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Expression of Bovine Interleukin-1β in a Bovine Herpesvirus-1 Vector: *In Vitro* Analysis

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In order to evaluate whether bovine herpesvirus-1 (BHV-1) could be used as a live viral vector for the expression of cytokines, we constructed a recombinant BHV-1 expressing bovine interleukin-1**β** (boIL-1**β**). The boIL-1**β** coding sequence, corresponding to the cleaved mature product, was fused with the BHV-1 glycoprotein C (gC) signal peptide sequence; the resultant gC-boIL-1**β** fusion gene was recombined into the gC locus of the BHV-1 genome. Southern blot analysis confirmed the proper genomic configuration of the recombinant virus. Results from transcript analysis showed that boIL-1**β** was expressed in infected cells with kinetics similar to that of gC. Indirect immunofluorescence and immunoprecipitation assays showed that the recombinant protein was produced in both cell-associated and secreted forms. Western blot analysis detected a 19.3-kDa protein. Further analysis, using an IL-1**β** bioassay demonstrated that both the cellular and secreted forms of recombinant boIL-1**β** possessed biological activity. The expression of the boIL-1**β** protein did not affect the *in vitro* growth efficiency of the virus, which exhibited similar growth kinetics to that of a simple gC deletion mutant. The results from this study demonstrate that BHV-1 can be used to express a functional cytokine, thereby establishing the basis to further study recombinant BHV-1 expressing cytokines as an alternative means to attenuate the virus and also as a potential *in situ* cytokine delivery system to modulate immune responses against BHV-1 and other cattle pathogens.
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INTRODUCTION

Cytokines have a central role in the initiation and differentiation of the immune system and in regulating both the type and magnitude of the immune response. Cytokine regulation during disease progression has been well demonstrated in both parasitic and bacterial infections (Powrie and Coffman, 1993). For example, in *Mycobacterium leprae* infections, T helper subset 1 (TH1) cells secrete interleukin-2 (IL-2) and interferon-γ, resulting in the induction of a cell-mediated response that is associated with resistance. In contrast, TH2 cells that produce IL-4 and IL-10 are associated with progressive disease (Seiling and Modlin, 1994). Because of the central role that cytokines play in the immune response, researchers have attempted to use cytokines to enhance the immune response in both therapeutic treatments and in vaccine formulations (Heath and Playfair, 1992). However, since cytokines have a short half-life and largely function within a limited microenvironment, their usefulness has been hampered by the need for large doses administered systemically which can sometimes result in toxic side effects (Haworth and Feldman, 1991). Therefore, new methods to deliver cytokines to the site of infection are being investigated. These include the creation of chimeric molecules, delivery vehicles, and the use of viral vectors to express cytokines (Hughes *et al.,* 1992; Pardoll, 1995; Ramshaw *et al.,* 1992).

Bovine herpesvirus-1 (BHV-1) belongs to the subfamily *Alphaherpesvirinae* of the family Herpesviridae. BHV-1 causes infectious bovine rhinotracheitis, a contagious viral disease that produces substantial economical loss in the cattle industry (Wyler *et al.,* 1989). BHV-1 is also the major viral pathogen in the development of the respiratory disease complex commonly referred to as shipping fever (Yates, 1982). Current interest in vaccine development has focused on the use of subunit vaccines, gene deleted, killed, or live viral vaccines (van Drunen Littel-van den Hurk *et al.,* 1993; van Oirschot, 1994). Attempts at attenuating BHV-1 for use in vaccines have focused on the creation of different deletion mutants (Kit *et al.,* 1985; Liang *et al.,* 1991; Kaashoek *et al.,* 1994). The expression of cytokines by a recombinant virus could potentially function as an alternative means to attenuate and increase the immune response to the recombinant virus.

Interleukin-1β (IL-1β) has been used as an immunological adjuvant because of its ability to influence a broad spectrum of biological activities within the haematopoietic and immunological systems (Dinarello, 1991). For example, it has been shown that the coadministration of recombinant bovine IL-1β (boIL-1β) and

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a modified live BHV-1 vaccine was able to enhance both the humoral and cellular immune response against BHV-1 (Reddy *et al.,* 1990, 1993). Therefore, we have chosen boIL-1β to evaluate the potential of utilizing BHV-1 for the expression of cytokines. This would create a system for investigating immunomodulation caused by cytokine delivery at the site of infection, and also, for investigating possible attenuation of BHV-1 and reducing opportunistic pathogens associated in the development of the respiratory disease complex initiated by BHV-1. As a first step, we constructed a BHV-1 recombinant virus expressing boIL-1β at the gC locus and characterized its *in vitro* properties.

MATERIALS AND METHODS

Virus and cells

The Cooper strain of BHV-1, obtained from the National Veterinary Services Laboratory (Ames, Iowa), was used as the prototype virus; a gC-negative mutant was previously produced in this laboratory (Liang *et al.,* 1991). The viruses were propagated in Madin–Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL).

