuum tubes (Becton Dickinson). Total and differential white blood cell counts were determined by Prairie Diagnostic Services (Saskatchewan, Canada).

For flow cytometric analysis, leukocytes were incubated with MAbs specific for the following bovine leukocyte surface markers: CD3 (clone MM1A), CD4 (ILA12), CD8 (clone CACT80C), IgM (clone PIG45A), B-cell (BAQ44A) and granulocytes/monocytes (DHH59B). Leukocytes were also dual-labelled for the MHC class II molecule (TH14B) and CD4 (ILA12). Antibodies were obtained from VMRD. The IL12A (CD4) monoclonal antibody was a generous gift from J. Ellis (Western College of Veterinary Medicine, Saskatoon, Canada). Labelling by MAbs was evaluated using a FACScan (Becton Dickinson) and the Lysis II program.

■ Cattle challenge model. All animals were cared for under the guidelines provided by the Canadian Council on Animal Care (1993). BHV-1 sero-negative 6-month-old Holstein calves were randomly divided into three groups. At day 0, animals were intranasally challenged with 4 x 10⁶ p.f.u./ml of either wild-type Cooper strain, gC–/LacZ– mutant or recombinant BHV-1/IFNγ aerosol using a Devilbiss nebulizer model 65. Approximately 3 x 10⁶ p.f.u. of virus was administered by aerosol during a 5 min period. Each experimental group was housed separately to prevent cross-infection by different virus strains. Animals were monitored daily for 10 days by a veterinarian who assessed clinical responses. Thirty-five days after the primary infection, animals were treated for 5 days with 0.1 mg/kg dexamethasone to reactivate latent virus infections (Homan & Easterday, 1983). Clinical responses were monitored and nasal swabs were taken to detect virus shedding for 10 days after dexamethasone treatment.
Virus isolation and mucus collection. Samples to evaluate virus shedding were collected by inserting a cotton swab into the nasal cavity and rubbing the mucosa. The cotton swab was then placed into 1 ml MEM and stored at -70 °C until virus titre was determined. To collect nasal secretions, a cotton tampon was inserted into one nostril for 20 min and then placed into a 50 ml sterile syringe to extract the fluids.

ELISA. To detect BHV-1 antibody levels, Immunolon-II microtitre plates (Dynatech) were coated overnight with 0.05 μg per well of purified gD protein (van Drunen Littel-van den Hurk et al., 1984) in 50 mM carbonate buffer (pH 9.6). Bound IgG was detected by adding a 1:10000 dilution of phosphatase-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratory). Plates were developed by the addition of substrate p-nitrophenyl phosphate diTris salt (Sigma) in 0.5 mM MgCl₂ (pH 9.8) with 1% diethanolamine. A BioRad 3550 microplate reader was used to read plates at a wavelength of 405 nm (reference of 490 nm).

The gD-specific IgA was detected in a similar manner using a mouse anti-bovine IgA (clone M67) MAb at a 1:5000 dilution, followed by a biotinylated horse anti-mouse IgG at 1:5000 (Vector Laboratories) and a 1:1000 dilution of streptavidin–alkaline phosphatase (SAP) for 1 h (Gibco/BRL).

BoIFN-γ was detected with a capture ELISA. Immunolun II plates were coated with MAb (clone 2-2-1) diluted 1:1000 in 50 mM carbonate buffer (pH 9.6). Samples were serially diluted, added to the wells and incubated for 2 h at room temperature. After washing, rabbit anti-boIFN-γ polyclonal antiserum (lot 92-131), diluted 1:2000, was added for 1 h. Biotinylated goat anti-rabbit IgG, diluted 1:1000, was used for developing with the SAP system. To calculate the concentration of IFN-γ in a test sample, recombinant boIFN-γ (Ciba Geigy) was used to generate a standard curve. The 36 h growth kinetics curve of culture supernatant from vector-produced IFN-γ was compared to the recombinant boIFN-γ standard curve and calculated to contain 512 U/ml activity and a protein concentration of 182 ng/ml (sp. act. 2.8 × 10⁶ U/mg).

