THE CONSTRUCTION AND CHARACTERIZATION OF BOVINE HERPESVIRUS-1 EXPRESSING CYTOKINES

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SUMMARY OF DISSERTATION

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By

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ABSTRACT

This thesis investigated the potential of using Bovine Herpesvirus-1 (BHV-1) virus as a viral vector to express cytokines and analysed the effects of these cytokines on BHV-1 infection and host immunity. Two constructs were generated by homologous recombination by using transfer vectors containing either IL-1β or bovine IFN-γ flanked by the 5' and 3' ends of glycoprotein C gene. Recombination occurred within the gC locus which created a recombinant virus with a gC minus (gC^-) background phenotype that expressed the cytokine using the gC promoter. Molecular characterization showed that the recombinant cytokine genes were expressed with similar kinetics to a late BHV-1 protein; mRNA expression was detected at 5 hour post-infection followed by the detection of biologically active protein. These recombinant proteins had biological activity comparable to a recombinant protein standard. Both recombinant viruses, BHV1/IL1β and BHV-1/IFNγ, exhibited *in vitro* growth characteristics similar to a gC^- minus virus. This indicated that the expression of these cytokine did not affect BHV-1 growth.

To analysis the *in vivo* effects of these recombinant viruses, two experimental models were used. First, cattle were used to analyse the immune responses and viral pathogenicity during infection by recombinant BHV-1/IFNγ. Similar levels of virus shedding and similar clinical responses were observed for both recombinant BHV-1/IFNγ and gC-/LacZ+ viruses. BHV-1 was shown to be a potent inducer of boIFN-γ in

the nasal cavity and IFN-γ secretion correlated with the onset and duration of viral shedding. Analysis of cellular and humoral responses did not reveal any significant immune modulation during infection by BHV-1/IFN-γ. Serum IgG, mucosal IgA and antibody neutralization titers were similar between BHV-1/IFNγ and control gC⁻/LacZ+ virus. In addition, gD specific proliferative responses and IFN-γ ELISPOTs did not reveal any differences. After re-activation from latency with dexamethasone, viral shedding was similar for both BHV-1/IFNγ and gC-/LacZ+ viruses. These results suggested that during a respiratory infection, the production of exogenous IFN-γ did not provide an advantage to the host. Also, BHV-1/IFNγ was a stable vector during latency and recrudescence and expressed biologically active IFN-γ protein throughout the experimental period.

A sheep model for BHV-1 infection was also used to evaluate the recombinant BHV-1 vector. A preliminary experiment showed that both wild-type BHV-1 Cooper virus and gC-/LacZ+ infected sheep, caused mild clinical signs and generated BHV-1 specific immune responses. Recombinant BHV-1/IL-1β and BHV-1/IFNγ were then used to infect sheep. In this experiment, there was no difference in viral pathogenesis or the immune responses to the recombinant viruses and gC-/LacZ+ virus. Results in both sheep and cattle indicated that using BHV-1 as a viral vector to express bovine IFN-γ or IL-1β did not alter pathogenesis or the host immune response.

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a. a.	amino acid
ADCC	antibody-dependent cell-mediated cytotoxicity
AM	alveolar macrophages
boIL-1β	Bovine interleukin-1β
boIFN-γ	Bovine interferon-γ
BHV-1	Bovine herpesvirus-1
BSA	bovine serum albumin
CD4	Cluster designation 4
Con A	concanavalin-A
CPE	cytopathic effect
CTL	cytotoxic lymphocytes
DMSO	dimethyl sulfoxide
Endo H	endoglycosidase H
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FCM	flow cytometric analysis
FBS	fetal bovine serum
gC	glycoprotein C
gD	glycoprotein D
gE	glycoptetein E
gC⁻	gC minus virus
gC-/LacZ+	BHV-1δgIII/LacZ
GM-CSF	granulocyte/macrophage colony stimulating factor
HSV	herpes simplex virus
IBR	infectious bovine rhinotracheitis

IL-1	interleukin-1
IL-1Ra	interleukin-1 receptor antagonist
IL-2	interleukin-2
ICE	IL-1 converting enzyme
IE	immediate early
IgA	Immunoglobulin A
IgG	immunoglobulin G
IFN-γ	Interferon-gamma
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
JAK	Janus kinase
KDa	kilo-Dalton
LAT	latency-associated transcript
LAK	lymphokine activated killers
LCMV	lymphocytic choriomeningitis virus
LR	latency-related transcript
mAb	monoclonal antibody
MDBK	Madin-Darby bovine kidney cells
MEM	minimum essential medium
MHC	major histocompatibility complex
MNC	mononuclear cell
MOI	multiplicity of infection
Mu.	Mutant
NK cells	natural killer cells
ORF	open reading frame
PBS	phosphate-buffered saline
PBSA	phosphate-buffered saline without Ca^{++} and Ma^{++}
PBST	PBS-Tween-20
PBMC	Peripheral blood mononuclear cells
PFU	plaque forming units

p. i.	Post-infection
PGE_2	Prostaglandin E_2
PrV	Pseudorabies virus
PMN	polymorphonuclear cells
S.D.	standard deviation
SDS	sodium dodecyl sulphate
SI	stimulation index
SN	serum neutralization
UV	ultraviolet radiation
VSV	Vesicular Stomatitis Virus
WBC	white blood cell
Wt.	Wild-type virus

1. 0 INTRODUCTION

Current interest in vaccine development has focused on the use of subunit vaccines, gene deleted viruses, killed and live viral vectors (van Drunen Little-van den Hurk *et al.*, 1993; van Oirschot, 1996). Attempts at attenuating BHV-1 for use in vaccines have focused on the creation of a variety of deletion mutants (Kit *et al.*, 1985; Liang *et al.*, 1992; Kaashoek *et al.*, 1994). The deletion mutants were used as marker vaccines to distinguish vaccinated animals from naturally infected animals by performing a relatively simple ELISA against the deleted protein. In addition, other genes can be inserted into the deleted gene location. However, these mutants can still become latent and cause clinical disease especially in susceptible animals such as neonatal calves. The expression of cytokines by a recombinant virus could potentially function as an alternative means to attenuate and increase the immune response to a recombinant virus.

A number of different cytokines have been investigated as possible adjuvants in animal vaccine (Heath and Playfair, 1992). 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 haematopoiestic and immunological systems (Dinarello, 1991). Interferon-γ (IFN-γ) has been tested for its function as both an anti-viral molecule and its activity as a regulator of various immunological responses. The construction of recombinant BHV-1 vectors containing bovine IL-1β and IFN-γ made it possible to investigate this novel vaccine approach in a respiratory infection model.

2.0 LITERATURE REVIEW

2.1 Biology of BHV-1

Bovine herpesvirus virus 1 (BHV-1) is a member of the sub-family *Alphaherpesvirinae*, genus varicellovirus. BHV-1 is commonly referred to as infectious bovine rhinotracheitis (IBR) virus because of its ability to cause respiratory infections and rhinitis. BHV-1 can also cause conjunctivitis, genital infections and systemic infections that result in abortions and fetal deaths and can be a major pathogen in initiating the bovine respiratory disease complex (Comprehensively reviewed in Gibbs and Rweyemamu, 1977; Yates *et al.*, 1982; Wyler *et al.*, 1989).

The entire BHV-1 genome has been sequence with 135105 bases and 71 open reading frames (compiled by Schwyzer *et al*, 1997). BHV-1 shares a number of characteristics with other alphaherpesviruses including gene homologs, a variable host range, relatively short reproductive cycle, temporal control of gene expression, cytolytic properties in cell culture and the capacity to establish latency (Roizman, 1996).

2.1.1 Clinical pathogenesis

During respiratory infections, BHV-1 can be transmitted by aerosolization or direct contact between animals. BHV-1 attaches to the mucosal epithelium and replicates creating micro-focal necrotic lesions. An inflammatory response is initiated

resulting in fever and respiratory distress; conjunctivitis may also be present. At this point, the severity of respiratory distress can be compounded by secondary infections including viral super-infections or more commonly by bacterial colonization, primarily with *Pasteurella haemolytica*. This may result in a pneumonia syndrome known as 'shipping fever' (Babiuk *et al.*, 1988).

In genital infections, infectious pustular vulvovaginitis and infectious balanoposthitis (bull), BHV-1 can be transmitted by direct contact or by semen. Viral incubation occurs for 1-3 days in the genital mucosa followed by the appearance of pustles and painful inflammation (Wyler *et al.*, 1989). Similar to respiratory infections, secondary bacterial infections may occur.

Systemic spread in pregnant cows and new-born calves can cause a substantial loss through abortions and neonatal death, respectively (Mechor *et al.*, 1987; Bryan *et al.*, 1994). The actual mechanisms by which virus spreads systemically has not been conclusively defined, but the finding that monocytes and lymphoctyes are infected *in vitro* by BHV-1 suggests a possible mechanism of transmission to the fetus and central nervous system (Nyaga and Mckercher, 1979; Forman and Babiuk, 1982; Denis *et al.*, 1994b).

2.1.2 Latency

One of the hallmarks of Herpesviridae is the ability to become latent in the host. This probably accounts for its evolutionary success in spreading to most animal species (Roizman, 1996). In BHV-1, latency occurs soon after infection. After local viral replication in the mucosal epithelium, the virus enters the axonal nerve and is then

retrograde transported to the sensory gangalia, primarily the trigeminal and lumbrosacral ganglia (reviewed in Rock, 1994; Schang *et al.*, 1997). In genital infections, the virus is transported to the sacral ganglia (Ackerman and Wyler, 1984).

One of the interesting problems in herpesvirus latency is uncovering the mechanisms by which the virus can go from a lytic life cycle into a latent stage and vice versa. Recent work on the molecular basis for this phenomenon has focussed on the only RNA transcript expressed during latency, the latency-related transcript (LR) or latency-associated transcript (LAT) in HSV (Rock *et al.*, 1987a; Rock *et al.*, 1987b; Stevens *et al.*, 1987; Rock, 1994). Since the LAT (LR) gene is anti-parallel, overlapping the IE 2.9/ E2.6 region, it had originally been proposed that LR RNA functions to interfere with IE/E expression (Stevens *et al.*, 1987). Recently, the LR gene was expressed in a mammalian expression system. This revealed a 41 kDa protein representing a poly(A)+ transcript from the ORF 2 region (Hossain *et al.*, 1995). In the rabbit latency model, the LR protein was co-precipitated with cyclin A, a protein involved in the S phase of the cell cycle. It was suggested that the association between these proteins may inhibit progression of the cell through the cell division cell, and may enhance neuronal viability during latency by preventing apoptosis. Cyclin A was also shown to be induced during acute infection and reactivation (Schang *et al.*, 1996). However, the finding that a BHV-1 LR promoter deletion mutant is still able to become latent and undergo reactivation suggests that other factors are also involved in latency (Rock, 1994). Moreover, studies in HSV on the LAT gene have been unable to detect a protein; and LAT null mutants still become latent giving credence to the possibility of other mechanisms being involved in induction and maintenance of

latency (Javier *et al.*, 1988; Block *et al.*, 1993). Recent investigations into the mechanism of BHV-1 latency have shown that the improper expression and polyadenylation of immediate early genes (IE) genes in the neurons may be one of the factors that leads to the establishment of latency in trigeminal ganglia of cattle (Schang *et al.*, 1997).

2.1.3 Reactivation

BHV-1 can reactivate spontaneously or reactivation can be induced by natural stimuli - stress, injury, infection- or experimentally by the administration of the glucocorticoid steriod -dexamethasone (Sheffy and Davies, 1972; Thiry *et al.*, 1987; Wyler *et al.*, 1989). Following reactivation, the virus travels back to the primary site of infection. The actual mechanisms of reactivation are still under intensive research.

The glucocorticoid steriod, dexamethasone, has been widely used in the study of latency and re-activation in Herpesviradae. In general, corticosteriods suppress numerous immunological functions, including cytokine production, and can alter cellular metabolism within the cells in such a way that there is viral replication from a latent state. The characteristic suppression of macrophages and neutrophils by glucocorticoids increases the host's susceptibility to bacterial infections (Roth and Frank, 1989). In bovine cells, the *in vitro* addition of dexamethasone to polymorphonuclear neutrophils reduced migratory function, decreased phagocytosis activity and affected cytotoxicity mediated by antibodies or complement (Bielefeldt Ohmann *et al.*, 1987a). In addition, both *in vitro* and *in vivo* treatments of peripheral blood lymphocytes reduced IL-2 induced proliferation. However, dexamethasone did

not affect the *in vitro* replication of BHV-1 (Bielefeldt Ohmann *et al.*, 1987a).

Because corticosteriods have major effects on cellular populations, it was suggested that the maintenance of latency involves immune surveillance (Babiuk and Rouse, 1979; Bielefeldt Ohmann *et al.*,1987a). However, recent work on LR gene function and the direct effects of dexamethasone on neurons suggest a more probable hypothesis for reactivation where dexamethasone has a direct effect through the binding of the dex receptor on neurons. This may alter LR gene expression and cell cycle progression (Schang *et al.*, 1996). Indirect evidence was found in bovine cells where *in vitro* dexamethasone treatment causes decreased LR promoter function (Jones *et al.*, 1990) and in the rabbit model of latency, viral transcriptional differences were detected 15 to 18 hours after dexamethasone treatment (Rock *et al.*, 1992). Recently, HSV-1 was found to contain a glucocorticoid response element within the origin of DNA replication – Ori L region. The addition of dexamethasone to neurons enhanced replication while the mutation of the glucocorticoid response element abolished this effect; the authors suggested that dexamethasone may play a direct role in *in vivo* viral reactivation within neurons (Hardwicke and Schaffer, 1997).

2.2 Immune response to BHV-1

A number of comprehensive reviews on BHV-1 immunology have been written in the last few years(Denis *et al.*, 1994b; Tikoo *et al.*, 1995a; Babiuk *et al.*, 1996). This section will not be an extensive review but will highlight the important aspects of the host immune response, including new literature in this area.

The immune response to BHV-1 can be functionally classified into non-specific

and specific host defence mechanisms based on our understanding of innate and adaptive immunity. A variety of cell types such as alveolar macrophages, natural killer cells (NK), NK-like cells and polymorphonuclear cells (PMN) have been studied for their ability to generate non-specific host mechanisms. The specific host defence mechanisms are based on the specificity and memory generated by B cells and T cells which produce humoral immunity and cytotoxic lymphocytes (CTL), respectively.

2.2.1 Cell mediated immunity

After BHV-1 infection, a number of sequential events occur that influences the type and magnitude of the immune response. From the viral view-point, the virus undergoes attachment, penetration, temporal gene expression - immediate early (IE), early (E) and late (L) – followed by egress of new viral particles leading to viral spread by either extracellular virus or by cell to cell spread (Babiuk *et al.*,1996). These events result in the production of a number of viral antigens to which the immune system can respond. Of these, the viral surface glycoproteins contain the major T and B cells epitopes to which the specific immune system response is directed.

Peripheral blood lymphocyte activation occurs soon after infection as shown by proliferative responses, cytotoxicity and cytokine production (Campos and Rossi 1986a; Hutching *et al.*, 1990a; Denis *et al.*, 1993). Proliferative responses have been recognised as an important indicator for cell mediated immunity in primary and recurrent BHV-1 infections, since peak proliferative responses are correlated with the cessation of viral shedding and clinical signs (Davies and Carmicheal, 1973). Studies of proliferative responses have shown that the major surface glycoproteins gB, gC, gD

and one viral tegument protein VP8 induce good proliferative responses (Hutching *et al.*, 1990b; Leary and Splitter, 1990a). Furthermore, proliferative responses by peripherally isolated lymphocytes were shown to be limited by CD4+ helper T cells (Leary and Splitter, 1990b; Denis *et al.*, 1994b). In addition, peptide analysis methods have defined certain epitopes on gC and gD that are recognised by stimulated cells (Leary and Splitter, 1990b; Tikoo *et al.*, 1995b). It must be emphasized that a proliferative response against BHV-1 antigens does not correlate directly with a protective response, but does reveal the activation of specific T cells that recognise specific BHV-1 antigens.

The generation of major histocompatibity class I (MHC I) restricted killing has been difficult to reproduce in a CTL based assay. A detectable level of MHC restricted cytotoxicity was observed after various *in vitro* stimulation methods (Campos and Rossi, 1986a; Lawman *et al.*, 1987/88; Splitter *et al.*, 1988). A more reproducible observation has been the detection of other cytocidal cells - macrophages, neutrophils, NK and lymphokine activated killers (LAK) cells (see next sections). Differences in stimulation methods may reflect differences in antigen presentation by different cell types and various antigen combinations (Denis *et al.*, 1994b). Denis *et al.* showed that depending on the stimulation method, cytotoxicity varied between the major glycoproteins gC and gD. CTL's stimulated by BHV-1 infected autologous cells efficiently killed Vaccinia-gD infected targets whereas UV-irradiated BHV-1 stimulated CTL's killed Vaccinia-gC infected targets with higher specificity (Denis *et al.*, 1993).

The difficulty in generating classic CTL activity may also suggest that CTLs are

produced at a low frequency. Traditionally, the inability to generate a strong CTL reaction has been thought to arise from a lack of a helper signal from CD4+ T cell or macrophage populations (Dr. Philip Griebel, personal communication). Recent evidence supports this view. Eskra and Splitter showed that a large portion of Con-A activated CD4+ lymphocytes, when infected with BHV-1, express early virus glycoproteins. On average, 38 % of CD4+ cells can be infected *in vitro*, but 71 % of these cells undergo apoptosis after 48 hours (Eskra and Splitter, 1997). This confirms earlier findings that BHV-1 can induce apoptosis in lymphocyte populations (Greibel *et al.*, 1990; Hanon *et al.*, 1996). Another factor that has made the detection of CTL problematic may be the capacity of most herpesviruses to modulate MHC antigen processing and presentation pathways (Hill and Masucci, 1998). Although no protein that interferes with BHV-1 antigen processing and presentation has been characterised, the phenomena of MHC I down-regulation can be detected as early as 8 hour after infection while MHC II expression remains unaffected (Nataraj *et al.*, 1997). The authors also showed a reduction in the synthesis of class I heavy chains and transport into the Golgi suggesting that BHV-1 may evade a CD8+ CTL response during a primary infection and after reactivation by down-regulating MHC class I (Nataraj *et al.*, 1997).

Another aspect of BHV-1 infection is that the interaction between BHV-1 and T cells can cause immunosuppression or alterations in immune functions during the early stages of BHV-1 infection. *In vivo*, this apparent decreased response was suggested to be caused by altered T lymphocyte re-circulation in the PBMC population (Griebel *et al.*, 1987/88b; Bielefeldt Ohmann *et al.*, 1990). *In vitro*, live BHV-1 has been shown to

inhibit PBMC proliferative responses induced by either IL-2 or Con-A (Filion *et al.*, 1983; Hutching *et al.*, 1990a; Carter *et al.*, 1989; Griebel *et al.*, 1990). In addition, it has been shown that BHV-1 is able to infect activated lymphocytes, primarily CD4+, decreasing IL-2 proliferative responses and inducing apoptosis (Griebel *et al.*, 1990; Hanon *et al.*, 1996; Eskra and Splitter, 1997). This suggests a direct role for BHV-1 in the immune suppression phenomena. The infection of activated lymphocytes is abortive since late proteins cannot be detected (Eskra and Splitter, 1997). The ability of U.V. inactivated BHV-1 to inhibit proliferation of peripheral blood leukocytes is more controversial (Hutching *et al.*, 1990a; Carter *et al.*, 1989; Griebel *et al.*, 1990). Hutching *et al.*, (1990a) have shown that U.V. inactivated virus inhibits the *in vitro* proliferative response and this inhibition could be blocked with antibodies against gB and gD (Hutching *et al.*, 1990a). However, in different model systems, U.V. inactivated virus did not induce suppression (Carter *et al.*, 1989; Griebel *et al.*, 1990) or induce apoptosis of CD4+ T cells (Eskra and Splitter, 1997). The significance of the findings of Hutching *et al.* (1990a) is that the phenomena of U.V. inactivated BHV-1 inhibition indicates that BHV-1 induces suppressive factors – cellular mediators or cytokine - that indirectly affect T lymphocytes (Hutching at *et al.*, 1990a, Denis *et al.*, 1994; Tikoo *et al.*, 1995a). Overall, these mechanisms provide a theoretical basis for understanding the observed immunosuppression on lymphocyte populations (Greibel *et al.*, 1990; Eskra and Splitter, 1997).

T cells are also important in regulating an effective immune response by producing cytokines at the site of infection, especially IL-2 and IFN-γ which can

activate non-specific immune cells to attack infected cells. IL-2 has been used to generate both non-specific cytotoxicity mechanisms -NK and LAK- as well as BHV-1 specific CD8+ T cells (Campos and Rossi,1986a; Campos *et al.*, 1992b; Lawman *et al.*, 1987/88; Denis *et al.*, 1993). IFN-γ is also an important cytokine in the immune response to BHV-1. Increased levels of IFN-γ can be detected in the supernatant of BHV-1 *in vivo* stimulated PBMC within 3 days post-infection (p. i.) (Campos *et al.*, 1992a). In addition, IFN-γ activity was detected through an up-regulation of MHC II and an increased frequency of cytotoxic cells in lung parenchyma after a primary infection (Campos *et al.*, 1992a). IFN-γ is important for the generation of non-MHC cytotoxicity activity by both peripheral macrophages and alveolar macrophages (Campos *et al.*, 1989; Campos *et al.*, 1992b).

2.2.2 Antibody response to BHV-1

During the primary immune response, B cells generate high levels of antibodies to the three major BHV-1 surface glycoproteins gB, gC and gD (Collins *et al.*, 1985, Babiuk *et al.*, 1987a). After a high level of antibody is generated to the major surface proteins, then antibodies to minor glycoproteins and other structural proteins can be detected (van Drunen Littel-van den Hurk *et al.*, 1995). Neutralizing antibodies are detected 8 to 12 days after infection and may persist for years if re-activation occurs (Gibbs and Rweyemamu, 1977, Wyler *et al.*, 1989).

Mucosal IgA antibodies can be detected in the nasal and genital secretions and are important in protection from re-infection or re-activation from latency (Agiular-Setien *et al.*, 1980). IgA is very effective in neutralizing viruses both on mucosal

surfaces and within epithelial cells by secretory IgA molecules that are being transported through the epithelium to the mucosal surface (Tomasi, 1992).

Functionally, antibodies participate in (1) antibody dependent cellular cytotoxicity (ADCC) by interacting with Fc receptors on relevant effector cells (Rouse *et al.*, 1976), (2) in direct virus neutralization or (3) complement-facilitated neutralization (Dubuisson *et al.*, 1992). Antibody responses are considered less effective in primary infections relative to a good cell mediated response, but do play an important role in viral clearance and preventing re-infection by BHV-1 (Babiuk *et al.*, 1996).

2.2.3 Macrophages

Cells from the monocyte/macrophages lineage perform a central role in host defences. They participate in antigen presentation to CD4+ cells, phagocytosis, direct killing of viral infected cells, and secrete cytokines that promote leukocyte infiltration and activation of neighbouring cells (Babiuk and Rouse, 1996; Bielefeldt Ohmann *et al.*, 1991). During primary infection, macrophages play a central role in the acute inflammatory response by releasing cytokines including IL-1, TNF-α, IL-6 and chemotactic proteins that include IL-8 and IL-12. After an active immune response is generated, macrophages secrete a second group of cytokines that are anti-inflammatory and inhibit lymphocyte responses - IL-10, TGF-β and IL-1 receptor antagonist (Unanue, 1993). This indicates that monocyte/macrophages play an important role as immune regulators. The high number of alveolar macrophages in ruminants have an important role in controlling infectious agents and removing particulate material

encountered in the natural environment (Goddeeris *et al.*, 1998).

The effects of BHV-1 infection on macrophages vary between *in vitro* and *in vivo* infection models. Forman *et al.*, showed that during an *in vitro* BHV-1 infection, the majority of macrophages (90%) can be infected and both early and late viral proteins were produced in the cells (Forman *et al.*, 1982c). However, relative to MDBK cells, the authors indicated that they achieved low yields of infectious virus in macrophages with a 1.5 -2 $\log_{10}$ increase during a 24-hour period. In an attempt to explain their low yield, an infectious center assay was carried out which showed < 5% of cells produced infectious centers while 100 % infectious centers were achieved with MDBK cells. One possible factor limiting BHV-1 infections was the production of interferon by BHV-1 infected macrophages. Assaying the supernatant of infected macrophages revealed 162 units of interferon activity per ml after the first 24 hour period (Forman *et al.*, 1982c). In contrast, during the BHV-1 infection of animals, few infected macrophages were detected. At day 4 post-infection, 5 % of lung lavage cells were BHV-1 positive by immunoflourescence. Only 0.1 % of lavage cells produced infectious centers (Forman *et al.*, 1982b). This lack of BHV-1 infection was confirmed by Bielefeldt Ohmann and Babiuk, (1986), who reported that < 0.05 % alveolar macrophages were productively infected when assayed by infectious center assay.

A number of points may be raised that could alter the above interpretation. Firstly, it was clearly shown for the *in vitro* infection of macrophages that the infectious center assay was not detecting all infected macrophages. If only 5 % of infected macrophages produced infectious virus, as indicated by the infectious center assay, then it would not be expected that BHV-1 replication in cultured macrophage

could produce a 1.5 –2 $\log_{10}$ increase in virus during a 24 hour period. Secondly, the infectious center assay used an overlay, that contained anti-IBR serum instead of methylcellulose, to prevent extracellular viral spread. The addition of anti-IBR antibodies may have no effect on fibroblasts, but the immunoglobulin could interact with Fc receptors on macrophages and cytocidal activity could limit viral spread. In contrast, when a methyl cellulose overlay was used in an infectious center assay to studying *in vitro* infection of Con-A stimulated PBMC cultures, <0.05 % of these cells produced infectious virus and 90% of these cells could be removed by nylon wool (Carter *et al.*, 1989). This result suggests that the adherent population, possibly macrophages, produced an active infection. Similarly, the infectious center assay for detection of *in vivo* infectious macrophages also contained anti-IBR serum (Bielefeldt Ohmann and Babiuk, 1986). Furthermore, it has been reported that after a BHV-1 infection, BHV-1 infected macrophages could be isolated for up to 10 days post-infection (Bielefeldt Ohmann and Babiuk, 1986). This may indicate a productive and self-sustaining infection, since an abortive or low level infection would not be expected to persist this long.

BHV-1 infection of isolated alveolar macrophages can alter their ability to secrete cytokines such as TNF-α, downregulates C3b receptors and decreases ADCC and bacterial phagocytosis functions (Bienhoff *et al.*, 1992; Brown and Ananaba, 1988; Forman and Babiuk, 1982a). In contrast, blood monocytes from BHV-1 infected animals did not have altered bacterial phagocytosis (Brown and Shin, 1990).

Alveolar macrophages isolated from BHV-1 infected animals are able to secrete

IFN-α, function in Fc mediated phagocytosis and showed increased MHC II expression. This suggests that the vast majority of macrophages are functional after an experimental BHV-1 infection (Bielefeldt Ohmann and Babiuk, 1986). However, super-oxide anion generation was significantly decreased on days 1 and 2 p. i. and ADCC was impaired on days 1 and 4 post-infection. This indicates some impairment of macrophage function which may be associated with the development of secondary bacterial infections (Bielefeldt Ohmann and Babiuk, 1986). Historically, the problems associated with secondary bacterial infections have been attributed to other immune factors and mechanism and not directly to BHV-1 replication (Denis *et al.*, 1994). Indirect effects of BHV-1 infection may include an altered ability of macrophages to secrete cytokines. This may alter cell trafficking and possibly increase pathology of the lung by promoting leukocyte infiltration (Griebel *et al.*, 1990; Bielefeldt Ohmann *et al.*, 1991).

During viral infections, macrophages are often the major producers of IFN-α, the primary anti-viral interferon. In experimental BHV-1 infections, IFN-α production peaks at days 3-4 post-infection (Straub and Ahl, 1976). The production of IFN-α acts to prevent viral spread by inducing an antiviral state in uninfected cells and up-regulating the effector functions of PMN, NK and macrophages (Bielefeldt Ohmann *et al.*, 1984; Babiuk *et al.*, 1985; Babiuk *et al.*, 1987b; Jenson and Schultz, 1990).

After the production of antibody, macrophages can participate in ADCC to kill viral infected cells (Bielefeldt Ohmann *et al.*, 1984; Grewal *et al.*, 1977; Rouse *et al.*, 1977). Direct macrophage cytocidal function is also activated by the production of

IFN-γ by T cells (Campos *et al.*, 1989; Campos *et al.*, 1992b). Thus macrophages may play a variety of roles during BHV-1 infection.

2.2.4 Polymorphonuclear cells

During a BHV-1 infection, a massive infiltration of polymorphonuclear cells (PMN) occurs in the lung. This results in activation of alveolar macrophages and epithelial cells and the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα. These pro-inflammatory cytokines induce intercellular adhesion molecules and other cellular factors, which increase vascular permeability and promote leukocyte adhesion (Durum and Oppenheim, 1993). In addition, these early cytokines activate lung parenchymal cells and infiltrating lymphocytes to release cytokines, including granulocyte/macrophage colony stimulating factor (GM-CSF), which contributes to macrophage differentiation and activation (Babiuk *et al.*, 1996). In turn, these macrophages can release more TNF-α which increases inflammation and, if not controlled, this inflammation can result in immunopathology in the lung (Bienhoff *et al.*, 1992).

PMN play an important role in the clearance of bacteria in the lung, primarily gram-negative bacteria such as *Pasteurella haemolytica*. During BHV-1 infection, the clearance of *Pasteurella haemolytica* was delayed and this reduced bacterial clearance resulted in pasteurella pneumonia (McGuire and Babiuk, 1983/1984). Moreover, following BHV-1 infection, PMN have decreased chemotactic and anti-microbial phagocytic ability, which may also contribute to the susceptibility to secondary infections (McGuire and Babiuk, 1984; Bielefeldt Ohmann and Babiuk, 1985a).

In viral infections, PMN are also important in antibody-dependent cell-mediated cytotoxicity (ADCC) (Rouse *et al.*, 1976; Grewal *et al.*, 1977). Bovine neutrophils contain Fc receptors for IgM which may allow neutrophils to play an important role in viral clearance during a primary BHV-1 infection (Goddeeris, 1998). PMN can also bind complement and mediate complement–dependent cell mediated cytotoxicity against virally infected cells (Grewal *et al.*, 1980; Bielefeldt Ohmann and Babiuk, 1988). The proposed mechanism for this interaction involves the binding of complement by glycoprotein C (gC), which contains a complement binding motif (C3b), and the complement receptor for C3b on PMN. This creates a bridge between the infected cell and the PMN that results in lysis of the infected cell (Bieflefeldt Ohmann and Babiuk, 1988; Huemer *et al.*, 1993).

PMN have been shown to release an antiviral substance known as polyferon which inhibits viral replication but it is not related to classic interferon molecules (Rouse *et al.*, 1980; Bielefeldt Ohman *et al.*, 1989). However, the mechanism for this inhibition is thought to be ADCC since when PMN and infected cells are separated by a permeable membrane the influence of polyferon is lost (Babiuk *et al.*, 1996).

2.2.5 Natural killer cells and NK-like cytotoxicity

Natural killer cells (NK) are one of the major cell populations that mediate non-MHC restricted cytotoxicity against virally infected cells. They are part of the first line of defence against viral infection, being most active 1-3 days after infection and level off after the appearance of CTL at 5-8 days post-infection (Kos and Engleman, 1996). In addition, NK cells produce interferon-γ which activates the immune system to

control certain bacterial, parasitic and viral infections (Biron, 1997). NK cells are activated by type I interferons (α/β) and a variety of viral glycoproteins (Biron, 1997).

NK cells were originally defined as a large granular lymphocyte population that could mediate cytolytic activity against various heterologous cell targets including tumor cells, virus infected cells and various normal cell lines (Hercend and Smith, 1988). Originally, natural cytotoxicity had been detected in both adherent and non-adherent PBMC that were incubated for 3-7 days for optimal acitvation (Brigham and Rossi, 1986; Campos and Rossi, 1986b). Bovine NK cells are now defined as a population of non-adherent low density lymphocytes that do not express T cell markers (minus CD3, CD4, CD5, CD8 and $\gamma\delta$ markers) or B cell markers and which can lyse virally infected allogeneic and xenogeneic target cells (Cook and Splitter, 1989; Roth *et al.*, 1994). A second cell population with NK-like cytotoxicity has been defined as CD3+, CD45+, FcR+ which may possibly be a subset of $\gamma\delta$ T cells (Amadori *et al.*, 1992).

It has been difficult to demonstrate classical NK activity against tumor cells with bovine NK cells. However, NK activity was detected when PMBC cells were incubated with various cytokines that induce lymphokine activated killers (LAK) (Brigham and Rossi, 1986; Campos and Rossi, 1986a; Cook and Splitter, 1989; Jensen and Schultz, 1990). Cytokines that induce NK activity include - IL-2, IFN-γ, IFN-α and IL-4 (Jensen and Schultz, 1990). Treatment with IL-2 activates a $CD2^{+}CD4^{-}CD8^{-}$ population of cells to kill virus infected cells (Campos *et al.*, 1992b). The reports that a variety of cell types can be activated by various methods suggest that NK and NK-like

activity is associated with more then one cell type.