An IL-1 responsive cell-line (LM-1) was subcloned from the murine helper T-cell line D10.G4.1 (Kaye *et al.,* 1983) as previously described (Orencole and Dinarello, 1989). Briefly, D10.G4.1 cells, which grew in the absence of conalbumin and spleen feeder cells, were subcloned and analyzed for sensitivity to IL-1. One subclone, designated LM-1, was used for the IL-1 bioassay. LM-1 cells were maintained in Click's Media (Irvine Scientific, Santa Ann, CA) supplemented with 10% FBS (Hyclone, Logan, Utah), 2 m*M* L-glutamine, and 10% Con A-stimulated rat spleen supernatant.

Construction of transfer vector

To construct the transfer vector, p113RI-Bgl3.0, a subclone derived from pSD113 (Mayfield *et al.,* 1983) containing the gC sequence, was digested with *Bam*HI and *Pvu*II; the plasmid backbone was treated with Klenow fragment and ligated with a Klenow-treated, 1-kb *Bam*HI–*Pvu*II gC 5′ fragment from pSD113. This extended the 5′ flanking sequence and converted the *Bam*HI site 61 bp upstream of the gC translation initiation codon into a *Cla*I site. The resultant plasmid is named as pSD113EClaH/P (Fig. 1A). pSD113EClaH/P was digested with *Sal*I, blunted with Klenow fragment, and ligated with a *Bam*HI linker (CGGGATCCCG) (Fig. 1, step 1), resulting in pSD113Sal-Bam+. A 68-bp *Nco*I–*Bam*HI fragment containing the gC signal peptide sequence was isolated from plasmid pRSVgCSSbam (Fitzpatrick, unreported results) and used to replace the *Nco*I and *Bam*HI fragment of pSD113Sal-Bam+ (step 2). This resulted in pSD113SSBam, which contains the 1-kb gC 5′ flanking sequence, gC signal peptide sequence, signal peptide cleavage site, and a *Sma*I–*Bam*HI–*Sma*I cloning site and an 1-kb 3′ gC flanking sequence. The boIL-1β coding sequence was isolated from plasmid pBIL1β3.3 (CIBA-GEIGY, Basel, Switzerland) by Mae III and Mae II digestion (Leong *et al.,* 1988). After treating with Klenow fragment, the IL-1β fragment was cloned into the *Bam*HI site of pSD113SSBam which had been blunted with Klenow fragment (step 3). This resulted in pSD113SSIL-1β, a transfer vector that contains the mature sequence of boIL-1β fused with the gC signal peptide sequence (Fig. 1B).

Purification of virus and viral DNA

Virus was purified as previously described (Babiuk *et al.,* 1975). Briefly, MDBK cells were infected at a multiplicity of infection (m.o.i.) of 1. After the cells exhibited complete cytopathology, the supernatants containing the virus were clarified by low-speed centrifugation at 1000 *g.* Virus was harvested by pelleting the clarified supernatant through a 30% sucrose in phosphate-buffered saline (PBS) cushion at 100,000 *g* for 60 min. The virus pellet was then resuspended in 0.05 *M* Tris–HCl, 0.15 *M* NaCl, 10 m*M* EDTA (pH 8.0) and applied to a 20 to 50% potassium-sodium tartrate discontinuous gradient and centrifuged at 100,000 *g* for 90 min. After centrifugation, the virus band was collected, diluted in PBS, and pelleted at 75,000 *g* for 60 min. Purified virus was stored at −70°C.

Viral DNA was purified according to the method of Summers and Smith (1987). Purified virus was suspended in extraction buffer (0.1 *M* Tris–HCl, 0.15 *M* NaCl, 0.1 *M* EDTA, 0.1 *M* KCl, pH 7.5) containing 45 μg of proteinase K (Sigma Chemical Co., St. Louis, MO) per milliliter and incubated at 50° for 1 hr. Sarkosyl was added to a final concentration of 1%, and the sample was incubated at 50° for an additional 1 hr. The sample was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. The DNA pellet was resuspended in 0.05 *M* Tris–HCl, 10 m*M* EDTA, pH 8.0, aliquoted, and stored at −70°.

Recombinant virus production

Recombinant viruses were produced by cotransfection of MDBK cells with pSD113SSIL1-β plus naked BHV-1 genomic DNA by electroporation (Chu *et al.,* 1987). Briefly, MDBK cells were trypsinized, resuspended in ice-cold HEPES-buffered saline (HeBS; 20 m*M* HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* $NaHPO_4$, 5 m*M* D-glucose, pH 7.1), and washed three times in HeBS at 4°. After the final wash, cells were resuspended to 3×10^6 cells/ml in HeBS and transferred to an electroporation cuvette (Bio-Rad Laboratories, Richmond, CA) containing 10 μg of linearized plasmid DNA and 20 μg of viral DNA. Elec-

troporation was performed at 500 μF and 200 V using a Bio-Rad Gene Pulser. The cells were then transferred to a 100-mm culture dish containing 15 ml of MEM with 10% FBS, incubated at 37° for 5 hr, and overlaid with 1% agarose containing 2% FBS in MEM. gC-negative virus plaques were screened by an antibody-based black-plaque assay.