BoIFN-γ enzyme-linked immunospot assay (ELISPOT). ELISPOT was used to determine the number of IFN-γ-secreting cells present in the blood. Blood mononuclear cells (PBMCs) were isolated by layering buffy coat cells over 60% Percoll (Pharmacia). PBMCs were cultured in 24-well plates at a concentration of 6 × 10⁶ cells per well in culture medium containing 5% FBS, 2 mM glutamine, 50 mM 2-mercaptoethanol and 5 ng/ml dexamethasone in the presence or absence of 0.4 μg per well of purified gD protein. Dexamethasone was added to the culture medium to decrease the background frequency of non-specific IFN-γ-secreting cells. Nitrocellulose plates (Amicon) were coated with a MAb for boIFN-γ (clone 2-2-1) diluted 1:400 in 50 mM carbonate buffer (pH 9.6) at 37 °C. Following a 24 h culture, PBMCs were harvested and viable cells were plated at 1 × 10⁵, 5 × 10⁵ and 2.5 × 10⁶ cells per well in the pre-coated 96-well nitrocellulose-based microtitre plates. After incubation for 8–16 h, the plates were washed twice with cold H₂O and five times with PBS. To detect bound boIFN-γ, rabbit anti-boIFN-γ antiserum (lot 92-131) was added at 1:100 dilution for 2 h at room was titred by plaque assay in 24-well plates. Cooper strain wild-type; gC−, a gC-negative mutant; BHV-1/IFNγ; gC-negative mutant expressing boIFN-γ.
temperature. Plates were washed and incubated with a 1:2500 dilution of biotinylated rat anti-rabbit IgG (Zymed) for 2 h, followed by incubation with a 1:1000 dilution of SAP for 1 h. IFN-γ spots were developed with 5-bromo-4-chloro-3-indolyl phosphate Nitro Blue Tetracium substrate (Sigma). The number of gD-specific IFN-γ-secreting cells was determined by subtracting the number of spots in wells with PBMCs cultured in the absence of purified gD protein.

**Statistical analysis.** Statistical analysis was performed by the GraphPad PRISM program (GraphPad Software). Groups were compared by Student's t-test, analysis of variance or the Mann–Whitney rank-sum test as indicated in the figure legends.

**Results**

Recombinant BHV-1 was generated by co-transfection of MDBK cells with transfer plasmid pSD113SSIFNγ and purified DNA from BHV-1-lgIII/LacZ (Liang et al., 1992). Recombinant viruses were screened by blue plaque assay. White plaques were identified and plaque-purified twice. A purified progeny virus was designated BHV-1/IFNγ and was used for further characterization. To confirm the genomic rearrangement of the boIFN-γ gene in the gC region, Southern blot analysis was carried out using total cellular DNA from both wild-type virus and recombinant virus (Fig. 1A). The gC probe containing both 5′ and 3′ ends hybridized to a 30 kb fragment in the wild-type lane, whereas the lane containing recombinant BHV-1/IFNγ had two fragments of 1.5 kb and 0.7 kb. The insertion of boIFN-γ created a unique EcoRI site in front of the gC signal sequence, which resulted in two fragments. The boIFN-γ probe did not hybridize to wild-type DNA; a 1.5 kb fragment was detected with BHV-1/IFNγ DNA.

**BHV-1/IFNγ produces biologically active IFN-γ**

To ascertain whether BHV-1/IFNγ-produced recombinant boIFN-γ protein had biological activity, the supernatant from cell cultures infected with BHV-1/IFNγ virus was tested in a standard anti-virus VSV assay. IFN-γ activity was detected as early as 6 h post-infection (p.i.) and this activity was detected throughout the sampling period (Fig. 1B). At 36 h p.i., the supernatant fraction contained the majority of antiviral activity (512 U/ml) while activity in the cellular fraction remained constant after 24 h p.i. (256 U/ml). Furthermore, the specific activity of the recombinant boIFN-γ produced by BHV-1/IFNγ was calculated to be 2.8 × 10⁶ U/mg using a boIFN-γ capture ELISA. This specific activity is comparable to the E. coli-produced recombinant boIFN-γ standard of 3 × 10⁶ U/mg (Ciba Geigy).

**Expression of boIFN-γ does not alter in vitro virus growth**

To assess the in vitro growth efficiency of the recombinant BHV-1/IFNγ, a growth kinetics experiment was performed. MDBK cells were infected with either wild-type Cooper, gC−/LacZ+ or BHV-1/IFNγ virus. Both recombinant viruses exhibited similar growth kinetics with virus yields approximately 10- to 100-fold lower than wild-type virus (Fig. 1C).

