Bovine interleukins 2 and 4 expressed in recombinant bovine herpesvirus 1 are biologically active secreted glycoproteins

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The open reading frames encoding bovine interleukin 2 (boIL-2) and bovine interleukin 4 (boIL-4) were integrated into the unique short segment of the genome of bovine herpesvirus 1 (BHV-1) and expressed under control of the murine cytomegalovirus (MCMV) immediate-early 1 (ie1) enhancer–promoter element or the MCMV early 1 (e1) promoter. Madin–Darby bovine kidney cells infected with the recombinant viruses secreted boIL-2 or boIL-4 into the culture medium. Secretion was inhibited by the presence of brefeldin A during the infection, indicating that export from the cells was dependent on a functional Golgi apparatus. Treatment of the secreted interleukins with N-glycosidase F reduced the apparent molecular mass of recombinant BHV-1-expressed boIL-2 from 22 kDa to 16 kDa and that of boIL-4 from 20 kDa to 13 kDa, which demonstrated that both cytokines contain N-linked oligosaccharides. Digestion with neuraminidase and O-glycosidase had no detectable effect on the apparent molecular masses, suggesting that BHV-1-expressed boIL-2 and boIL-4 are not, or only slightly, O-glycosylated. In vitro experiments demonstrated the biological activity of recombinant BHV-1-expressed boIL-2 and boIL-4 by their ability to maintain the proliferation of bovine 4325 T cells and activated bovine B cells, respectively. In conclusion, we show that boIL-2 and boIL-4 are secreted from recombinant BHV-1-infected cells as biologically active glycoproteins.

Introduction

Bovine herpesvirus 1 (BHV-1), a member of the subfamily Alphaherpesvirinae with a double-stranded DNA genome of approximately 138 kbp (Mayfield et al., 1983; Roizman et al., 1992), is an economically important pathogen. Infectious rhinotracheitis and infectious pustular vulvovaginitis are the most common clinical symptoms induced in cattle (Gibbs & Rweyemamu, 1977). Vaccination is widely used to control the disease and to reduce the concomitant financial losses. Although the currently available live attenuated and inactivated vaccines are efficacious, they have the disadvantage that animals have to be revaccinated repeatedly to achieve full protection.

Strategies in the development of next-generation vaccines need to consider the balance of CD4+ T helper (Th) cell subsets they induce. The present perception of the function of these T lymphocytes in immunity to infectious diseases is that Th1 cells, which secrete interleukin 2 (IL-2) and interferon-γ, are associated with the induction of cellular immune responses (Cher & Mosmann, 1987), while Th2 cells, which secrete IL-4, IL-5, IL-6 and IL-10, invoke high-titre antibody responses and poor cellular immune responses (Coffman et al., 1988). Depending on the type of Th cell bias required, it is possible to direct the immune response to a peptide or protein by employing one or more of a number of strategies, including immunization in the presence of cytokines (Romagnani, 1994). Recently, strategies which permit the delivery of antigen and cytokine have involved the construction of recombinant viral vectors encoding both antigen and cytokine genes (Leong et al., 1994). Such model systems have provided clear evidence of a crucial involvement of distinct cytokines in mediating host protection against pathogens and have shown that manipulation of the immune response through the Th cell–cytokine axis can favour either the host or the pathogen.

Because of the functional significance of IL-2 and IL-4 in determining the development of the CD4+ Th2 subset of murine and human lymphocytes (Mosmann & Coffman, 1989; Parronchi et al., 1992) and to investigate the potential of a herpesvirus vector for the release of cytokines in vitro and ultimately in vivo, the open reading frames (ORFs) encoding
bovine (bo) IL-2 and boIL-4 were selected for expression by recombinant BHV-1.

Here we report the construction of boIL-2- and boIL-4-expressing BHV-1 recombinants, the characterization of the expressed cytokines and describe in vitro experiments designed to test the biological activity of boIL-2 and boIL-4 produced by cells infected with the recombinants.