Black-plaque assay

The black-plaque assay was carried out by the method of Johnson *et al.* (1986). Briefly, the MDBK cell monolayer with an appropriate number of viral plaques were fixed with 0.25% glutaraldehyde (Sigma) in PBS, pH 7.2, for 3 min, washed three times with PBS, and blocked for 1 hr with 1% bovine serum albumin (BSA; Sigma) in PBS (BSA-PBS). An anti-gC monoclonal antibody (MAb) pool (van Drunen Littel-van den Hurk *et al.,* 1984), diluted to 1:1000 in BSA-PBS, was added to the plates and incubated for 1 hr at room temperature. Cells were then washed three times with PBS, followed by the addition of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; Boehringer Mannheim, Dorval, Quebec, Canada, 1:2000 in BSA-PBS). After an additional hour of incubation, the plates were again washed three times with PBS, followed by the addition of 0.01% 4-chloronaphthol (Sigma) and 0.0003% H_2O_2 (Sigma) in water. The plates were incubated at room temperature until black plaques developed.

Southern analysis

Viral DNA was isolated from wild-type and recombinant virus as previously described (Liang *et al.,* 1992). The DNA was digested with *Eco*RI and *Bgl*II restriction endonucleases, run on a 1% agarose gel, and transferred to nitrocellulose filter using standard procedures (Maniatis *et al.,* 1982). The nitrocellulose filter was hybridized with ^{32}P-labeled DNA probes corresponding to the boIL-1β gene and the 5′ noncoding region of the gC gene (0.6-kbp *Sma*I–*Bam*HI fragment from pSD113SSIL-1β).

Northern blot hybridization analysis

RNA preparation was performed as previously described (Kowalski and Denhardt, 1989). Briefly, confluent MDBK cells in 100-mm plates were infected with recombinant virus at a m.o.i. of 10. At various time points, cells were washed once in phosphate-buffered saline lacking calcium and magnesium and harvested into 200 μl of TSM [30 m*M* Tris–HCl (pH 8), 100 m*M* NaCl, 1.5 m*M* $MgCl_2$]. Nonidet P-40 was added to a final concentration of 0.5%, and the sample was incubated on ice for 30 min and pipetted with a 200-μl Gilson disposable tip. Nuclei were removed by centrifugation at 15,000 *g* for 10 min. The cytoplasmic extract was added to 200 μl of lysis buffer [2% sodium dodecyl sulfate (SDS), 7 *M* urea, 350 m*M* NaCl, 2 m*M* EDTA, 10 m*M* Tris–HCl (pH 8)] and extracted once with 400 μl of phenol-chloroform (1:1) saturated with 100 m*M* Tris Chloride (pH 8). The aqueous phase was equilibrated to 0.3 *M* in sodium acetate (pH 5.5) by adding 40 μl of 3 *M* sodium acetate. The RNA was precipitated with ethanol and resuspended in H_2O. Equal amounts of infected cell RNA (3 μg) purified at various times postinfection were separated on a 1.2% agarose-formaldehyde gel and hybridized as previously described (Maniatis *et al.,* 1982).

Indirect immunofluorescence assay

MDBK cells cultured in chamber slides (Nunc Inc., Naperville, IL) were infected with recombinant virus at a m.o.i. of 0.01 for 16 hr, fixed with 2% paraformaldehyde for 5 min, and permeabilized with methanol at −20° for 15 min. Nonspecific reactions were blocked by treating slides with 2% BSA and 2% normal goat serum in PBS (blocking solution) for 30 min. Afterwards, slides were incubated with rabbit anti-boIL-1β serum at 1:500 dilution for 1 hr. Rabbit anti-boIL-1β serum was produced against recombinant bovine IL-1β (American Cynamid, Princeton, New Jersey). After washing, slides were stained with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody at an 1:80 dilution in blocking solution (Bio-Rad, Mississauga, Canada). Slides were mounted in citifluor glycerol (Ted Pella, Inc., Redding, CA) and examined with a fluorescence microscope.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Liang *et al.,* 1991). Briefly, subconfluent monolayers of MDBK cells were infected with respective viruses at a m.o.i. of 10 and labeled with 50 μCi of L-[^{35}S]methionine (Amersham, Oakville, Ontario, Canada) per milliliter of medium, for 24 hr. After labeling, the cellular and supernatant fractions were harvested separately and precipitated with rabbit anti-boIL-1β antibody. The antibody precipitated samples were separated on SDS–10% polyacrylamide gels under reducing conditions.

Western blot analysis

Western blot procedures were carried out as described previously (van Drunen Littel-van den hurk *et al.,* 1984). Briefly, samples were prepared by concentrating 36-hr-postinfection supernatant by ultrafiltration using a Centricon-10 (Amicon, Beverly, MA). Samples containing 2 ng of boIL-1β were denatured and resolved by electrophoresis in a 10% polyacrylamide–SDS gel (Mini-protean, Bio-Rad) and transferred to a nitrocellulose sheet. The protein was detected with a 1:500 dilution of a rabbit polyclonal antibody raised against rBoIL-1β. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG at a 1:1000 dilution (Bio-Rad).

Single step growth curve

Confluent MDBK cells grown in 6-well plates were infected at an m.o.i. of 5 with either gC$^-$, wild-type virus

or BHV-1 expressing IL-1β (BHV/IL1β) or. After 1 hr adsorption at 37°, the virus inoculum was removed; cells were washed with MEM and incubated with 2 ml of MEM supplemented with 10% FBS. At various times postinfection, aliquots of culture media were collected and assayed for viral titers.