The maximal yield for recombinant gC-negative phenotype viruses was approximately 4 × 10⁵ p.f.u./ml while wild-type Cooper virus produced greater than 2 × 10⁶ p.f.u./ml. Therefore, the production of biologically active boIFN-γ did not alter the in vitro growth of recombinant BHV-1. The observation that recombinant boIFN-γ did not alter BHV-1 growth is consistent with previous reports that herpesviruses are generally resistant to the antiviral activity of interferons (Babiuk et al., 1985; Bielefeldt Ohmann et al., 1984).

**Infection of cattle with BHV-1/IFNγ**

Since in vitro growth curve studies of BHV-1/IFNγ showed no inhibitory effect on virus growth, three different groups of sero-negative calves were challenged with either BHV-1/IFNγ, gC-negative parental virus or wild-type BHV-1 virus. All calves responded to infection with an increased body temperature (> 39.5 °C). Elevated rectal temperatures began 2 days p.i. and peaked 4–5 days p.i. (Fig. 2A). Clinical scores were also elevated 2 days p.i. and peaked at days 4–6 p.i. for all groups. Clinical symptoms included increased nasal discharge, coughing, nasal mucosa hyperaemia and plaque formation in the nares of all animals. Overall, changes in body temperature and clinical score were similar in the three groups and paralleled what would be expected in the BHV-1 challenge.
model (Wyler et al., 1989; Yates, 1982). Thus, the expression of boIFN-γ by BHV-1/IFNγ had no detectable effect on the clinical response to virus infection.

Virus was isolated in nasal swabs from all animals. The majority of virus shedding occurred between days 4 and 6 p.i. (Fig. 2B). A comparison of virus shedding among groups showed that animals infected with both gC-negative phenotype viruses shed 10- to 100-fold less virus than those infected with wild-type Cooper virus. This difference in shedding was similar to the growth differences observed in vitro (Fig. 1C). A comparison between recombinant gC-negative viruses, gC−/LacZ+ and BHV-1/IFNγ, showed no significant differences in virus shedding, indicating that the in vitro expression of recombinant boIFN-γ did not have a significant effect on virus replication. The decreased shedding of the gC-deleted strains also appeared to correlate with smaller plaques in the nasal cavity and earlier resolution of nasal lesions as indicated by the clinical score (data not shown).

During a BHV-1 infection, changes in circulating leukocytes occur within the blood (Griebel et al., 1987). To study the effects of recombinant BHV-1/IFNγ on blood leukocyte population dynamics, we monitored changes by flow cytometric analysis and by total and differential white blood cell counts. However, there were no significant differences in total and differential blood counts among groups (data not shown). Similarly, FACS analysis of leukocyte subpopulations 3 days p.i. revealed no significant differences in leukocyte subpopulations or MHC class II expression on CD4+ T-cells (see Methods).

To determine whether boIFN-γ was secreted in nasal secretions, we collected nasal secretions every second day p.i. and analysed IFN-γ levels by ELISA. As shown in Fig. 3, IFN-γ was detectable in nasal secretions and the level of IFN-γ paralleled virus replication and clinical signs. The level of IFN-γ in nasal secretions peaked on day 4 p.i. for all animals and by day 6 p.i. the level of IFN-γ exceeded 100 pg/ml in nasal secretions of all animals. However, on day 6 p.i. IFN-γ levels in nasal secretions from all the animals in the BHV-1/IFNγ group exceeded 1000 pg/ml, but only a single animal from the other two groups had a similar level of IFN-γ in nasal secretions. A direct comparison between gC−/LacZ+ and BHV-1/IFNγ by Student’s t-test (P = 0.0567) and the Mann–Whitney rank-sum test (P = 0.050) indicated a statistical difference for IFN-γ produced in the nasal cavity. Given that IFN-γ has a half-life of approximately 30 min, we would expect the level of IFN-γ assayed to reflect production during the day of sampling (Kurzrock et al., 1985). Thus, the elevated level of IFN-γ in BHV-1/IFNγ-infected animals supports the conclusion that
BHV-1/IFNγ virus expressed recombinant IFN-γ during infection.