Non-stimulated bovine NK cells are able to lysis BHV-1 infected target cells (Amadori *et al.*, 1992; Cook *et al.*, 1989; Cook and Splitter, 1989). Analysis of this activity, by using target cells transfected with BHV-1 glycoprotein, showed gB and gD were the major inducers of cytotoxicity with gC being a partial inducer (Palmer *et al.*, 1990). These experiments led to the conclusion that NK activity is virus dependent. Similarly, parainfluenza-3 and bovine leukaemia virus infected cells are also lysed by non-activated bovine NK cells (Goddeeris *et al.*, 1998). It is now generally believed that the major function of NK cells is to attack virus infected cells (Roth, 1994; Biron, 1997).

Studies on NK cell recognition of target cells have shown that NK cells express several receptor families (inhibitory receptor super-family) which bind polymorphic MHC I on normal cells. Engaging these receptors causes a non-activation signal to be transmitted to the NK cell. In contrast, when cells become infected with virus or transformed into tumor cells, they fail to express polymorphic MHC molecules 'missing self-MHC theory' and such cells cannot signal NK cells. This lack of signalling initiates an activation signal for NK cell cytolytic function (Ljunggren and Karre, 1990; Kos and Engleman, 1996; Lanier and Philips, 1996; Lanier, 1998). The MHC requirement for NK recognition of BHV-1 infected cells has not been defined (Denis *et al.*, 1994).

2.3 Early and Late Cytokines

To conceptualize the role of cytokines in BHV-1 pathogenesis and immunity, a model for expression of cytokines during BHV-1 infection has been developed.

'A typical initial response to pathogens is characterized by early inflammatory signs, followed by infiltration and activation of inflammatory cells (i.e., neutrophils and macrophages), and initiation of specific immune responses by B and T lymphocytes. These responses are orchestrated by the production of cytokines in a sequential manner in response to the invading pathogens. Thus according to their time of production, cytokines can be grouped as early and late cytokines. In general early cytokines are produced by cells present at the site of infection and they are responsible for the initiation of the inflammatory response, recruitment and activation of inflammatory cells, and in conjunction with antigen, effective T and B cell stimulation. In contrast, late cytokines are produced mainly by T lymphocytes after activation by antigen and early cytokines, and are responsible for the differentiation, amplification, and fine tuning of the immune response towards a given pathogen.' (Campos *et al.*, 1994).

Research into the role of cytokines in BHV-1 pathogenesis and immunity can be interpreted within the context of this conceptional model. Early cytokines include IFN-α and IFN-β that are released by infected fibroblasts, as well as pro-inflammatory IL-1β and TNF-β which is released by macrophages. In general, late cytokines such as IL-2 and IFN-γ are released by T lymphocytes a few days after infection. Although this temporal model presents a foundation for understanding the immunomodulation of the host response by cytokines, the pluripotential nature and redundant functions of cytokine has shown that most cytokines are active throughout an active infection and can play different roles at different times.

A number of good reviews on the biological functions of bovine cytokines and their effects during a respiratory disease have been recently written (Godson *et al.*,

1997; Hanon *et al.*, 1997). The availability of purified recombinant bovine cytokines (IFN-α, IFN-γ, TNF-α, IL-1β, IL-2, and colony-stimulating factor) during the 1980's and 1990's have been used to extensively study the *in vitro* and *in vivo* biological effects. They have been tested as both potential immunomodulators and adjuvants in cattle diseases, including a BHV-1 infection model (Godson *et al.*, 1997).

The following sections will give an overview of the major findings for different groups of cytokine in the respiratory infection model. Afterwards, a more detailed discussion of the cytokines used in this study will be outlined.

2.3.1 BHV-1 and interferons

The bovine interferon family is classified into two types. Type 1 contains four sub-type: (1) IFN-α contains 10-12 sub-family types, (2) IFN-β has 3 sub-family types, and newly designated subtypes (3) IFN-ω (omega; previously called IFN-α_{II} genes that included 15-20 sub-family types) and (4) IFN-tau, trophoblast interferons (Bielefeldt Ohmann *et al.*, 1987b; Li and Roberts, 1994; Senda *et al.*, 1995; Ryan and Womack, 1997). IFN-α and IFN-β interferons share a common receptor. Type II interferon includes only IFN-γ and contains a single gene with a unique receptor. This section will concentrate on IFN-α and IFN-β, while IFN-γ will be covered in a separate section.

IFN-α is produced by macrophages, T cells, B cells and a variety of other cells. IFN-β is produced by fibroblasts and numerous other cell types in response to viral infections (Bielefeldt Ohmann *et al.*, 1987b; Durum and Oppenheim, 1993). In general, cells that predominately produce one type of interferon may also produce the other at

low levels. For example, fibroblasts which produce predominately IFN-β also produce 20% IFN-α and macrophages which produce high levels of IFN-α will also secrete low levels of IFN-β (Durum and Oppenheim, 1993).

Traditionally, interferons have been defined by their ability to inhibit viral replication. Interferons capacity to inhibit viral replication is effected by an number of different mechanisms including the activation of inducible genes such as a double stranded RNA activated protein kinase (PKR), 2'-5' oligoadenylate synthetase (2-5A synthetase) and dsRNA specific adenosine deaminase (dsRAD) (reviewed in Boehm *et al.*, 1997; Bielefeldt Ohmann *et al.*, 1987b).

In BHV-1 viral infections, BHV-1 is relatively resistant to low concentrations of interferon (Bielefeldt Ohmann *et al.*, 1984; Babiuk *et al.*, 1985). However, *in vivo* there is a clear relationship between high interferon levels (> 1000 units/ml) in nasal secretion and maximal viral titers (Todd *et al.*, 1972; Le Jan and Asso, 1981; Straub and Ahl, 1976; Bielefeldt Ohmann and Babiuk, 1985a; Babiuk *et al.*, 1985). In evolutionary terms, the production of interferon during a viral infection could protect against viral superinfections associated with bovine respiratory disease complex. Virus associated with bovine respiratory disease include parainfluenza type 3, bovine virus viral diarrhea, rhinovirus, adenovirus, reovirus and respiratory syncytial virus (Todd *et al.*, 1972; Ghram *et al.*, 1989). For example, calves experimentally infected with BHV-1 were protected by endogenous interferon against a secondary bovine rhinovirus infection (Cummins and Rosenquist, 1980).

The availability of recombinant IFN-α_1 facilitated studies of its anti-viral

properties and immunomodulatory potential in a BHV-1 respiratory infection model (review in Bielefeldt Ohmann *et al.*, 1987b). *In vitro*, the addition of IFN-α activates macrophages to increase their cytolytic and anti-microbial functions (Bielefeldt Ohmann *et al.*, 1984). In the BHV-1/ P. *haemolytica* respiratory model, the intra-nasal injection of exogenous IFN-α (10mg/animal) reduced the overall mean viral titer but not to a statistically significant level. However, immune modulation was detected as indicated by reduced clinical signs, decreased morbidity and mortality and increased PMN function (Babiuk *et al.*, 1985; Babiuk *et al.*, 1987b).

2.3.2 BHV-1 and pro-inflammatory IL-1β and TNF-α

A general overview of both IL-1β and TNF-β roles in BHV-1 infections will be given here. Both of these cytokines belong to a class of mediators involved in the initiation and progression of the inflammatory response during respiratory diseases (Durum and Oppenheim, 1993; Gallin, 1993; Wyler *et al.*, 1989).

Macrophages are high producers of both IL-1β and TNF-α, but other cells including lymphocytes and epithelial cells can also produce these cytokines (Bielefeldt Ohmann *et al.*, 1991; Durum and Oppenheim, 1993). The production of IL-1 by alveolar macrophages is impaired during the first 6 days of a BHV-1 infection, while prostaglandin E_2 levels are increased (Bielefeldt Ohmann and Babiuk, 1986). Similarly, *in vitro* infection with BHV-1 impaired TNFα production by alveolar macrophages (Beinhoff *et al.*, 1992). However, the administration of intra-nasal TNF-α alone or in combination with IFN-γ did not protect calves from bacterial disease (Bielefeldt Ohmann *et al.*, 1991). Thus, BHV-1 impairment of alveolar macrophage function

during BHV-1 infection could contribute to increased lung injury, especially, if a secondary viral or bacterial infection were present (Bielefeldt Ohmann and Babiuk, 1986; Bielefeldt Ohmann *et al.*, 1991).

2.3.3 BHV-1 and T-cell derived cytokines

IL-2 and IFN-γ are both produced by T cells. The role of IFN-γ as an adjuvant and immunomodulator will be covered in a following section. Since IL-2 has pleotrophic effects on B and T cell differentiation and expansion, it has been assessed as an adjuvant with both subunit gD vaccine and a live modified vaccine (Reddy *et al.*, 1989; Hughes *et al.*, 1991; 1992). The administration of multiple doses of IL-2 in concert with a modified live virus BHV-1 vaccine increased cytotoxic function in both vaccinated and non-vaccinated cattle (Reddy *et al.*, 1989). This increased activity was suggested to be through IL-2 activation of specific lymphokine activated killer cells (Campos *et al.*, 1992b). Calves treated with a high dose of IL-2 (0.25, 2.5, 25 μg/kg/day) showed enhanced serum neutralization titers and shed less virus then untreated animals (Reddy *et al.*, 1989). However, calves treated with 25 μg/kg/day developed diarrhea and mild fever (Reddy *et al.*, 1989). Similarly, the administration of multiple low dose of IL-2 with Avridine and a subunit gD vaccine acted as an effective adjuvant, increasing cell mediated immunity and antibody responses (Hughes *et al.*, 1991; 1992).

2.4 Biology of Interleukin-1β

2.4.1 Gene

Interleukin-1β is a potent, multifunctional cytokine with inflammatory, metabolic, physiological, hematopoietic and immunological properties (Reviewed in Dinarello, 1991; Arend, 1993; Dinarello, 1996).

Three distinct genes (IL-1α, IL-β and IL-1Ra) and two receptors (IL-1RI and IL-1RII) define the Interleukin-1 family. Both IL-1α and IL-1β can activate similar biological activities by binding the IL-1RI receptor. The Interleukin-1 receptor antagonist (IL-1Ra) blocks signalling by binding to IL-1RI receptors (Arend *et al.* 1998). IL-1α/β, interleukin-6 and TNF form a group of pro-inflammatory cytokines which share numerous overlapping biological activities that revolve around the acute inflammation response (Durum amd Oppenheim, 1993).

Both bovine interleukin-1 genes have been cloned from LPS-stimulated alveolar macrophages (Maliszewski *et al.*, 1988). The IL-1α gene encodes a 268 amino acid protein with 73% and 62 % homology with human and murine IL-1α, respectively and the IL-1β gene encodes a 288 a.a. protein with 62% and 57% homology with human and murine IL-1β, respectively (Maliszewski *et al.*, 1988). Both interleukins are produced as precursor proteins with the approximate molecular weight of 31 kDa and are proteolytically cleaved into mature proteins. IL-1β is cleaved by a novel protease, IL-1β converting enzyme (ICE), to generate the mature protein which is the major biological form (Kostura *et al.*, 1989; Black *et al.*, 1989; Thornberry *et al.*, 1992;

Cerretti *et al*, 1992). In contrast, IL-1α is biologically active in both precursor and mature forms; precursor IL-1α (ProIL-1α) can remain intracellular and function as an autocrine growth factor, regulating cellular differentiation (Mosley *et al.*, 1987; Durum and Oppenheim, 1993; Dinarello, 1996). The lack of a traditional secretory signal sequence has been a distinct feature of IL-1 biology. Recently, it has been shown that IL-18 also lacks a signal sequence and uses the ICE molecule to produce an active molecule (Dinarello, 1998; Tone *et al.*, 1997).

IL-1 signal transduction occurs by the binding of the high affinity receptor IL-1RI, which is a member of the immunoglobulin super-family. IL-1RI is found in high numbers on fibroblasts, synovial lining cells, endothelial and smooth muscle cells (Dinarello, 1991; Arend, 1993; McMahan *et al.*, 1991). In contrast, the IL-1RII acts as a regulated 'decoy' receptor to IL-1 production by binding to IL-1β but not transmitting a signal (Colotta *et al.*, 1994). IL-1RI contains 213 a.a. on the C-terminal end of the intracellular domain, while IL-1RII contains only 29 a.a. on the C-terminal end and has not been shown to mediate a signalling function (Colotta *et al.*, 1994). IL-RII is expressed on predominantly B cells, monocytes, PMN and bone marrow cells (McMahan *et al.*, 1991).

2.4.2 Producers

Numerous cell types have been shown to produce IL-1 including monocytes/macrophages, neutrophils, endothelial cells, fibroblasts, dendritic cells, keratinocytes, T cells, B cells, NK cells and central nervous sytem microglia (Durum and Oppenheim, 1993). Of these, monocytes are the major sources of IL-1β, particularly

when exposed to microbial products such as endotoxins (Hsi and Remick, 1995). Monocytes and keratinocytes also produce IL-1Ra (Dinarello, 1998).

2.4.3 Biological Effects

IL-1 production is tightly regulated by negative feedback loops consisting of prostaglandins produced from mononuclear phagocytes and glucocorticoids produced by the central nervous system (Dinarello, 1991). In addition, the IL-1Ra binds IL-1RI and directly competes with IL-1, and IL-1RII binds IL-1β but does not transmit a signal (Colotta *et al.*, 1994). The need for tight regulation of IL-1 is evident in the immunopathological consequences of unregulated IL-1 production. IL-1 is associated with chronic inflammation including rheumatoid arthritis, inflammatory bowel disease and such diseases as artherosclerosis, sepsis, impaired haematopoiesis, acute and chronic myelogenous leukemia and IL-1 dependent neoplasia (Dinarello, 1993; Durum and Oppenheim, 1993; Dinarello, 1996). The wide variety of biological activities of IL-1 can be grouped into two basic functions: (1) Immunological effects and (2) Pro-inflammatory properties (Dinarello, 1991).

IL-1 pro-inflammatory properties involve the induction of diverse cellular mediators. IL-1 effects on the central system include modulation of the hypothalamus to produce fever, suppression of appetite and induction of slow wave sleep. The liver responds to IL-1 by increasing the production of acute phase proteins and reducing zinc and iron levels. IL-1 also causes neutrophilia by inducing the bone marrow to release neutrophils (Durum and Oppenheim, 1993). In addition, IL-1 induces prostaglandins (PGE_2) from various cells including fibroblasts, skeletal muscle cells,

endothelial cells and macrophages. IL-1 increases the production of proteases, increases cartilage and bone catabolism and induces the growth of fibroblasts (Durum and Oppenheim, 1993). Endothelial cells also produce platelet activitation factor and cell adhesion molecules in response to IL-1 (Durum and Oppenheim, 1993).

The cataloguing of IL-1's biological effects is complicated by the simultaneous induction of other cytokines including IL-6, TNF, IFN-γ, IL-2 and the release of the growth factors GM-CSF, G-CSF, M-CSF (Dinarello, 1991).

In the immune system, IL-1 was originally described as a co-mitogen for the proliferation of thymocytes (Gery and Waksman, 1972). Both immature, double negative thymocytes and double positive thymocytes respond to IL-1 (Rothenberg *et al.*, 1990; Gotlieb *et al.*, 1990). During later steps of lymphocyte development, IL-1 is able to act synergistically with other cytokines to mediate T cell activation and B cell development (Mannel *et al.*, 1985; Dinarello, 1996). Because of its ability to synergize with other cytokines, IL-1 has been used as an immunological adjuvant in various vaccine formulations.

During B cell development, IL-1 acts as a co-factor with IL-2, IL-4, IL-5 and IL-6 to increase B cell proliferation and antibody production (Hoffman *et al.*, 1987; Durum and Oppenheim, 1993). In adjuvant formulations, IL-1β has been shown to enhance IgA and IgG production, presumably by inducing IL-6 (Staruch and Wood, 1983; Gao *et al.*, 1995).

IL-1 plays a role in activating immune cells such as NK, LAK and macrophages. IL-1 induces macrophages to produce increased amounts of IL-1 itself as well as IL-6,

IL-8, TNF and prostaglandins - PGE_2. During infection, the increase in IL-1 production by macrophages may have a positive effect on hematopoiesis by inducing granulocyte-colony stimulating factor (G-CSF), GM-CSF and M-CSF release from endothelial and bone marrow stromal cells (Zsebo *et al.*, 1988; Zucali *et al.*, 1986). This may have a positive effect during microbial infections by increasing the number of macrophages and granulocytes (neutrophils and eosinophils) in circulation (Ulich *et al.*, 1987). Neutrophilia is a common side-effect following injection of IL-1β into animals (Godson *et al.*, 1995).

2.4.4 Clinical and adjuvant effects of bovine IL-1β

Interleukin-1β has been tested as an adjuvant in cattle because it can influence a broad spectrum of biological activities within the haemotopoietic and immune systems (Dinarello, 1991; Godson *et al.*, 1997). In a modified-live BHV-1 vaccine trial, the vaccine was co-administerered with an intramuscular injection of 33, 100 or 330 ng/ml of rBoIL-1β (Reddy D.N. *et al.*, 1990). The injection of recombinant rBoIL-1β (330 ng/kg) increased total blood leucocyte counts by increasing both neutrophils and lymphocytes. CD4/CD8 ratios also increased in rBoIL-1β treated animals. Increased non-MHC restricted cytotoxicity occurred in rBoIL-1β treated calves when compared to control animals given modified-live BHV-1 vaccine alone (Reddy D.N. *et al.*, 1990). In a subsequent experiment, calves were injected with 100, 500 or 2500 ng/kg of rBoIL-1β to determine the immunoadjuvant potential of rBoIL-1β (Reddy D.N. *et al.* 1993). The non-MHC restricted cytotoxicity activity in PBMC was again enhanced. The authors hypothesized that a lymphokine-activated $CD2^+$ cytotoxic population was

responsible for this cytotoxic activity (Reddy P.G. *et al.*, 1989; Reddy D.N. *et al.*, 1990; Campos *et al.*, 1992b). Moreover, calves injected with a BHV-1 vaccine and 2500 ng/kg of IL-1 had increased serum neutralization and antibody titers but calves treated with low IL-1 doses had serum neutralization titers similar to control animals.

More detailed studies have been completed to determine the effects of IL-1 on host immune mechanisms, physiology and IL-1 effects during BHV-1 infection (Godson *et al.*, 1995; Baca-Estrada *et al.* 1995; Van Kessel *et al.*, 1996). A single injection of various concentration of IL-1β ranging from 10 ng/kg to 1000 ng/kg induced dose dependent clinical signs, including inappetance, depression, and increased body temperature (Godson *et al.*, 1995). IL-1 enhanced the number of circulating mature and immature neutrophils which resulted in elevated WBC counts (Godson *et al.*, 1995; Van Kessel et a., 1996). A dose of 333 ng/kg significantly enhanced haptoglobin and fibrinogen levels for two days after a single or multiple injection protocol. This demonstrated that IL-1 was involved in the induction of an acute phase response in cattle (Godson *et al.*, 1995). In a BHV-1 infection model, a single dose of 300 ng/kg did not enhance immunological responses (Van Kessel *et al.*, 1996). In contrast to previous experiments (Reddy *et al.*, 1989; Reddy *et al.*, 1993), ADCC and non-MHC cytotoxicity were not increased following IL-1 treatment (Van Kessel *et al.*, 1996).

In a respiratory disease model, consisting of a BHV-1 challenge followed four days later with a *P. haemolytica* aerosol challenge, the repeated injection of 300 ng of IL-1/kg for 5 days, elevated polymorphonuclear cell and monocyte numbers in the

blood and enhanced phagocytic activity in the monocyte population (Baca-Estrada *et al.*, 1995). The ability of lymphocytes to be stimulated to produce IFN-γ was significantly decreased but no significant effects on BHV-1/*P. heamolytica* infection were recorded. It was concluded that the administration of IL-1 could be used as a safe adjuvant during an active respiratory disease without increasing immunopathology (Baca-Estrada *et al.*, 1995).

Gao *et al.* tested the potential use of boIL-1β to enhance immune responses to a subunit vaccine (Gao *et al.*, 1995). In their studies, the use of BHV-1 gB and a mixture of incomplete Freund's adjuvant and boIL-1β (500 ng/kg) was tested. The vaccine was given subcutaneously at the base of the ear. This formulation enhanced both mucosal IgA responses and systemic immunity (Gao *et al.*, 1995). This study re-enforces the role of IL-1β as an effective immunomodulator in vaccine formulations.

2.5 Biology of Interferon-γ

Interferon-γ is classified as a type II interferon. IFN-γ binds to a unique IFN-γ receptor and is structurally unrelated to type I interferons, IFN-α and IFN-β (reviewed in Bach *et al.*, 1997). It is both an important anti-viral cytokine, as well as an immune cytokine regulating numerous immunological functions. In general, IFN-γ has four basic immune functions: (1) cytokine network regulation, (2) activation of phagocytic cells, (3) processing and presentation of antigens by MHC and (4) direct anti-viral action (reviewed in Farrar and Schreiber, 1993; Boehm *et al.*, 1997; Billiau, 1996).

2.5.1 Bovine Interferon-γ gene

Bovine IFN-γ cDNA encodes a precursor protein of 166 amino acids containing a 23 a.a. signal sequence and a 143 a.a. mature protein with a predicted molecular weight of 16.8 kDa. In most cross-species experiments, interferon-γ has been shown to be species specific. Bovine IFN-γ has a 63 % a.a. similarity with human IFN-γ and 47 % a.a. similarity with murine IFN-γ (Cerritti *et al.*, 1986). Similar to human and murine IFN-γ, bovine IFN-γ has two potential N-linked glycosylation sites which occur at positions 39 and 106 a.a. (Cerritti *et al.*, 1986). Biologically active human IFN-γ exist as a non-covalent homodimer (Ealick *et al.*, 1991; Langer *et al.*, 1994). The homodimer binds a two chain IFN-γ receptor (α and β chain) activating JAK (Janus kinases) and STAT (signal transduction and activators of transcriptor) signalling pathways (Bach *et al.*, 1997). The list of cellular genes that are upregulated by IFN-γ has grown to over 200 genes since an initial list of 20 genes was compiled in 1990 (Staeheli *et al.*, 1990; Boehm *et al.*, 1997).

The main producers of IFN-γ are NK cells and T cells (Handa *et al.*, 1983; Bancroft *et al.*, 1987; Farrar and Schreiber, 1993). For T cells, IFN-γ is mainly induced by the cross-linking of the TCR receptor; while NK cell induction occurs primarily following stimulation by macrophage derived cytokines such as TNF-α and IL-12 and autostimulation by IFN-γ (Hardy and Sawada, 1989; Trinchieri, 1995). Although T cells from CD4+ and CD8+ lineages and NK cells have been shown to be the major producers of IFN-γ, the role of γδ T cells as IFN-γ producers and important regulatory cells between innate and adaptive immunity is now becoming more appreciated. γδ T

cells may play an important role in viral recovery by secreting cytokine that activate other immune cells (Maloy *et al.*, 1998). This is an important area of investigation for cattle diseases, since newborn ruminants have a high percentage of γδ T cells (Hein *et al.*, 1994).

2.5.2 Immune regulation.

IFN-γ is one of the major immunoregulatory molecules responsible for polarizing the immune response within context of the Th1/Th2 paradigm. Although IL-12 has emerged as the primary determinant of Th1 differentiation, IFN-γ still augments differential induction, including the upregulation of IL-12 on monocytes and macrophages (Trinchieri, 1995). In gene knock out studies, the disruption of the IL-12 signal pathway resulted in a complete loss of Th1 differentiation (Thierfelder *et al.*, 1996; Kaplan *et al.*, 1996; Schijns *et al.*, 1998). In contrast, disruption of the IFN-γ gene or IFN-γ receptor did not result in the loss of Th1 differentiation (Graham *et al.*, 1993; Bach *et al.*, 1995; Schijns *et al.*, 1994; Swihart *et al.*, 1995). Aside from its role in the generation of a cell mediated immune response, IFN-γ is a natural antagonist of IL-4 by upregulating genes that are functionally part of the Th1 polarity including IgG2a and the FcγRI receptor in mice (Boehm *et al.*, 1997).

Although the Th1/Th2 paradigm still needs to be clearly established for ruminants, the availability of new bovine and ovine cytokine reagents may help determine the role of individual cytokines in regulating the immune response in ruminants (Wood and Seow, 1996). Recently, recombinant boIFN-γ has been shown to induce bovine B cells to produce antibodies of the IgG2 subclass (Estes *et al.*, 1994).

Studies with the parasitic infections, *Babesia bovis* and *Fasciola hepatica*, have shown bovine T cells with characteristic Th0, Th1 and Th2 functions(Brown *et al.*, 1993; 1994). However, as more bovine T cell clones undergo analysis, it appears that the Th1/Th2 paradigm may be an 'oversimplification of a much more complex immunoregulatory network' (Brown *et al.*, 1998). The majority of bovine T cells against parasitic antigens have been shown to express both IL-4 and IFN-γ (Brown *et al.*, 1998).

2.5.3 Activation of phagocytic cells

IFN-γ was originally named 'macrophage activating factor' for its ability to heighten macrophages from a resting state to an activated state - 'angry macrophages'. Activated macrophages have enhanced endocytic capacity, including increased pinocytosis and phagocytosis via the upregulation of complement receptors and IgG2a receptors (Billau, 1996). This enhanced microcidal activity is accompanied by the release of oxygen intermediates and nitric oxide (Boehm *et al.*, 1997). The importance of this microcidal action has been clearly shown by the use of anti-IFN-γ monoclonal antibodies which leads to increased severity of *Listeria monocytogenes, Toxoplasma gondii and Leishmania major* infections (Farrer and Schreiber, 1993).

Freshly isolated bovine monocytes stimulated with recombinant boIFN-γ, with or without co-stimulation with LPS, produced nitric oxide presumably by an inducible L-arginine-dependent pathway (Zhao *et al.*,1996). The induction of nitric oxide in bovine alveolar macrophages in response to *Pausteurella haemalytica* has also been reported (Yoo *et al.*, 1996).

2.5.4 Antigen Presentation

The upregulation of major histocompatability class I and II antigens is an important function in the surveillance of infectious agents and the mounting of a specific immune response. IFN-γ upregulates a number of genes involved in MHC class I and MHC class II molecule assembly. MHC I is constitutively expressed on most tissues, with higher levels on mature lymphomyeloid cells (Boehm *et al.*, 1997). After cell activation with IFN-γ or IFNα/β the expression of MHC is increased by activating a number of genes containing transcriptional elements, interferon-sensitive response elements (ISRE) and enhancer A elements with a NF-κB consensus motif. In addition, TNF-α acts syngergistically with IFN-γ in the induction of MHC class I expression, as well as in the induction of microcidal activity by macrophages (Boehm *et al.*, 1997; Billiau, 1996).

The upregulation of Class II MHC is more tightly regulated with constitutive expression restricted to professional antigen presenting cell - dendritic and B cells. IFN-γ enhances MHC class II expression by upregulating a single transcription factor CIITA (class II transactivator); upon IFN-γ activation, class II negative cells can be induced to produce class II molecules (Steimle *et al.*, 1994; Mach *et al.*,1996). In cattle, MHC II can be induced by the addition of recombinant boIFN-γ to PBMC (Campos *et al.*, 1989). It has been suggested that the induction of Class II is the most distinct and specialised function of IFN-γ (Boehm *et al.*, 1997).

2.5.5 Antiviral activity

The anti-viral activity of IFN-γ is controlled by three major inducible genes: (1) double stranded RNA activated protein kinase (PKR), (2) 2'-5' oligoadenylate synthetase (2-5A synthetase) and (3) double stranded RNA specific adenosine deaminase (dsRAD). Functionally, PKR inhibits protein synthesis by phosphorylating the α-subunit of eukaryotic initiation factor 2 which inhibits both RNA and DNA virus replication. Similarly, viral production of dsRNA intermediates results in the activation 2-5A synthetase. 2-5A synthetase activates RNaseL which degrades single-stranded viral and cellular RNAs. Finally, dsRAD uses dsRNA to catalyze the deamination of adenosine to isosine (reviewed in Boehm *et al.*, 1997).

The use of transgenic mice with either a deletion of the interferon receptors (α/β or γ) and the IFN-γ gene has refined our view of viral susceptibility to IFN-α/β and IFN-γ antiviral activity. Both IFN-γR$^{-/-}$ and IFN-αβR$^{-/-}$ mice show increased disease when infected with either Vaccinia virus (VV) or Lymphocytic choriomeningitis virus (LCMV) (Huang *et al.*, 1993; Muller *et al.*, 1994). In contrast, vesicular stomatatis virus (VSV) and Semliki Forest virus (SFV) had increased disease progression in IFN-α/βR$^{-/-}$ mice while IFN-γR$^{-/-}$ mice were not significantly different with respect to viral induced disease (Muller *et al.*, 1994).

In an IFN-γR$^{-/-}$ knock-out mouse model, pseudorabies virus did not exhibit an increase in disease progression (Schijns *et al.*, 1994). Mouse mammary tumour virus infection of IFN-α/βR$^{-/-}$ and IFN-γR$^{-/-}$ mice also showed no difference in the humoral immune response or the amount of virus detected when compared to normal mice

(Maillard *et al.*, 1998). In contrast, in an HSV-1 stromal keratitis model, there was increased virus persistence and increased periocular skin lesions in the IFN-γ gene knock-out mouse (Bouley *et al.*, 1995).

Overall, these studies indicate that differences exist in how viruses are affected by IFN-α/β and IFN-γ. They also suggest that both antiviral activities are essential in viral control and that the anti-viral activities of IFN-γ and IFN-α/β are not redundant (van den Broek *et al.*, 1995). An analysis of interferon signalling pathways has revealed similarities and differences in the expression of JAK-STAT family members during receptor signalling by IFN-γ and IFN-α/β. The different signalling events result in the activation of different proteins and differential expression of the major anti-viral inducible genes. This may account for some of the difference detected in the above studies (Staehli, 1990; Bach *et al.*, 1997).

2.5.6 Clinical and adjuvant effects of Bovine IFN-γ

Recombinant boIFN-γ has been used to test the immunomodulation potential of IFN-γ for disease therapy and enhancing immune responses to vaccines. The production of IFN-γ by lymphocytes following BHV-1 infection plays an important role in the activation of non-MHC cytotoxicity (Campos *et al.*1989). IFN-γ up-regulates various immune molecules including MHC II, Fc receptor, C3b receptor and influences a variety of effector functions (Bielefeldt Ohman *et al.*, 1987b). The *in vivo* administration of bovine IFN-γ significantly decreased PMN migration and enhanced O^{-}_{2} generation (Bielefeldt Ohman and Babiuk, 1986).

In addition, bovine IFN-γ has been shown to enhance both macrophage and

neutrophil functions (Canning and Roth, 1989; Steinbeck *et al.*, 1989). BoIFN-γ has been evaluated for its potential to increase resistance to bacterial infections in normal and immunocompromised cattle (Roth and Frank, 1989; Chiang *et al.*, 1990). Treatment of calves with 2,200 units of IFN-γ/kg (2 μg/kg) for 2 days before *Haemophilus somnus* challenge resulted in a reduction in pneumonic lung volume in calves immunocompromised with dexamethasone (Chiang *et al.*, 1990). However, at the dose given, animals developed a fibrile response 24 hours after administration of the initial dose. Similarly, a high dose of 0.5 and 2.5 mg/animal injected subcutaneously caused fever within 24 hrs (Roth and Frank, 1989). An optimal dose of 0.5 mg/animal (approximately 1.25 μg/kg) caused reduced neutrophil migration, enhanced microbial phagocytosis and ADCC in normal and dexamethasone treated cattle (Roth and Frank, 1989).

In a BHV-1/P. *haemolytica* challenge experiment, calves treated prior to infection by intramuscular injection or intranasal routes with 10,000 units of IFN-γ/kg had some reduced disease. However, calves treated with IFN-γ after BHV-1 infection showed no enhanced protection (Bielefeldt Ohman *et al.*, 1991). The overall mean survival scores and clinical scores were lower in pre-treated animals suggesting a beneficial immunomodulatory role for IFN-γ. In contrast, the addition of the pro-inflammatory cytokine TNF-α alone or in combination with IFN-γ enhanced clinical score and decreased mean survival time (Bielefeldt Ohman *et al.*, 1991). However, the lack of sufficient animals to generate statistically significant results makes the interpretation of the above experiment equivocal.

2.6 BHV-1 Vectors

The construction of herpesvirus with deletions in non-essential genes provides new vaccine candidates that promise to solve problems associated with previous modified live virus vaccines and killed vaccines. Four basic deletion mutants have been proposed. These include deletions in genes encoding (1) glycoproteins, (2) tegument proteins, (3) nucleic acid metabolism proteins and (4) viral assembly proteins (reviewed in Babiuk and Rouse, 1996; van Drunen Little-van den Hurk *et al.*, 1993).