Bioassay for IL-1 activity

LM-1 cells (see above) cultured for 5 to 7 days were washed three times in Click's media and resuspended in Click's media with 5% FBS and 2 m*M* L-glutamine. The samples to be tested were filtered through Centricon-30 ultrafiltration devices (Amicon) to remove virus from the supernatant and serially diluted in a 96-well plate. Bioassays using LM-1 cells (2 to 4 $\times 10^5$ cells/ml) were performed in triplicate in 96-well polystyrene flat bottom microtiter plates at volumes of 200 μl/well and incubated for 72 hr at 37° in 5% CO_2. Biological activity was determined by adding [^{3}H]thymidine (Amersham, 0.4 μCi/well) 18 hr prior to harvest. Incorporation of radioactivity by LM-1 cells was determined by scintillation counting (Beckman). Relative activity of each sample was calculated as previously described (Maliszewski *et al.,* 1988). One unit of IL-1 activity was defined as the amount of lymphokine that induced 50% of maximal proliferation in 200-μl cultures.

Recombinant bovine IL-1β 6.8 mg/ml (American Cyanamid Co., Princeton, NJ) was used as our standard. The rBoIL-1β standard had a specific activity of 4.6 $\times 10^8$ U/mg when assayed with LM-1 cells. The specific activity of recombinant boIL-1β produced by BHV/IL1β was calculated using the LM-1 bioassay and a capture ELISA consisting of an ovine Mab 3.41 (gift from Dr. Andrew Nash) and a polyclonal rabbit anti-boIL-1β antibody. The capture ELISA was performed by coating a 96-well plate with 100 μl of a 1:250 dilution of MAb 3.41 ascites in carbonate buffer overnight at 4°. After three washes with PBS + 0.05% Tween 20, plates were blocked with 1% w/v gelatin in PBST for 1 hr at room temperature, and then samples and rBoIL-1β standard were serially diluted in duplicate and incubated for 2 hr at room temperature. After washing, the polyclonal rabbit anti-boIL-1β antiserum was added at a 1:3000 dilution and incubated for 1 hr. After further washing, a 1:1000 dilution of horseradish peroxidase-congated goat anti-rabbit IgG (Bio-Rad) was added for an additional 1 hr. The assay was developed using an ABTS substrate.

RESULTS

Construction of recombinant virus

IL-1β is initially synthesized as a 31-kDa precursor which is cleaved by an IL-1β converting enzyme (ICE) into a 17.5-kDa mature cytokine (Kostura *et al.,* 1989; Cerretti *et al.,* 1992; Thornberry *et al.,* 1992). Unlike most secretory proteins, IL-1β does not contain a signal peptide sequence for secretion (Dinarello, 1991). Considering these unique characteristics of IL-1β, we chose to fuse a gC signal peptide to the amino terminus of the cleaved functional protein, thereby generating a gC-IL1β fusion molecule that could be efficiently secreted. Our cleaved product would be predicted to retain the 153 amino acids associated with mature bovine IL-1β plus three additional amino acids from the gC signal sequence (Fig. 1B). Recombinant BHV-1 virus expressing bovine interleukin 1β was produced by homologous recombination between the transfer vector pSD113SSIL-1β and purified BHV-1 DNA cotransfected into MDBK cells. Progeny viruses were screened by a black plaque assay utilizing a pool of monoclonal antibodies against gC. A gC-negative white plaque was identified and designated "BHV/IL1β." After an additional two rounds of plaque purification, the gC-negative mutants were used for further characterization.

To ascertain that the gC-negative mutant indeed contained the boIL-1β gene and also to confirm the genomic arrangement at the gC region, Southern blot analysis was carried out. Viral DNA from both wild-type virus and recombinant virus (BHV/IL1β) was digested with *Eco*RI and *Bgl*II, separated on a 1% agarose gel, and probed with a 0.6-kbp *Sma*I–*Bam*HI gC 5′ fragment from pSD113SSIL-1β (Fig. 2). The results of the Southern analysis were consistent with what would be predicted from the transfer vector. In the wild-type viral DNA, a 3.0-kb fragment hybridized to the 5′ gC probe, whereas the mutant virus DNA had a 2.3-kb fragment. When BoIL-1β was used as a probe, no signal was detectable in the wild-type BHV-1 DNA; in contrast, a band of approximately 2.3-kb was detected in the mutant DNA. The results from the Southern blot analysis confirmed that BHV-1/IL1β contained the boIL-1β gene in the expected configuration.

Detection of boIL-1*β* transcripts in virus infected cells

As a first step to assess the expression of boIL-1β, as well as to define the kinetics of IL-1β expression during viral infection, boIL-1β transcripts were analyzed in BHV/IL1β-infected cells by Northern analysis. In order to control for the amount of RNA in each lane, a parallel gel was run and stained with ethidium bromide to highlight the 28S and 18S bands (top panel Fig. 3). As shown in Fig. 3 (bottom panel), boIL-1β transcripts were first detected at 6 hr postinfection followed by a gradual increase until 10 hr.