Serum antibody titres for gD were determined by ELISA (van Drunen Littel-van den Hurk et al., 1984). After the primary intranasal challenge, all animals became sero-positive by week 2 p.i. Peak IgG titres occurred 4 weeks after the primary challenge period (Fig. 4A). A secondary rise in serum IgG antibody titres followed dexamethasone reactivation of latent infections and there was no significant difference for total IgG between gC−/LacZ− and BHV-1/IFNγ-infected animals (Fig. 4A). Furthermore, a comparison of the IgG isotypes, IgG1 and IgG2a, revealed no significant differences in the IgG1:IgG2a ratios of gD-specific antibody induced by the two recombinant viruses (data not shown). Similarly, no statistically significant differences in virus neutralization titres were detectable (data not shown).

Nasal IgA was detected using gD-coated plates and an antitbovine IgA MAb (Fig. 4B). During the first 5 weeks after primary infection, there were no significant differences in nasal IgA titres for animals infected with the recombinant BHV-1. In contrast, following dexamethasone treatment there was a significant increase in nasal IgA titres in animals infected with wild-type BHV-1 and gC−/LacZ+ viruses but not in animals infected with BHV-1/IFNγ (Fig. 4B).

To detect virus-specific T-cell responses, peripheral blood lymphocytes were isolated and assayed for gD-specific IFN-γ-secreting cells. The frequency of gD-specific IFN-γ-secreting cells was comparable among all experimental groups, especially the gC−/LacZ+ and BHV-1/IFNγ groups (Fig. 5). The frequency of gD-specific IFN-γ-secreting cells decreased by week 5 after the primary infection but IFN-γ-secreting cell frequency was elevated after reactivation of the latent infection. Similarly, gD-induced proliferative responses of blood lymphocytes were not significantly different among the three groups after either the primary infection or following reactivation of the latent infection (data not shown).

Reactivation of latent virus

Calves were treated with dexamethasone to determine if recombinant BHV-1/IFNγ established a latent infection and if the IFN-γ gene insert was stable. All animals were clinically normal before dexamethasone treatment but following treatment there was an increase in clinical scores and rectal temperatures that correlated with virus shedding (Fig. 6). Calves from all three groups shed virus for at least 5 days following treatment and virus shedding peaked 3 days after dexamethasone treatment. As observed during the primary infection, animals infected with wild-type Cooper virus shed 10- to 100-fold more virus than animals infected with recombinant gC-negative viruses. The Cooper group shed a maximum titre of 2.7 × 10^6 p.f.u./ml per swab, while recombinant gC−/LacZ+ shed a maximum of 3.6 × 10^4 p.f.u./ml per swab and BHV-1/IFNγ shed a maximum of 2.3 × 10^4 p.f.u./ml per swab. Virus shedding was monitored for 7 days (data not shown), but only one calf from the BHV-1/IFNγ group shed detectable virus (40 p.f.u./ml). Neither virus titre nor the duration of virus shedding was significantly different between recombinant BHV-1/IFNγ- and gC−/LacZ+ infected animals.

Biologically active IFN-γ was expressed by reactivated virus

To determine whether latent BHV-1/IFNγ retained a functional IFN-γ gene, the virus was re-isolated from nasal swabs of animals treated with dexamethasone. This virus was grown in MDBK cells and the supernatant was analysed for biologically active IFN-γ. As shown in Fig. 7, supernatant from BHV-1/IFNγ-infected cells up-regulated MHC class II ex-
expression. Moreover, this activity could be neutralized with anti-IFN-γ antibodies (Fig. 7F). The biological activity of recombinant IFN-γ was also confirmed in an anti-virus VSV assay (data not shown). These observations confirmed that latent BHV-1/IFNγ retained a functional boIFN-γ gene.

### Discussion

In this report, we established that BHV-1 was a stable vector for cytokine expression. The expression of biologically active IFN-γ was confirmed both in vitro and in vivo during a primary infection and following reactivation of a latent infection. The latent BHV-1/IFNγ recombinant virus retained a functional IFN-γ gene that produced functional IFN-γ protein with both antiviral activity and the ability to up-regulate MHC class II expression (Fig. 7). These observations confirmed that recombinant BHV-1 is a stable vector. Other virus vectors have not always displayed the same stability. For example, when a recombinant SIV vector expressing IFN-γ was used to infect macaque monkeys, there was a deletion of the IFN-γ gene within 12 weeks p.i. (Giavedoni et al., 1997). The retrovirus vector tended to be unstable and deleted genetic material when it did not confer an evolutionary advantage (Giavedoni et al., 1997).