Methods

- **Viruses and cells.** Madin–Darby bovine kidney cell clone Bu100 (MDBK-Bu100, kindly provided by W. Lawrence and L. Bello, Philadelphia, USA) was grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum, 350 μg/ml glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

- **BHV-1 wild-type strain Schönbbeken** (obtained from O. C. Straub, Tübingen, Germany) was propagated on MDBK-Bu100 cells. The glycoprotein D+ (gD+) mutant BHV-1/80-221 was grown on the gD-expressing cell line BUV3-7 (Fehler et al., 1992).

- **Isolation of ORFs encoding boIL-2 and boIL-4.** Because plasmid pACIL-2.B222 (Collins et al., 1994) encompasses only codons 21–155 of the boIL-2 gene (Cerretti et al., 1986), the boIL-2 ORF was completed using synthetic oligonucleotides and standard cloning procedures (Sambrook et al., 1989). The sequence contained in the resulting plasmid, pSPI1-2OK, was verified by dideoxy chain-termination sequencing (T7 sequencing kit; Pharmacia). Plasmid pSPI1-2OK was sequenced with [α-32P]dCTP using the ‘Ready To Go’ DNA labelling kit (Pharmacia).

- **Construction of cytokine integration plasmids.** BHV-1 transfer vectors pROMie and pROMe were designed to integrate heterologous ORFs into the genome of BHV-1/80-221. Their construction will be described in detail elsewhere. Briefly, both vectors contain the ORF for gD (Fehler et al., 1994) encompassing only codons 21–155 of the boIL-2 gene (Cerretti et al., 1986), the boIL-2 ORF was completed using synthetic oligonucleotides and standard cloning procedures (Sambrook et al., 1989). The sequence contained in the resulting plasmid, pSPI1-2OK, was verified by dideoxy chain-termination sequencing (T7 sequencing kit; Pharmacia). Plasmid pSPI1-2OK was sequenced with [α-32P]dCTP using the ‘Ready To Go’ DNA labelling kit (Pharmacia).

- **RNA isolation and Northern blot hybridization.** Cytoplasmic RNA was isolated essentially as described by Sambrook et al. (1989). Glyoxal-denatured RNA (5 μg) was separated in 1% formaldehyde gels and transferred to nitrocellulose filters and hybridized following standard procedures (Sambrook et al., 1989). DNA probes used for hybridization were labelled with [α-32P]dCTP using the ‘Ready To Go’ DNA labelling kit (Pharmacia). For estimation of RNA sizes, Escherichia coli 16S and 23S rRNAs and murine 18S and 28S rRNAs were used as size markers. Hybridization procedures were as described by Keil et al. (1984).

- **Production of anti-boIL-2 and anti-boIL-4 serum.** The expression vector pATH2, which contains a part of the TrpE ORF downstream of the inducible TrpE promoter (Dieckmann & Tzagoloff, 1985), was digested with BamHI and a 355 bp BamHI fragment from plasmid pACIL-2.B222 encompassing codons 21–155 of the boIL-2 ORF was inserted in-frame. The resulting plasmid, pFusIL-2, encodes a fusion protein which consists of 334 amino acids of the TrpE protein and 135 amino acids of boIL-4. Plasmid pFusIL-4 was obtained after cleavage of pATH11 with Smal and in-frame insertion of the entire boIL-4 ORF and encodes a fusion protein which consists of 334 amino acids of the TrpE protein and 135 amino acids of boIL-4.

- **Immunoprecipitation and glycosidase treatment.** Confluent monolayers of MDBK-Bu100 cells grown in six-well tissue culture dishes were infected at an m.o.i. of 10. After 1 h adsorption, the inoculum was
Map units