One of the primary advantages of gene deleted strains is that they can function as marker vaccines distinguishing vaccinated animals from naturally infected animals through a relatively simple ELISA assay against the deleted protein. In addition, other genes can be inserted into the deleted gene location, and these new genes can include candidate antigens from related pathogens or immunostimulatory molecules that can function as adjuvants. For example, gene deleted BHV-1 viruses have been constructed to express foot-and-mouth disease virus protein VP1, Psuedorabies virus glycoproteins and bovine respiratory syncytial virus (Kit *et al.*, 1991; Otsuka *et al.*, 1996; Taylor *et al.*, 1998). Potential immunostimulatory cytokines have also been inserted into the unique short segment of the genome of BHV-1 (Kuhnle *et al.*, 1996).

In Bovine herpes virus-1, the number of non-essential genes that have been deleted and tested as possible vaccines include thymidine kinase (TK-) (Kit and Qavi, 1983), various glycoprotein mutants (Denis *et al.*, 1996), a tegument protein UL49 mutant and deoxyuridine triphosphatase (dUTPase) mutant (Liang *et al.*, 1997). The most widely studied mutants have been TK- mutants and various glycoprotein deleted

viruses (Kit *et al.*, 1985; Liang *et al.*, 1992; Denis *et al.*, 1996). The glycoprotein deleted mutants comprise gC, gD, gE, gI or gG deleted virus (Liang *et al.*, 1992; Kaashoek *et al.*, 1994; van Engelenburg *et al.*, 1994; Denis *et al.*, 1996; Schroder *et al.*, 1997).

So far, the most commercially successful deletion mutant has been the gE minus based mutant (Rijsewijk *et al.*, 1993; Kaashoek *et al.*, 1994). Both live gE- and killed gE- vaccine have undergone field trials in Germany (van Oirschot *et al.*, 1996; Strube *et al.*, 1996). In challenge experiments, animals infected with gE negative strains have exhibited reduced clinical signs and reduced weight loss during initial inoculation. After challenge, vaccinated animals show decreased viral shedding both in total titer and duration (Kaashoek *et al.*, 1994; van Engelenburg *et al.*, 1994; Bosch *et al.*, 1997). However, similar to other herpesviruses, gE mutants are also able to become latent (van Engelenburg *et al.*, 1994). The gE minus vaccines and a gD subunit vaccine have been incorporated into the European Unions BHV-1 vaccine program (Strube *et al.*, 1996; Bosch *et al.*, 1998). Currently, a gE minus vaccine is available in Germany (Bayer AG) with a gE antibody based detection kit (HerdCheck® Anti-IBR gE (IDEXX Inc)) (Kaashoek *et al.*, 1995; Van Oirschot, 1997).

2.6.1 BHV –1 glycoprotein C .

BHV-1 glycoprotein C, previously named gIII and a homolog of the HSV gC, has undergone extensive molecular and immunological analysis. Glycoprotein C is one of the most abundant viral glycoproteins expressed both in the viral envelope and on the surface of infected cells (Marshall *et al.*, 1986; van Drunen Little-van den Hurk *et*

al., 1984; van Drunen Little-van den Hurk and Babiuk, 1986b). Structurally, gC is a 95-99 kDa protein that forms a protuding homodimer spike (20-25 nm) on the surface of the virion (van Drunen Little-van den Hurk *et al.*, 1986b; M. Redmond, unpublished observations). Primary structure analysis revealed a 521 a.a. sequence with an amino-terminal hydrophobic signal sequence followed by a highly hydrophilic segment containing N-linked and O-linked glycosylation sites and a carboxy-terminal hydrophobic transmembrane anchor (Fitzpatrick *et al.*, 1989; 1990b). Secondary structure analysis revealed a C1-set domain of the immunoglobulin super-family with homology to class II MHC within a conserved region that is shared by other herpesvirus members (Fitzpatrick *et al.*, 1989; 1990a; 1990c).

One of the major functions of gC is its involvement as a major attachment protein that interacts with cellular heparan sulfate proteoglycans (Okazaki *et al.*, 1987; Okazaki *et al.*, 1991; Liang *et al.*, 1991a; 1993). After binding occurs, gC also participates in virus penetration (Liang *et al.*, 1991b). Aside from its important structural functions, gC minus mutants have been shown to be non-essential for both *in vitro* and *in vivo* viral growth (Liang *et al.*, 1991a; 1992). This highlights the multi-functionality of the herpesvirus envelope glycoproteins which can compensate for lost functions in viral attachment, penetration and cell to cell spread during viral evolution (Mettenleiter, 1994). Glycoprotein B has also been shown to bind heparan receptors (Bryne *et al.*, 1995; Li *et al.*, 1995). Recently, a PrV gB and gC double deletion mutant was shown to retain infectivity, indicating that other receptor binding proteins exist that can attach to cellular receptors (Karger *et al.*, 1998).

Immunologically, gC is a major target for neutralizing antibodies and cytotoxic

responses (van Drunen Little-van den Hurk and Babiuk, 1986a; Fitzpatrick *et al.*, 1988). Moreover, comparable to HSV, PRV and equine herpesvirus, BHV-1 gC has complement C3 binding activity (Huemer *et al.*, 1993; 1995). Although it has not been shown experimentally, BHV-1 gC may play a role in inhibiting complement-mediated virus neutralization as seen in HSV gC (Friedman *et al.*,1984; Friedman *et al.*, 1996; McNearney *et al.*, 1987; Hidaka *et al.*, 1991). Antibodies against gB, gC and gD, when mixed with complement enhance the reduction of plaque formation during an antiserum neutralization assay (Liang *et al.*,1991b).

Despite its structural and immunological functions, gC has been shown to be non-essential for both *in vitro* and *in vivo* viral growth (Liang *et al.*, 1991a; 1992). Therefore, gC deletion mutants have been used to create recombinant vaccine candidates for testing in cattle (Liang *et al.*,1992; Denis *et al.*, 1996). In an intra-nasal cattle challenge experiment, a mutant gC minus virus (gC^-/LacZ+) expressing *Escherichia coli* LacZ gene was able to replicate, spread to sentinel animals and establish latency (Liang *et al.*, 1992). Clinically, gC^-/lacZ+ virus infected calves were not distinguishable from control wild-type challenged calves. Both groups developed clinical signs and nasal lesions (Liang *et al.*, 1992). Nevertheless, peak viral titer and the duration of viral shedding were significantly lower in the gC^-/LacZ+ group when compared to wild-type BHV-1 infection. Calves developed serum neutralization antibodies against BHV-1 and the LacZ gene. When challenged by BHV-1 108 strain, all animals were protected (Liang *et al.*, 1992).

In a BHV-1 challenge experiment, animals immunized with a thymidine kinase negative and gC negative double deletion mutant BHV-1, shed no virus during initial

inoculation and developed only mild clinical signs relative to control animals following Cooper BHV-1 challenge 4 weeks later (Flores *et al.*, 1993). In addition, viral titers in nasal secretions and the duration of viral shedding was reduced (Flores *et al.*, 1993). Subsequent experiments confirmed that the double deletion mutants established latent infections (Galeota *et al.*, 1997).

In a recent study which compared four glycoprotein vaccine candidates (gC, gE, gI, gG) in an intranasal challenge model, the gC$^-$ mutant shed less virus (approximately 10^6 versus 10^8) for a shorter duration (10 days versus 14+ days) than wild-type virus (Denis *et al.*, 1996;Kaashoek *et al.*, 1998). The lower shedding for the gC$^-$ mutant was confirmed in a second experiment that compared gC$^-$ and wild-type only (Kaashoek *et al.*, 1998). Moreover, only the gC$^-$ mutant displayed clinical signs that were similar to the wild-type control; the three other glycoprotein mutants exhibited a less virulent phenotype (Kaashoek *et al.*, 1998). This increased virulence correlated directly with an increased immunogenicity and was reflected in higher virus neutralizing antibodies and enhanced control of viral replication during BHV-1 challenge three weeks after initial inoculation. In addition, gC$^-$ immunized animals were protected after challenge as assessed by reduced clinical signs (Kaashoek *et al.*, 1998). When comparing the cell-mediated immune response generated by the various deletion mutants, the gC$^-$ virus exhibited both the highest proliferative response and the highest non-MHC restricted cytotoxicity that equalled responses in animals infected with wild-type virus (Denis *et al.*, 1996). Limiting dilution analysis of specific proliferative lymphocyte frequency showed a lower frequency in only the gC$^-$ groups. This indicated that gC was an antigen for the CD4+ T cell response and confirmed previous findings by Leary and

Splitter, 1990 (Denis *et al.*, 1996). Subsequent experiments with hyperimmune catttle revealed that gE was also an antigen recognized by CD4+ T cells (Denis *et al.*, 1996).

In conclusion, these experiments have shown that although gC is a major attachment protein and a major antigen for both antibody and cell-mediated responses, a gC^- virus still retains its immunogenicity and could be used as marker vaccine (Liang *et al.*, 1992; Flores *et al.*, 1993; Kaashoek *et al.*, 1998; Denis *et al.*, 1996).

2.7 Viral vectors expressing cytokines

In the last 10 years, the use of viral vectors to express foreign proteins has risen exponentially. The novel use of a viral vector, expressing cytokines to modulate a viral infection began in 1987 with the publication of two papers reporting the construction of vaccinia virus expressing IL-2. This vaccinia vector was attenuated as shown by reduced virus replication in a typically lethal mouse infection model (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987). Table 1.1 is a partial survey of virus vectors that have been constructed to express different cytokines. A variety of viral vectors using vaccinia, adenovirus, retroviruses and herpesvirus have been developed as potential therapeutic agents to treat cancers or hereditary diseases. The approach to construct viral vectors for cytokine therapy will not be reviewed since it has been extensively reviewed previously (Yanez and Porter, 1998; Robbins *et al.*, 1998; Kovesdi *et al.*, 1997; Trapnell and Gorziglia, 1994).

The total number of cytokines expressed in different viral vectors has increased dramatically and for each new promising cytokine a viral vector is rapidly constructed. Viral vectors expressing cytokines can be classified into two general categories: (1)

vectors which alter virus pathogenicity and (2) vectors that alter the immune response against the virus itself or a co-expressed antigen (Ramshaw *et al.*, 1992).

2.7.1 Anti-viral activity of viral vectors

IL-2, IFN-γ and TNF expressed in recombinant vaccinia virus (VV) are able to control viral growth and prevent a lethal infection in nude or athymic mice (Ramshaw *et al.*, 1987; Flexner *et al.*, 1987; Kononen-Corish *et al.*, 1990; Sambhi *et al.*, 1991). The local expression of IL-2 during a recombinant vaccinia virus encoding IL-2 (VV-HA-IL-2) infection attenuates viral growth in ovaries, lungs, spleen and brain and this protection was reversed by anti-IL-2 and anti-IFN-γ antibody treatment (Karupiah *et al.*, 1990b; Karupiah *et al.*,1991). The anti-IFN-γ antibody treatment showed that vaccinia expressing IL-2, attenuated viral growth by enhancing IFN-γ production by NK and non-NK cells (Karupiah *et al.*, 1990b). In addition, during wild-type VV infection, exogenous IL-2 or IFN-γ had no effect on the mortality rate (Karupiah *et al.*, 1990b). Similarly, VV-HA-TNF and VV-muIFNγ-TK displayed decreased viral growth in ovaries, the main tissue used as an indicator of viral attenuation (Kohonen-Corish *et al.*, 1990; Sambi *et al.*, 1991). In contrast, vaccinia virus constructs expressing IL-1α, IL-3, IL-4, IL-5 and IL-6 were not attenuated *in vivo* (reported in Sambi *et al.*, 1991).

The actual mechanisms of vaccinia attenuation are still being debated. Two general mechanisms of action have been proposed which may complement each other (Ramsay *et al*, 1993a). The first mechanism involves the direct up-regulation of effector cells (NK, NK-like and T cells) by recombinant virus infected cells. These

effector cells may either directly lyse the infected cell or produce cytokines which regulate viral replication directly or indirectly through their immunomodulatory activity. The second mechanism involves the production of IFN-γ and TNF which acts on target cells to induce an anti-viral state (Ramsay *et al.*, 1993a). In an athymic mouse model, both NK activity and the local production of antiviral cytokines (IFN and TNF) are increased (Karupiah *et al.*, 1990a; Karupiah *et al.*, 1991). Enhanced NK cell activity during recombinant vaccinia infection may be activated by IFN-γ. In athymic mice, both VV-HA-IL-2 and VV-HA-IFNγ vaccinated mice had decreased survival rates and increased viral replication when treated with anti-IFN-γ antibody (Karupiah *et al.*, 1990b).

Due to the success of recombinant vaccinia virus in controlling viral infection, a number of other prominent viruses have been constructed to express cytokines (Table 1.1). In an attempt to produce a safe and efficacious live attenuated vaccine for AIDS, researchers have constructed simian immunodeficiency virus (SIV) expressing IL-2 or IFN-γ (Giavedoni *et al.*, 1996; Gundlach *et al.*, 1997). Both constructs are based on a nef gene deletion background, since nef minus viruses are unable to cause clinical disease, but can induce protection (Daniel *et al.*, 1992; Kestler *et al.*, 1991). SIV expressing IFN-γ (SIV-hyIFNγ) has similar *in vitro* growth and cytopathological characteristics to the parental strain in tissue culture (Giavedoni *et al.*, 1996). Unlike vaccinia, SIV-hyIFNγ is unstable during tissue passage, losing the IFN-γ gene within six passages. This instability may be caused by the limited capacity of retroviruses to accommodate extra genomic material or a tendency to delete sequences that do not

provide a selective advantage (Giavedoni *et al.*, 1996). Nevertheless, in vivo infection of rhesus macaques with SIV-hyIFNγ significantly lowered the viral load in blood as compared with a SIVΔnef mutant (Giavedoni *et al.*, 1997). Although, SIV-hyIFNγ infected animals developed an overall lower antibody response compared with SIVΔnef mutant, they still had a significantly lower viral load following challenge with virulent SIVmac (Giavedoni *et al.*, 1997).

In contrast to SIV-hyIFNγ, SIV-IL2 has an *in vitro* growth advantage in stimulated PBMC cultures over wild-type SIVmac virus (Gundlack *et al.*, 1997). However, *in vivo* infection of rhesus macaques, SIV-IL2 did not exhibit a reduced viral load relative to a SIVnef control and both had lower viral titer then wild-type parental virus (Gundlack *et al.*, 1997). Similar to SIV-hyIFNγ, the IL-2 gene was lost within 3 months post-vaccination (Giavedoni *et al.*, 1997; Gundlack *et al.*, 1997). Analysis of the immune response induced in animals following infection with SIV-IL2 revealed no difference in antibody titers, proliferative responses or CTL activity when compared to SIVnef (Gundlack *et al.*, 1997). The only significant difference occurred in higher urinary neopterin/creatine levels for SIV-IL2 infected animals which is associated with activated T-cells and macrophages releasing this factor (Gundlack *et al.*, 1997).

A different viral approach, with modulation potential, involves the use of replication defective viral vectors expressing cytokines. One such vector is a defective-interfering RNA vector expressing IFN-γ in murine coronavirus (Zhang *et al.*, 1997). In co-infection experiments with both a defective virus expressing IFN-γ and a replication competent strain, a decrease in viral titer was observed and this

corresponded with an increase in survival rate (Zhang *et al.*, 1997). Histological examination revealed increased mononuclear cell infiltration suggesting a possible modulation by the IFN-γ expressing vector (Zhang *et al.*, 1997). Similarly, wild-type and defective herpesvirus vectors have been engineered to express cytokines and were tested for their immunomodulation (Mester *et al.*, 1995). The expression of IFN-α by the KOS strain or a defective d120 strain did not inhibit *in vitro* viral growth. However, pre-treatment of cells with culture supernatant from IFN-α producing virus inhibited viral replication (Mester *et al.*, 1995). Similarly, herpesvirus vector d120 expressing either IL-4 or IFN-γ induced a quantitatively lower systemic and mucosal immune response after enteric immunization (Kuklin *et al.*, 1998). In addition, recombinant d120IFN vector induced a significantly higher number of IFN-γ secreting cells in splenocyte cultures, but after challenge only partial protection was achieved. This was similar to the control d120 virus, but differed from the complete protection generated by wild-type HSV (Kuklin *et al.*, 1998).

2.7.2 Modulation of the immune system by viral vectors

The construction of viral vectors with immunomodulatory cytokines has provided valuable information regarding the potential role of these cytokines in the generation of an immune response (Ramshaw *et al* 1997). Studies with recombinant viruses expressing IL-4 or IL-5 has provided *in vivo* evidence for the involvement of these cytokines in the generation of mucosal IgA (Ramsay *et al.*, 1993a; Ramsay *et al.*, 1994). The intranasal injection of VV-HA-IL5 increases the number of IgA secreting cells as well as the total IgA response. This enhancement can be blocked with anti-IL-5

antibody (Ramsay *et al.*, 1993). Moreover, intranasal challenge of IL-6 deficient transgenic mice with a VV-HA-IL6 vector restored both IgA and IgG responses (Ramsay *et al.*, 1994). In contrast, recombinants containing IL-2, IL-3 and IL-5 could not restore antibody levels (reported in Ramsay *et al.*, 1994).

Based on this success, constructs with IL-4 and IFNγ have been tested for immunomodulation potential. VV-HA-IL-4 recombinant virus increased viral growth in the ovaries, while antibody and NK levels were similar to control virus (Sharma *et al.*, 1996). In addition, CTL and CTL precursor frequencies were significantly lower throughout the infection period. This correlated with decreased levels of IL-2, IL-12 and IFN-γ in splenocytes (Sharma *et al.*, 1996). In contrast, the construction of a VV vector with high levels of IL-4 expression from the VP37 locus and co-expression of protein F, from respiratory syncytial virus, failed to modulate RSV-specific CTL memory immune response or alter IFN-γ production by splenocytes (Bembridge *et al.*, 1998). However, antibody production was biased towards IgG1 with a reduction in IgG2 antibody responses (Bembridge *et al.*, 1998).

In conclusion, the use of viral vector expressing cytokines has expanded our understanding of how cytokines interact with the immune system. It has confirmed the direct anti-viral roles of IFN-γ and TNF-α acting in a localised microenvironment. Viral vectors have demonstrated the immunomodulation potential of many factors to induce host defense mechanisms (IL-2, IL-4, IL-5, IL-6, IL-12) that are important in viral recovery and pathogenicity.

Table 2.1 Recombinant viruses expressing cytokines

Virus	Cytokine Inserted	Immunomodulation	Author
Vaccinia virus (VV)	IL-2	Attenuated viral growth Stimulates NK cells to produce IFN-γ Chemotactic signal? Enhances NK killing	Flexner *et al.*, 1987 Ramshaw *et al.*, 1987 Flexner *et al.*, 1990 Karupiah *et al.*, 1990a Karupiah *et al.*, 1990b Karupiah *et al.*, 1991
VV	IFN-γ	Attenuated viral growth Upregulates anti-viral mechanism and induces $NO^{\cdot}$ MHC upregulation	Kohonen-Corish *et al.*, 1990. Giavedoni *et al.*, 1992.
VV	IL-1α	Enhances antibody memory response	Ruby *et al.*, 1991
VV	IL-1β	Inhibit experimental autoimmune encephalomyelitis (EAE) No change in viral replication	Willenborg *et al.*, 1995 Ruby *et al.*, 1991
VV	IL-3	Induces haemopoiesis	Reported in Ramshaw *et al.*, 1992
VV	IL-4	Enhances viral pathogenicity Enhances EAE	Sharma *et al.*, 1996 Ramshaw *et al.*, 1997 Bembridge *et al.*, 1998 Willenborg *et al.*, 1995
VV	IL-5	Enhances mucosal IgA	Ramsay and Kohonen-Corish, 1993b.
VV	IL-6	Enhances mucosal IgA No change in viral replication	Ramsay *et al.*, 1994 Willenborg *et al.*, 1995.
VV	IL-7	Accelerates viral clearance	Ramshaw *et al.*, 1997 Leong *et al.*, 1997
VV	IL-10	Inhibits EAE	Willenborg *et al.*, 1995.
VV	IL-12	Attenuates viral growth	Ramshaw *et al.*, 1997

VV	TNF-α	Attenuates viral growth Inhibits EAE	Sambhi *et al.*, 1991. Willenborg *et al.*, 1995.
VV	TGF-β	N.D.	Reported in Ramshaw *et al.*, 1992
VV	IFN-γ/HIVgp160	N.D.	Giavedoni *et al.*, 1992.
Fowlpox virus	IL-6/HA IFN-γ//HA	Intranasal application augments IgA; primes for enhanced secodary immune response Decrease antibody response	Leong *et al.*, 1994.
Simian immunodef-ieciency virus (SIV)	IFN-γ	Attenuated viral load and rechallenge virus load Decreased antibody response	Giavedoni *et al.*, 1996. Giavedoni *et al.*, 1997.
SIV	IL-2	No effect on viral load	Gundlach *et al.*, 1997.

N.D. represents not determined.

3.0 SPECIFIC AIMS AND HYPOTHESIS

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-gamma resulting in the induction of a cell-mediated response that is associated with resistance. In contrast, Th2 cells that produce IL-4, IL-5 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, DNA immunization and the use of viral vectors to express cytokines (Hughes *et al.*, 1992; Pardoll., 1995; Ramshaw *et al.*, 1992; Lewis *et al.*, 1997; Babiuk *et al.*, 1998). In addition, cytokine therapy can be achieved by the addition of anti-cytokine molecules including anti-cytokine antibodies

and natural inhibitors i.e. IL-1 receptor antagonist (Dinerallo, 1996).

Cytokine modulation may be achieved by the addition of exogenous cytokine near or at the site of infection. This can be done by the in vivo expression of cytokine genes incorporated in vectors (recombinant viruses, bacteria, DNA immunization). Viral vectors expressing different cytokines have been very effective in the modulation of vaccinia virus infections (Ramsey *et al.*, 1997). The generation of a recombinant BHV-1 vector containing cytokines will allow us to investigate this novel vaccine approach in a respiratory disease model. Furthermore, the construction of a BHV-1 vector expressing different cytokines would allow us to study the effects of cytokine expression on BHV-1 pathogenesis and the generation of an immune response. Our hypothesis was that the incorporation of cyokines into a BHV-1 virus would either attenuate viral growth or enhance the immune response. The selection of IL-1β and IFN-γ for incorporation into the BHV-1 genome was based on previous usage of these cytokines as immunological adjuvants and the limitations of gene insertion into our BHV-1 vector. Although other cytokines may be equally important during BHV-1 infection, we focused on two cytokines that had been used in previous investigations.

Therefore, the specific aims are the following:

1) The construction of recombinant BHV-1 expressing bovine cytokines IL-1β and IFN-γ.
2) The characterisation of cytokine expression *in vitro*.
3) The investigation of the immunomodulation potential of recombinant BHV-1 vectors expressing IL-1β and IFN-γ *in vivo*.
4) The examination of an alternative BHV-1 infection model in sheep to investigate the recombinant vaccine candidates.

4.0 MATERIALS AND METHODS

4.1 Virology

4.1.1 Virus and cells

The Cooper strain of BHV-1 was used as the prototype virus; Cooper virus was obtained from the National Veterinary Services Laboratory (Ames, Iowa). A gC negative mutant (gC^-) and a gC negative mutant expressing LacZ gene (BHV-1δgIII/LacZ) were previously produced in this laboratory, denoted gC^- and gC-/LacZ+, respectively (Liang *et al.*, 1991; Liang *et al.*, 1992). Field isolate strain 108 was obtained from the Animal Disease Research Institute, Lethbridge, Alberta, Canada. The viruses were propagated in Madin-Darby bovine kidney (MDBK) cells grown in minimum essential medium (MEM) (GIBCO laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL). MDBK cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

An IL-1 responsive cell-line was subcloned from the murine helper T-cell 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, California) supplemented with 10% FBS (Hyclone, Ont. Can.), 2 mM L-glutamine and 10 % concanavalin A (Con A) stimulated rat spleen supernatant.

4.1.2 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 pSD113EClaH/P (Figure 5.1.1). pSD113EClaH/P was digested with *Sal*I, blunted with Klenow fragment and ligated with a *Bam*HI linker (CGGGATCCCG) (Figure 5.1.1, 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, 1988) 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 (Figure 5.1.1.B).

To construct a transfer vector with the boIFN-γ coding sequence, the boIFN-γ gene was isolated from plasmid pTrpBoγCAAXPG (CIBA-GEIGY, Basal Switzerland) by an *Eco*RI and *Ssp*I digest followed by treatment with Klenow fragment to repair the protruding end. A *Bam*H1 Linker was added (dCCGGATCCG) for insertion into the *Bam*H1 Site of SD113Bam resulting in transfer plasmid pSD11SSIFNγ. DNA sequencing of the gC-boIFNγ junction confirmed the proper coding sequence.

4.1.3 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 (MOI) of 1 of PFU. After the cells exhibited complete cytopathology, the supernatants containing the virus were clarified by low-speed centrifugation at 1000 x g. Virus was harvested by pelleting the clarified supernatant through a 30% sucrose in phosphate-buffered saline (PBS) cushion at 100,000 x g for 60 min. The virus pellet was then resuspended in 0.05 M Tris-HCl, 0.15 M NaCl, 10 mM EDTA (pH 8.0) and applied to a 20 to 50 % potassium-sodium tartrate discontinous gradient and centrifuged at 100,000 x g for 90 minutes. After centrifugation, the virus band was collected, diluted in PBS, and pelleted at 75,000 x g for 60 minutes . Purified virus was stored at -70°C.

Viral DNA was purified according to the method of Summers and Smith (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 ml and incubated at 50°C for 1 h Sarkosyl was added to a final concentration of 1%, and the sample was incubated at 50°C for an additional 1 h . 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 mM EDTA, pH 8.0, aliquoted and stored at -70°C.

4.1.4 Recombinant virus BHV1/IL-1β 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 mM HEPES [*N*-2hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 137 mM NaCl, 5 mM KCl, 0.7 mM $NaHPO_4$, 5 mM D-glucose, pH 7.1), and washed three times in HeBS at 4°C. After the final wash, cells were resuspended to 3 x 10^6 cells/ml in HeBS and transferred to an electroporation cuvette (Bio-Rad Laboratories, Richmond, Calif.) containing 10 μg of linearized plasmid DNA and 20 μg of viral DNA. Electroporation 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°C for 5 hours, and overlaid with 1 % agarose containing 2 % FBS in MEM. gC negative virus plaques were

screened by an antibody-based black-plaque assay.

4.1.5 Recombinant virus BHV1/IFN-γ production

BHV-1δgIII/LacZ DNA was purified according to methods previously described in section 4.1.3. To generate recombinant virus, MDBK cells were co-transfected with 1.5 μg of pSD113SSIFNγ and 10 μg of BHV-1δgIII/LacZ DNA by lipofection. Briefly, plasmid and viral DNA was added drop-wise to 35 μl of dH_2O plus 40 μl of lipofectin (GIBCO/BRL), and allowed to stand for 15 minutes at room temperature. MDBK cells grown in 4-well plates were washed twice with Opti-MEM (GIBCO) and then 5 mls of Opti-MEM was added. The DNA/lipofection mixture was added drop-wise. The cells were incubated overnight at 37°C in 5% CO_2 . Next day, 2% fetal bovine serum was added and cells were incubated for another day. Cells were then washed twice with MEM, trypsinized and resuspended into a 96-well plate. Positive CPE wells were harvested and screened for β-galactose production by a blue plaque assay (Liang *et al.* 1992).

4.1.6 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 h 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 1:1000 in BSA-PBS, was added to the plates and incubated for 1 h 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. Plaques developed by recombinant viruses should contain a gC deletion and appear white against non-recombinant black plaques. Positive white plaques were plaque purified three times for analysis.

4.1.7 Blue Plaque assay

A 30 mg/ml stock solution of Bluo-gal (Bethesda Research Laboratories, Gaithersburg, MD) in dimethyl sulfoxide (DMSO) was used. To screen for blue plaques, a confluent monolayer of MDBK cells was incubated with virus inoculum for 1 hour. After adsorption, the monolayer was overlayed with a MEM solution containing 0.8% agarose, 2% FBS and 300 μg/ml of Blue-gal. Plates were incubated at 37 °C until plaques developed. Since parental virus contained the LacZ gene, blue plaques were non-recombinant viruses and white plaques had undergone a recombination event. Positive plaques were plaque purified for analysis.

4.1.8 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 a nitrocellulose filter using standard procedures (Maniatis *et al.*, 1982). The nitrocellulose filter was hybridized with ^{32}P-labelled DNA probes corresponding to the boIL-1β gene and the 5' non-coding region of the gC gene (0.6 kbp *Sma*I-*Bam*HI fragment from pSD113SSIL-1β).

Total cellular DNA was isolated from MDBK cells infected at a MOI of 10 PFU with either wild-type and recombinant virus. Briefly, cells were harvested at 20 h postinfection by scraping cells in 5 mls of Tris-buffered saline (pH 8.0) and pelleted at 2000 rpm for 5 min at 4 °C in a microcentrifuge. Cells were resuspended in 0.5 ml TE (pH 8.0), then 5 mls of extraction buffer (10 mM Tris-HCl pH 8.0; 0.1 M EDTA, 20 μg/ml Rnase, 0.5% SDS) was added. After incubating 1 h at 37 °C, proteinase K (100μg/ml) was added and the sample was incubated for 3 hrs at 50 °C. Samples were extracted three times with phenol (pH 8.0), followed by the addition of a 0.5 volume of 7.5 % ammonium acetate and 2.0 volume of absolute ethanol. Samples were centrifuged, washed twice in 70% ethanol, dried and resuspended in 0.5 ml of 10 mM Tris-HCl–1mM EDTA (pH 8.0). The DNA was digested with *Eco*RI and *Bgl*II restriction endonucleases, run on a 1% agarose gel and transferred to nitrocellulose. The nitrocellulose filter was hybridized with a ^{32}P-labelled DNA probe corresponding to the boIFN-γ gene (*Eco*RI - *Ssp*I fron pSD11SSIFN-γ) and a probe corresponding to the gC gene.

4.1.9 Northern blot hybridization analysis

RNA preparation was performed as previously described (Kowalski *et al*, 1989). Briefly, confluent MDBK cells in 100 mm plates were infected with recombinant virus at a M.O.I. of 10 PFU. At various time points, cells were washed once in phosphate-buffered saline lacking calcium and magnesium and harvested into 200 µl of TSM [30 mM Tris-HCl (pH 8), 100 mM NaCl, 1.5 mM $MgCl_2$]. 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 X g for 10 min. The cytoplasmic extract was added to 200 µl of lysis buffer [2% sodium dodecyl sulfate (SDS), 7 M urea, 350 mM NaCl, 2 mM EDTA, 10mM Tris-HCl (pH 8)] and extracted once with 400 µl of phenol-chloroform (1:1) saturated with 100 mM 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 hydridized as previously described (Maniatis *et al.*,1982).

4.1.10 Indirect Immunofluorescence assay

MDBK cells cultured in chamber slides (Nunc Inc., Naperville, Ill.) were infected with recombinant virus at a M.O.I of 0.01 for 16 h, fixed with 2% paraformaldehyde for 5 minutes and permeabilized with methanol at -20°C for 15 minutes. Nonspecific reactions were blocked by treating slides with 2% BSA and 2%

normal goat serum in PBS (blocking solution) for 30 minutes. Afterwards, slides were incubated with rabbit anti-boIL-1β serum at 1:500 dilution for 1 h . Rabbit anti-boIL-1β serum was produced against recombinant bovine IL-1β (American Cyanamid, 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, Calif.) and examined with a fluorescence microscope.

4.1.11 Immunoprecipitation

Immunoprecipitation was performed as previously described (Liang *et al.*, 1991). Briefly, subconfluent monolayers of MDBK cells were infected with respective viruses at a MOI of 10 and labelled with 50 μCi of L-[^{35}S] methionine (Amersham, Oakville, Ontario, Canada) per ml, for 24 hours. After labelling, 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.

4.1.12 Glycosidase digestion

Immunoprecipitation was performed as described above. Briefly, MDBK cells were infected at a MOI of 10 PFU and labelled with 50 μCi of L-[^{35}S] methionine (Amersham, Oakville, Ontario, Canada) per milliliter for 18 hrs. The cellular and supernatant fractions were harvested separately and precipitated with rabbit anti-

boIFN-γ antibody. Proteins were collected by boiling in 0.8 % SDS.

For endoglycosidase H treatment, the proteins were resuspended in 0.125 M Sodium citrate, pH 5.5 and digested with endoglycosidase H (endo H) (Boeringer-Mannheim, Laval, Quebec) at 37°C overnight. For N-glycosidase F (PNGase F) treatment, the proteins were resuspended in 0.2 M sodium phosphate buffer, pH 8.6 and digested with PNGase F (Boeringer-Mannheim, Laval, Quebec) at 37°C overnight. Samples were precipitated with acetone, pelleted and separated on SDS-PAGE.

4.1.13 Growth Kinetics curve

Confluent MDBK cells grown in 6-well plates were infected at an MOI of 5 with either gC⁻, wild-type virus, BHV-1 expressing IL-1β (BHV/IL1β) or BHV-1 expressing IFN-γ (BHV-1/IFNγ). After 1 h adsorption at 37°C, the virus inoculum was removed; cells were washed with MEM and incubated with 2ml of MEM supplemented with 10% FBS. At various times post-infection, aliquots of culture media were collected and assayed for viral titers. Samples were collected in triplicate.