The growth kinetics curve confirmed that recombinant BHV-1/IFNγ was able to replicate in vitro and this replication was comparable to gC′/LacZ′ virus (Fig. 1). This observation suggested that an analysis of the in vivo effects of IFN-γ production would not be complicated by differences in recombinant BHV-1 replication. Therefore, this appeared to be a valid system for evaluating the in vivo expression of recombinant cytokine expression during BHV-1 infection. Nevertheless, deletion of gC, an important glycoprotein for attachment and penetration, resulted in decreased virus replication both in vitro and in vivo (Liang et al., 1991a, b).

The primary infection of animals with control virus and recombinant BHV-1/IFNγ showed a similar fever response and clinical signs of infection. The systemic injection of recombinant boIFN-γ can induce a fever response in cattle (Roth & Frank, 1989; Chiang et al., 1990), but there was no indication that production of exogenous IFN-γ by the recombinant BHV-1 vector had significant systemic activity. For example, the level of acute phase protein haptoglobin was similar in the serum of animals from all three groups and there were no significant differences in blood leukocyte populations during primary infection (data not shown). The apparent lack of activity by BHV-1/IFNγ during a primary infection may be explained by the level of endogenous IFN-γ production during an acute BHV-1 infection. All BHV-1-challenged calves produced 2000 pg/ml of IFN-γ in nasal secretions on day 4 p.i. (approximately 6000 U/ml based on 3-0 x 10^5 U/mg standard) and produced over 100 pg/ml on day 6 p.i. (Fig. 4). This indicates that BHV-1 is a potent inducer of IFN-γ production and endogenous IFN-γ production may exceed IFN-γ production by the BHV-1/IFNγ vector. The detection of IFN-γ in nasal secretions correlates with earlier findings of total interferon levels in nasal secretions (Babiuk et al., 1985; Bielefeldt Ohmann & Babiuk, 1985). In these previous studies, the interferon activity was quantified by inhibition of VSV replication. Therefore, the contribution of each type of interferon to this antiviral activity was not assessed. Traditionally, interferon in nasal secretions has been assumed to be IFN-α (Straub & Ahl, 1976). The present finding that high levels of IFN-γ are present in nasal secretions following a primary infection suggests that there may be a high number of infiltrating NK cells. The availability of an ELISA for both bovine IFN-α/β and IFN-γ would help quantify the relative contribution of each type of IFN.

In two previous reports, both recombinant VV and SIV displayed reduced virus growth in vivo when expressing recombinant IFN-γ (Kohonen-Corish et al., 1990; Giavedoni et al., 1997). One question that arises from our study is why BHV-1 expressing recombinant IFN-γ did not exhibit an

---

**Fig. 7.** Up-regulation of MHC on alveolar macrophages by culture supernatants of cells infected with dexamethasone-reactivated virus. Bovine alveolar macrophages (AM) were isolated by lung lavage. Recombinant BHV-1/IFNγ was isolated from a nasal swab taken after dexamethasone treatment of a BHV-1/IFNγ-infected calf. Virus was grown in MDBK cells and the culture supernatant was assayed for IFN-γ activity by assaying changes in MHC expression. The culture supernatants from infected MDBK cells were ultra-centrifuged to remove virus before being incubated with 4 x 10^6 AM/ml. The AM were then washed and dual-stained with monocyte- (clone DH59B) and MHC class II- (clone TH14B) specific MAbs. Staining was as follows: (A) AM cultured for 72 h in MEM, (B) AM cultured for 72 h in MEM with rabbit anti-IFN-γ antisera (1/20), (C) AM cultured for 72 h with 100 ng/ml recombinant boIFN-γ, (D) AM cultured for 72 h with 100 ng/ml recombinant boIFN-γ plus rabbit anti-IFN-γ antisera (1/20), (E) AM cultured for 72 h with culture supernatant from MDBK cells infected with BHV-1/IFNγ isolated after dexamethasone treatment, (F) AM cultured for 72 h with culture supernatant from MDBK cells with re-isolated latent BHV-1/IFNγ plus rabbit anti-IFN-γ antisera (1/20). The percentage of monocyte/macrophages expressing detectable MHC class II on their surface is indicated in the top right corner of each panel.
attenuated phenotype. One possible explanation may be that unique aspects of VV and SIV life-cycles made this strategy successful. A BHV-1 respiratory infection is limited to primarily nasal, trachea and lung epithelial cells but VV has a wider tropism, infecting cells in various tissues which include ovaries, lungs, spleen and brain (Karupiah et al., 1990; Sambhi et al., 1991). This broader tropism results in a high level of VV replication and subsequently the amount of recombinant cytokine produced may be much greater. For example, infection by certain VV vectors expressing IL-2 and IL-4 can result in clinical symptoms that mimic the administration of large amounts of recombinant cytokine (Bembridge et al., 1998). Similarly, SIV infects a high number of lymphocytes both in the blood and in the lymph nodes and these infected cells can interact with dendritic cells (Wood, 1990; Fauci et al., 1996). This particular cell tropism would be an asset when expressing recombinant cytokines, since expression could be targeted to the lymph nodes and dendritic cells. This localized expression may increase the potential for immunomodulation by a recombinant cytokine. Furthermore, both VV and SIV may have different sensitivities to the antiviral effects of IFN-γ. For example, transgenic mice without the IFN-γ receptor gene show increased susceptibility to VV infection (Muller et al., 1994).