Expression of boIL-1*β* protein by recombinant virus

Having identified the transcripts of boIL-1β, we wanted to determine whether the protein was produced in infected cells. To achieve this, we first reacted infected cells with rabbit antibody against boIL-1β followed by

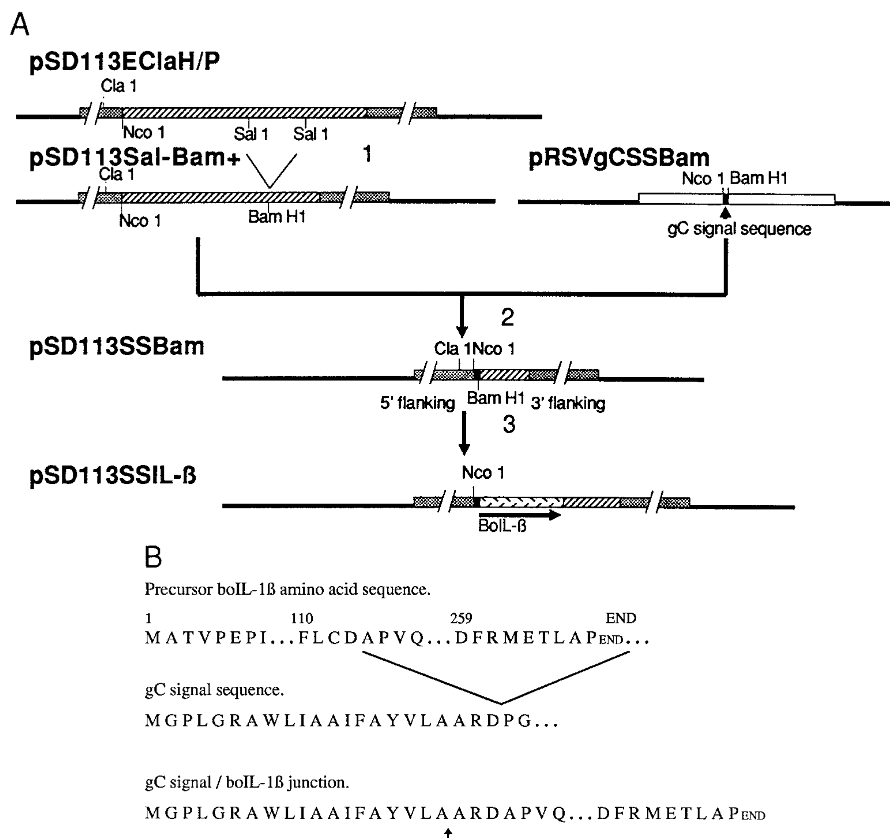

FIG. 1. Construction of transfer vector pSD113SSIL-1*β*. (A) Striped bars represent the gC gene fragment, solid bars represent the gC signal sequence, dotted bars represent the gC flanking sequence, tire marks represent boIL-1*β*, and the solid line signifies the pBR322 backbone (see details for procedures under Materials and Methods). (B) Amino acid sequences at the junction between the gC signal peptide and mature BoIL-1*β* protein. The fusion protein retains the Asp and Ala sites associated with cleavage of the precursor protein into the 153 amino acid mature cytokine. The predicted signal sequence cleavage site is indicated by an arrow.

reaction with fluorescein-labeled goat anti-rabbit antibody. At 16 hr postinfection, infected cells showed bright cytoplasmic fluorescence (Fig. 4). No fluorescence was detected in control slides infected with wild-type virus.

To confirm that the boIL-1*β* expressed by the recombinant virus had the predicted molecular weight, an immunoprecipitation assay was performed. After 24 hr postinfection, cellular and supernatant fractions were collected separately and immunoprecipitated with anti-boIL-1*β* antibody. A polypeptide with an apparent molecular weight between 17 and 20 kDa was recognized in both the supernatant and cellular fraction of BHV1/IL1*β*-infected cells, but not in the wild-type virus-infected cells (Fig. 5A). This indicated that boIL-1*β* was indeed produced by the recombinant virus and was also secreted into the medium, as would be predicted considering that the boIL-1*β* protein contained a gC signal peptide. To further confirm the molecular weight of the recombinant boIL-1*β* protein and to assess whether the signal sequence was cleaved, a Western blot was performed (Fig. 5B). BoIL-1*β* produced by BHV1/IL1*β* had a molecular weight comparable to our rBoIL-1*β* standard of 19.3 kDa, indicating that the signal sequence was cleaved.

BHV/IL1*β* produced a biologically active molecule

To determine whether the boIL-1*β* protein expressed by the recombinant virus possessed biological function, a bioassay for IL-1 was carried out using a subclone of D10.G4.1 (LM-1) (Kaye *et al.*, 1983). Since the boIL-1*β* was secreted into the medium, we collected supernatant samples from BHV1/IL1*β*, gC^-, wild-type virus, and mock-infected MDBK cells. As shown in Fig. 6, IL-1 activity was detected 24 hr postinfection in BHV/IL-1*β*-infected supernatant while no activity was detected in the controls. Based on our LM-1 bioassay, we calculated the level of boIL-1*β* expressed at 24 and 36 hr to be approximately 160 units ml^{-1} and 320 units ml^{-1}, respectively. In addition, we calculated the specific activity of the boIL-1*β* produced by BHV/IL-1*β* to 4.3×10^8 U/mg which was similar to the specific activity of our rBoIL-1*β* standard produced in *Escherichia coli* (4.6×10^8 U/mg).