4.1.14 Bioassay for IL-1 activity

LM-1 cells (see 4.1.1) cultured for 5 to 7 days were washed three times in Click's media and re-suspended in Click's media with 5% FBS and 2mM L-glutamine. The samples to be tested were filtered through Centricon-30 ultra-filtration devices to remove virus from the supernatant (Amicon, Beverly, MA) and serially diluted in a 96-well plate. Bioassays with LM-1 cells (2 to 4 × 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 hours at 37°C in 5% CO_2. Biological activity was determined by adding [^{3}H] thymidine (Amersham, 0.4 µCi/well) 18 hours 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 stock) was used as a positive control (American Cyanamid, Princeton, New Jersey). The rBoIL-1β standard had a specific activity of 4.6×10^8 U/mg when assayed with LM-1 cells.

4.1.15 Bioassay for IFN-γ activity

Bovine interferon gamma activity was determined by a standard vesicular stomatitis virus (VSV) plaque inhibition assay as previously described (Campos *et al.*, 1989). Briefly, confluent MDBK cells in a 96-well flat bottom plate (Nunc, DK) were incubated with two-fold dilutions of various culture supernatants and a control dilution of 100 U/ml of recombinant boIFN-γ (American Cyanamid, Princeton, New Jersey). After an overnight incubation at 37 °C, 100µl of MEM containing 100 plaque forming units (PFU) of VSV was added to each well for 1 h at 37°C. The virus inoculum was removed and replaced with a 2% FBS– 1% methyl cellulose MEM overlay solution; and plates were incubated overnight. Next day, the plates were stained with crystal violet. One unit of boIFN-γ activity was taken as the dilution of supernatant at which

50% of the VSV plaques were inhibited.

4.1.16 MHC up-regulation by IFN-γ

Alveolar macrophages were isolated from 2-3 month old calves by lung lavage. After lung removal, 1 litre of MEM was poured into the trachea and the lung lobes were gently massaged. The lung was inverted to collect MEM solution that contained alveolar macrophages. This procedure was repeated three times. Afterwards, the collected medium was centrifuged at 1500 rpm in a Beckman GH-3.7 rotor for 10 minutes to collected cells. Cells were washed two times with MEM, counted and resuspended to 5×10^6 cells/ml. Alveolar macrophages were incubated in 6-well plates at 15×10^6 cells/well in MEM containing 5 % FBS.

To detect IFN-γ induced MHC II up-regulation, 3 ml of isolated alveolar macrophages were incubated with 2 ml supernatant from either Wild-type Cooper virus, gC-/LacZ+ or BHV-1/IFNγ that had been centrifuged at 75,000 x g for 2 h to remove active virus. Control wells were incubated with 100 ng/ml of rboIFN-γ or MEM. After a 24-hour incubation, cells were collected from the wells by incubating adherent cells in PBSA with 0.05% EDTA for 10 minutes. Cells were washed once and counted for staining.

Cell suspensions at 5×10^5 cells/well were placed into a 96 well plate and incubated for 30 min with lineage-specific monoclonal antibodies: BoLa class II (nonpolymorphic determinant; TH14B) (VMRD, Inc., Pullman, Wash.) and monocyte-macrophage marker DH59B (VMRD). After two washes with PBS, cells were

incubated for 30 min with flourescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, CA.), then washed twice and fixed with 2 % formaldehyde. Cells were stored in the dark at 4°C until analyzed. The flourescent intensity was evaluated using a Becton Dickinson FACScan. Non-specific staining was determined with an isotype-matched irrelevant monoclonal antibody and 5,000 cells were analyzed for the percentage positive staining for each antibody tested.

4.2 *in vivo* procedures

4.2.1 Animal Care compliance

All animals were cared for under the guidelines provided by the Canadian Council on Animal Care (1993).

4.2.2 Sheep challenge model

4.2.2.1 Cooper and gC-/LacZ+ challenge

Twelve 3-month-old male lambs were obtained from the Department of Animal and Poultry Science, University of Saskatchewan. All animals were sero-negative for BHV-1. The lambs were divided into two isolation rooms. Each group was challenged by intranasal aerosolization with either wild-type Cooper strain or a mutant BHV-1 gC-/LacZ+ strain (Liang *et al.*, 1992). Three animals received approximately 4×10^7 PFU (1 min.) and 3 animals received 4×10^8 PFU (10 min.). A Devilbiss Nebulizer model 65 (Devilbiss, Barry, Ont.) generated the aerosol which was delivered into the

nostril. To collect nasal secretions, a cotton applicator (Staplex Scientific, Etobicoke, Ont.) was used to swab the mucosa and then placed in 1 ml of MEM. Nasal samples were collected for 10 days and animals were monitored daily during the initial challenge period.

At week 4, all animals were challenged with 1 x 10^7 PFU/ml of Strain 108; three naive animals were included as controls. Sera were taken weekly.

4.2.2.2 Recombinant challenge

Fifteen 3-month-old male lambs were obtained from the Department of Animal and Poultry Science, University of Saskatchewan. All animals were sero-negative for BHV-1, and five lambs were housed as a group in each of three isolation rooms. One group was challenged by intranasal aerosolization with a gC minus BHVgC-/LacZ+ strain (Liang *et al.*, 1992), one group was challenged with BHV1/IL-1β recombinant strain and the final group was challenged with BHV-1/IFNγ recombinant strain. Each animal was initially challenged by intranasal aerosolization with approximately 4×10^7 PFU/ml for 5 minutes for a total dose of 2×10^8 PFU calculating 1ml per min at maximum flow. At week 5, all animals were re-challenged with 1×10^8 PFU of BHV-1 Strain 108. Five naive animals were included as naive controls for the secondary challenge.

4.2.3 Cattle challenge model

Nine 6-month-old crossbred holstein dairy calves were randomly divided into three groups. At day 0, animals were intranasally challenged for 5 minutes with 4×10^7

PFU/ml of either wild-type Cooper strain, gC-/LacZ+ mutant strain (Liang *et al.* 1992) or recombinant BHV-1/IFNγ strain. Groups were housed in individual isolation rooms to avoid any cross infection by recombinant viral strains. Animals were monitored daily for 10 days by a veterinarian who assessed the clinical score for each group without knowledge of the specific virus used for challenge. For the first 10 days post-infection, nasal samples were collected with both nasal swabs and cotton tampons. Blood samples were also collected in citrate for FACS analysis of leukocyte phenotype and serum isolation. After the initial clinical period, animals were bled weekly to assess BHV-1 specific proliferative responses, IFN-γ secreting cell frequency and serum antibody titers. After 35 days, animals were treated for 5 days with 0.1 mg/kg of dexamethasone to reactivated latent virus (Homan and Easterday, 1983). Animals were clinically monitored for 10 days after reactivation and nasal swabs were taken to detect viral shedding.

4.2.4 Clinical Observation

Clinical examination included rectal temperatures, assessing depression and an examination for nasal lesions. A sick score was calculated by adding the scores for nasal lesions and depression which were ranked from 0 to 4 (0= Normal and 4= severe). Nasal lesions scores were based on nasal discharge, mucosal hyperaemia and nasal discharge. Depression score was based on decreased appetite and responsiveness

4.2.5 Virus isolation and mucus collection

Virus shedding was monitored by inserting a cotton swab into the nasal cavity and rubbing the mucosa. The cotton swab was then placed into 1 ml of MEM and stored at –70°C until viral titer was determined. To collect nasal secretions, a cotton tampon was inserted into one nostril for 20 minutes then removed and placed into a 50 ml sterile syringe to squeeze out the nasal fluids.

4.2.6 Isolation of Peripheral Blood Lymphocytes

Blood was collected into 20 ml collection tubes containing 10% Citrate. Blood from an individual animal was transferred into a 50 ml tube and centrifuged for 20 min at 2500 rpm in a Beckman GH-3.7 rotor. Buffy coat cells were collected, mixed with an equal amount of PBS-EDTA (0.1%) and layered on 60% Percoll (Pharmacia). Samples were centrifuged at 3000 rpm for 20 min with no brake. Cells at the interphase were collected, washed three times, and then resuspended in the appropriate assay medium.

4.3 Immunology

4.3.1 Total and differential leukocyte count

Venous blood samples were collected in EDTA vacuum tubes (Becton Dickinson, Rutherford, NJ). Total and differential white blood cell counts were determined by the Department of Clinical Pathology, WCVM, U of S.

4.3.2 Flow Cytometric Analysis

Peripheral blood leukocytes were obtained by a red blood lysis method. Briefly, 250 µl of blood in 96-well plates were spun for 20 min at 2000 rpm in a Beckman GH 3.7 rotor. 150 µl of plasma was removed. Cells were re-suspended in 150 µl of ammonium chloride lysis buffer [0.15M NH_4Cl, 0.10 mM Tris-HCL (pH 7.2)] and incubated at 37°C for 5 min. Lysis process was repeated. Cell were washed and resuspended in FACola for staining. Leukocytes were incubated with monoclonal antibodies (mAb) specific for the following bovine leukocyte surface markers: CD3 (MM1A), CD4 (ILA12), CD8 (CACT80C), IgM (PIG45A), B cell (BAQ44A), total B cell (DU2-104; CD72), and granulocytes/monocytes (DH59B). Leukocytes were also dual-labelled for major histocompatibility complex (MHC) classII (TH14B) and CD4 (ILA12). Antibodies were obtained from Veterinary Medical Research and Development (VMRD) Inc. (Pullman, WA, USA). IL12A (CD4) was a generous gift form Dr. J Ellis (WCVM, Saskatoon) and DU2-104 was a generous gift from Dr. Wayne Hein (Basal, Switzerland).

After incubation with the mAb, cells were washed and incubated with a fluorescein isothyiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, Ca). For dual-labelling studies isotype specific goat anti-mouse Ig, conjugated to either FITC or PE, were used. Cells were then washed, fixed with 2% formaldehyde. Labelling by mAbs was evaluated using a Becton Dickinson FACScan and the Lysis II program. Non-specific labelling was excluded by using an irrelevant, primary mAb. For each sample, 10,000 cells were analyzed for the

percentage of specific staining after substracting non-specific labelling.

4.3.3 Histopathology and Immunohistochemistry

Tracheal, lung and lymph node samples were removed and fixed in 10% neutral buffered formalin and embedded in paraffin wax. Samples were sectioned and stained with hemtoxylin and eosin by the Veterinary Pathology Department, WCVM.

Tissue sections were stained for BHV-1 antigen at the Veterinary Microbiology Department by using a cocktail of monoclonal antibodies against BHV-1 and developing with an avidin-biotin complex method (Haines *et al.*, 1989). A BHV-1 infected bovine lung section was used as a positive control.

4.3.4 BHV-1 specific lymphocyte proliferative response

Peripheral blood lymphocyte proliferative responses were measured by incubating 3×10^5 cells/well in round-bottomed 96-well microtiter plates (Nunc, DK) with 200 µl MEM supplemented with 5% FBS, 2 mM glutamine, 50 mM 2-mercaptoethanol and 5 ng/ml of dexamethasone. All samples were assayed in triplicate. Samples were cultured in medium alone and with either purified gD protein at 0.1µg/ml, 0.01 µg/ml or irradiated (2000 mjoules) Cooper BHV-1 at 10^5 PFU/ml. Cultures were incubated for 72 h at 37°C in 5% CO_2. During the last 8 hrs of culture, 0.4 µCi [methyl-^{3}H]-thymidine (Amersham, Canada) was added. Cells were harvested with a Skatron harvester (Skatron, Sterling, Va.) and [^{3}H]-thymidine incorporation was determined with a Beckman scintillation counter (Beckman, Fullerton, California).

Proliferative responses were expressed as a stimulation index (SI) (counts per minute of antigen stimulated culture/counts per minute of non-stimulated culture).

4.3.5 ELISA

A number of enzyme-linked immunosorbent assays (ELISA) were employed. To detect BHV-1 antibody titers, Immulon-II microtiter plates (Dynatech Laboratories, Alexandra, VA) were coated overnight with 0.05 ug/well of purified tgD in 50 mM carbonate buffer (pH 9.6). Plates were washed with PBS-0.05% Tween-20 (PBS-T). Serum was serially diluted in PBS-T and incubated in the plate for 2 h at room temperature. After washing, bound IgG was detected by adding a 1:10000 dilution of phosphatase-conjugated goat anti-bovine IgG (Kirkegaard and Perry Laboratory, Gaithersburg, MD) and incubating for 2 hrs. Plates were developed by the addition of the substrate *p*-nitrophenylphosphate di(TRIS) salt (Sigma) in 0.5 mM $MgCl_2$ (pH 9.8) with 1 % diethanolamine and read at a wavelength of 405 (reference of 490 nm) on a Bio-Rad 3550 microplate reader.

The gD specific IgA was detected in a similar manner to IgG using a mouse anti-bovine IgA (M67) monoclonal at a 1:5000 dilution. This reaction was detected with a biotinylated horse anti-mouse IgG 1:5000 (Vector Laboratories, Burlingame, CA) followed by a 1:1000 dilution of strepavidin-alkaline phosphatase for 1 h (Gibco/BRL). Plates were developed with the streptavidin-alkaline phosphatase system (see above).

For the detection of boIFN-γ, a capture enzyme-linked immunosorbent assay was

used. Immunolon II plates were coated with mAb (2-2-1) at a 1:1000 dilution. Samples were serially diluted, added to the wells, and incubated for 2 h at room temperature. After washing, rabbit anti-boIFNγ polyclonal (92-131) at 1:2000 was incubated for 1 h. Biotinylated goat anti-rabbit IgG diluted 1:1000 was used for developing with the streptavidin-alkaline phosphatase system. *E. coli* produced boIFN-γ (Ciba Geigy, Basel, Switzerland) was used to generate a standard curve to calculate the concentration of IFN-γ in the test sample. The 36 h sample contained 512U/ml of activity with a concentration of 182 ng/ml.

For the detection of boIL-1β, a capture enzyme-linked immunosorbent assay was used. Briefly, a 96-well plate was coated overnight at 4°C with 100 µl of a 1:250 dilution of anti-ovineIL-1β monoclonal antibody (3.41) (generous gift from Dr. Andrew Nash – Andrews *et al.*, 1994) in carbonate buffer. After three washes with PBS + 0.05% Tween 20, plates were blocked with 1 % gelatin in PBST for 1 h at room temperature, then samples and recombinant boIL-1β (American Cyanamid Co., Princeton NJ) were serially diluted and incubated in duplicate wells for 2 h at room temperature. After washing, a rabbit anti-bovine Il-1β antiserum was added at a 1:3000 dilution and incubated for 1 h, followed by the addition of a 1:1000 dilution of horse-radish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h . The assay was developed using an ABTS substrate. Recombinant bovine IL-1β (American Cyanamid, Princeton, New Jersey) was used as our standard. The specific activity of recombinant boIL-1β produced by BHV/IL1β was calculated using the LM-1 bioassay and the capture ELISA, with a sample that contained 2500 units/ml of activity at a

concentration of 5.9 ng/ml.

Haptoglobin levels were detected with an ELISA as previously described (Godson *et al.*, 1996).

4.3.6 BHV-1 Neutralization Titer

Neutralization assays were performed as previously described (Babiuk *et al.*, 1975). Briefly, serial dilutions of the heat inactivated serum (56°C for 30 min) were mixed with an equal amount of medium containing 100 PFU of BHV-1. After incubation for 1 h at 37°C, each sample was added to a well in a 96-well flat-bottom microtiter plate (Nunc, DK) coated with confluent MDBK cells. After a 48-h incubation, plates were stained with crystal violet. Serum neutralization titers were defined as the reciprocal of the highest serum dilution that effected a 50 % reduction of viral plaques.

4.3.7 IFN-γ ELISPOT

An enzyme-linked immunospot (ELISPOT) assay was used to determine the number of IFN-γ secreting cells present in peripherial blood mononuclear cells (PBMC). PBMC isolated on a Percoll gradient and incubated in culture medium containing 5 % FBS, 2 mM glutamine, 50 mM 2-mercaptoethanol, 5 ng/ml dexamethasone and with or without 0.4 μg/well of purified gD. Cells were cultured in 24-well plates at a concentration of 6×10^6 cells per well. Nitro-cellulose plates (Amicon) were coated with a monoclonal antibody for boIFN-γ (mAb 2-2-1, diluted 1:400) in 50 mM carbonate buffer, pH 9.6 at 37 °C. After overnight incubation, cells

were harvested and viable cells were plated at 1×10^6, 5×10^5 and 2.5×10^5 cells per well into pre-coated 96-well nitro-cellulose based microtiter plates. After incubation for 8-16 hrs, the plates were washed twice with cold H_20 and 5x with PBS. To detect bound boIFN-γ, rabbit anti-boIFN-γ antibody (92-131) was added at 1:100 dilution for 2 h at room temperature. Plates were washed and incubated with a biotinylated rat anti-rabbit IgG (Zymed, San Francisco, CA) at a 1:2500 dilution for 2 hrs, followed by incubation with streptavidin-alkaline phosphatase at 1:1000 for 1 h. Spots were developed with 5-bromo-4-chloro-3-indoyl phosphate Nitro Blue Tetrazolium (BCIP/NBT) substrate (Sigma). The numbers of spots were counted in duplicate cultures.

4.3.8 Mucus Assay

To analyze the effects of mucus on *in vitro* viral replication, nasal mucus was obtained from BHV-1 sero-negative calves. Mucus was mixed with a fixed number of viral particles of either Cooper or gC-/LacZ+ viruses resulting in a 90% mucus solution. Samples were incubated at 37 °C for 1 h and then titered in 24-well plates. Each sample represented nasal mucus from an individual animal. Four different nasal samples were used per group. Analysis was performed by student t-test.

4.4 Statistical Analysis

Statistical analysis was performed by GraphPad PRISM program (GraphPad Software, Inc. San Diego, CA). Groups were compared by t-test, analysis of variance or Mann-Whitney rank-sum test as indicated in the figure legends.

5.0 RESULTS

5.1 Expression of Bovine Interleukkin-1β in a Bovine Herpesvirus-1 Vector

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 co-administration of recombinant bovine IL-1β (boIL-1β) and a modified live BHV-1 vaccine was able to enhance both the humoral and cellular immune response against BHV-1 (Reddy, *et al.*, 1990; Reddy *et al.*, 1993). Therefore, we chose boIL-1β to evaluate the potential of utilising BHV-1 as an expression vector for cytokines. This would create a system for investigating immunomodulation caused by cytokine delivery at the site of infection, and also, for investigating a possible mechanism to attenuate BHV-1 virulence. Consequently, this attenuation could reduce the risk of developing respiratory disease complex caused by opportunistic pathogens. As a first step, we constructed a BHV-1 recombinant virus expressing boIL-1β at the gC locus and characterized the *in vitro* properties of the recombinant virus.

5.1.1 Construction of recombinant virus

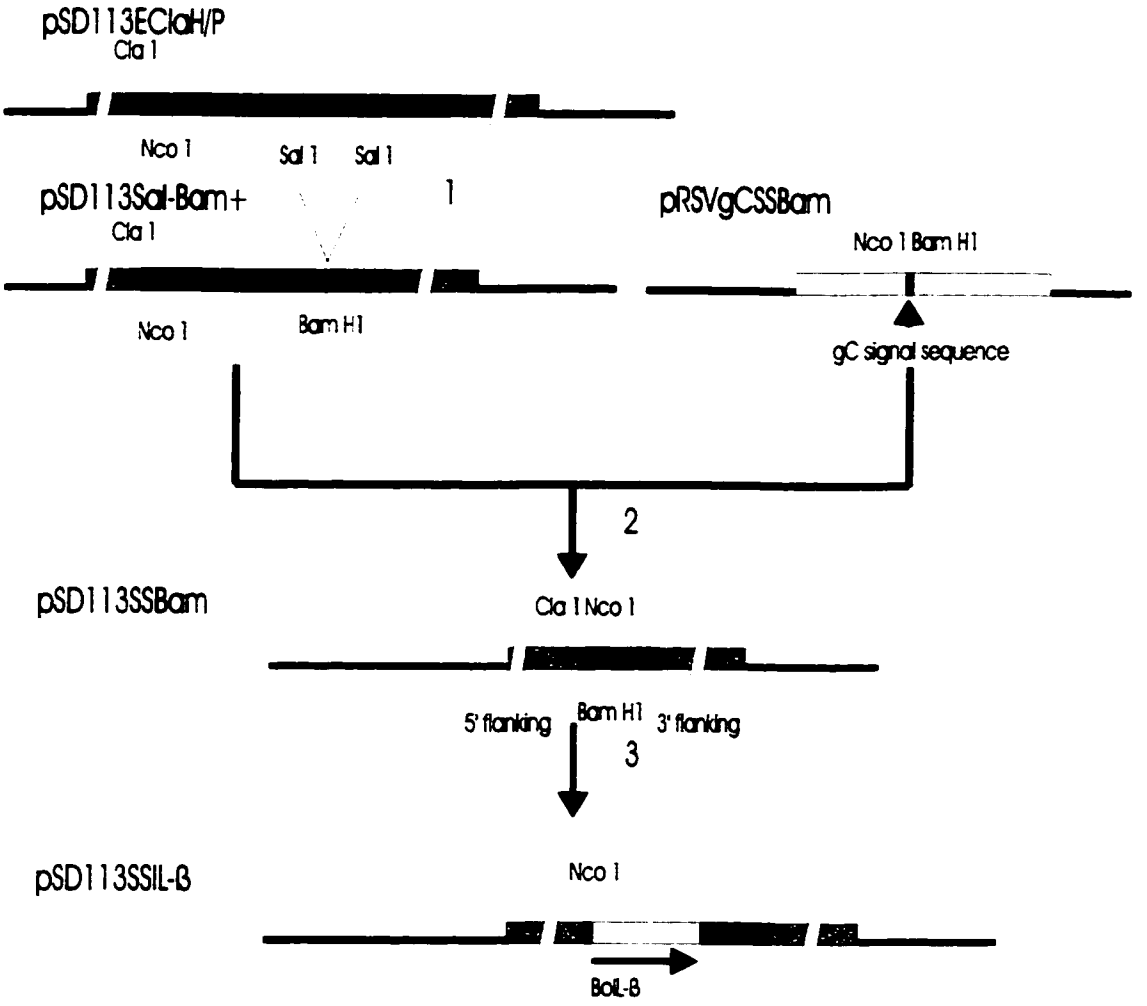
IL-1β is initially synthesized as a 31 kDa precursor which is cleaved by an IL-1β converting enzyme (ICE) into a 17 kDa mature cytokine (Kostura *et al.*, 1989; Cerretti *et al.*, 1992; Thornberry *et al*, 1992). Unlike most secretory proteins, IL-1β does not

contain a signal peptide sequence for secretion (Dinarello, 1991). The actual mechanism of how IL-1β secretion occurs is still unknown, but it has been suggested to be linked to the ICE protein (Howard *et al.*, 1995). 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 (Fig. 5.1.1). 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$^-$ mutant was further characterized.

To ascertain that the gC$^-$ mutant indeed contained the boIL-1β gene and also to confirm the genomic arrangement at the gC region, Southern blot analysis was done. Viral DNA from both wild type virus and recombinant virus (BHV/IL1β) was digested with EcoRI and BglII, separated on a 1% agarose gel, and probed with a 0.6 kbp SmaI-BamHI gC 5' fragment from pSD113SSIL-1β (Fig. 5.1.2). 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 a 2.3 kb was detected in the mutant DNA. The results from the Southern

Figure 5.1.1 Construction of transfer vector pSD113SSIL-1β. (A). Dark grey bars represent the gC gene fragment, solid black bars represent the gC signal sequence, light gray bars represent the gC flanking sequence, clear bar represents boIL-1β 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-1β 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.

A



B

Precursor boIL-1ß amino acid sequence.

1 110 259 END

M A T V P E P I . . . F L C D A P V Q . . . D F R M E T L A P END . . .

gC signal sequence.

M G P L G R A W L I A A I F A Y V L A A R D P G . . .

gC signal / boIL-1ß junction.

M G P L G R A W L I A A I F A Y V L A A R D A P V Q . . . D F R M E T L A P end

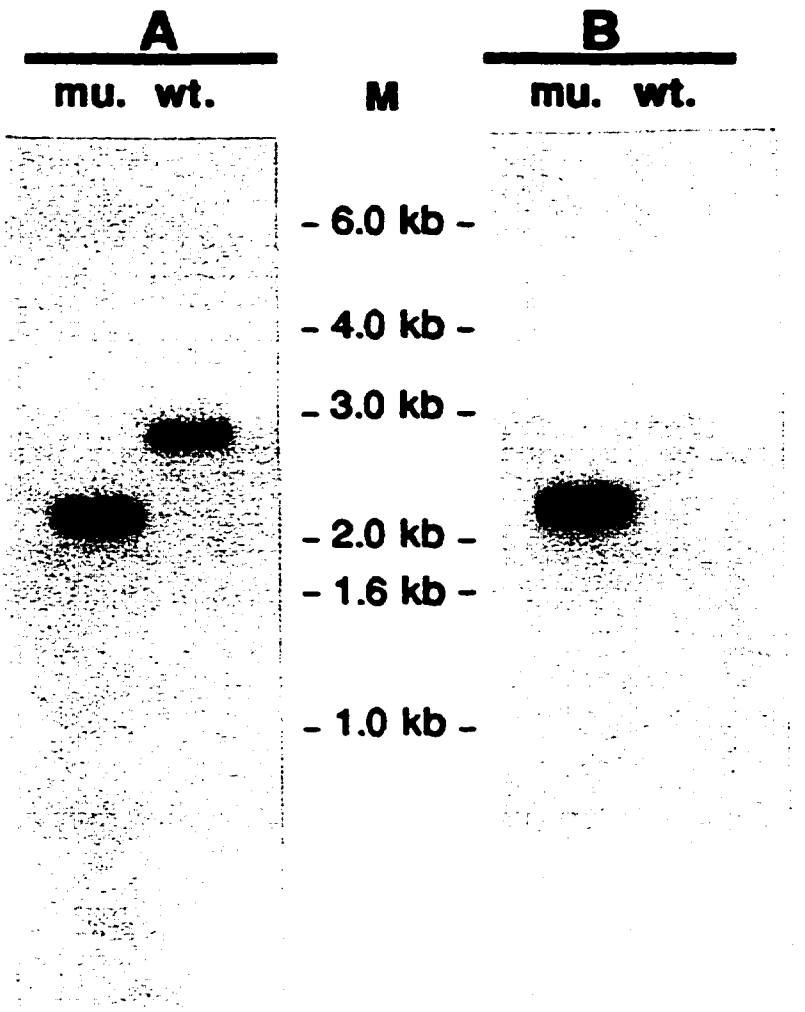


Figure 5.1.2 Southern blot analysis of BHV/IL1β and wild type BHV-1 DNA. Wild type genomic DNA (wt.) and BHV/IL1β genomic DNA (mu.) were digested with *Eco*RI and *Bgl* II. Two sets of digested samples, along with DNA size markers were separated on a 1% agarose gel and transferred to a nitrocellulose 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).

blot analysis confirmed that BHV-1/IL1β contained the boIL-1β gene in the expected configuration.

5.1.2 Detection of boIL-1β 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 transcription in BHV/IL1β infected cells was analyzed 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. 5.1.3). As shown in Fig. 5.1.3 (bottom panel), boIL-1β transcripts were first detected at 6 hrs p. i. followed by a gradual increase in expression until 10 hrs p. i..

5.1.3 Expression of boIL-1β 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 reacted infected cells with rabbit antibody against boIL-1β followed by reaction with fluorescein labelled goat anti-rabbit antibody. At 16 hours post-infection, infected cells showed bright cytoplasmic fluorescence (Fig. 5.1.4). No fluorescence was detected in control cells infected with wild type virus.

To further confirm that boIL-1β expressed by the recombinant virus had the predicted molecular weight, an immunoprecipitation assay was performed 24 hrs p. i... Metabolically labelled cellular and supernatant fractions were collected separately and immunoprecipitated with anti-boIL-1β antibody. A polypeptide with an apparent

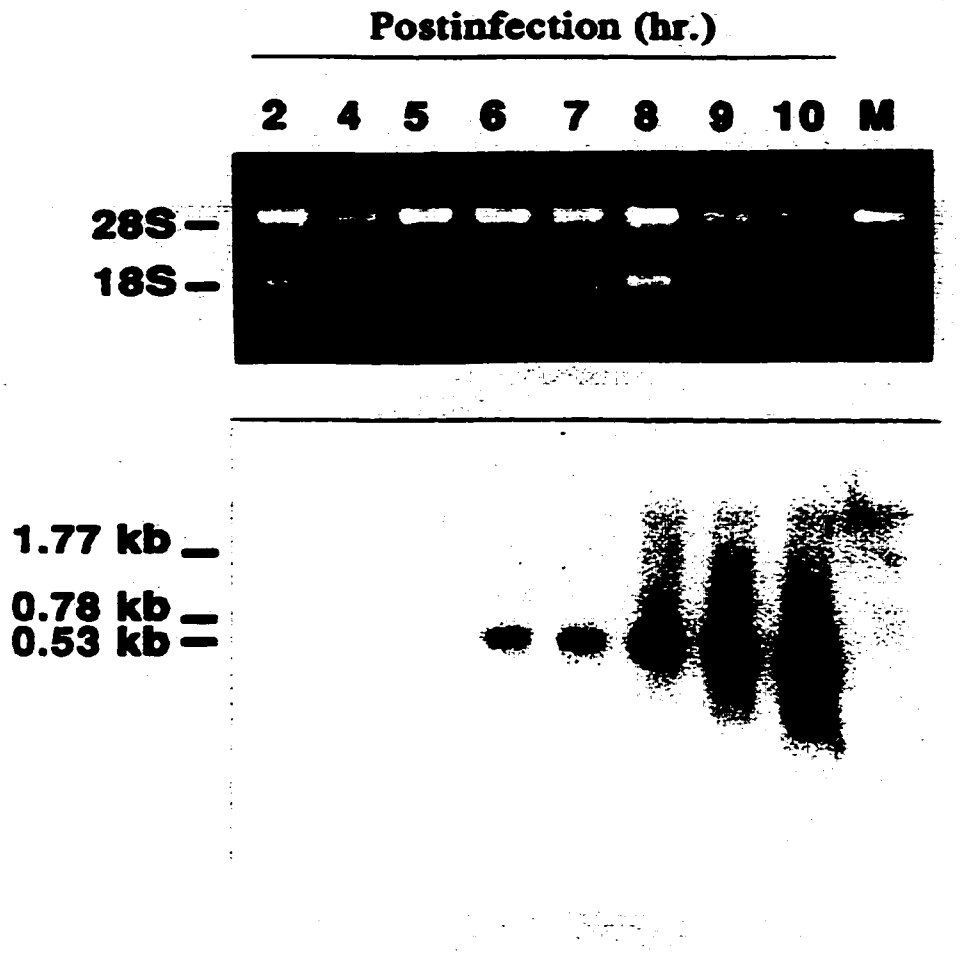


Figure 5.1.3 Transcription kinetics of recombinant boIL-1β in virus infected cells. At various time points post-infection, RNA samples were prepared and run on a 1.2% agarose gel (bottom figure). To confirm RNA integrity and approximate concentrations per lane, duplicate samples were stained with ethidium 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 collected at 10 hours.

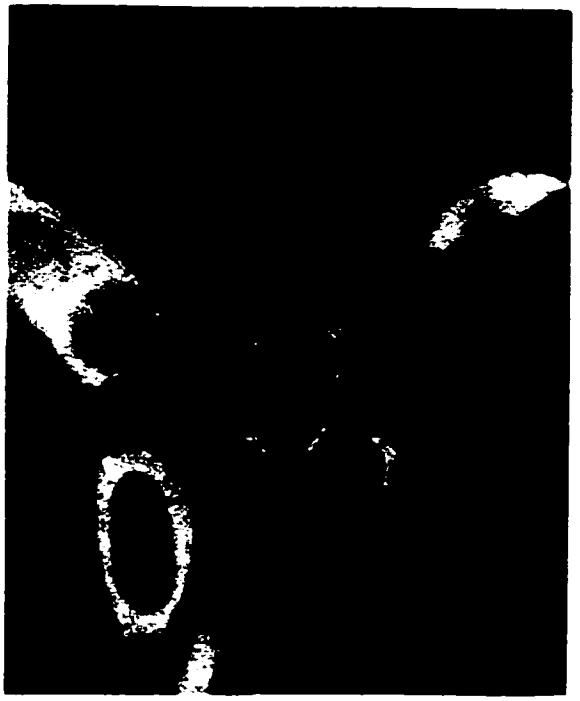


Figure 5.1.4 Indirect immunofluorescence demonstrating subcellular location of boIL-1β. MDBK cells were infected with BHV/IL1β at a M.O.I. of 0.01 for 16 h . Cells were fixed with 2% paraformaldehyde, permeabilized with methanol and incubated with rabbit anti-boIL1β serum at a 1:500 dilution followed by incubation with fluorescein labeled goat anti-rabbit antibody. Magnification 375X.

molecular weight between 17-18 kDa was recognized in both the supernatant and cellular fraction of BHV1/IL1β infected cells, but not in the BHV-1 wild-type virus infected cells (Fig. 5.1.5A). This indicated that boIL-1β was indeed produced by the recombinant virus and was also secreted into the medium, as would be predicted for a boIL-1β protein that contained a gC signal peptide. To further confirm the molecular weight of the recombinant boIL-1β protein and to assess whether the signal sequence was cleaved, a Western blot was performed (Fig 5.1.5B). Recombinant boIL-1β produced by BHV/IL-1B had a similar molecular weight to the 19.3 kDa rBoIL-1β standard. This indicated that the signal sequence was cleaved from the virus produced boIL-1β protein.