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0

I l I l I l I l I l I l I l

Organization

UL IR US TR

Hind III

NJ M I O E G A B K C LP H

10kb

wt BHV-1

Hind III Sma I Sma I Sal I Nru I Hind III

gE gI gD gG PK

1kb

gD 'lacZ' BHV-1

lacZ

gD poly A cytokine ORF MCMV promoter MCMV iε2 poly A gD ORF gD TATA

Fig. 1. Construction of recombinant BHV-1. The HindIII restriction fragment map of BHV-1 strain Schönböken is shown below a schematic diagram of the prototype orientation of the genome (Engels et al., 1986; Mayfield et al., 1983). The wild-type (wt) HindIII L fragment is expanded and the location and direction of transcription of genes encoding the putative protein kinase (PK) and glycoproteins G (gG), D (gD), I (gI) and E (gE) are indicated by arrows (Leung-Tack et al., 1994). Relevant restriction enzyme cleavage sites are marked. The corresponding HindIII fragment of the gD 'lacZ' mutant BHV-1/80-221 is depicted below. The location (dotted area) and direction of transcription of the lacZ cassette that replaces the gD ORF is indicated by an arrow. The lacZ cassette is not drawn to scale. At the bottom, a schematic representation of the integration fragment contained in the cytokine recombination vectors is shown. The gD TATA and gD poly(A) segments indicate BHV-1 sequences that contain the gD promoter and the gD polyadenylation signal, respectively, which provide homologous regions for recombination. The cytokine ORFs used in this report are the boIL-2 and boIL-4 ORFs, whose transcription is directed either by the MCMV ie1 enhancer–promoter or by the MCMV e1 promoter. Expression of the gD ORF in the constructs used is under control of the authentic gD promoter.

replaced by 500 μl DMEM lacking methionine and cysteine. After addition of [35S]methionine (500 μCi/ml) and [35S]cysteine (250 μCi/ml), cells were incubated for the times indicated and immunoprecipitations of proteins from cell lysates or cell culture supernatants were carried out as described by Keil et al. (1985). Labelled proteins were visualized after SDS–PAGE by fluorography. To remove N- or O-linked carbohydrates, immunoprecipitated proteins were incubated overnight at 37 °C with 0.4 U N-glycosidase F (Boehringer) or 1.5 mU O-glycosidase (Boehringer). All deglycosylation reactions were performed under conditions recommended by the supplier.

Determination of the biological activity of boIL-2 and boIL-4 expressed by recombinant BHV-1. The murine monoclonal antibodies (MAbs) IL-A58 (lgG2a) and IL-A111 (lgG1), kindly provided by the International Livestock Research Institute, Kenya, are specific for bovine immunoglobulin light chain and the alpha subunit of the IL-2 receptor (CD25), respectively. Recombinant bovine (rbo) IL-2 and rboIL-4 were produced in the baculovirus–insect cell system (Collins et al., 1994). Antibodies directed against rboIL-2 and rboIL-4 that were able to block the biological activity of IL-2 and IL-4, respectively, were raised in rabbits. Cell phenotype was determined using fluorescein-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates). Culture medium consisted of RPMI 1640 medium with glutamax (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum, 5 × 10⁻⁵ M-2-mercaptoethanol, 200 U penicillin and 100 μg/ml streptomycin.

Peripheral blood cells were used as a source of B lymphocytes for the boIL-4 assay. Essentially, blood was collected into heparin (10 U/ml) from a conventionally reared Bos taurus heifer. Whole peripheral blood leukocytes were separated by density centrifugation on Histopaque (Sigma), washed and adjusted to 3 x 10⁶ cells/ml in PBS containing 0.5% BSA, stained with IL-A58 and incubated with superparamagnetic particles (Miltenyi Biotech). The labelled cells were isolated on a MiniMacs column (Miltenyi Biotech) following the manufacturer’s instructions and adjusted to 1.5 x 10⁶ cells/ml. The purity of the population was assessed using a FACScan (Beckton Dickinson) and was shown to contain greater than 90% B cells. The viability of cells was assessed by trypan blue dye exclusion. To determine boIL-4 activity, 10⁵ purified B cells in 100 μl medium were dispensed into wells containing 100 μl of fivefold serial dilutions of supernatants from recombinant BHV-1-infected cells or dilutions of rboIL-4. For the boIL-2 bioassay, the long-term rboIL-2–maintained bovine cell line 4325, isolated from peripheral blood cells, was used. The 4325 cells, which are αβ TCR⁺ and CD4⁺, were stimulated with rboIL-2, washed 3 or 4 days later, and 100 μl cell suspension containing 5 x 10⁶ cells was added to 100 μl fivefold serial dilutions of
supernatants from recombinant BHV-1-infected cells or dilutions of rboIL-2. In some assays, 20 μl anti-CD25 MAb (IL-A111), diluted 1:600, was added. After incubation for 18–24 h, cultures were labelled with [3H]thymidine (NEN, DuPont) for 6 h and the incorporated radioactivity was determined by liquid scintillation counting.