Expression of boIL-1*β* lacked effect on virus growth in cell culture

To assess the growth efficiency of the recombinant BHV/IL1*β* virus, a single-step growth experiment was

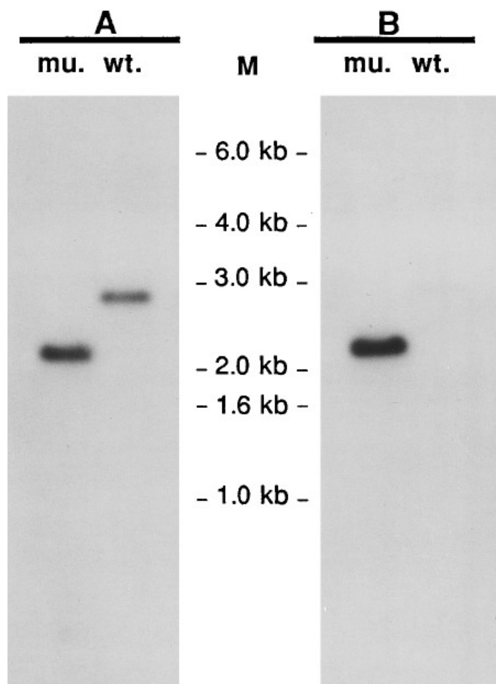

FIG. 2. Southern blot analysis of BHV/IL1β and wild-type BHV-1 DNA. Wild-type genomic DNA (wt.) and BHV/IL1β genomic DNA (mu.) were digested with *Eco*RI and *Bgl*II. Two sets of digested samples, along with DNA size markers were separated on a 1% agarose gel and transferred to a nitrocellulose filter. One set of samples was probed with a 0.6-kbp *Sma*I–*Bam*HI fragment from pSD113SSIL-1β corresponding to the 5′ region of the gC gene (A); and a second set of samples was probed with a 0.5 *Sma*I–*Bam*HI fragment coding for the IL-1β gene from pSD113SSIL-1β (B).

performed. MDBK cells were infected with wild-type BHV-1, gC-negative mutant and BHV/IL1β. At various time points postinfection, supernatant samples were harvested and assayed for virus. As shown in Fig. 7, the gC deletion mutant and BHV/IL1β exhibited comparable growth kinetics; compared with wild-type virus, both mutants yielded about a 10-fold lower virus titer at 24 hr postinfection. Although BHV/IL1β appears to replicate slightly better than gC-negative virus in this experiment, multiple experiments showed no statistically significant differences between these viruses. The observation that BHV/IL1β and the gC deletion mutant had similar growth curves indicates that the expression of boIL-1β does not affect the virus growth *in vitro*.

DISCUSSION

The use of cytokines as adjuvants for modulating the immune response against BHV-1 has been investigated in both subunit and modified live vaccines (Reddy *et al.*, 1993; Hughes *et al.*, 1992; Gao *et al.*, 1995). Although the results have been promising, the systemic administration of rBoIL-1β to develop an adjuvant effect usually requires a large dose which sometimes results in toxic side-effects (Reddy *et al.*, 1993; Godson *et al.*, 1995). In addition, administering multiple injections at a localized site is required for an adjuvant effect (Gao *et al.*, 1995). The construction of a recombinant BHV-1 expressing boIL-1β cytokine has the potential to overcome these drawbacks by delivering the cytokine to the site of infection. This would allow the cytokine to act within a limited microenvironment, reducing dose requirements and thereby decreasing the chances of possible side-effects.

Our recombinant BHV/IL1β virus expressed the boIL-1β gene from the gC locus similar to a late class gene by utilizing the gC promoter (Fig. 3). This parallels gC mRNA kinetics where gC transcripts are first detected at 5 hr followed by a sharp increase at 6 hr coinciding with DNA synthesis (Seal *et al.*, 1992). The transcripts also produced a boIL-1β protein with a molecular weight of 19.3 kDa (Fig. 5B). This was comparable to both our rBoIL-1β standard and the molecular weight previously reported for the recombinant mature form of boIL-1β (Maliszewski *et al.*, 1988).