5.1.4 BHV/IL1β produces a biological active molecule

To determine whether the boIL-1β 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-1β was secreted into the medium, we collected culture supernatant from BHV/IL1β, gC$^-$, wild-type virus and mock infected MDBK cells. As shown in Fig. 5.1.6, IL-1 activity was detected 24 hours post-infection in BHV/IL-1β infected supernatant while no activity was detected in the controls. Based on our LM-1 bioassay, we calculated the level of boIL-1β expressed at 24 and 36 h to be approximately 160 units ml^{-1} and 320 units ml^{-1}, respectively. In addition, we calculated the specific activity of the boIL-1β produced by BHV/IL-1β to be 4.2×10^8 U/mg which was similar to the specific activity of the rBoIL-1β standard produced in *Escherichia coli*. (4.6×10^8 U/mg).

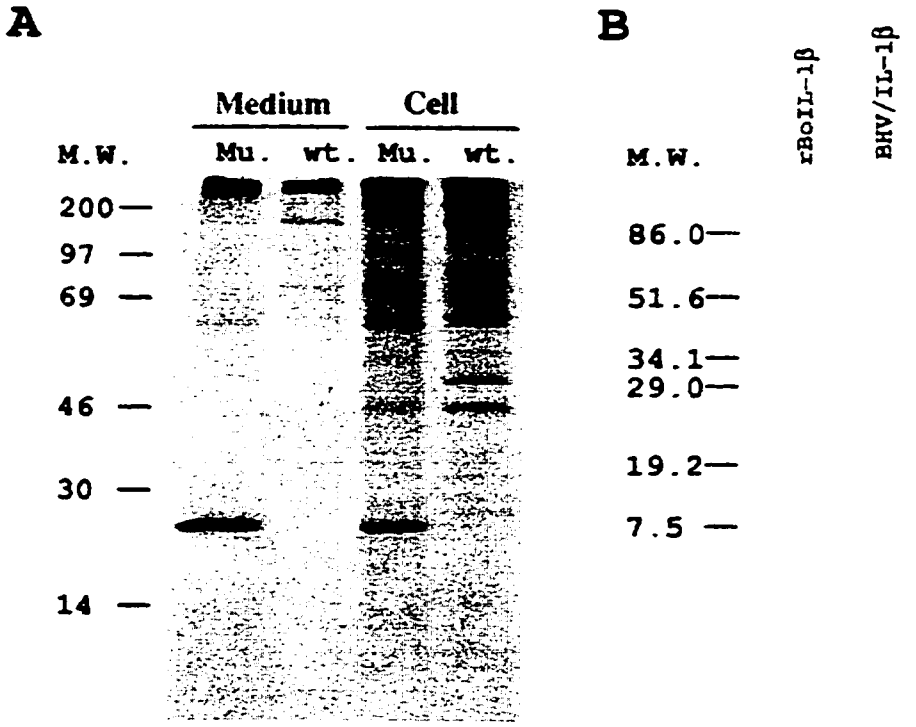


Figure 5.1.5 Detection of bovine interleukin-1β by immunoprecipitation assay and Western blot. MDBK cells were infected with either BHV1/IL1β (mu.) or wt BHV-1 (wt.) at an M.O.I. of 10 and labelled with 50 μCi [^{35}S] methionine per milliliter. After 24 hours of labelling, cellular and medium fractions were harvested separately and immmunoprecipitated using bovine IL-1β specific rabbit antiserum. (B) Western blot analysis of boIL-1β. BHV/IL1β supernatant was concentrated to 2 ng of boIL-1β per lane, denatured, and resolved by electrophoresis on a 10 % polyacrylamide-SDS gel. rBo-IL-1β at 2 ng per lane was used as a control. Molecular weight markers are indicated on the left margin.

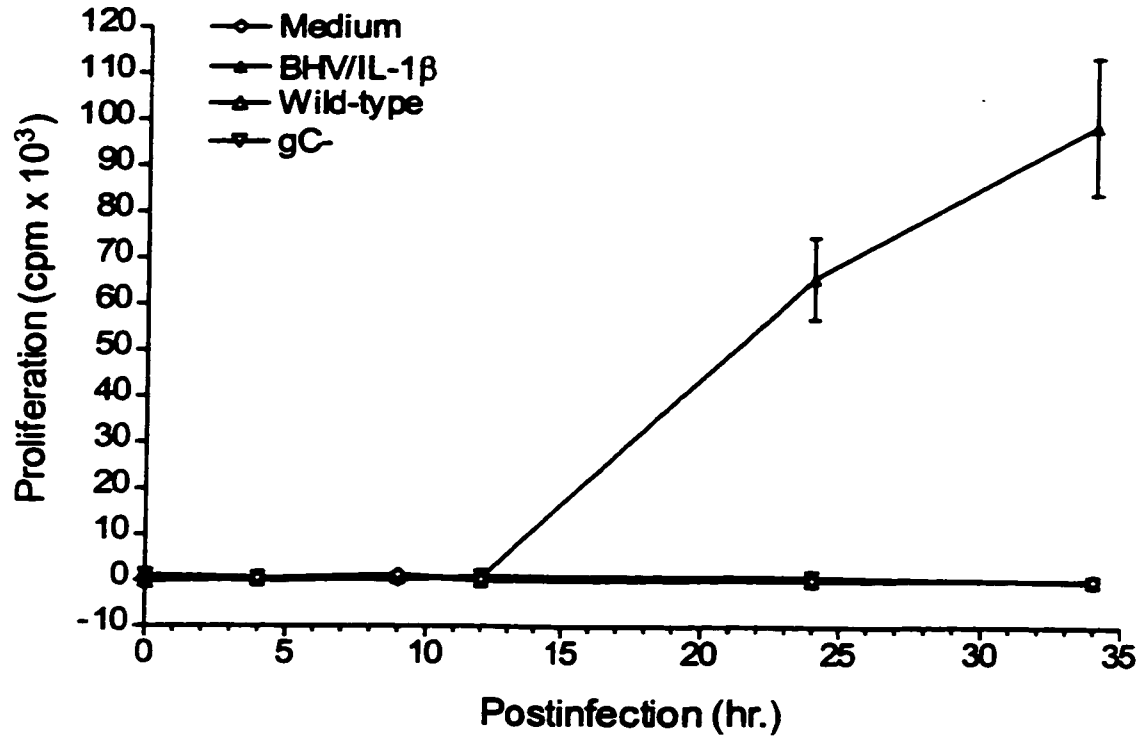


Figure 5.1.6 Bioassay for bovine IL-1β expression. Confluent monolayers of MDBK cells in 100 mm plates were infected with wild-type BHV-1, gC⁻ mutant and recombinant BHV/IL1β at a MOI of 5. At indicated times, the culture supernatants were harvested. Samples were assayed for IL-1β activity using a subclone (LM-1) of the D10.G4.1 ATCC cell line sensitive to interleukin-1. The results represent means ±S.D. of triplicate samples.

5.1.5 Expression of boIL-1β does not alter virus growth in cell culture.

To assess the growth efficiency of the recombinant BHV/IL1β virus, a growth kineics experiment was performed. MDBK cells were infected with wild-type BHV-1, gC⁻ mutant and BHV/IL1β. At various time points post-infection, supernatant samples were harvested and assayed for virus titer. As shown in Fig. 5.1.7, the gC⁻ deletion mutant and BHV/IL1β exhibited comparable growth kinetics; compared with wild type virus, both mutant viruses produced about a 10 fold lower virus titer at 24 hrs p. i.. Although BHV/IL1β appears to replicate slightly better than gC⁻ virus in this experiment, multiple experiments showed no statistically significant difference in growth between these viruses. The observation that BHV/IL1β and the gC⁻ mutant had similar growth curves suggests that boIL-1β expression did not affect virus growth *in vitro*.

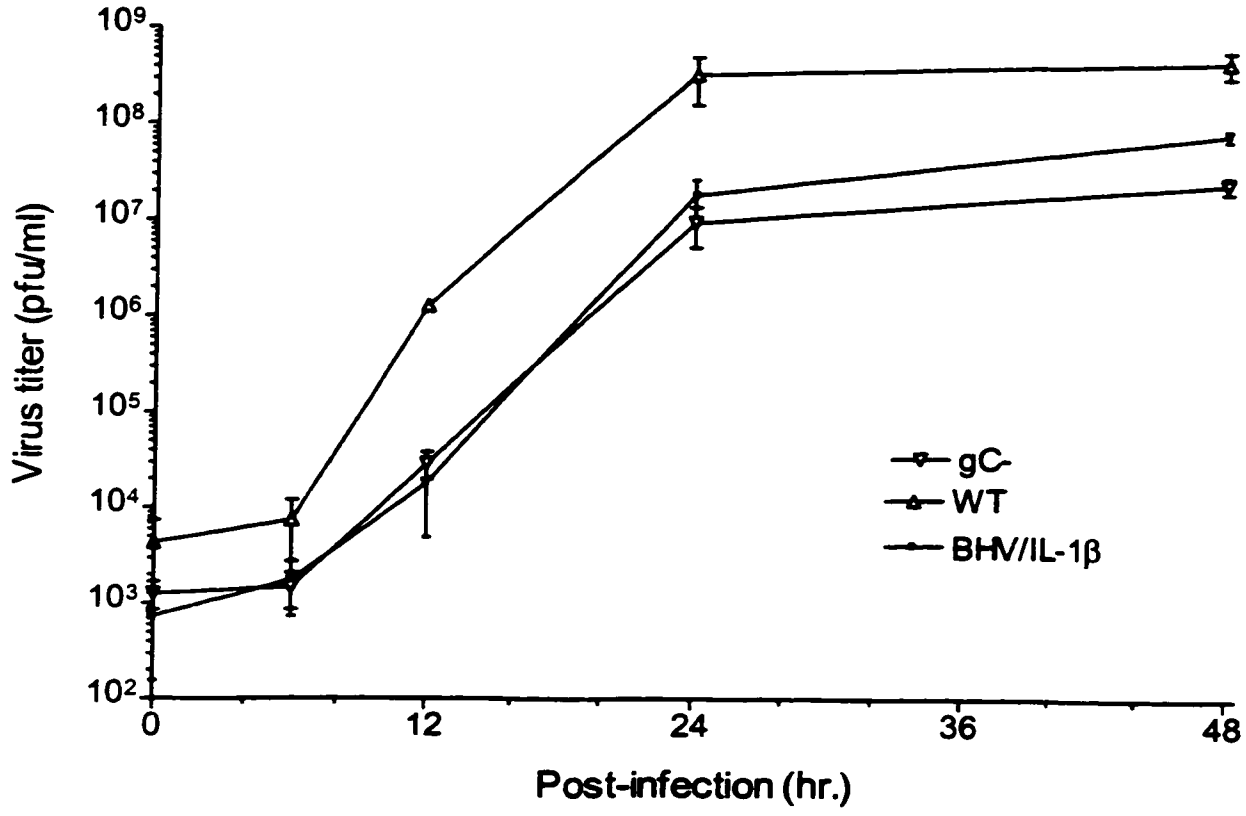


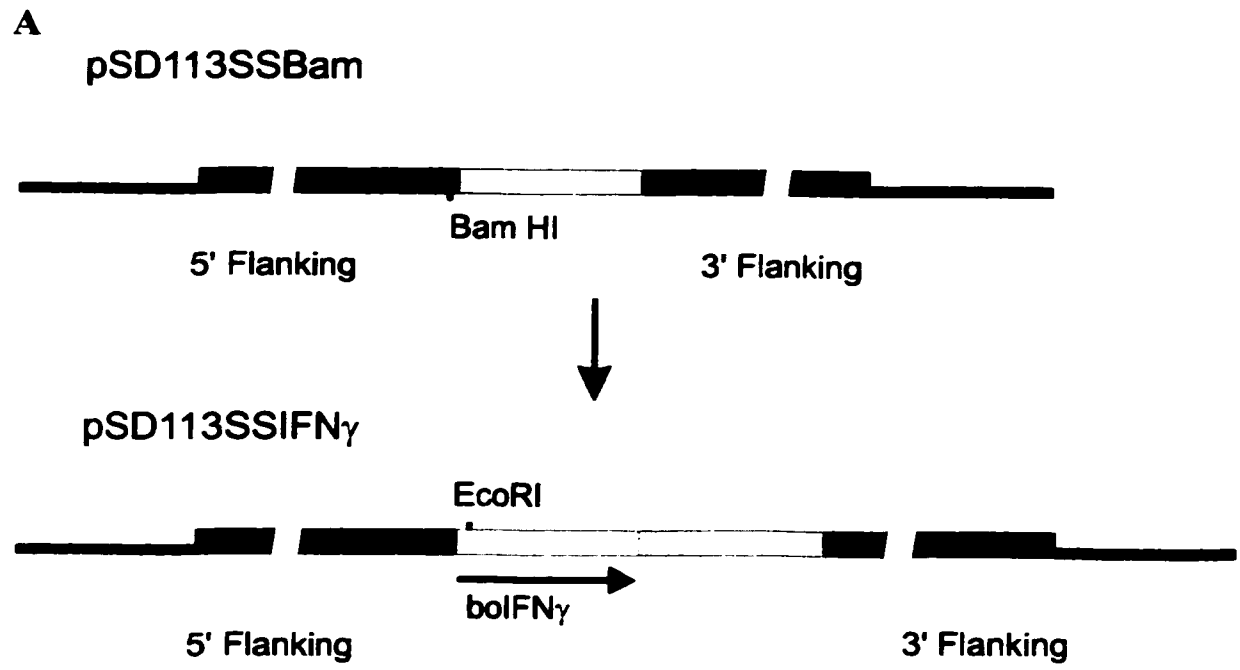
Figure 5.1.7 Growth kinetics experiment. Confluent MDBK cells in 6 well culture plates were inoculated at a M.O.I. of 5. After 1 h adsorption at 37°C, virus inoculum was removed, cells were washed three times with MEM and incubated in 2 ml of MEM supplemented with 10% FBS. At various times post-infection, 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.

5.2 Expression of Bovine Interferon-γ in a Bovine Herpesvirus-1 Vector

Interferons have been used as immunological adjuvants in cattle because of their ability to influence a variety of immunological regulatory activities (Babiuk *et al.*, 1985, 1987; Chiang *et al.*, 1990; Bielefeldt Ohmann *et al.*, 1987, 1991). Interferon-γ has numerous functions which may be beneficial in modulating viral diseases in cattle. These include: (1) Fc receptor upregulation; (2) activation of macrophages and PMN phagocytic functions; (3) upregulation of MHC class I and class II; and (4) inhibition of viral replication (Campos *et al.*, 1989). Therefore, the construction of a BHV-1 vector expressing bovine interferon-γ could potentially have beneficial immunomodulating effects during a BHV-1 infection and a secondary bacterial infection.

5.2.1 Construction of transfer vector pSD1113SSIFN-γ

Bovine interferon-γ is initially produced as a 166 amino acid precursor that is cleaved into a 143 amino acid mature protein with a predicted molecular weight of 16.8 kDa (Cerreti *et al.*, 1986). To express boIFN-γ, we used our gC transfer plasmid pSD113SSBam and inserted the mature sequence of boIFN-γ into the gC signal cassette (Figure 5.2.1A). Our cleaved product was predicted to retain the 143 a.a. from the mature sequence plus 5 a.a. from the gC fusion protein at the amino terminus (Figure 5.2.1B). Recombinant BHV-1 was generated by co-transfection of MDBK cells with transfer plasmid pSD113SSIFN-γ and purified DNA from BHV-1/δgIII/LacZ (Liang *et al.*, 1992). Progeny viruses were screened by blue plaque assay.



B

gC signal/ boIFN-γ junction

MGPLGRAWLIAAIFAYVLAARDPEFMQGQFF ...

*

Figure 5.2.1 Construction of transfer vector pSD113SSIFN-γ. (A) Solid line represents the pBR322 backbone; Clear bar represents boIFN-γ; Grey Bar represents the gC flanking sequence; light gray represents the gC fragment (see Materials and Methods for detail procedures). (B) Amino acid sequence at the junction between the gC signal cleavage site (Indicated by arrow) and the start of the mature sequence of boIFN-γ (Indicated by star).

White plaques, that had lost the lacZ insert, 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 bovine interferon gene into the gC region, Southern blot analysis was carried out using total cellular DNA. DNA from both wild-type virus and recombinant BHV-1/IFNγ was prepared 20 hours p. i., digested with *Eco*RI and *Bgl*II, separated on a 1 % agarose gel, transferred to nylon membrane and hybridized with either a ^{32}P-labelled DNA probe corresponding to the gC gene or a probe from the boIFN-γ gene (Figure 5.2.2). The results were consistent with what would be predicted. The gC probe containing both 5' and 3' ends hybridized with a 3.0 kb fragment in the wild-type lane, whereas recombinant BHV-1/IFNγ had two fragments of 1.5-kb and 0.7-kb. The insertion of the boIFN-γ created a unique *Eco*RI site in front of the gC signal sequence which resulted in two fragments. The boIFN-γ probe (*Eco*RI-*Ssp*I) did not hybridize wild-type DNA; while, a 1.5 kb fragment was detected with BHV-1/IFNγ DNA. This band corresponded with the gC region in the wild-type lane. The results from our Southern analysis confirmed that the recombinant BHV-1/IFNγ virus contained the boIFN-γ gene in the expected configuration.

5.2.2 Expression and modification of boIFN-γ protein

It has been determined that the human IFN-γ gene produces a protein with either one or two N-glycosylations resulting in a 20 kDa or 25 kDa molecule (Rinderknecht *et al.*, 1984). Naturally occurring boIFN-γ has two possible N-glycosylated sites (Cerriti *et al.*, 1986). To determine whether recombinant boIFN-γ protein was

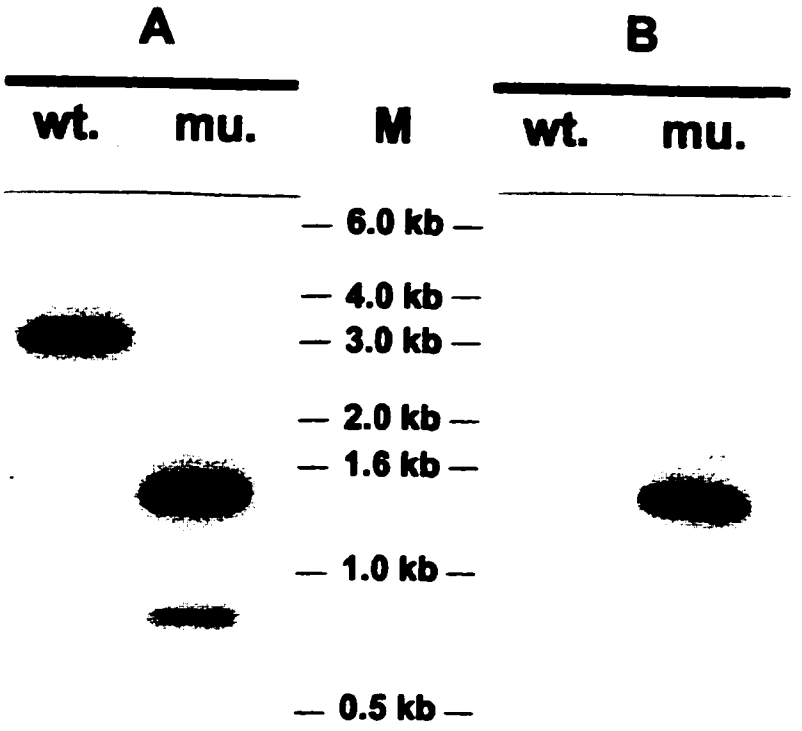


Figure 5.2.2 Southern blot analysis of BHV-1/IFN-γ and wild-type BHV-1 DNA. Wild-type genomic DNA (wt.) and BHV-1/IFNγ genomic DNA (mu.) were digested with *Eco*RI and *Bgl* II. Two sets of digested samples and a DNA marker were separated on a 1% agarose gel. (A)One set of samples was hybridized to a probe corresponding to the gC gene. (B) The second set was probed with an *Eco*RI – *Ssp*I fragment from pSD113SSIFN-γ.

expressed and retained N-linked glycosylation, immunoprecipitated proteins were treated with N-glycosidases. After labelling, cellular and supernatant fractions were divided and immunoprecipitated with anti-IFN-γ antibody (Figure 5.2.3). Untreated samples (lanes 2 and 6) contained a polypeptide with a molecular weight between 25-28 kDa indicating that rboIFN-γ was expressed and was glycosylated at both sites since the predicted molecular weight, without glycosylation, is 17 kDa. Control wild-type Cooper virus samples were negative (lanes 1and 5). After treatment with endoglycosidase H, there was no shift in the cellular fraction (lane 3) indicating that the protein is Endo H resistant. However, the untreated and endo H treated samples from the supernatant fractions contained an additional 17 kDa product. This suggested that a natural IFN-γ degraded product occurs in the supernatant since no comparable product existed in the cellular fraction. After treatment with N-glycosidase F, the 25-28 kDa protein completely shifted down to the predicted 17 kDa molecular weight of rboIFN-γ. This molecular shift confirmed that BHV-1IFNγ produced a protein with two N-linked complex glycans, if we assume that each glycosylation added approximately 5 kDa to the molecular weight of the protein (Rinderknecht *et al.*, 1984). The protein in panel 8 produced a diffuse band which may have been due to the ability of boIFN-γ to form non-covalent dimers (Ealick *et al.*, 1991; Langer *et al.*, 1994).

5.2.3 BHV-1/IFNγ produced biologically active IFN-γ

Next, we determined if recombinant boIFN-γ protein, produced from BHV-1/IFNγ infected cells, was biologically active. MDBK cells were infected at a MOI of

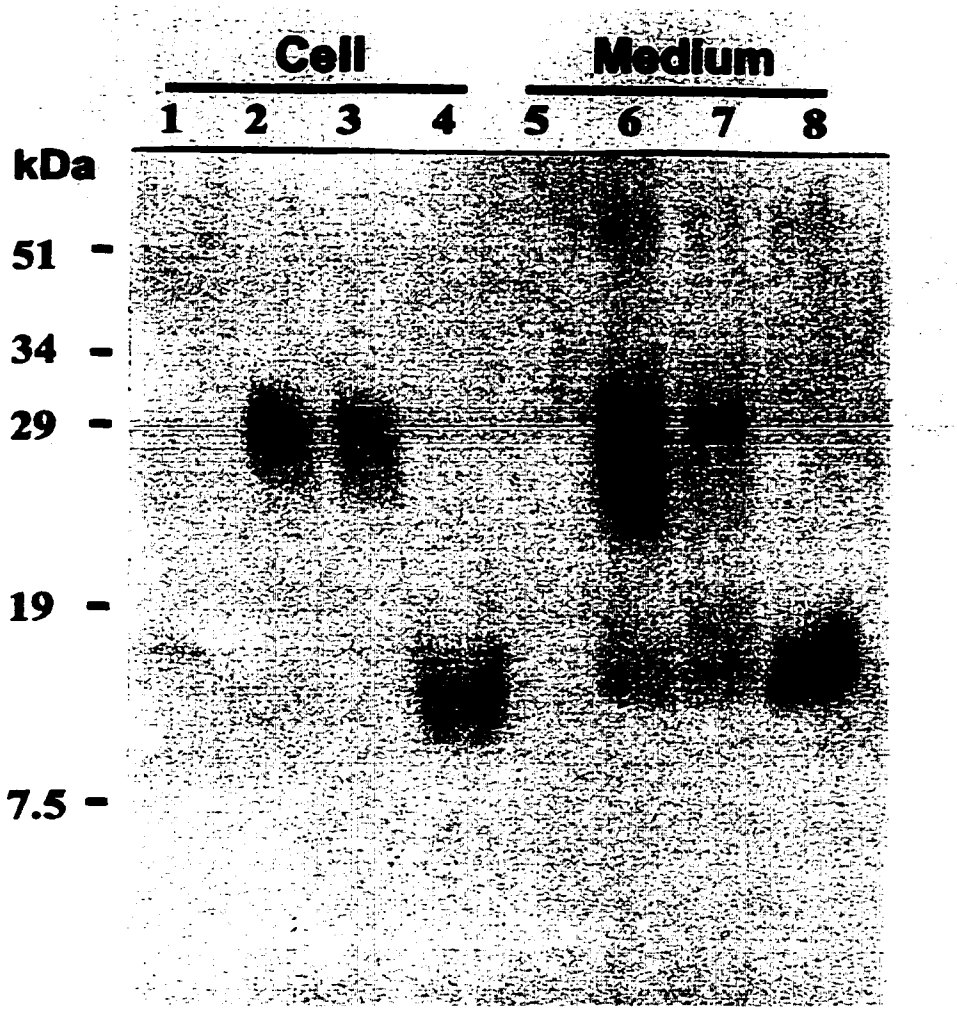


Figure 5.2.3 Glycosylation analysis of IFN-γ from BHV-1/IFNγ. MDBK cells were infected with wild-type Cooper or BHV-1/IFN-γ at a MOI of 10 PFU and metabolically labeled with 50μCi of [^{35}S] methionine. After labeling, cellular and medium fractions were immunoprecipitated with rabbit anti-boIFN-γ sera. Lanes 3 and 7 were treated with endoglycosidase H (Endo H); and lanes 4 and 8 were treated with N-glycosidase F (PNGase F). Lanes 1 and 5 contained wild-type Cooper virus. Lanes 2 and 6 contained untreated BHV-1/IFNγ. Precipitated proteins were analysed by SDS-PAGE. Molecular weight markers are indicated in the left margin.

5 PFU. Samples were collected from culture supernatants and cellular fractions at various times post-infection. To remove viable BHV-1 virus from the vesicular stomatis virus (VSV) interferon inhibition assay, samples were exposed to U. V. irradiation to inactivate the BHV-1 virus. Interferon activity was detected as early as 6 hrs p. i. and this activity was detected throughout the sampling period (Fig. 5.2.4). At 36 hrs p. i.., the supernatant fraction contained the majority of anti-viral activity (512 U/ml) while activity in the cellular fraction remained constant after 24 hrs (256 U/ml). Furthermore, by using a boIFN-γ capture ELISA, the specific activity of the recombinant boIFN-γ produced by BHV/IFN-γ was calculated to be 2.8×10^6 U/mg (see Material and Method for calculation). This specific activity is comparable to the *E. coli* produced rboIFN-γ standard of 3×10^6 U/mg (Cyanamid). Therefore, BHV-1/IFNγ produced an active interferon molecule with anti-viral biological activity and specific activity comparable to *E. coli* recombinant boIFN-γ.

To ascertain whether recombinant boIFN-γ from BHV-1/IFN-γ virus had immunological activity, alveolar macrophages (AM) from a BHV-1 sero-negative calf were incubated with supernatant from cultures infected with BHV-1/IFNγ virus and the up-regulation of MHC II was monitored by FACS analysis. As shown in Figure 5.2.5, AM incubated with control media had MHC II expression on 28.95 % of cells. Incubation with 100 ng/ml of recombinant boIFN-γ, induced MHC expression on over 78.5 % of the AM. The addition of Cooper and gC-/LacZ+ BHV-1 supernatant to the AM cultures did not significantly change MHC II expression, relative to the control culture. In contrast, the addition of supernatant from BHV-1/IFNγ virus infected

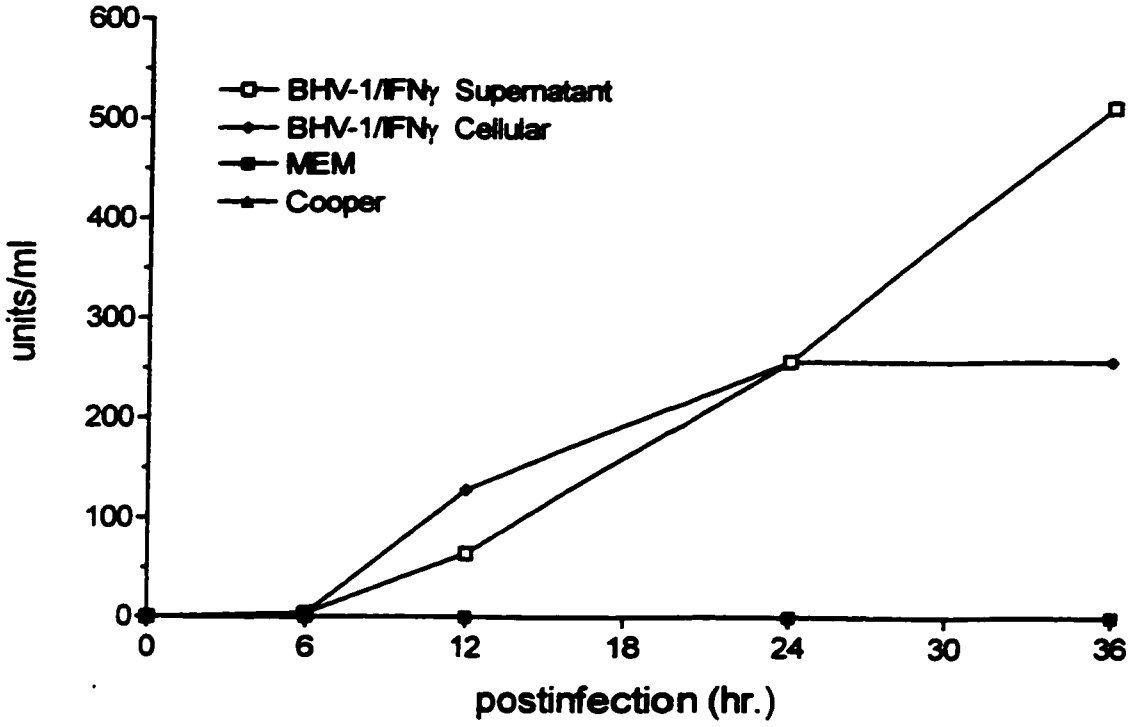
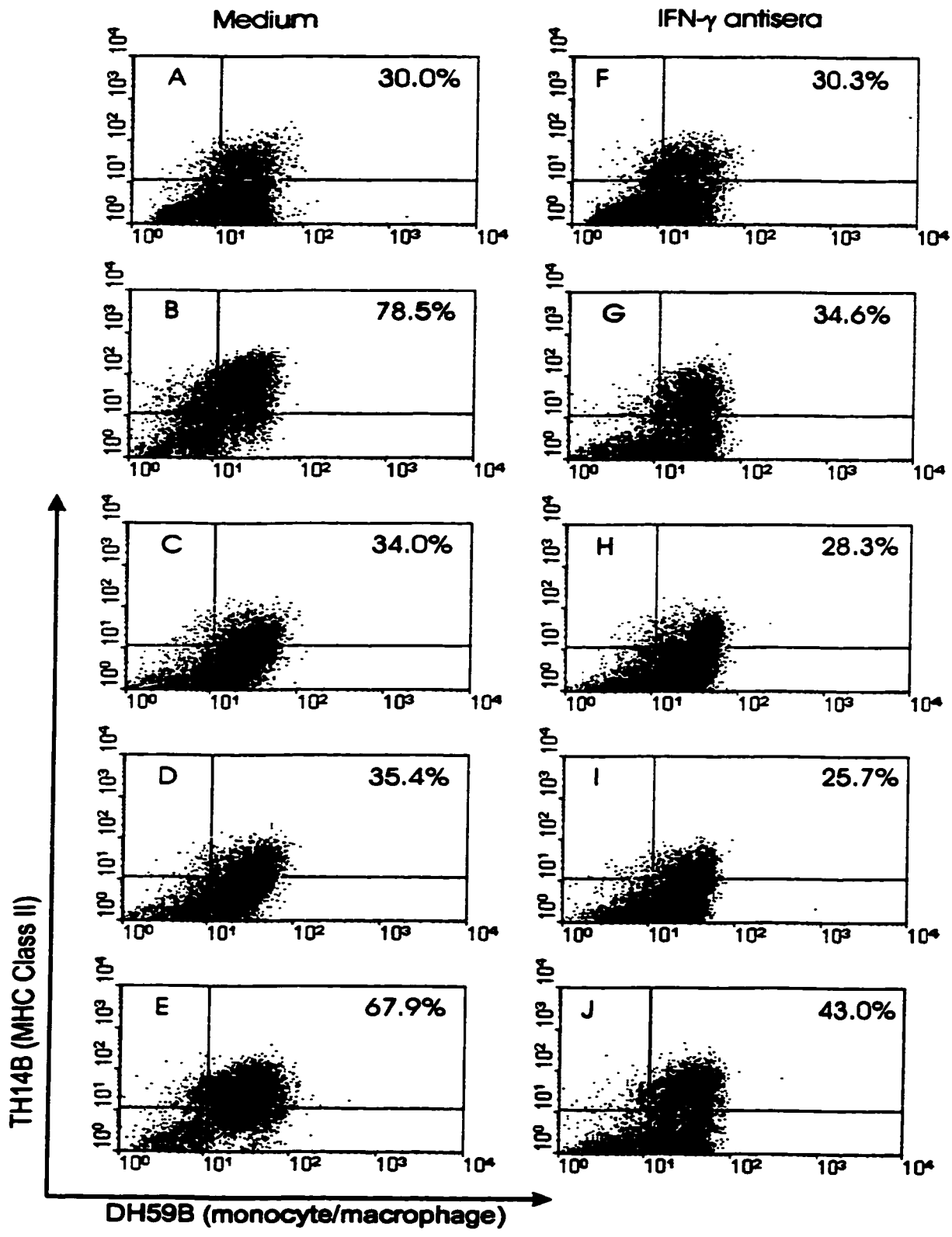


Figure 5.2.4 Bioassay for bovine IFN-γ activity. Confluent monolayers of MDBK cells in a 6-well plates were infected with wild-type Cooper BHV-1 and recombinant BHV-1/IFNγ. At times indicated culture supernatant and cellular fractions were harvested. Samples were assayed for the boIFN-γ activity using a standard VSV plaque inhibition assay.