Results
Isolation of bolL-2- and bolL-4-expressing BHV-1 recombinants
Recombination plasmids pROMiel-L2, pROMeil-L2, pROMiel-L4 and pROMeil-L4 were cotransfected with purified BHV-1/80-221 into MDBK-Bu100 cells and the progeny virus were serially diluted and plated on MDBK-Bu100 cells. Plaques that did not stain blue under a Bluo-Gal-containing agarose overlay were picked and virions were again titrated on MDBK-Bu100 cells. Plaques which produced only 'white' plaques were plaque purified once more and further characterized. The resulting recombinants were named BHV-1/eiIL-2, BHV-1/eil-L2, BHV-1/eiIL-4 and BHV-1/eIL-4. To demonstrate integration of the gD–cytokine expression cassettes into the genomes of the respective viruses, MDBK-Bu100 cells were

Fig. 3. Transcription of BHV-1-expressed cytokine ORFs. MDBK-Bu100 cells were infected with BHV-1/eiIL-2 (lane 1), BHV-1/eiIL-2 (lanes 2 and 3), BHV-1/eIL-4 (lane 6), BHV-1/eIL-4 (lanes 7 and 8) and wild-type BHV-1 (lanes 4 and 5) with (lanes 1, 2, 4, 6 and 7) or without (lanes 3, 5 and 8) cycloheximide (100 μg/ml). Cells were harvested at 6 h p.i., and 5 μg of cytoplasmic RNA was size fractionated in denaturing 1% agarose gels. After transfer to nitrocellulose, filters were hybridized to 32P-labelled DNA fragments encompassing the bolL-2 (lanes 1–5) or bolL-4 (lanes 6–8) ORFs. Bound radioactivity was visualized by autoradiography. The size of the transcripts is indicated in kb.

Fig. 2. Integration of the bolL-2 and bolL-4 ORFs into the genome of BHV-1. MDBK-Bu100 cells were infected with BHV-1/eiIL-2 (lanes 1), BHV-1/80-221 (lanes 2), wild-type BHV-1 (lanes 3) or BHV-1/eIL-2 (lanes 4). Infected-cell DNA was prepared 20 h p.i., cleaved with HindIII and fragments were transferred to nitrocellulose after size separation in a 0·6% agarose gel. Filters were hybridized to 32P-labelled DNA of the IL-2 ORF (b), the gD ORF (c), the gI ORF (d) or the lacZ ORF (e). (a) Gel after staining with ethidium bromide. Fragment sizes are indicated.
Fig. 4. BHV-1-expressed bolL-2 and bolL-4 are secreted from infected cells. MDBK-Bu100 cells were infected with BHV-1/eIL-2 (a, lanes 1, 2, 5 and 6), BHV-1/eIL-2 (a, lanes 3, 4, 7 and 8), BHV-1/eIL-4 (b, lanes 1, 2, 5 and 6) and BHV-1/eIL-4 (b, lanes 3, 4, 7 and 8) in the presence of [35S]methionine–cysteine with (lanes 5–8) or without (lanes 1–4) brefeldin A (2.5 μg/ml). At 16 h p.i., cells (lanes 2, 4, 6 and 8) and supernatants (lanes 1, 3, 5 and 7) were harvested and proteins were immunoprecipitated with the anti-bolL-2 serum (a) or the anti-bolL-4 serum (b). Precipitated proteins were analysed by SDS-PAGE followed by fluorography. The apparent molecular masses of the proteins are indicated.