The bioassay results confirmed that indeed the boIL-1β protein was biologically active possessing a specific activity similar to rBoIL-1β produced in *E. coli.* We also tested the cellular fraction and detected biological activity that was comparable to the supernatant fraction (data not shown). Although IL-1 activity was not detectable at 12 hr within the supernatant, the kinetics of mRNA expression indicates that boIL-1β could be produced in small amounts after 6 hr. We speculate that since the expression of IL-1 will occur at the site of infection, a

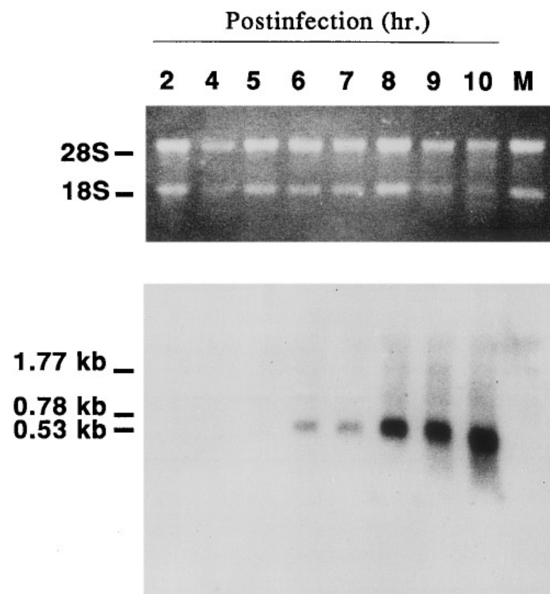

FIG. 3. Transcription kinetics of BoIL-1β in virus-infected cells. At various time points postinfection, RNA samples were prepared and run on a 1.2% agarose gel (bottom figure). To confirm RNA integrity and approximate concentrations per lane, duplicate samples were stained with ethidium bromide; 18S and 28S RNA bands are shown on top. Molecular size markers in kilobases are noted to the left. M indicates mock-infected cells at 10 hr.

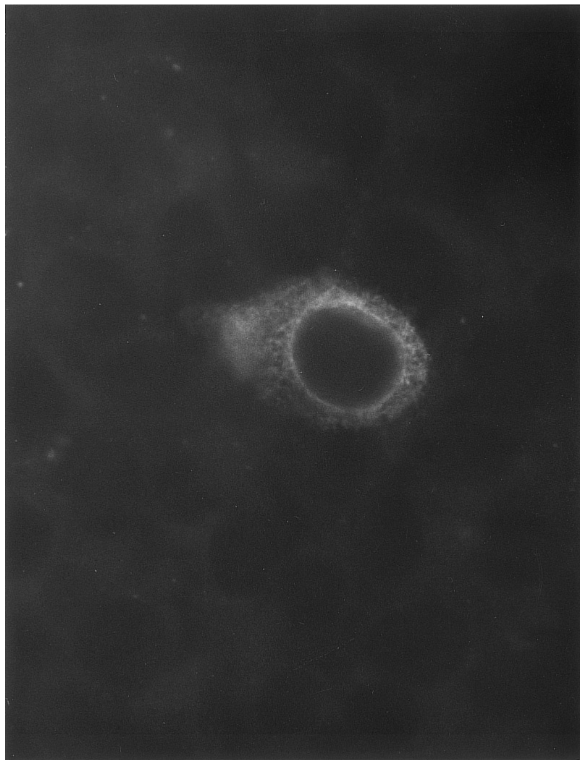

FIG. 4. Indirect immunofluorescence demonstrating subcellular location of boIL-1β. MDBK cells were infected with BHV/IL1β at a m.o.i. of 0.01 for 16 hr. Cells were fixed with 2% paraformaldehyde, permeabilized with methanol, and incubated with rabbit anti-boIL1β serum at a 1:500 dilution followed by incubation with fluorescein-labeled goat anti-rabbit antibody. Original magnification, 250×.

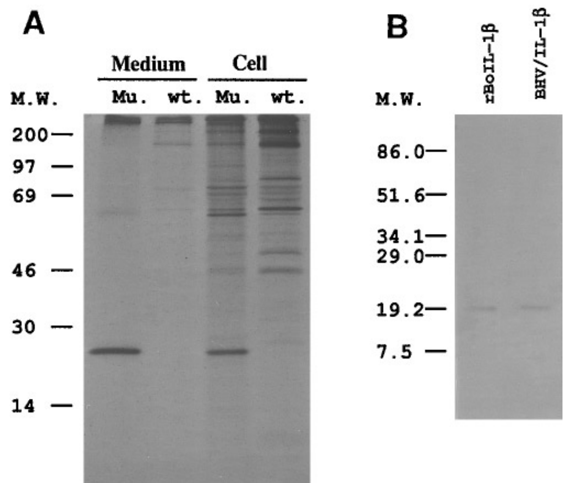

FIG. 5. Detection of bovine interleukin-1β by immunoprecipitation assay and Western blot. (A) MDBK cells were infected with either BHV1/IL1β (mu.) or wt BHV-1 (wt.) at an m.o.i. of 10 and labeled with 50 μCi of [^{35}S]methionine per milliliter. After 24 hr of labeling, cellular and medium fractions were harvested separately and immunoprecipitated using bovine IL-1β-specific rabbit antiserum. (B) Western blot analysis of boIL-1β. BHV/IL-1β supernatant was concentrated to 2 ng of boIL-1β per lane, denatured, and resolved by electrophoresis on a 10% polyacrylamide–SDS gel. rBo-IL1β at 2 ng per lane was used as a control. Molecular weight markers are indicated on the left margin.

small amount of cytokine produced by BHV1/IL-1β should create a milieu consisting of infected cells plus cells which are directly or indirectly stimulated by the cytokine.