Figure 5.2.5 Induction of MHC II expression on alveolar macrophages. Lung alveolar macrophages were isolated from a 3 month old calf by lung lavage (see material and methods). Alveolar macrophages were pre-incubated for 72 hours with culture supernatants being tested. MHC II expression on Am was quantified by dual staining with monocyte specific mAb (DH59B) and MHC Class II specific mAb (TH14B). Samples were as follows: Panels A and F were negative controls with MEM medium; panels B and G were positive control medium containing 100 ng/ml of boIFN-γ. Panels C and H contained Cooper virus supernatant; Panels D and I contained gC-/LacZ+ virus supernatant and panels E and J contained BHV-1/IFNγ virus supernatant. In addition, panels F to G contained 1/20 dilution of anti-IFN-γ polyclonal antibody. The percentages of monocyte/macrophages with MHC class II expression are indicated in the upper right corners of each panel.


Medium
IFN-γ antisera
A
30.0%
F
30.3%
B
78.5%
G
34.6%
C
34.0%
H
28.3%
D
35.4%
I
25.7%
E
67.9%
J
43.0%
TH14B (MHC Class II)
DH59B (monocyte/macrophage)

cells, induced MHC II expression on 67.8 % of the AM. To confirm that up-regulated MHC II expression was due to IFN-γ, neutralizing anti-IFN-γ antibody was added. As shown in Figure 5.2.5. (panel F and G), the anti-IFN-γ antibody did not alter MHC expression in the control culture. However, MHC II expression on AM incubated with 100 ng/ml rboIFN-γ was reduced to the background level of 34.6%. Similarly, addition of anti-IFN-γ antibody to cultures containing Cooper and gC-/LacZ+ supernatant did not change MHC expression significantly (panel H and I). However, BHV-1/IFNγ induction of MHC II expression was reduced from 67.9% to 43 % on AM following the addition of anti-IFNγ antibody (panel E and J). This neutralization of IFN-γ activity confirmed that recombinant BHV-1/IFNγ produced biologically active IFN-γ protein.

5.2.4 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 wild-type Cooper, gC-/LacZ+, and BHV-1/IFN-γ. Both recombinant viruses exhibited similar growth kinetics with viral yields approximately 1-2 $\log_{10}$ lower than wild-type virus (Figure 5.2.6). The maximal yield for recombinant gC$^-$ phenotype viruses was approximately 4×10^7 PFU/ml while wild-type Cooper produced greater than 2×10^8 PFU/ml . Therefore, the production of biologically active boIFN-γ did not alter the *in vitro* growth of recombinant BHV-1.

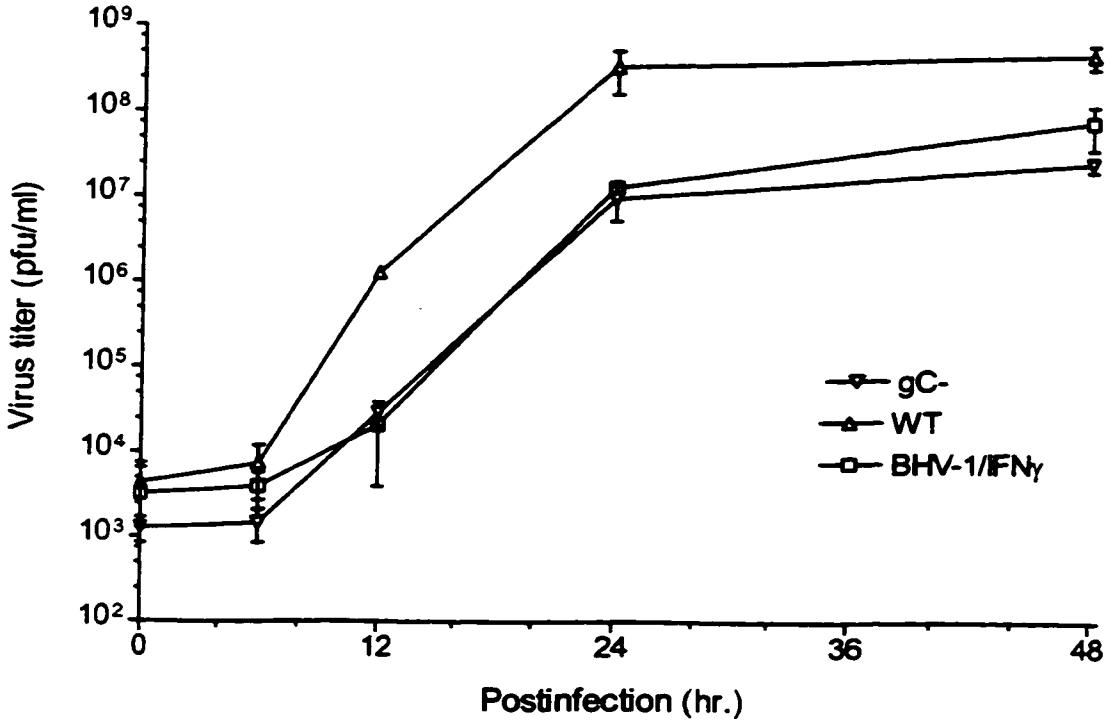


Figure 5.2.6 Growth kinetics experiment. Confluent MBDK cells were inoculated at a m.o.i. of 5. After 1 h , cells were washed three times and incubated with MEM supplemented with 10% FBS. At various times p. i. , medium was collected and the titer for BHV-1 assayed. The supernatant was titered by plaque assay in 24-well plates. Wt, wild-type Cooper strain; gC⁻, a gC negative mutant; BHV-1/IFNγ ; gC⁻ negative mutant expressing boIFN-γ.

5.2.5 The *in vitro* effect of interferon on BHV-1 growth

To study the effects of recombinant bovine interferon-α or recombinant bovine interferon-γ on the *in vitro* growth of BHV-1, MDBK cells were cultured overnight with IFN-γ and IFN-α at various concentrations ranging from 0.1 units/ml to 1000 units/ml. Following the overnight incubation, cells were infected with a fixed amount of BHV-1 virus or control VSV virus. To analyze viral growth inhibition, the number of viral plaques were counted (see Figure 5.2.7). Control wells contained 100 units/ml of serially diluted IFN-γ and 100 pfu of VSV virus. One unit of biological activity was defined as a 50 % inhibition of viral growth. A 50% inhibition of VSV growth was evident with 1 to 10 units/ml of both IFNα and IFN-γ. However, there were only a 9% and a 23 % reduction in BHV-1 plaques with 1 unit/ml of IFN-γ and IFN-α, respectively. A two $\log_{10}$ higher IFN concentration was required to inhibit BHV-1 to a level comparable to VSV. Only at 1000 units/ml could IFN significantly inhibit BHV-1 plaque formation with a 68 % reduction by IFN-γ and a 62 % reduction by IFN-α. Plaque size was also slightly smaller than in control wells at IFN concentrations higher then 100 units/ml. Therefore, unlike VSV replication, which is inhibited at low concentrations of IFN, BHV-1 replication is much more resistant to IFN-α and IFN-γ inhibition.

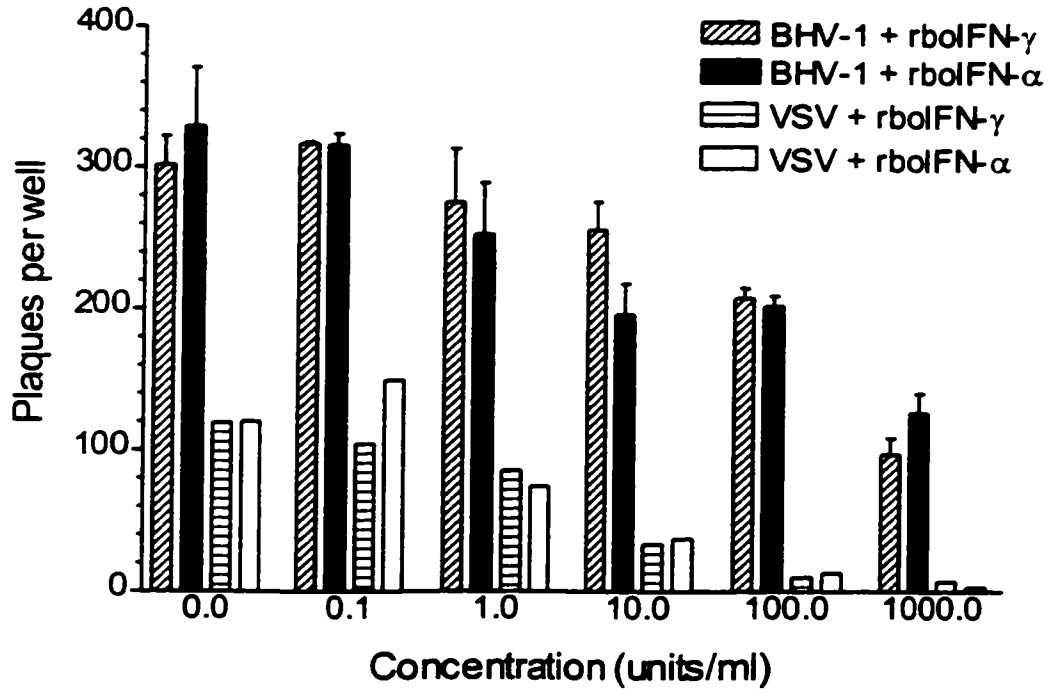


Figure 5.2.7 Interferon inhibition of viral growth. MDBK cells cultured in 24 –well plates were pre-incubated overnight with various concentrations of recombinant bovine IFN-α and recombinant bovine IFN-γ. The following day, cells were infected with 300 pfu of BHV-1 or 100 pfu of VSV virus for 1 h at 37 °C. The inoculum was removed and replaced with a methyl-cellulose overlay. After plaques had developed, the cells were stained with crystal violet and plaques were counted. Results are expressed as mean ±S.D. of triplicate cultures.

5.3 Cattle challenge

One of the major problems associated with BHV-1 vaccine development has been that modified-live virus vaccines can produce clinical symptoms and become latent (van Drunen Little-van den Hurk *et al.*, 1993). Upon reactivation, the transmission to naive animals may result in serious clinical problems, especially in new-born calves (Bryan *et al.*, 1994). One of the rationales for investigating new vaccine approaches has been to resolve these critical problems. In addition, the production of cytokines at the site of viral replication could have significant immunomodulation potential which may have beneficial effects assisting the host to control viral replication, prevent latency and possibly prevent secondary bacterial infections. Therefore, to determine the immunomodulation potential of recombinant BHV-1/IFNγ, we intranasally challenged calves and monitored the clinical and immune responses. After infection, dexamethasone treatment was used to determine the latency status of recombinant virus.

Three groups of 3 calves were challenged intranasally for 5 minutes with a Devilbiss nebulizer containing 4×10^7 PFU/ml of either wild-type Cooper strain, gC-/LAC+ mutant strain or recombinant BHV-1/IFN γ. Each group of calves was housed in a separate isolation room to avoid viral cross contamination. Animals were monitored daily for 10 days by a veterinarian who, without knowledge of the virus used for infection, assessed the clinical score for each group. During the first 10 days p. i., nasal secretions were collected with nasal swabs and cotton tampons for virus isolation and

assay of BHV-1 specific immunoglobulin, respectively. Blood was collected for flow cytometric analysis of leukocyte populations and serum was collected for analysis of BHV-1 specific antibodies. After the initial clinical period, animals were bleed weekly to analyze lymphocyte proliferative responses, bovine IFN-γ ELISPOTs and serum antibody titer. To determine the latency status of each group, the calves were injected i.m. with dexamethasone (0.1 mg/kg) once daily for a 5-day period, starting at week 5 post-infection (Homan and Easterday, 1983).

5.3.1 Clinical response

All sero-negative calves responded to infection with an increased rectal temperature (≥39.5°C). Elevated rectal temperatures began on day 2 p. i. and peaked at days 4-5 p. i. (Figure 5.3.1A). One calf in the Cooper group (97-04) did not develop an increase in body temperature. BHV-1 specific antibody titer and lymphocyte proliferative responses conclusively showed that calf 97-04, whose BHV-1 serum antibody titre was at the cut-off level for sero-negative animals before challenge, had prior exposure to BHV-1. Therefore, analysis of the Cooper group excluded animal 97-04 unless otherwise indicated. Overall, body temperature increases were similar in the three groups and paralleled what would be expected in the BHV-1 challenge model (Wyler *et al.*, 1989; Yates, 1982). The expression of boIFN-γ by BHV-1/IFN-γ had no apparent effect on the amplitude or duration of the fever response.

Clinical symptoms were evident on day 2 p. i. and peaked at days 4-6 p. i. for all groups (Figure 5.3.1B). Clinical symptoms included increases in nasal discharge, coughing, nasal mucosa hyperemia and plaque formation in all groups. Lesions in

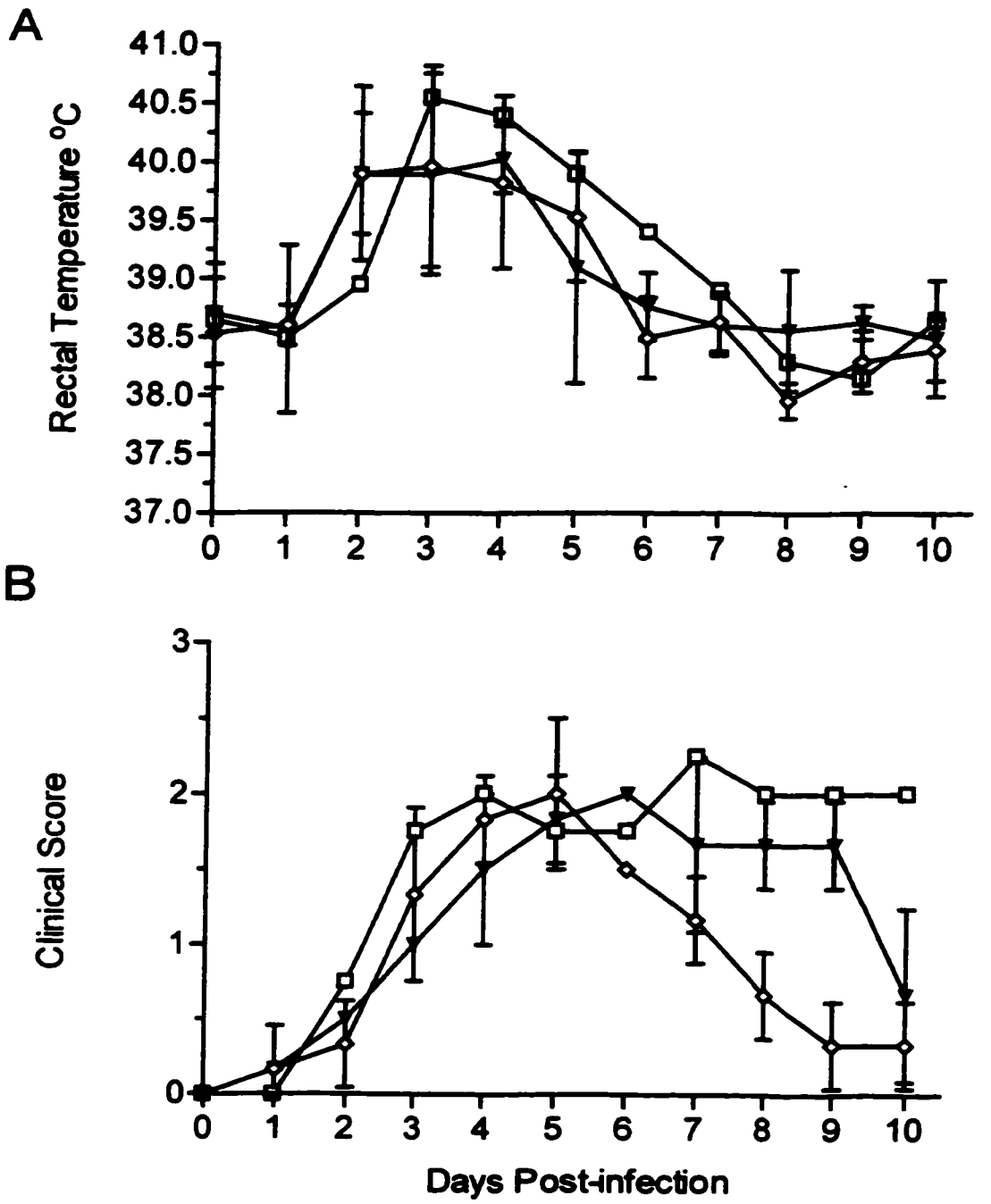


Figure 5.3.1 Clinical responses of BHV-1 infection. (A) Daily rectal temperatures. (B) Clincal scores. Calves were challenge with 4 $\times 10^7$ PFU/ml of Cooper (square), gC-/LacZ+ (diamond) and BHV-1/IFNγ (triangle) for 5 minutes.

calves infected with recombinant BHV-1 gC-/LacZ+ began resolving at day 7 p. i., while calves in the recombinant BHV-1/IFNγ and Cooper groups had clinical lesions that persisted until days 9 and 10 p. i., respectively. The higher clinical scores for the Cooper group may reflect a sight increase in lesion size which may correlate with increased viral growth relative to the gC minus phenotype of both recombinant viruses. The quantification of lesion size *in vivo* is problematic, since it relies on subjective observation.

5.3.2 Viral titers

Virus was isolated in nasal swabs from all sero-negative animals. Viral shedding peaked at day 6 p. i. (Figure 5.3.2). A comparison of viral shedding among groups showed that both gC$^-$ phenotype viruses shed 1-2 logs lower than wild-type Cooper virus. This difference in shedding was similar to the growth difference observed *in vitro* (see Figure 5.2.6). A comparison between recombinant gC deleted viruses (gC-/LacZ+ and BHV-1/IFN-γ) showed no significant differences in viral shedding, indicating that the expression of boIFN-γ at the site of infection did not have a significant effect on viral growth. 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.

The duration of virus shedding could not be effectively compared among groups, because the tampon collection method for nasal secretions did not consistently detect gC$^-$/LacZ+ or BHV-1/IFNγ viruses at low titers. Nevertheless, all animals were shedding virus on day 6 p. i.. This observation established that the duration of virus

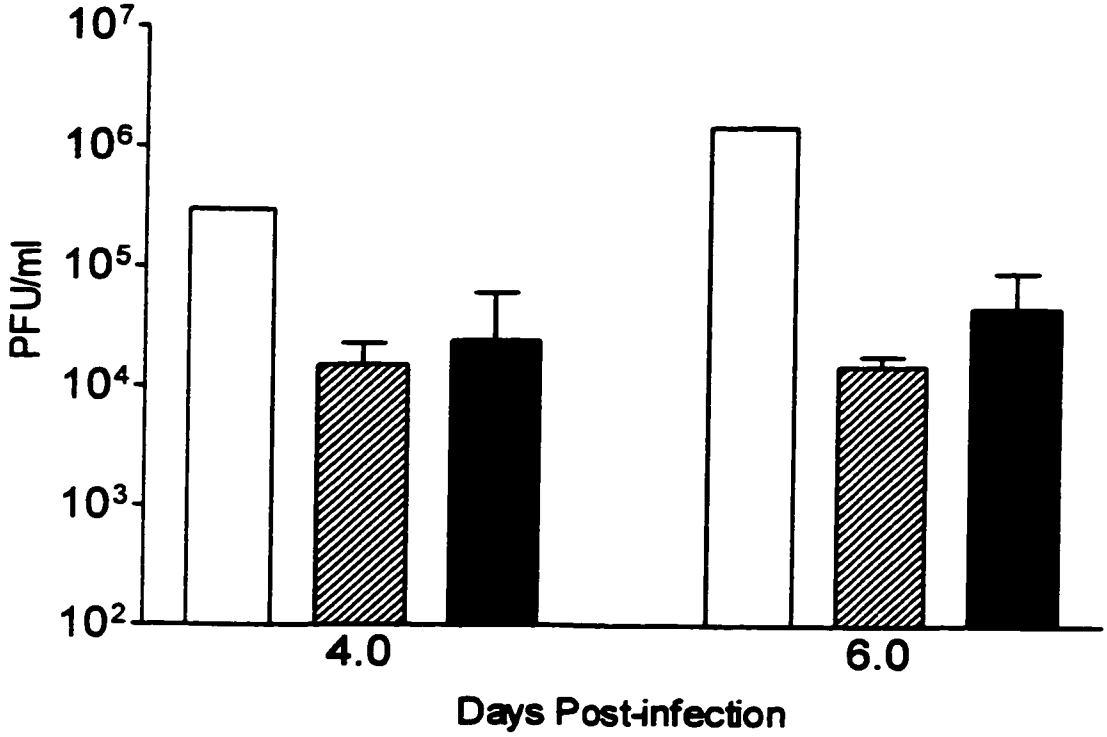


Figure 5.3.2 Comparison of viral shedding. Nasal secretions were titered for Cooper (open bar), gC-/LacZ+ (hatched bar) and BHV-1/IFNγ (solid bar). The mean value for the Cooper group represents two animals. Results are expressed as the mean ± S.D.

shedding was not significantly affected by the anti-viral activity of recombinant IFN-γ expressed at the site of infection. Furthermore, a comparison of tampon and nasal swab collection methods showed a significant difference in the detection of recombinant virus in nasal secretions when compared to wild-type Cooper virus. The significance of this observation will be discussed at the end of this section (see Figure 5.4.1).

5.3.3 Changes in blood leukocyte subpopulations

During BHV-1 infection, changes in circulating leukocytes occur within the peripheral blood mononuclear cell (PBMC) populations (Griebel *et al.*, 1987). To study the effects of recombinant BHV-1/IFN-γ on PBMC population dynamics, we monitored changes by flow cytometric analysis and by total and differential white blood cell counts. In Table 5. 3. 1., total and differential counts revealed a decrease in white blood cells, including lymphocytes, monocytes and total mononuclear cells. However, there were no significant differences when subpopulation changes, three days p. i., were compared between recombinant BHV-1 groups and the control wild-type Cooper virus.

Table 5.3.1. Changes in haematological profiles following recombinant BHV-1 challenge at day 0 and day 3 p. i. Cell number x 10^9/litre.

Virus	Day	WBC	Lymphocytes	Monocytes	MNC
Cooper	0	9.73 ± 1.16	5.51 ± 1.07	0.5 ± 0.15	6.01 ± 1.12
	3	7.67 ± 1.23	4.73 ± 0.88	0.25 ± 0.09	4.98 ± 0.96
gC-/LacZ+	0	8.47 ± 0.12	5.08 ± 1.13	0.36 ± 0.13	5.45 ± 1.23
	3	6.47 ± 0.12	4.73 ± 0.73	0.19 ± 0.17	4.92 ± 0.56
BHV-1/IFNγ	0	10.53 ± 1.35	7.21 ± 0.72	0.41 ± 0.15	7.62 ± 0.63
	3	8.50 ± 1.39	6.26 ± 0.93	0.27 ± 0.08	6.53 ± 0.86

Similarly, FACs analysis of leukocyte sub-populations 3 days p. i. revealed an overall decrease in T-lymphocytes (CD3, CD4. CD8), IgM B cells (PIg45A), and monocytes/granulocyte (DH59B). Total B cell number (CD72)/ml increased (DU2-104), while dual labelling for MHC class II and CD4 showed no changes among groups (Figure 5.3.3). A comparison of the changes in leukocyte number and phenotype among the different experimental groups revealed no significant differences in leukocyte subpopulations or MHC expression on CD4 cells. The pattern of change in cell number was similar to what has been previously reported for acute BHV-1 infection with a T cell lymphopenia and a relative increase in the percentage of B cells (Griebel *et al.*, 1987).

5.3.4 Haptoglobin response and IFN-γ levels in nasal secretions

IFN-γ activates a number of acute phase reactants in the liver by direct up-regulation or indirectly by synergizing with other cytokines including IL-6 and IL-1 (Boehm *et al.*, 1997). Similarly, IFN-γ has also been shown to repress the expression of a number of acute phase proteins including α1-antichymotrypsin and haptoglobin, that are up-regulated by IL-6 (Koj *et al.*, 1988; Magielska-Zero *et al.*, 1988). To assess whether recombinant BHV-1/IFNγ created a measurable difference in the acute phase response, we analysed serum haptoglobin levels by ELISA. As shown in Figure 5.3.4, haptoglobin levels increased at day 2 and levelled off by days 4-5, paralleling increased clinical symptoms. All animals had detectable levels of haptoglobin at day 4, but no difference among the groups was detected. Although the standard deviation was high,

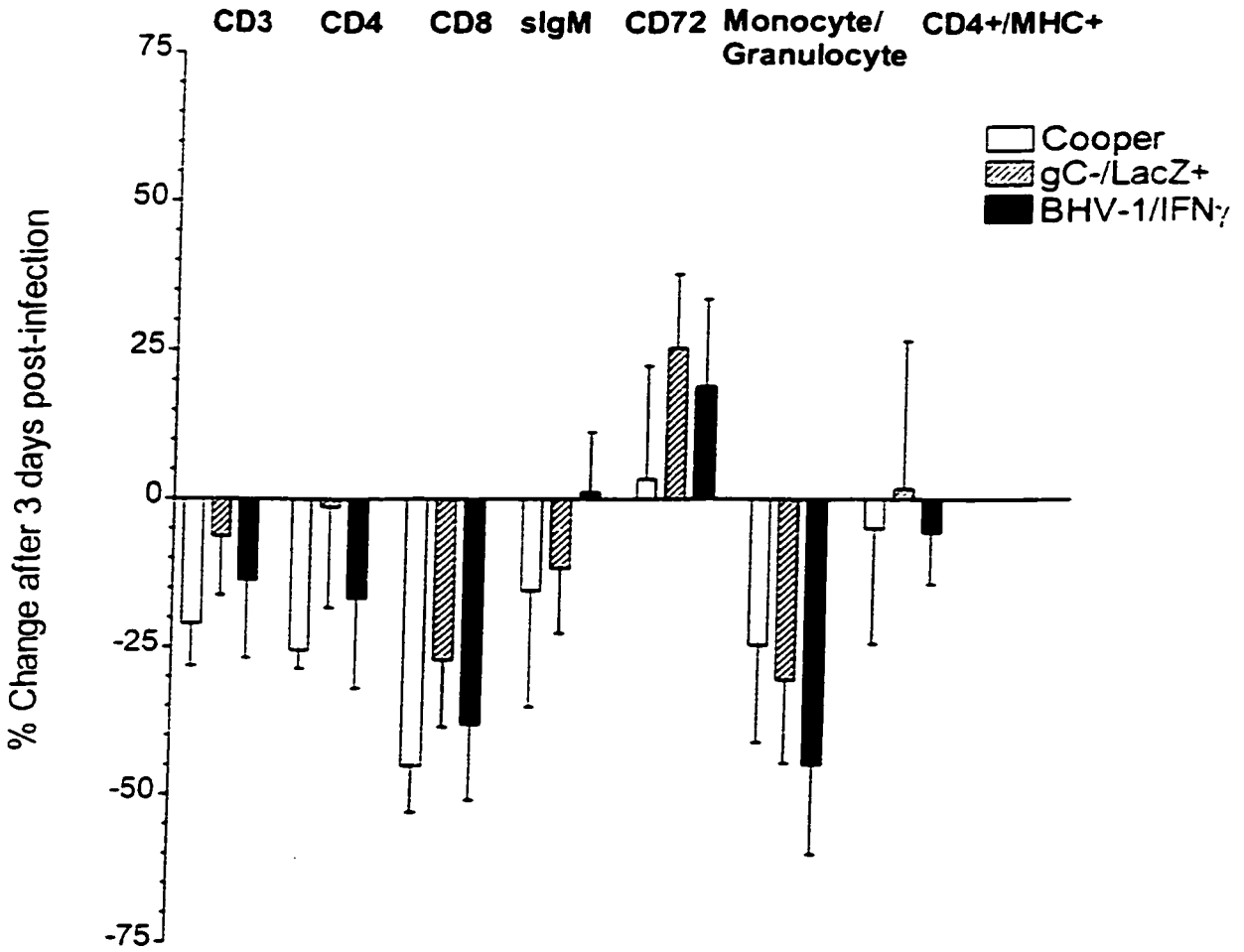


Figure 5.3.3 Changes in leukocyte subpopulation in peripheral blood at 3 days post-infection. T-lymphocyte markers (CD3, CD4, CD8), sIgM $^{-}$ Bcells (PIG45A), Total B cells CD 72 (DU2/104). monocyte and granulocyte (DH59B) and dual labelling for MHC II and CD4. Error bars represent one standard deviation

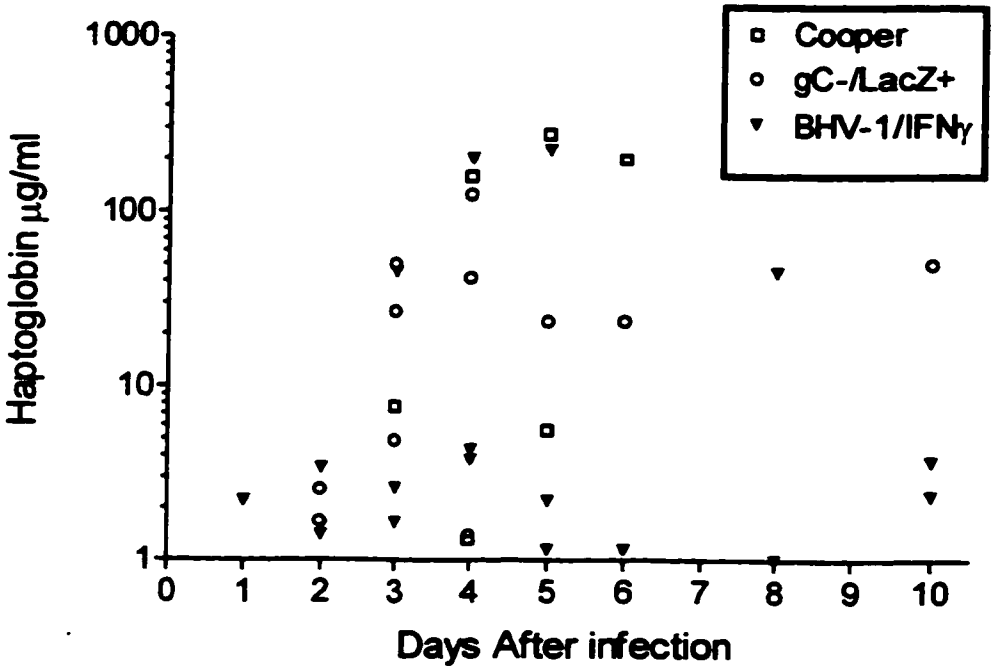


Figure 5.3.4 Serum haptoglobin response. Serum samples were analyzed by ELISA. Individual animals are shown with groupings indicated in the right margin.

the mean haptoglobin values were similar on day 4; 53.5 μg/ml for Cooper, 55.5 μg/ml for gC-/LacZ+ and 69.4 μg/ml for recombinant BHV-1/IFN-γ. Therefore, with the small group size used IFN-γ expression did not appear to have a systemic effect that could be detected by assaying haptoglobin levels.

To determine whether boIFN-γ was secreted in nasal secretions, we analysed nasal secretions collected every second day with tampons. As shown in Figure 5.3.5, IFN-γ was detectable in nasal secretions by ELISA. Similar to haptoglobin levels, the detection of IFN-γ parallels viral replication and clinical signs. Detectable levels of IFN-γ in nasal secretions peaked on day 4 p.i. for all animals. On this day, there was no significant difference in the levels among groups. On day 6 p.i., all animals exceeded 100 pg/ml of IFN-γ in nasal secretions. In addition, all three animals from the BHV-1/IFN-γ group had detectable IFN-γ levels over 1000 pg/ml, while only one animal from each of the other groups had IFN-γ concentrations that exceeded the cut-off of 1000 pg/ml. A direct comparison between gC-/LacZ+ and BHV-1 by T-test (P=0.0567) and Mann-Whitney rank-sum test (P= 0.050) indicates a statistical difference in the amount of IFN-γ produced in the nasal cavity. This confirmed that the recombinant BHV-1/IFNγ virus produced higher levels of IFN-γ. The duration of viral shedding and viral lesions seen in clinical score observations may correlate with detectable levels of IFN-γ in the nasal cavity, since by day 8-10 only two animals had detectable levels of IFN-γ and at days 12 and 14 no detectable IFN-γ was observed. However, there was not a direct linear correlation between the IFN-γ levels detected in nasal secretion and levels of viral titers achieved in individual animals (R=0.01).