infected with BHV-1/eIL-2 (Fig. 2, lanes 1), BHV-1/eIL-2 (Fig. 2, lanes 4), BHV-1/eIL-2-phenotypically complemented by propagation on gD-expressing cells (Fig. 2, lanes 2) and wild-type BHV-1 (Fig. 2, lanes 3). Whole-cell DNA was prepared 20 h p.i., cleaved with HindIII and transferred to nitrocellulose after size separation in 0.6% agarose gels. Filters were probed with 32P-labelled DNA from the bolL-2 ORF (Fig. 2b), the gD ORF (Fig. 2c), the gl ORF (Fig. 2d) or the lacZ ORF (Fig. 2e). The IL-2 probe only hybridized to 8.9 and 8.7 kb fragments present in BHV-1/eIL-2 DNA (Fig. 2b, lane 1) and BHV-1/eIL-2 DNA (Fig. 2b, lane 4), respectively; these fragments were also detected by the gD probe (Fig. 2c, lanes 1 and 4). The size of these fragments is as estimated, since substitution of the lacZ gene and the gl ORF with the gD-cytokine expression cassettes by homologous recombination into the 9.9 kb HindIII fragment of BHV-1/80-221 should result in a decrease in size of 1 and 1.2 kb, respectively. As expected, the gD probe bound to the 7.8 kb HindIII fragment of wild-type BHV-1 (Fig. 2c, lane 3) and not to DNA from BHV-1/80-221-infected cells (Fig. 2c, lane 2). Hybridization with gl- and lacZ-specific probes demonstrated that these ORFs were deleted from the genomes of BHV-1/eIL-2 and BHV-1/eIL-2 (Fig. 2d, e, lanes 1 and 4). The gl probe detected the 9.9 kb BHV-1/80-221 fragment (Fig. 2d, lane 2) and the 7.8 kb fragment of wild-type BHV-1 DNA (Fig. 2d, lane 3) but did not bind to any fragment of BHV-1/eIL-2 and BHV-1/eIL-2 DNA (Fig. 2d, lanes 1 and 4). The labelled lacZ DNA hybridized exclusively to a 9.9 kb fragment of BHV-1/80-221-infected cell DNA (Fig. 2e, lane 2). We conclude that BHV-1/eIL-2 and BHV-1/eIL-2 were generated by homologous recombination within the 9.9 kb HindIII fragment of BHV-1/80-221 DNA as envisaged. The presence of the gD-ieIL-4 and gD-eIL-4 expression cassettes in the genomes of BHV-1/eIL-4 and BHV-1/eIL-4 was demonstrated accordingly (data not shown).

The bolL-2 and bolL-4 ORFs are transcribed in recombinant BHV-1-infected cells

To test for expression of the bolL ORFs by the BHV-1 recombinants and to demonstrate ie transcription of bolL-2 and bolL-4 after infection with BHV-1/eIL-2 and BHV-1/eIL-4, cells were inoculated with the virus without or with cycloheximide to prevent de novo protein synthesis. Cytoplasmic RNA was isolated at 6 h p.i., size separated by agarose electrophoresis, and transferred to nitrocellulose. A 32P-labelled DNA probe representing the bolL-2 ORF detected an RNA of 1.1 kb after infection with BHV-1/eIL-2 in the presence of cycloheximide (Fig. 3, lane 1) and after infection with BHV-1/eIL-2 without cycloheximide (Fig. 3, lane 3). This transcript was not detected in cells infected with wild-type BHV-1 in the absence or presence of the inhibitor (Fig. 3, lanes 4 and 5) and after infection with BHV-1/eIL-2 in the presence of cycloheximide (Fig. 3, lane 2). Substantially the same results were obtained when RNA from cells infected with BHV-1/eIL-4 and BHV-1/eIL-4 in the presence or absence of cycloheximide was probed with 32P-labelled DNA encompassing the bolL-4 ORF (Fig. 3, lanes 6–8). Thus, the bovine IL-2 and IL-4 transcription units are expressed by the BHV-1 recombinants and the MCMV ie1 enhancer–promoter directs transcription under ie conditions in the BHV-1 genomic context.