We found that boIL-1β expressed by BHV/IL1β was both cell-associated and secreted into the medium (Fig. 5A). A significant proportion of boIL-1β still remained cell associated even after infection at a high m.o.i. Immunofluorescence showed that the majority of this cell-associated IL-1 was located in the cytoplasm. In this regard, expression of boIL-1β by the recombinant virus differs from normal IL-1β expression, since the 17.5-kDa mature cytokine is rarely found in high concentrations within the cell (Hazuda *et al.*, 1988). In monocytes, IL-1β is initially synthesized as a 31-kDa precursor that is cleaved by an ICE to generate the mature cytokine which is the major biological active form (Kostura *et al.*, 1989; Black *et al.*, 1989; Thornberry *et al.*, 1992; Cerretti *et al.*, 1992). The actual mechanism of transport outside of the cell is unknown, but it has been suggested to be linked with cleavage of the precursor into the mature form by ICE via a nonclassical pathway (Howard *et al.*, 1995). The finding that a significant proportion of the boIL-1β remained in the cell was unexpected since the mature sequence of the boIL-1β gene was fused to the gC signal peptide sequence (Fig. 1B). Based on the observation that a gC mutant lacking the transmembrane and cytoplasmic domain regions is effectively secreted from infected cells (Liang *et al.*, 1993), we speculate that the processing and secretion of gC-boIL-1β was either not interacting completely with the ER/Golgi complex or some other factor was interacting with the gC-boIL1β protein to prevent effective secretion.

In vitro growth experiments indicated that the production of boIL-1β was not toxic to the cells and did not

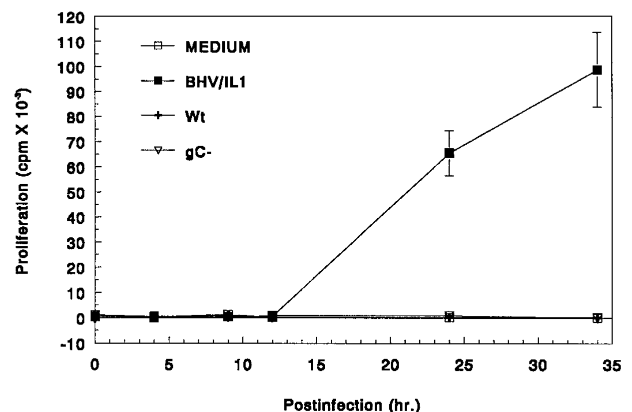

FIG. 6. Bioassay for bovine IL-1β expression. Confluent monolayers of MDBK cells in 100-mm plates were infected with wild-type BHV-1, gC-negative mutant, and recombinant BHV/IL1β at a m.o.i. of 5. At indicated times, the supernatants were harvested. Samples were assayed for IL-1β activity using a subclone (LM-1) of the D10.G4.1 ATCC cell line sensitive to interleukin-1. The results represent means ± S.D. of triplicate samples.

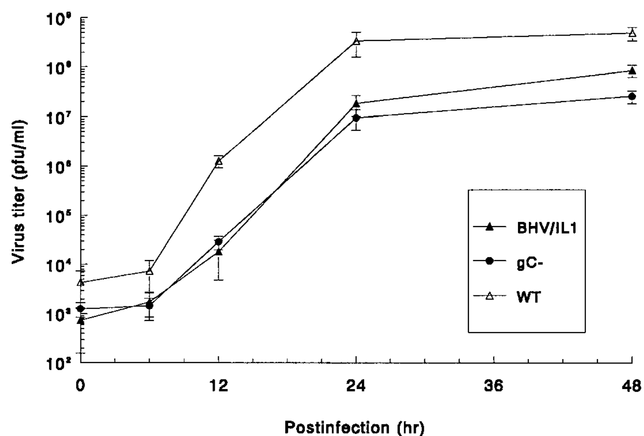

FIG. 7. Single step-growth experiment. Confluent MDBK cells in 6-well culture plates were inoculated at a m.o.i. of 5. After 1 hr adsorption at 37°, virus inoculum was removed, cells were washed three times with MEM, and incubated in 2 ml of MEM supplemented with 10% FBS. At various times postinfection, medium was collected and assayed for BHV-1 titers. The supernatant was titered by plaque assay. wt, wild-type BHV-1; gC−, a gC deletion mutant; BHV/IL1β, a gC-negative recombinant expressing bovine interleukin-1β.

affect viral growth. The single-step growth curve showed that the growth of recombinant BHV/IL1β was comparable *in vitro* to a gC-negative mutant (Liang *et al.,* 1991). This particular property is important since it provides an opportunity to compare BHV/IL1β and gC^--negative mutants *in vivo,* thereby determining the effects of local boIL-1β expression on BHV-1 infectivity.

In conclusion, we have described the construction and characterization of a recombinant BHV-1 expressing a biologically active BoIL-1β protein which can be both cell-associated or secreted into the medium. The expression of boIL-1β does not affect the *in vitro* growth of the virus. Overall, recombinant BHV-1 expressing cytokine has the potential to be used in the study of new approaches toward attenuation and immunomodulation of BHV-1 infections, including infections associated with respiratory disease complex in cattle. Such experiments are presently in progress.

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