The one observation that suggested immunomodulation by recombinant boIFN-γ was a reduced level of IgA in nasal secretions of calves infected with BHV-1/IFNγ after reactivation (Fig. 4B). In other animals models, both recombinant VV-IL-2 and VV-IFN-γ induced lower VV-specific antibody responses that correlated with a lower level of virus growth during a primary infection (Kohenen-Corish et al., 1990). Furthermore, intranasal immunization with a fowlpox virus vector co-expressing influenza virus haemagglutinin and IFN-γ produced no IgA antibody response, yet showed no difference in the CTL responses between recombinant and control (Leong et al., 1994). Our results with dexamethasone reactivation of BHV-1/IFNγ parallel these findings. However, unlike the fowlpox virus vector, we were able to detect BHV-1-specific IgA in nasal secretions after a primary infection. Since only a fourfold difference in IgA titre was found, further experiments will be necessary to establish if the lack of an anamnestic mucosal IgA response was a direct consequence of recombinant IFN-γ produced by the BHV-1/IFNγ vector.

The observed difference in nasal IgA modulation (Fig. 4B) may also be explained by differences in the host environment during a primary infection and reactivation. The primary respiratory infection was initiated with numerous cells being infected and a high level of virus replication until host factors were able to control virus growth. In contrast, reactivation of a latent infection affects a much smaller number of cells and virus replication occurs within the context of an active host immune response. Thus, production of recombinant IFN-γ may be able to activate virus-specific effector cells and limit virus replication in the reactivated area. However, our analysis of virus shedding in nasal secretions did not indicate that expression of recombinant IFN-γ reduced virus replication following reactivation of the latent infection.

The immunogenicity of gC-negative mutants was previously confirmed by the detection of antibody responses (Liang et al., 1992), as well as T-cell proliferative responses and NK-like responses (Denis et al., 1996). Assays for cell-mediated immune responses revealed no differences among the experimental groups for either BHV-1-specific proliferative responses or gD-specific IFN-γ-producing cells (Fig. 5). These observations indicated that the production of recombinant IFN-γ during a primary infection or recrudescence did not have detectable effects on T-cell responses.

In conclusion, we demonstrated that expression of boIFN-γ by a BHV-1 vector did not cause adverse clinical effects and that the vector expressed cytokine during both a primary and a recrudescence infection. Both gC-negative recombinant viruses (BHV-1/IFNγ and gC−/LacZ+) induced detectable immune responses but the expression of recombinant IFN-γ did not attenuate virus growth or significantly modulate systemic humoral and cellular immune responses. Analysis of nasal secretions revealed high levels of IFN-γ in calves infected by either wild-type or recombinant BHV-1 and revealed that endogenous IFN-γ production is a natural part of a primary BHV-1 infection. Finally, the expression of recombinant IFN-γ had no detectable effect on virus latency or reactivation of the latent virus. The observed stability of recombinant BHV-1, during a primary and latent infection, suggests that BHV-1 may provide a stable virus vector for the expression of cytokines and possibly other immunomodulating genes. Further studies are required to confirm vector stability following multiple passages within the host. The unique biology of herpesviruses may provide significant advantages for their use as virus vectors.

We would like to thank the animal care staff at VIDO for their excellent technical assistance. In addition, we would like to thank Marlene Snyder, Terry Beskorowany, Dr Dale Godson and Dr Xiaoping Liang for advice, reagents and assistance. The financial support for this work was provided by the MRC of Canada and the Natural Science and Engineering Council of Canada.

References


Received 8 May 2000; Accepted 25 July 2000