Identification of BHV-1-expressed bolL-2 and bolL-4

To identify and characterize the bolLs expressed by the recombinant BHV-1 strains, rabbit antisera, raised against E. coli-expressed TrpE–bolL-2 and TrpE–bolL-4 fusion proteins, were incubated with [35S]methionine–cysteine-labelled proteins from cell lysates and the corresponding cell culture media. After infection with BHV-1/eIL-2 (Fig. 4a, lanes 1, 2, 5 and 6), BHV-1/eIL-2 (Fig. 4a, lanes 3, 4, 7 and 8), BHV-1/eIL-2 (Fig. 4a, lanes 1, 2, 5 and 6), BHV-1/eIL-2 (Fig. 4a, lanes 3, 4, 7 and 8), BHV-1/eIL-2 (Fig. 4a, lanes 1, 2, 5 and 6), BHV-1/eIL-2 (Fig. 4a, lanes 3, 4, 7 and 8), BHV-1/eIL-2 (Fig. 4a, lanes 1, 2, 5 and 6), BHV-1/eIL-2 (Fig. 4a, lanes 3, 4, 7 and 8), BHV
Fig. 5. O-Glycosidase treatment of BHV-1-expressed bolL-2 and bolL-4. MDBK-Bu100 cells were infected with BHV-1/elL2 (a) and BHV-1/elL4 (b) in the presence of [35S]methionine-cysteine. Proteins from the supernatants were immunoprecipitated with the anti-bolL-2 serum (a, lanes 2-4) or the anti-bolL-4 serum (b, lanes 2-4) and incubated with PBS (lanes 2), neuraminidase (lanes 3) and neuraminidase followed by O-glycosidase (lanes 4). Lanes 1 show proteins precipitated by the matching preimmune sera. Precipitated proteins were analysed by SDS-PAGE followed by fluorography. The apparent molecular masses of the proteins are indicated.

Fig. 6. BHV-1-expressed bolL-2 and bolL-4 are N-glycosylated. MDBK-Bu100 cells were infected with BHV-1/elL2 (a) and BHV-1/elL4 (b) in the presence of [35S]methionine-cysteine. Proteins from the supernatants were immunoprecipitated with the anti-bolL-2 serum (a, lanes 2 and 4), the anti-bolL-4 serum (b, lanes 2 and 4) or the corresponding preimmune sera (lanes 1 and 3) and incubated with PBS (lanes 1 and 2) or N-glycosidase (lanes 3 and 4). Precipitated proteins were analysed by SDS-PAGE followed by fluorography. The apparent molecular masses of the proteins are indicated.

BoIL-2 and the 20 kDa anti-boIL-4-reactive protein from BHV-1/elL-4- and BHV-1/elL-4-infected cells constitutes the recombinant boIL-4. The differences in the calculated molecular masses of the cytokines (15.5 kDa for boIL-2 and 13.5 kDa for boIL-4) as compared to those found after expression in mammalian cells, as well as the processing by the Golgi apparatus-dependent secretion pathway, indicated that BHV-1-expressed boIL-2 and boIL-4 were associated with carbohydrates.

Analysis of carbohydrates

To determine whether BHV-1-expressed boIL-2 and boIL-4 contain O-linked carbohydrates as described for human IL-2 (Delves & Roitt, 1992), proteins immunoprecipitated from BHV-1/elL2 (Fig. 5a) and BHV-1/elL4 (Fig. 5b)-infected cell culture supernatants by the anti-boIL-2 serum and the anti-boIL-4 serum, respectively, were incubated with PBS (lanes 2), neuraminidase (lanes 3) or neuraminidase and O-glycosidase (lanes 4). Lanes 1 show proteins precipitated by the corresponding preimmune sera. The electrophoretic mobility of both BHV-1-expressed interleukins appeared unchanged after the enzyme treatment, indicating that they have not been modified by O-glycosylation.

Incubation of immunoprecipitated BHV-1-expressed boIL-2 and boIL-4 with N-glycosidase resulted in a decrease of the
apparent molecular mass of bolL-2 from 22 (Fig. 6a, lane 2) to 16 kDa (Fig. 6a, lane 4) and a decrease from 20 (Fig. 6b, lane 2) to 13 kDa (Fig. 6b, lane 4) for bolL-4, demonstrating that bolL-2 and bolL-4 secreted from recombinant BHV-1-infected cells contained N-linked glycans. The specificity of the reactions was shown by using the respective preimmune sera, which did not react with any of the mature ILs (Fig. 6a, b, lanes 1 and 3).