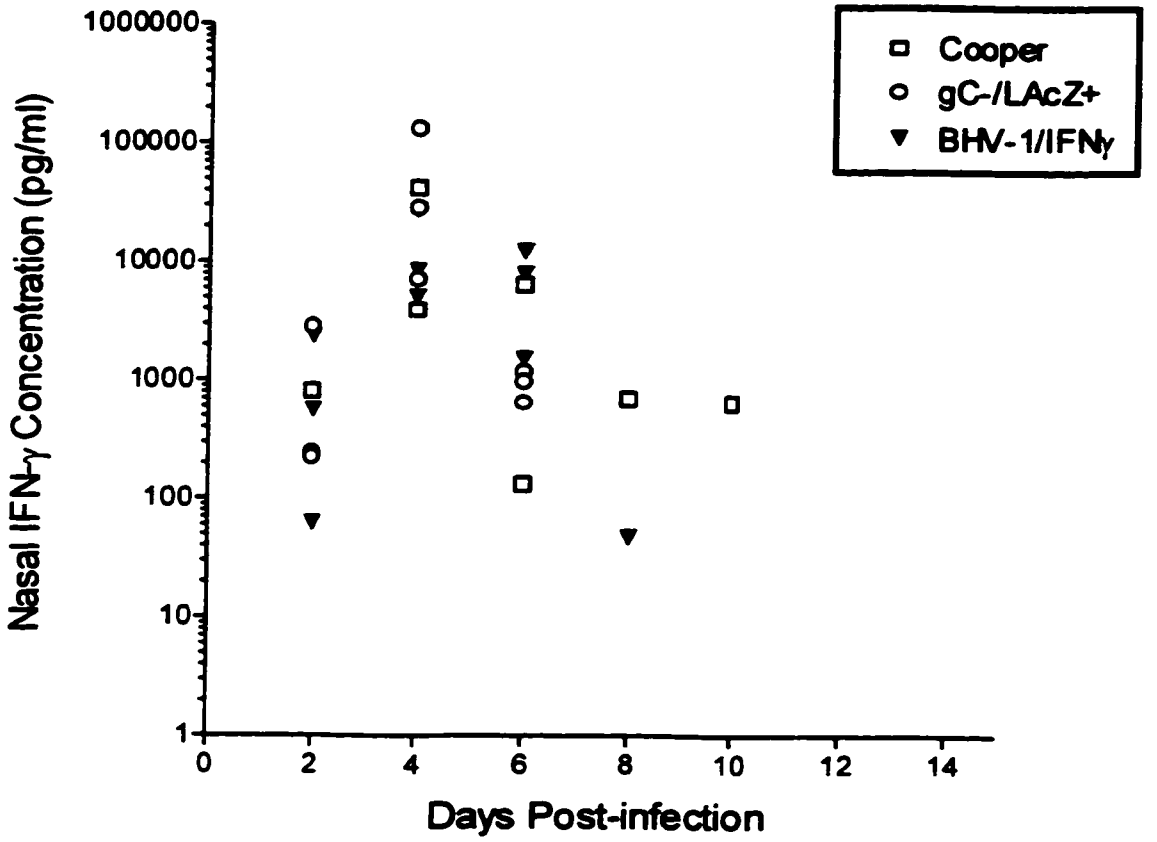


Figure 5.3.5 IFN-γ concentration in nasal secretions. Samples were collected with nasal tampons every second day p. i.. Bovine IFN-γ concentrations were determined by ELISA. Individual animals are shown with groupings indicated on the right hand margin.

5.3.5 Antibody response

Serum antibody titers for gD were determined by ELISA (van Drunen Little-van den Hurk *et al.*, 1984). After the initial intranasal challenge, all sero-negative animals were sero-positive by week 2 p. i.. As shown in Figure 5.3.6A, peak IgG titers occurred 4 weeks after the initial challenge period, with a second rise in titers following dexamethasone reactivation. There was no significant difference in the amount of total IgG between gC-/LacZ+ or BHV-1/IFNγ. Furthermore, comparison of the IgG isotypes, IgG1 and IgG2a, revealed no difference in the ratios generated by the different recombinant viruses during infection (Data not shown).

Virus neutralization titers were detectable by week 3 p. i., and there was a further increase after dexamethasone reactivation (Figure 5.3.6B). However, no statistically significant differences were found among the groups.

Nasal IgA was detected using gD coated plates and anti-bovine IgA antibody (M-67). Nasal IgA was detected at week 3 p. i. (Figure 5.3.7). Similar to the IgG results, there was an increase in nasal IgA after dexamethasone treatment. Interestingly, the BHV-1/IFN-γ had statistically lower IgA titers than the control groups at week 7 p. i..

5.3.6 PBMC proliferative response

To detect viral specific T-cells responses, peripheral blood lymphocytes were isolated for proliferation assays (Hutching *et al.*, 1990b). To control non-specific proliferative responses, dexamethasone (5 ng/ml) was added to the medium.

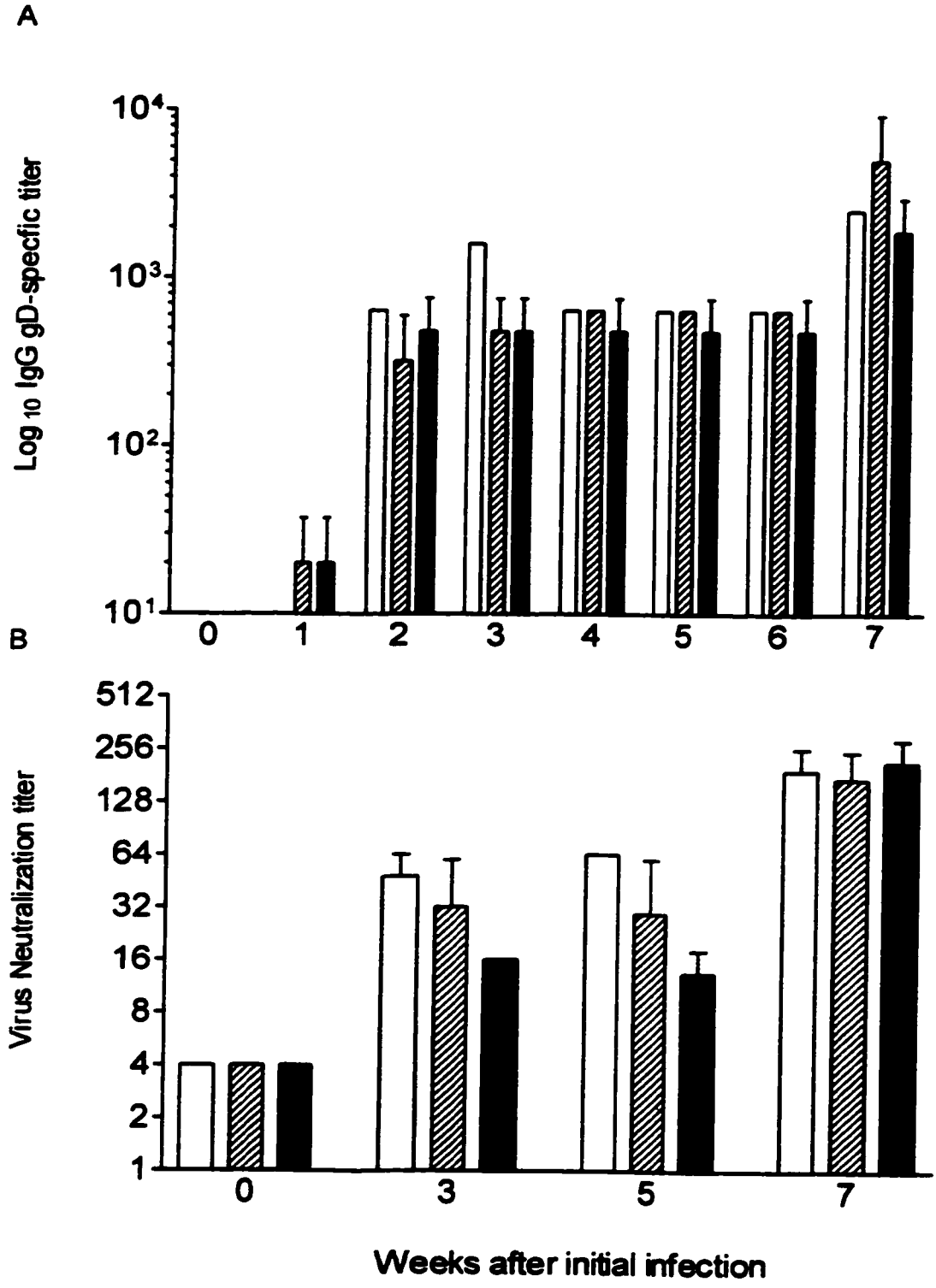


Figure 5.3.6 Serum antibody response. (A) gD specific ELISA titers expressed as reciprocal of highest dilution. (B) Virus neutralizing titer expressed as the reciprocal of the highest dilution. Cooper (open bar), gC-/LacZ+ (hatched bar) and BHV-1/IFNγ (solid bar). Dexamethasone treatment was started in week 5 p. i.. Error bars represent one standard deviation.

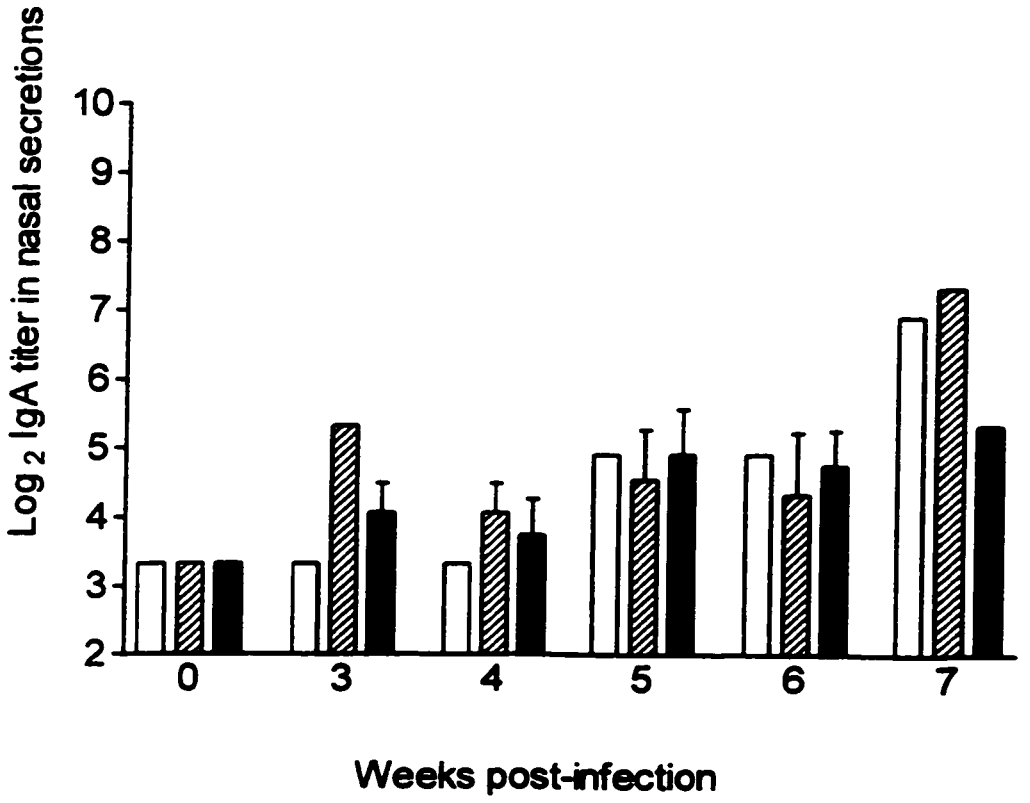


Figure 5.3.7 IgA Titer in nasal secretions. Titers were determined using gD coated ELISA plates. Mucosal samples obtain from tampons were analyzed for IgA in Cooper (clear bar), gC-/LacZ+ (hatched bar) and BHV-1/IFNγ (solid bar) groups. Dexamethasone treatment was started in week 5 p. i.. Error bars represent one standard deviation.

This resulted in lower background counts than traditionally recorded and increased the sensitivity of the assay. Results are expressed as a stimulation index (SI) (antigen induced CPM / background CPM). As shown in Figure 5.3.8, for all groups there was a strong gD specific proliferation for the first two weeks after primary BHV-1 infection. There was a further increase in the gD-induced proliferative responses at week 6 following BHV-1 reactivation with dexamethasone. The gD-induced proliferative responses were not significantly different among groups after either the primary infection or reactivation of the latent infection.

5.3.7 Cytokine secreting cell ELISPOT

Changes in the number of lymphocytes secreting IFN-γ in response to BHV-1 infection were assayed. As shown in Figure 5.3.9, the number of IFN-γ secreting cells specific for gD were comparable among all groups, especially gC-/LacZ+ and BHV-1/IFNγ. The proportion of lymphocytes secreting IFN-γ decreased by week 5 after the initial infection, but IFN-γ secreting cell frequency was boosted after reactivation of latent virus.

5.3.8 Reactivation of latent virus

To reactivate latent BHV-1, individual animals were treated with 0.1 mg/kg of dexamethasone for 5 days. All animals were clinically normal before dexamethasone treatment. After treatment, there was a slight increase in clinical scores and rectal temperature that correlated with viral recrudescence, but no group developed a fever (>39.5 °C) (Figure 5.3.10). Clinical symptoms included coughing, increased nasal

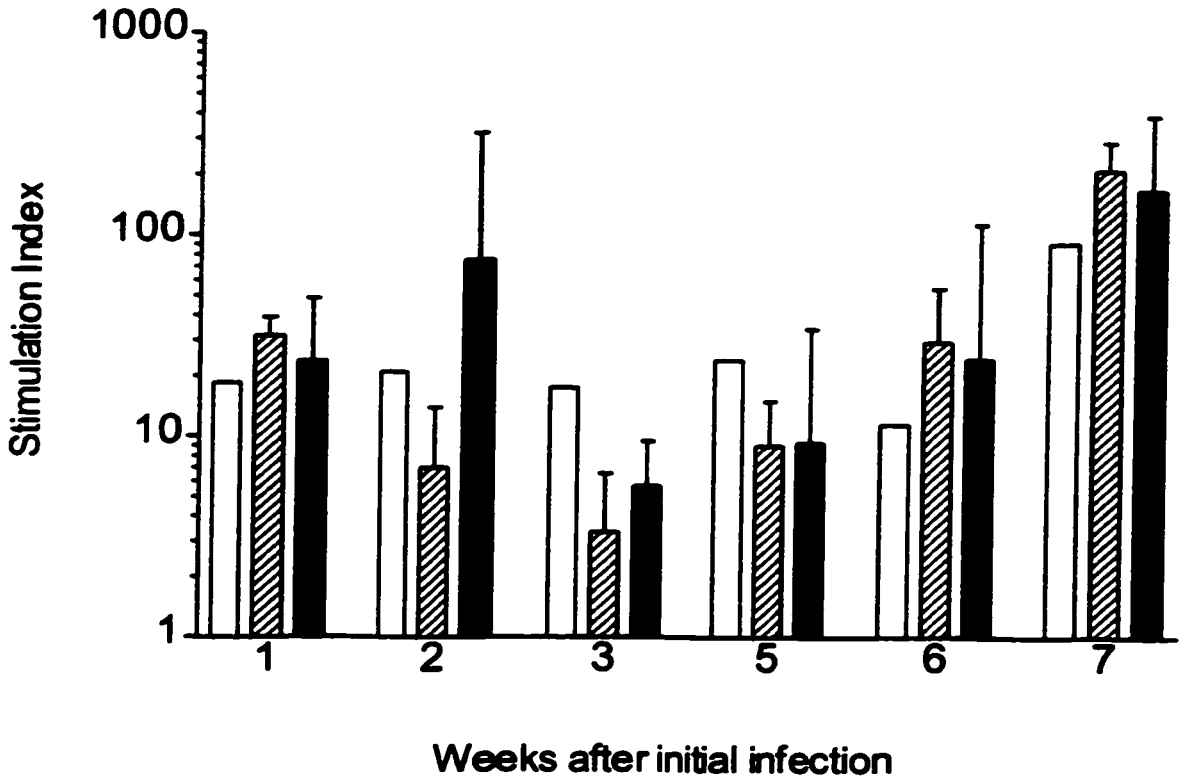


Figure 5.3.8 gD-induced proliferative responses. Isolated lymphocytes were cultured with 0.1 ug/ml of BHV-1 gD for 3 days. Groups were Cooper (clear bar), gC-/LacZ+ (hatched bar) and BHV-1/IFNγ (solid bar). PMBC were isolated weekly after infection. Results are expressed as mean ± one S.D.

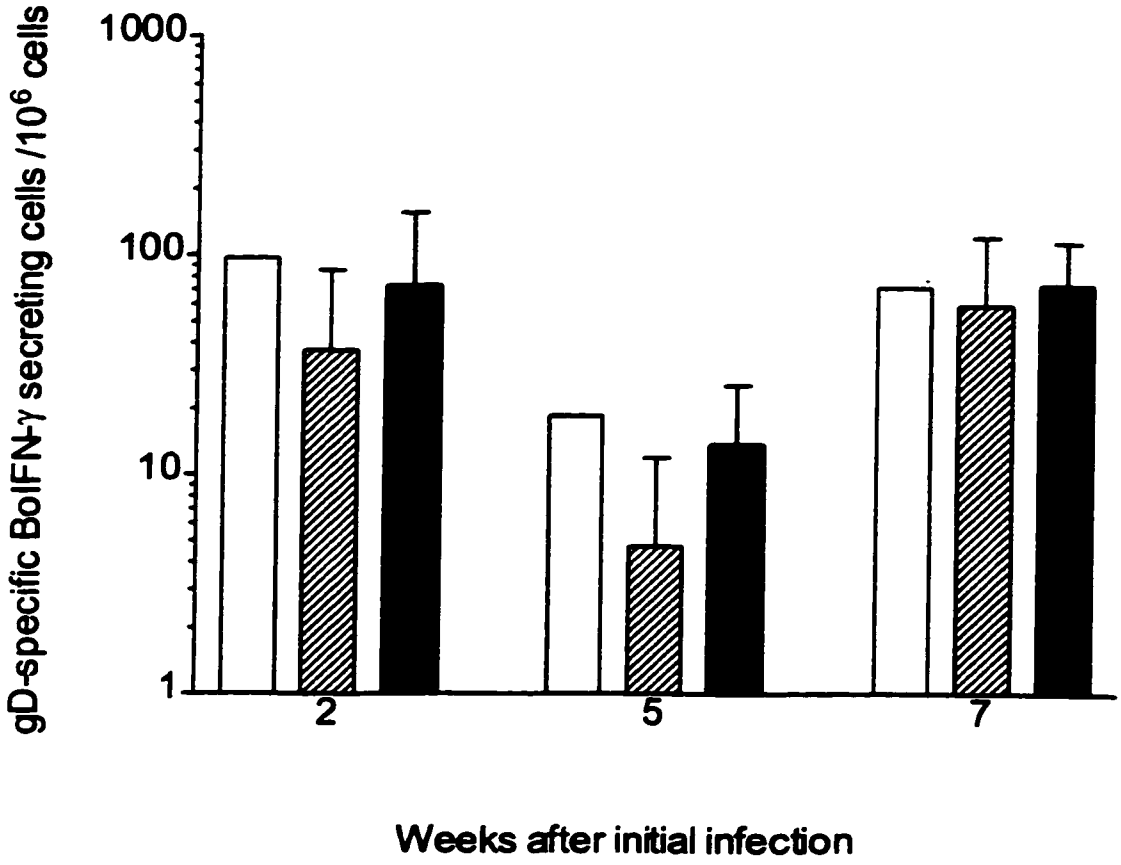


Figure 5.3.9 Bovine IFN-γ secreting cell ELISPOT. Peripheral blood mononuclear cells were isolated and cultured overnight with 0.1 ug/ml of gD before assaying IFN-γ secreting cells (SC). Results are expressed as gD stimulated IFN-γ SC minus IFN-γ SC from non-stimulated cell cultures. Cooper (open bar), gC-/LacZ+ (hatched bar) and BHV-1/IFNγ (solid bar). Dexamethasone treatment was started after week 5 p. i..

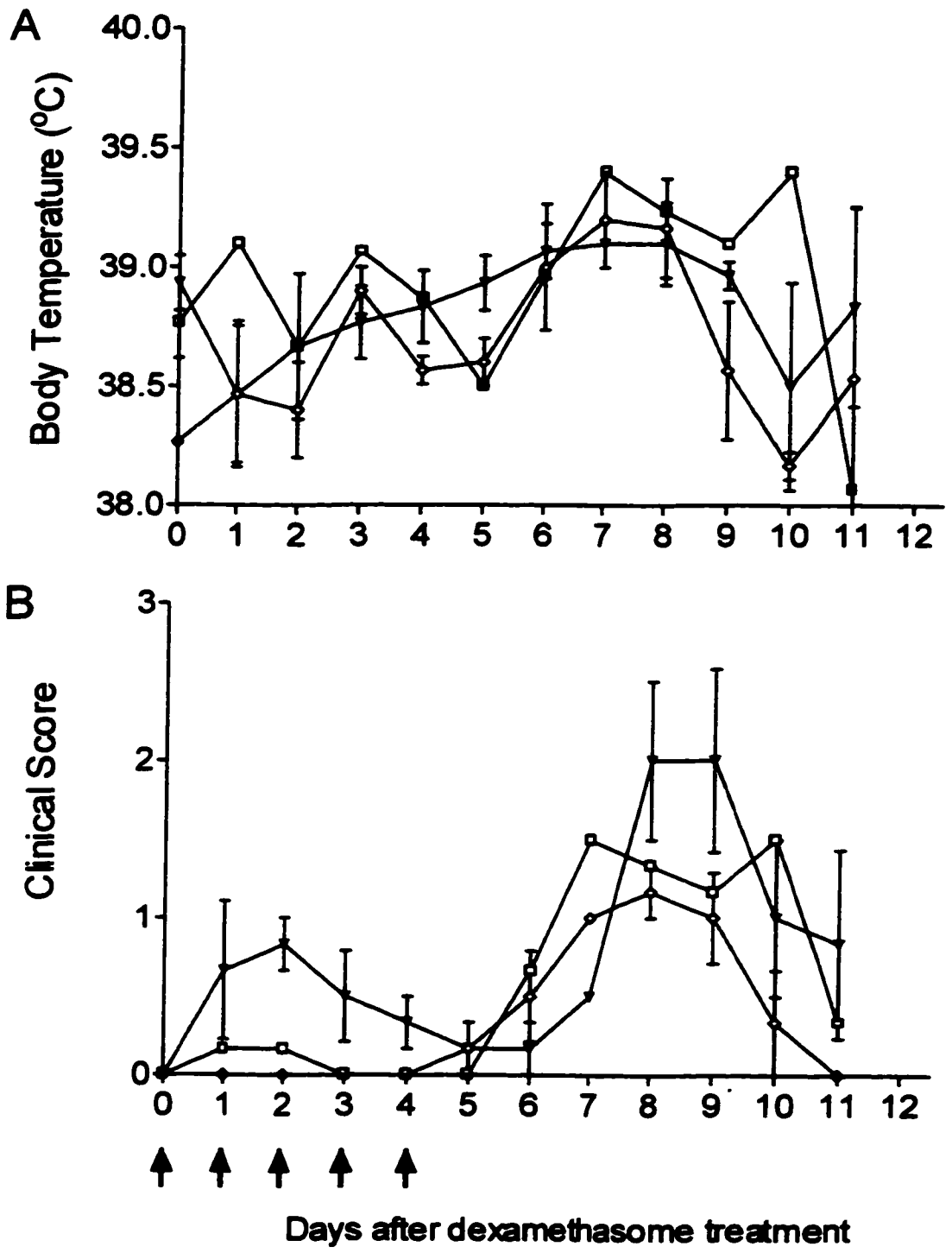


Figure 5.3.10 Clinical response following dexamethasone treatment. (A) Daily rectal temperatures. (B) Clincal scores. Calves were treated with 0.1 mg/kg of dexamethasone (arrows) Cooper (square), gC-/LacZ+ (diamond) and BHV-1/IFNγ (triangle). Clinical monitoring was stopped at day 11 after the start of treatment.

discharge and nasal lesions. Nasal lesions were slightly smaller in the gC-/LacZ+ group and the plaques disappeared more quickly than in the other groups. As shown in Figure 5.3.10B, the recombinant BHV-1/IFNγ group had the highest clinical score during the 5-day dexamethasone treatment period. Mild nasal mucosa hyperemia and inappetence were the predominant clinical signs recorded for the BHV-1/IFNγ group (Figure 5.3.10B).

Viral shedding was detected only after the 5-day dexamethasone treatment period (Figure 5.3.11). All animals that were sero-negative prior to the primary infection shed virus for at least 5 days. As observed during the primary infection, wild type Cooper virus was shed at 1-2 logs higher than recombinant gC$^-$ viruses during recrudescence. Viral shedding peaked 3 days after treatment. The Cooper group shed a maximum titer of 2.7×10^6 pfu/ml/swab, while recombinant gC-/LacZ+ shed a maximum 3.6×10^4 pfu/ml/swab and BHV-1/IFNγ shed a maximum of 2.3×10^4 pfu/ml/swab. However, there was no difference in viral titer or the duration of virus shedding between recombinant BHV-1/IFNγ and gC-/LacZ+ viruses. Only one calf shed virus at day 7 after dexamethasone treatment and this calf was from the BHV-1/IFNγ group.

5.3.9 Biological activity in reactivated virus

To determine whether latent BHV-1/IFNγ retained a functional IFN-γ gene the virus was re-isolated from nasal swabs collected from animals treated with dexamethasone. This virus was grown in MDBK cells and the supernatant was analysed for biologically active IFN-γ. As shown in Figure 5.3.12, supernatant from BHV-1/IFNγ infected cells could up-regulate MHC expression. Moreover, this activity could be neutralized with

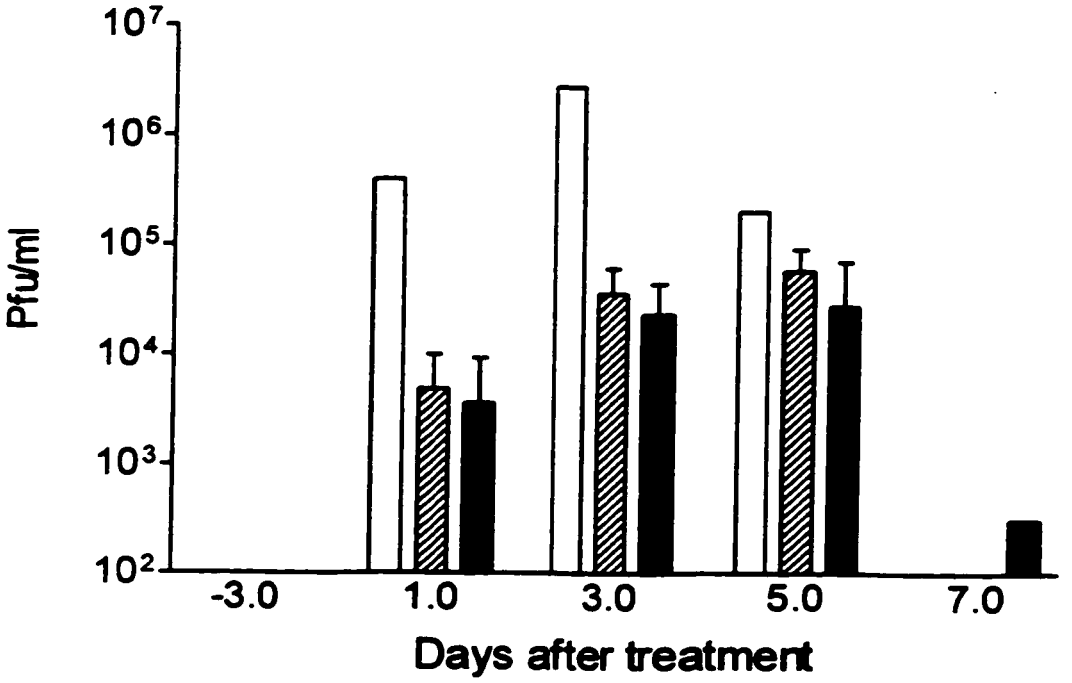


Figure 5.3.11 Viral reactivation. Calves were treated with 0.1 mg/kg of dexamethasone to reactivate BHV-1 - Cooper (open bar), gC-/LacZ+ (hatched) and BHV-1/IFNγ (solid). Viral shedding was sampled with nasal swabs. Results are expressed as mean ± S.D for each group.

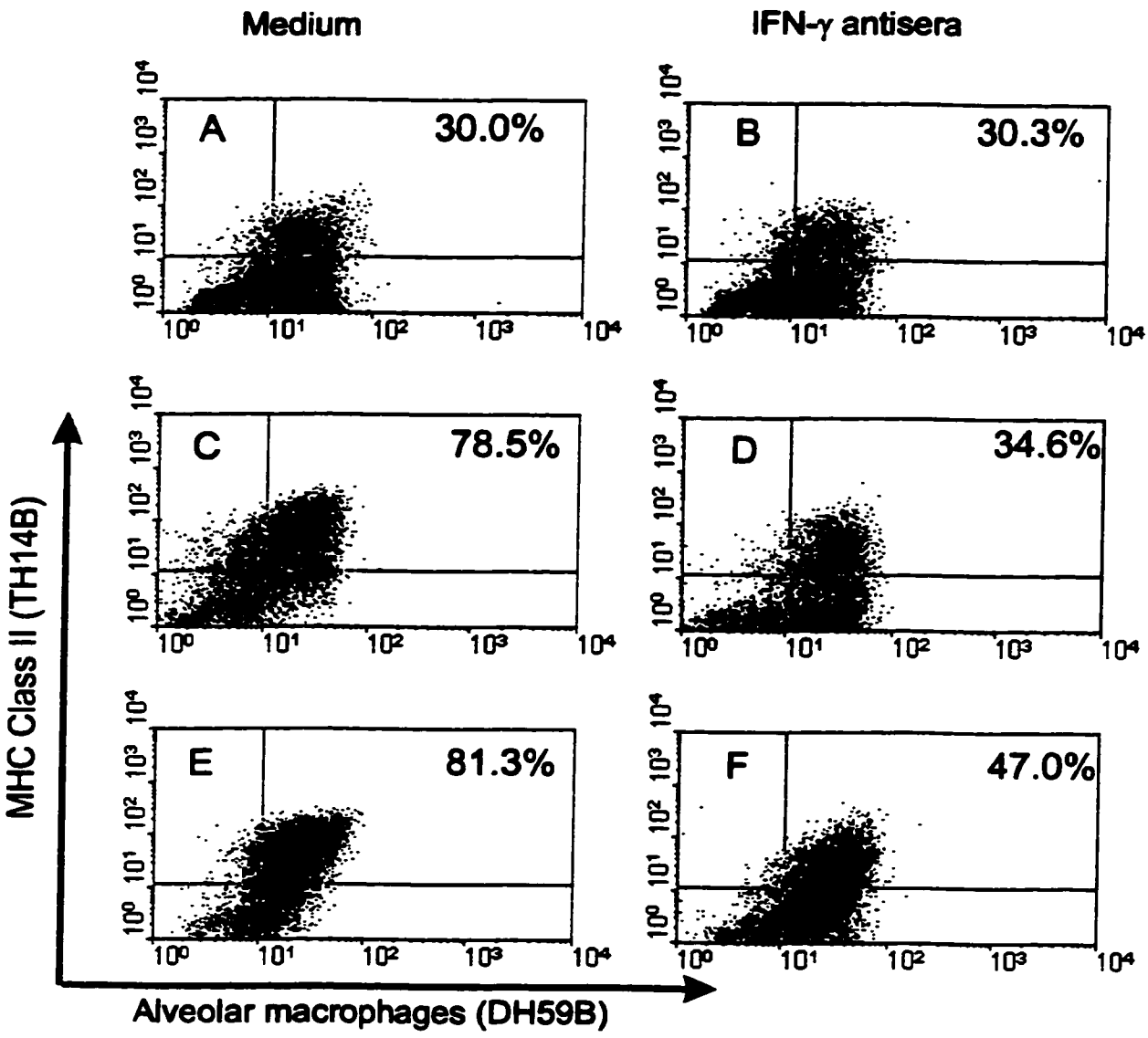


Figure 5.3.12 MHC up-regulation on alveolar macrophages by dexamethasone re-activated virus. Alveolar macrophages (AM) were isolated by lung lavage. Recombinant BHV-1/IFNγ was isolated form a nasal swab taken after dexamethasone and grown in MDBK cells. The culture supernatant was removed and analyzed for IFN-γ activity by *in vitro* MHC induction. Culture supernatant was incubated for 72 hours with 4 x 10^6 AM/ml. The AM were then washed and dual stained with monocyte (DH59B) and MHC class II (TH14B) specific monoclonal antibodies. Sample were as follows: (A) cells cultured in MEM; (B) cells cultured in MEM with anti-IFN-γ antibody (1/20). (C) Positive control: 100ng/ml recombinant boIFN-γ; (D) Recombinant boIFN-γ (100 ng/ml) plus anti-IFN-γ antibody (1/20). (E) Re-isolated latent BHV-1/IFNγ supernatant; (F) Re-isolated latent BHV-1/IFNγ supernatant plus anti-IFN-γ antibody (1/20). The percent monocyte/macrophages with MHC class II on their surface is indicated in the top right margin of each panel.

anti-IFN-γ antibodies (Figure 5.3.12., F).The biological activity of recombinant IFN-γ was also confirmed in an anti-viral VSV assay (data not shown). These observations confirmed that the *in vivo* passage and latency did not alter the function of the IFN-γ gene.