BHV-1-expressed bolL-2 and bolL-4 are biologically active

To determine whether bolL-2 and bolL-4 secreted from BHV-1-infected cells are biologically active, MDBK-Bu100 cells were infected with BHV-1/elL2 and BHV-1/elL-4 at 10 p.f.u. per cell, and phosphonoacetic acid (250 μg/ml) was added to prevent late gene expression and, thus, formation of progeny virions. Culture supernatants were clarified at 16 h p.i. by low-speed centrifugation and stored at −20 °C until use. As shown in Fig. 7, supernatants from cells infected with BHV-1/elL-2, but not BHV-1/elL-4 or BHV-1, stimulated the proliferation of 4325 cells, a long-term rboIL-2-maintained cell line. The level of activity in the medium of BHV-1/elL-2-infected cells was directly proportional to the dilution of the supernatant (Fig. 7a). There were no significant differences in the viability of 4325 cells following incubation in supernatants from MDBK-Bu100 cells infected with BHV-1/elL-2 or BHV-1/elL-4 compared with the cells cultured in medium (data not shown). The supernatant from BHV-1/elL-2-infected MDBK-Bu100 cells induced proliferation of 4325 cells comparable to that of 400 units rboIL-2/ml. Sufficient anti-bovine CD25 to block 400 units rboIL-2/ml was added to cultures to examine the neutralizing effect on the proliferative response. This amount of antibody completely inhibited the activity of the BHV-1/elL-2-infected culture supernatant and rboIL-2 (Fig. 7a).

To identify the presence of B cell growth factor activity, the culture supernatants from BHV-1-, BHV-1/elL-2- and BHV-1/elL-4-infected cells were incubated with bovine B cells stimulated with anti-Ig for 3 days. The supernatant from BHV-1/elL-4-infected cells induced a strong proliferative response, while the medium from BHV-1/elL-2-infected cells induced only a weak or modest response and the supernatant from BHV-1-infected cells contained no detectable activity (Fig. 7c, d). The B cell growth-promoting activity of the BHV-1/elL-4-infected cell supernatant displayed the same response relationship as the rboIL-4 standard and contained approxi-
mately 400 dilution units of boIL-4 activity/ml (Fig. 7d). The presence of sufficient polyclonal anti-boIL-4 antibody to completely inhibit the B cell proliferative activity of 1000 units/ml rboIL-4 also blocked the stimulatory activity of the BHV-1/eIL-4-infected cell supernatant. In contrast, the BHV-1/eIL-2-infected cell medium and the rboIL-2 standard only induced suboptimal or low stimulatory activity (10 dilution units of B cell proliferative activity/ml) with different response kinetics compared to rboIL-4 (Fig. 7c). These boIL-2-induced B cell proliferative responses were blocked by the addition of sufficient anti-bovine CD25 to block 400 units of boIL-2 activity. These results show that boIL-2 and boIL-4 expressed by recombinant BHV-1 are biologically active cytokines.

Discussion

The ORFs encoding boIL-2 and boIL-4 were cloned into plasmids pROMie and pROMe downstream of the well-characterized MCMV ie1 and e1 promoter (Dorsch-Häsler et al., 1985; Bühler et al., 1990), respectively. These heterologous promoters were chosen to test for their activity in the context of the BHV-1 genome and to avoid unwanted site effects which might occur by duplicating BHV-1 sequences using authentic promoter elements. Cotransfection of the recombination plasmids with purified genomic DNA of BHV-1 mutant 80-221 resulted in isolation of BHV-1 mutants BHV-1/eIL-2, BHV-1/eIL-2, BHV-1/eIL-4 and BHV-1/eIL-4. Replacement of the lacZ cassette and integration of the boIL ORFs into the unique short segment of the BHV-1/80-221 genome was demonstrated by Southern blot hybridizations. Northern blot analyses showed that the cytokine ORFs in BHV-1/eIL-2- and BHV-1/eIL-4-infected MDBK-Bul00 cells were transcribed under ie conditions. In contrast, expression in BHV-1/eIL-2- and BHV-1/eIL-4-infected cells was dependent on BHV-1 ie protein synthesis, indicating that the activity of the MCMV promoters in the context of the BHV-1 genome is comparable to the situation within the MCMV genome (Dorsch-Häsler et al., 1985; Bühler et al., 1990).

The proteins encoded by the cytokine ORFs were identified using rabbit polyclonal antisera against E. coli-expressed TrpE-boIL-2 and TrpE-boIL-4 fusion proteins. The boIL-2-specific antisera detected a 22 kDa protein in the supernatant of BHV-1/eIL-2- and BHV-1/eIL-2-infected cultures which was retained in the cells in the absence of brefeldin A. From the supernatants of BHV-1/eIL-4- and BHV-1/eIL-4-infected cells, the anti-boIL-4 serum precipitated a 20 kDa protein that also remained cell-associated when brefeldin A was present during the infection. Because brefeldin A disrupts the Golgi apparatus and thereby inhibits transport and secretion of newly synthesized glycoproteins and prevents complete O-glycosylation (Whealy et al., 1991), this result indicated that transport of BHV-1 expressed boIL-2 and boIL-4 is dependent on the Golgi apparatus and that both cytokines are not substantially O-glycosylated. This conclusion was confirmed by incubation of immunoprecipitated boIL-2 and boIL-4 with neuraminidase and O-glycosidase, which did not influence the migration of these cytokines in SDS-polyacrylamide gels. Treatment of immunoprecipitated boIL-2 and boIL-4 with N-glycosidase F, however, reduced the apparent molecular mass of boIL-2 from 22 to 16 kDa and the apparent molecular mass of boIL-4 from 20 to 13 kDa, demonstrating that BHV-1-expressed boIL-2 and boIL-4 contain N-glycans. The apparent molecular masses of the deglycosylated polypeptides are close to the calculated values of 15.5 and 12.7 kDa for the amino acid backbones of boIL-2 and boIL-4, respectively. Biological activity of the BHV-1-expressed cytokines was demonstrated by the stimulatory effect on activated bovine T and B cells in lymphoproliferative assays. Supernatants from cells infected with the recombinant BHV-1 constructs yielded comparable levels of bioactive boIL-2 and boIL-4, respectively, and antibodies which neutralized cytokine function provided a convenient way to confirm the specificity of these assays. In agreement with previous studies on the bioactivity of rboIL-2, BHV-1-encoded boIL-2 induced the proliferation of activated B (Collins & Oldham, 1995) and T (Collins et al., 1994) cells. This contrasted with the findings with boIL-4, which induced B cell proliferation, but was not capable of inducing T cell proliferation by itself, although it enhanced boIL-2-driven proliferation (data not shown). These observations are consistent with those recently published by Estes et al. (1995), who describe the lack of T cell growth in the presence of boIL-4 and suggest that bovine T cells require additional or different stimulatory signals to proliferate in response to boIL-4 and may differentiate bovine T helper cell requirements from those of murine T cells, but not human T cells (Van-Reijisjen et al., 1994).

Although only supernatants from BHV-1/eIL-2- and BHV-1/eIL-4-infected cells were tested for biological activity, it is reasonable to assume that also boIL-2 and boIL-4 expressed under the control of the MCMV ie1 promoter are biologically active because, in comparison to MCMV e1-driven expression, no differences in transport or processing of the respective cytokines could be detected (not shown).

The bovine regulatory pathways of the CD4-bearing T cell subset are poorly understood. However, the recent establishment of a small panel of bovine CD4+ T cell clones indicates a bias in the production of cytokines (Brown et al., 1994). In view of the major differences between Th1 and Th2 cytokine-synthesis patterns seen in other species, it would not be surprising if these T cells and their cytokines also differ markedly in function. Undoubtedly, studies that model the delivery of cytokines (and antigens) in recombinant virus vectors and evoke T cell-mediated immunity or antibody-dependent immune responses will help to identify those molecules dominant in the induction of Th1 and Th2 responses and might improve the efficiency of live vaccines against BHV-1 infection of cattle, thus leading towards the development of
vaccines that only have to be administered once. The use of bovine cytokine-expressing BHV-1 recombinants in current animal experiments will demonstrate whether this goal can be achieved and will also show to what extent the biological properties of the engineered viruses are affected by the modifications, a prerequisite also for the design of a safe vaccine. Furthermore, the co-expression of cytokines together with protective antigens from other pathogens should allow a more targeted approach to the development of vectored vaccines, which will be of broad relevance for a variety of disease problems in both veterinary and human medicine.

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