5.3.10 Viral collection methods

Previous experiments by Dr. Xiaoping Liang and workers showed that the recovery of virus from gC-/LacZ+ infected animals was problematic. Following infection, animals developed clinical symptoms and an antibody response, indicating a primary infection and an amnestic antibody response after dexamethasone reactivation, but virus isolation was difficult. The traditional tampon collection method was used in this study (Liang *et al.*, 1992). Therefore, to determine whether there was a substantial difference in collection methods, we compared recovery of shed virus by tampons or nasal swabs.

Usually a cotton swab is inserted into the nasal cavity to rub the mucosal surface to collect both mucosal secretions and cellular components. The swab is then placed into 1 ml of MEM medium and viral particles are released by vortexing or squeezing the fluid out of the cotton applicator. The second method uses a cotton tampon that is inserted for 20 minutes into the nasal cavity to absorb nasal secretions. The tampon is then removed and nasal secretions are squeezed out by compressing the tampon in a 50 ml syringe. Infectious BHV-1 in nasal secretions is then titered on MDBK cells.

As shown in Figure 5.3.13, there was a significant difference in viral titer between tampon and swab collection methods for gC^- mutants on days 4 and 6 p. i..

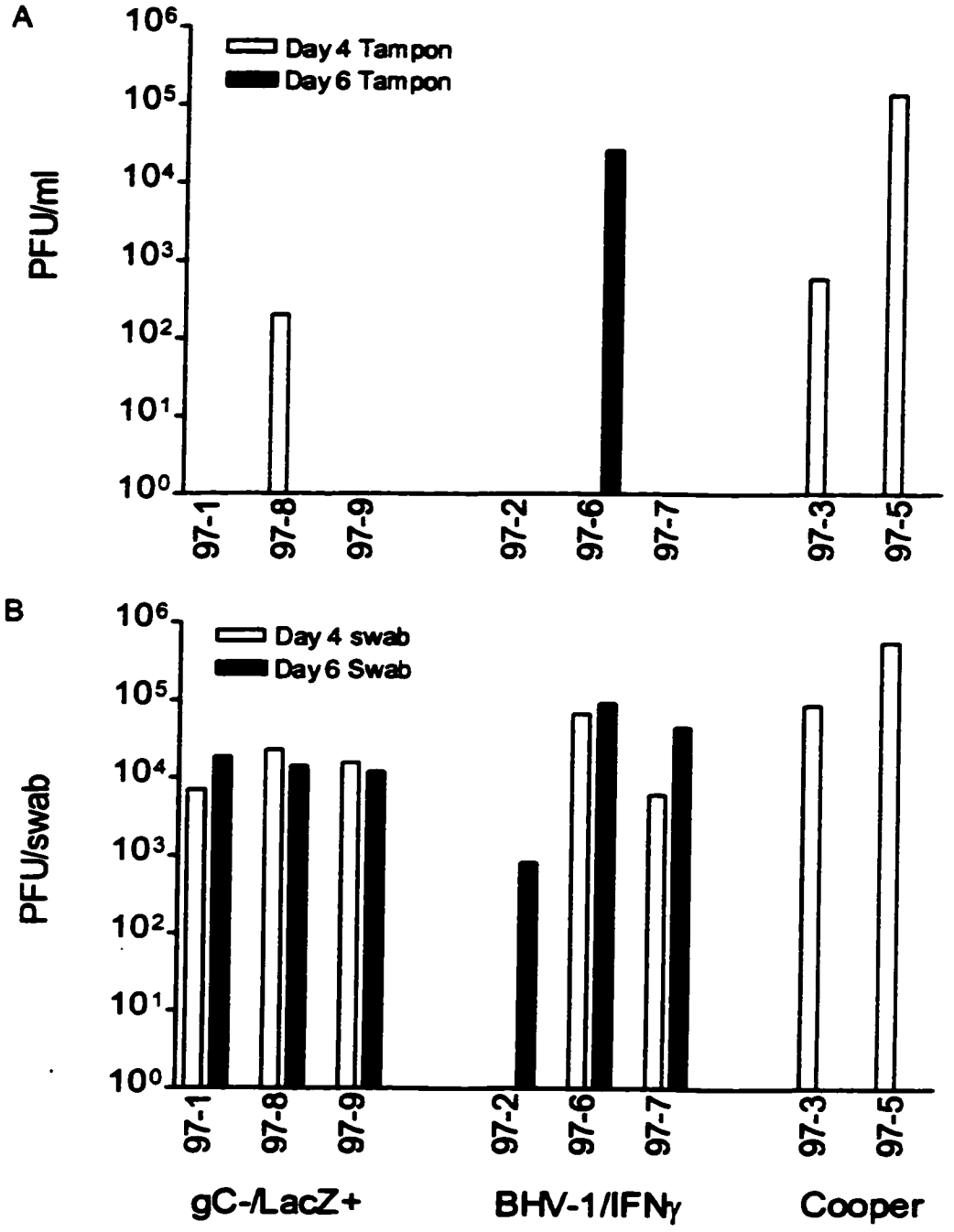


Figure 5.3.13 Comparison of virus collection methods. Samples were collected by two methods. The first method used a nasal swab to rub the mucosal surface. The swab was then placed into 1 ml of MEM. The second method used a nasal tampon inserted for 20 minutes into the nasal cavity to collect nasal secretions. Cooper samples were only taken on day 4 p. i.. Samples were frozen at –70°C until virus titer was assayed.

The tampon method detected virus in only 1 of 6 animals on both days tested. In contrast, samples from nasal swabs contained infectious virus particles from 5 of 6 animals on day 4 p. i. and all six animals on day 6 p. i.. In contrast, our analysis of Cooper virus shedding on day 4 revealed that both collection methods detected infectious virus. Moreover, with the Cooper group the tampon collection method was approximately a half to one log less sensitive than the swab method. The significance of this latter observation is difficult to explain since both methods involve different dilution effects during the collection, i. e. nasal secretions (tampon) and MEM medium (swab).

To clarify these observations, the influence of non-immune nasal secretions on the 'natural neutralization' of a gC-/LacZ+ mutant or wild-type Cooper virus was examined. A fixed amount of virus was incubated with non-immune nasal secretions from calves or control media and their effect on infectious BHV-1 titer was assayed. As shown in Figure 5.3.14, for both BHV-1 strains there was a significant ($p<0.05$) reduction in the amount of infectious virus recovered after a 1 hour incubation with mucus. This indicated that nasal secretions contain components that can neutralize BHV-1. This may explain the reduced viral titer in samples collected with tampons. Previous analysis of the gC gene have show that certain components of gC contain a motif that binds complement (Fitzspatrick *et al.*,1989; Huemer *et al.*, 1995). It's tempting to speculate this complement binding component may protect against complement mediated antibody neutralization and may also protect the virus particle from neutralization by other components in the nasal secretions. This would enhance the efficiency of viral transmission to another host.

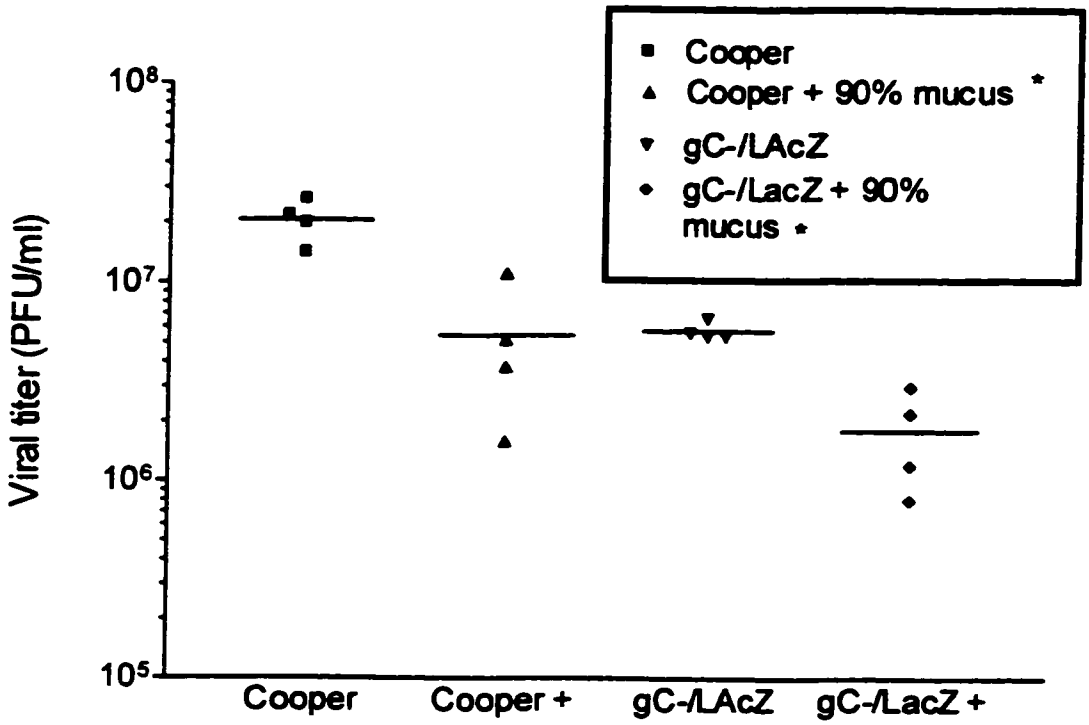


Figure 5.3.14. The effect of non-immune nasal mucous on *in vitro* viral replication. Virus was incubated with 90% mucus from BHV-1 sero-negative animals for 1 hour before performing a plaque assay on MDBK cells. Each sample represents mucus from an individual animal. The (*) symbol denotes a significant difference ($p < 0.05$).

5.4 Experimental BHV-1 infection in sheep

The development of an alternative animal model for BHV-1 would greatly advance the investigation of novel vaccine strategies, including recombinant BHV-1 expressing cytokines. Therefore, to investigate whether sheep could be used as model to study BHV-1 infections, we infected two groups of sheep with either recombinant gC-/LacZ+ virus or wild-type Cooper virus. Each group was challenge by intranasal aerosolisation with approximately 4×10^7 PFU (1 minute aerolisation - 3 animals) or 4×10^8 PFU (10 minutes -3 animals). Clinical symptoms were monitored for 7 days p. i.. After 4 weeks, protection was assessed by challenging with 4×10^7 PFU of BHV-1 strain 108. Three naive animals were included as controls.

5.4.1 Clinical response

As shown in Figure 5.4.1, following BHV-1 challenge body temperatures remained normal for both groups. The elevated temperatures on day 0 may reflect the initial stress experienced by the lambs during sampling and BHV-1 challenge. Some animals developed a fever, but there was not a difference between groups that received a high or low amount of virus. Similarly, clinical symptoms were minor, especially when compared to the BHV-1 infection model in cattle (Table 5.4.1) (Wyler *et al.*, 1989). The clinical signs in sheep included coughing, increased respiratory rates and mild mucosal hyperemia. The majority of clinical symptoms were recorded for the two Cooper groups, especially the group receiving the high dose challenge.

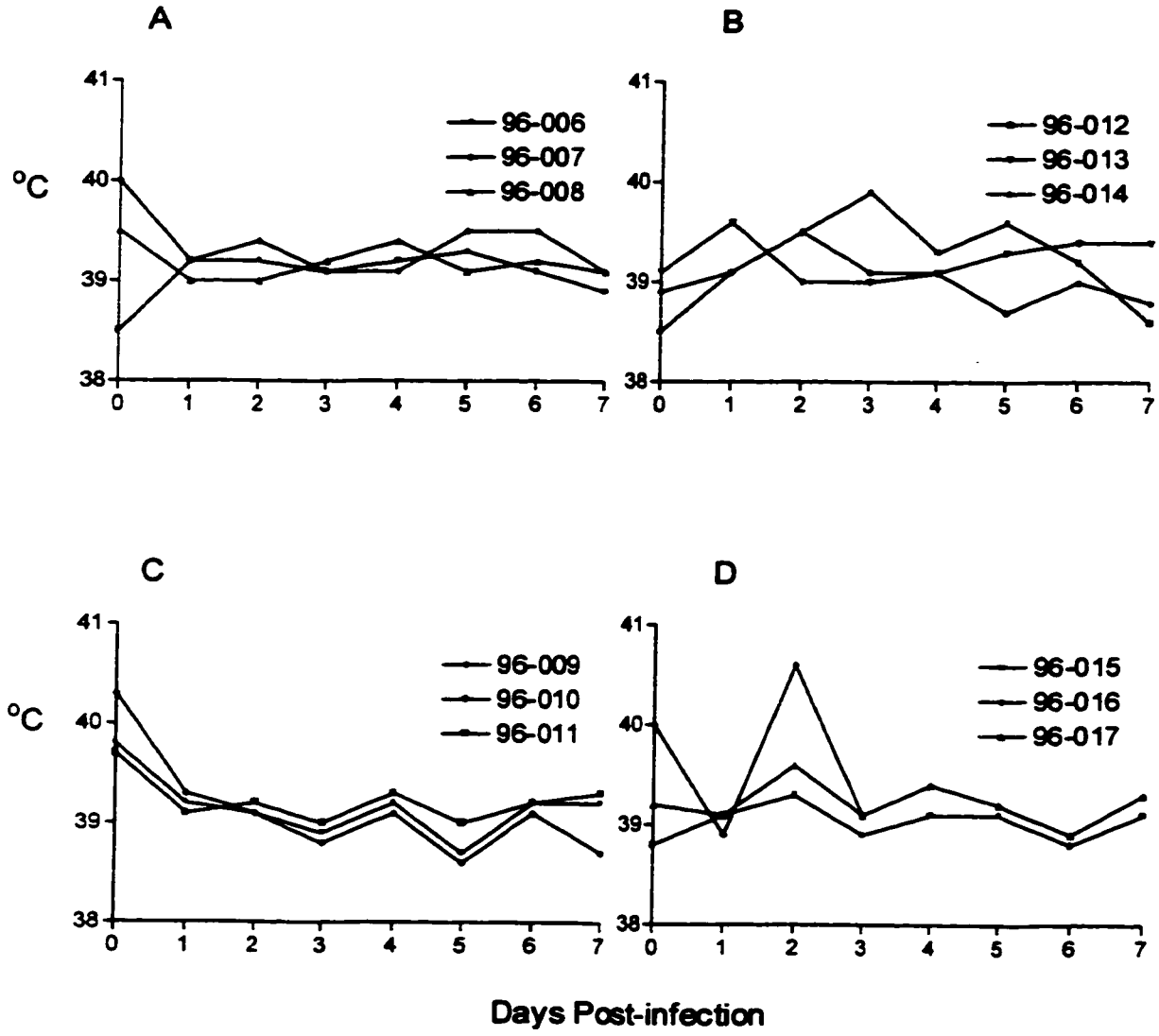


Figure 5.4.1 Body temperature following BHV-1 infection of sheep. Rectal temperatures were taken daily for 7 days p. i.. Individual animals are shown on the graphs. (A) gC-/LacZ+, 4×10^7 (B) gC-/LacZ+, 4×10^8 (C) Cooper, 4×10^7 (D) Cooper, 4×10^8.

Table 5.4.1 Clinical response after BHV-1 infection of sheep. Clinical monitoring was performed for 7 days p. i..

Challenge group	Animal number	Nasal Lesion	Mucous Discharge	Coughing	Respiratory Rate Increase	Mild Hyperemia
gC-/LacZ+ 4×10^7	96-006					
	96-007		Yes (5)			
	96-008	Yes (2)	Yes (5)			
gC-/LacZ+ 4×10^8	96-012	Yes (2)	Yes (2, 4, 5)			
	96-013					Yes (5)
	96-014					
Cooper 4×10^7	96-009					
	96-010		Yes (5)	Yes (2)	Yes (6)	
	96-011	Yes (2)	Yes (5)	Yes (4, 5)	Yes (4, 6)	
Cooper 4×10^8	96-015				Yes (6)	Yes (4)
	96-016	Yes (5)			Yes (6)	Yes (4)
	96-017				Yes (6)	Yes (4)

(#) denotes day of observation. Nasal lesions were noted only for the left nostril. Sampling for viral shedding was done in the right nostril.

5.4.2 Viral Shedding

Viral shedding was assessed by swabbing the right nostril with a cotton applicator. Peak virus shedding was recorded on day 2 p. i. (Figure 5.4.2). There was a two-fold difference between groups receiving the high dose challenge versus the low dose challenge of both types of viruses. The Cooper virus appeared to have a growth advantage over gC-/LacZ+ recombinant virus; the difference was approximately a 2 log difference on day 2 p. i.. There was no difference in the duration of virus shedding with both virus types being shed for 5 to 7 days p.i.. Relative to the cattle infection model, the amount of BHV-1 growth in sheep appeared to be less and of shorter duration (Wyler *et al.*, 1989).

5.4.3 Serum response

To determine the antibody response to BHV-1, sera were collected and analysed by gD ELISA or by virus neutralization assay. All animals were sero-negative for BHV-1 at the start of the experiment. As shown in Figure 5.4.3A, all groups developed an antibody response. The groups that received a high dose challenge developed significantly higher titers by week 4 p. i. than the animals challenged with a low dose. This correlates with the amount of viral replication in the initial challenge since both high dose groups had higher levels of viral shedding.

When we investigated the BHV-1 neutralization titer in serum, we found that there was a slight increase in neutralization antibody titer after challenge, but there was no significant differences among groups at week 3 and 4 p.i.. Unlike cattle, there was a

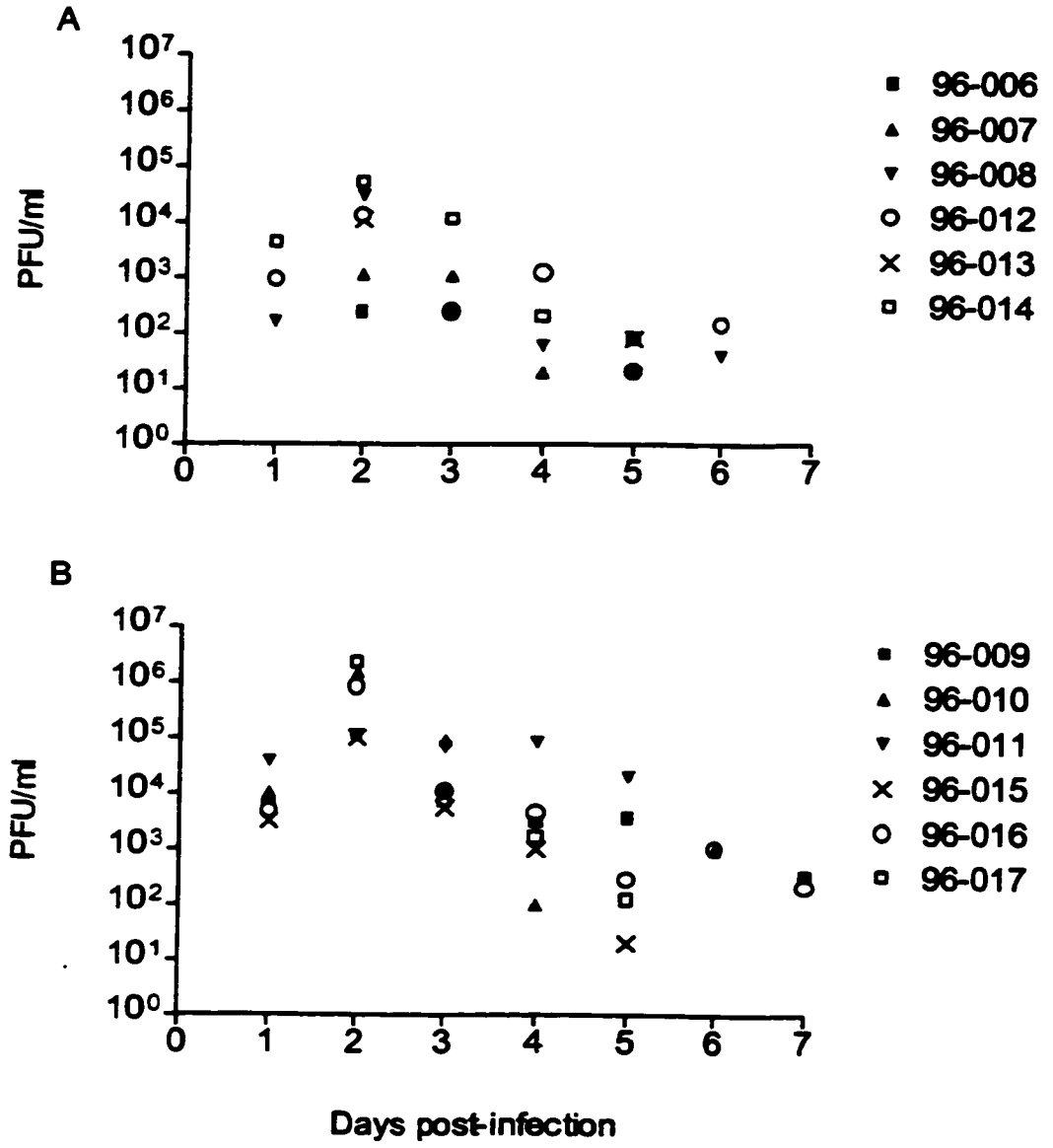


Figure 5.4.2 Comparison of viral shedding after infection. Virus was detected by swabbing the right nostril with a cotton applicator and then placed into 1 ml of MEM. Samples were titered in 24-well plates. Individual animals are indicated in the right margin. (A) gC-/LacZ+ (B) Cooper. Dark symbols denote animals challenged with a low dose ($4x10^7$ PFU) of virus and open symbols denote animals challenged with $4 x10^8$ PFU.

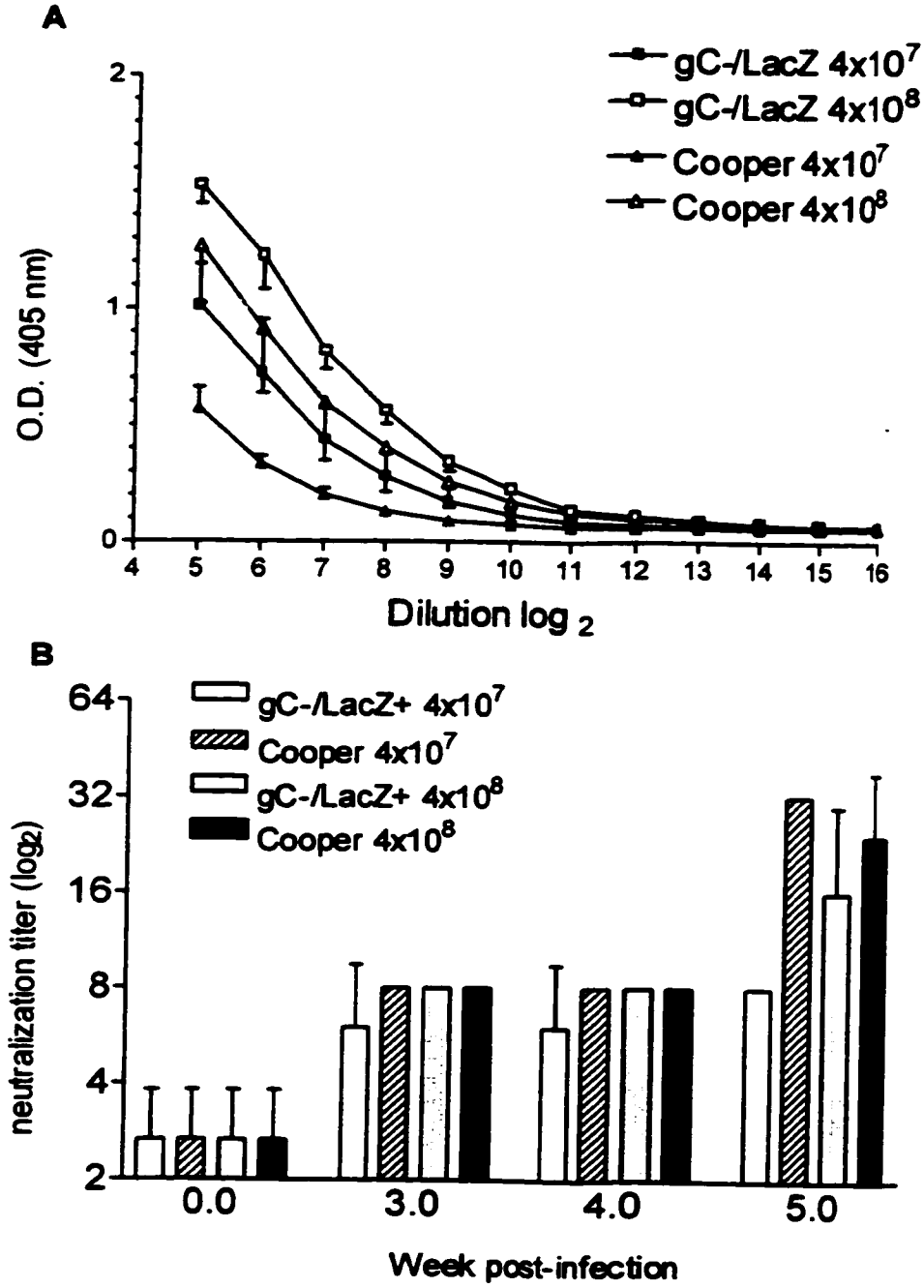


Figure 5.4.3 Serum antibody response. (A) gD specific IgG titer expressed as optical density (405 nm) at week 4 p. i.. Groups are indicated in the right margins. (B) Virus neutralizing titer expressed as the reciprocal of the highest dilution of serum that neutralized. Sheep were re-challenged at week 4 p. i.. Errors bars indicate one standard deviation.

slightly higher natural neutralization titer in approximately half of the naive animals. Only after secondary challenge did the low dose Cooper group develop distinctively higher titers then the gC$^-$ group counterpart (Figure 5.4.3B). These results demonstrated that BHV-1 infection induced an antibody response in sheep that correlated with the initial level of viral shedding.

5.4.4 Secondary challenge

To determine the degree of protection, all animals were re-challenged with BHV-1 strain 108 at week 4 p. i.; three naive animals were included as controls. As shown in Figure 5.4.4, only the high dose Cooper group (4×10^8 PFU) displayed complete protection while all the other groups achieved partial protection. The duration of viral shedding was limited to 2 days in all but one of the pre-exposed post-challenge animals. The animal that shed infectious 108 strain virus for more than 2 days also shed Cooper virus during the initial virus challenge. Thus, this animal showed a lack of immune protection, despite a clear infection during the initial infection. Two naive animals had virus shedding patterns similar to the initial Cooper and gC-/Lacz+ infections. Peaking viral shedding occurred on day 2 p. i. and virus shedding lasted for 5-7 days p. i. However, one of the naive sheep shed virus only on day 2 p. i..

5.4.5 Immunohistology of a BHV-1 infected sheep.

To further investigate the pathology of BHV-1 infection in the sheep lung and confirm the replication of BHV-1, a number of sheep were infected with BHV-1. Animals were infected either by aerosolization using a nebulizer or trans-tracheal

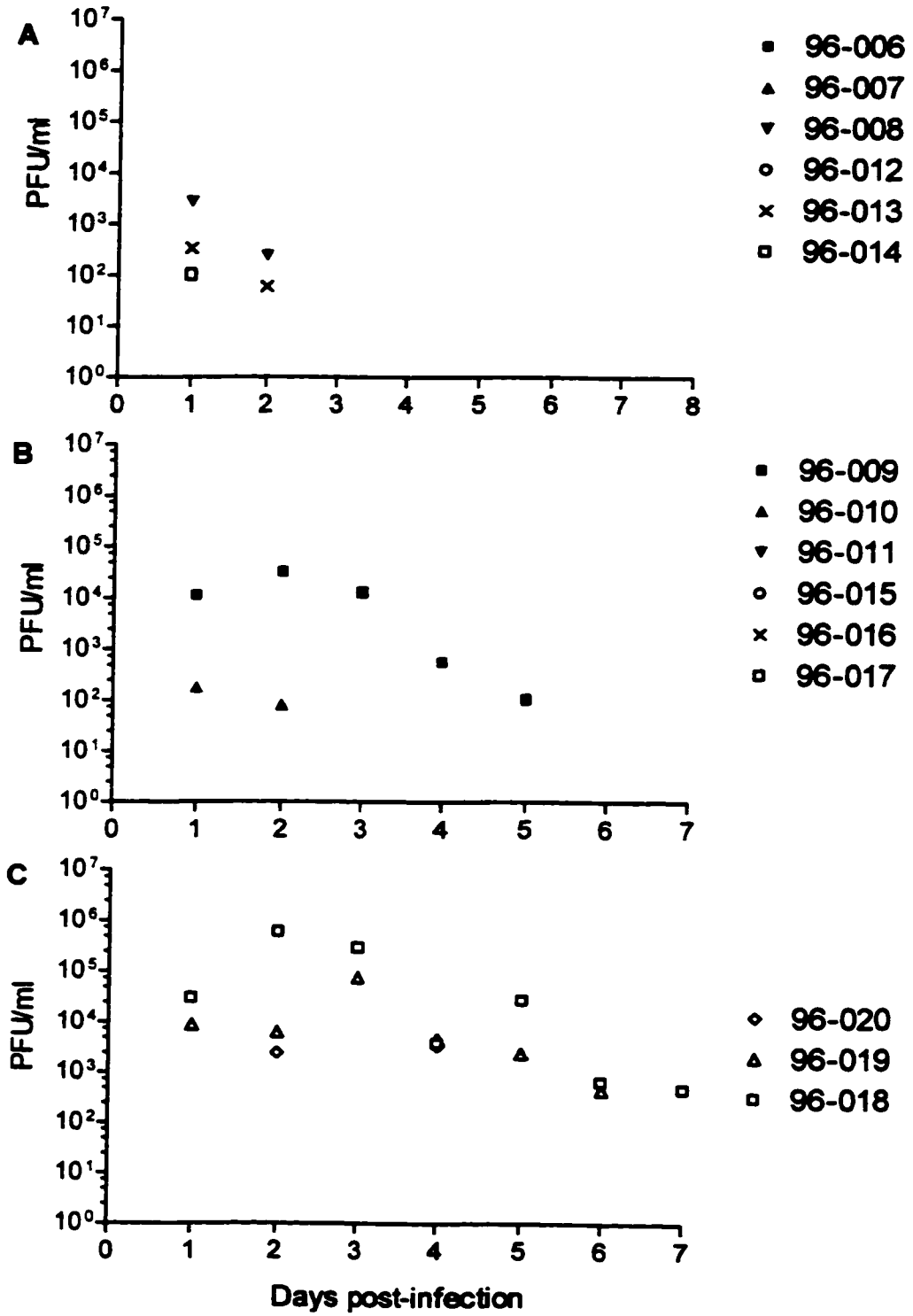


Figure 5.4.4 Viral titer shedding secondary challenge with strain 108. (A) pre-exposed gC-/LacZ+ animals.(B) pre-exposed Cooper animals (C) naive control animals. Individual animals are indicated on the right margins.

injection with 10 ml of virus medium and 1 ml of virus medium in each nostril containing 1 x10^8 PFU/ml. Lung, lymph nodes and tracheal samples were examined for the presence of BHV-1 by homogenizing tissue and lung tissue was collected on day 4 post-infection for immunohistochemistry.

Infectious virus was recovered from all tracheal samples by collecting four days after infection by aerosolization or trans-tracheal injection. Infectious virus was isolated from the lung following intra-tracheal challenge. Virus was recovered from both the cranial and medial lung lobes. A veterinary pathologist examined the lung histological sections to evaluate the effects of viral replication. As shown in Figure 5.4.5A, hemaoxylin and eosin (H & E) staining of the lung revealed multiple small foci of necrosis containing nuclear debris and increased infiltration of neutrophils and alveolar macrophages. There was also increased mononuclear cells within the interstitum. Alveolar spaces were largely obliterated by cellular debris and a thickened interstitum. The pathomorphologic diagnosis was a subacute, multifocal to coalescing necrotising mild pneumonia that was possibly due to infectious bovine rhinotracheitis. There also were tissue changes consistent with a chronic multifocal bronchointerstitial pneumonia that was suggestive of a bacterial infection.

Tissue sections were stained with a pool of monoclonal antibodies. This immunohistochemistry revealed multiple foci consisting of 6-7 positive cells (Figure 5.4.5B). These foci corresponded to multiple foci of necrosis seen in the H and E staining. This investigation confirmed that BHV-1 could replicate in sheep and caused some pathological changes similar to those reported for BHV-1 infection in cattle.

A)

B)

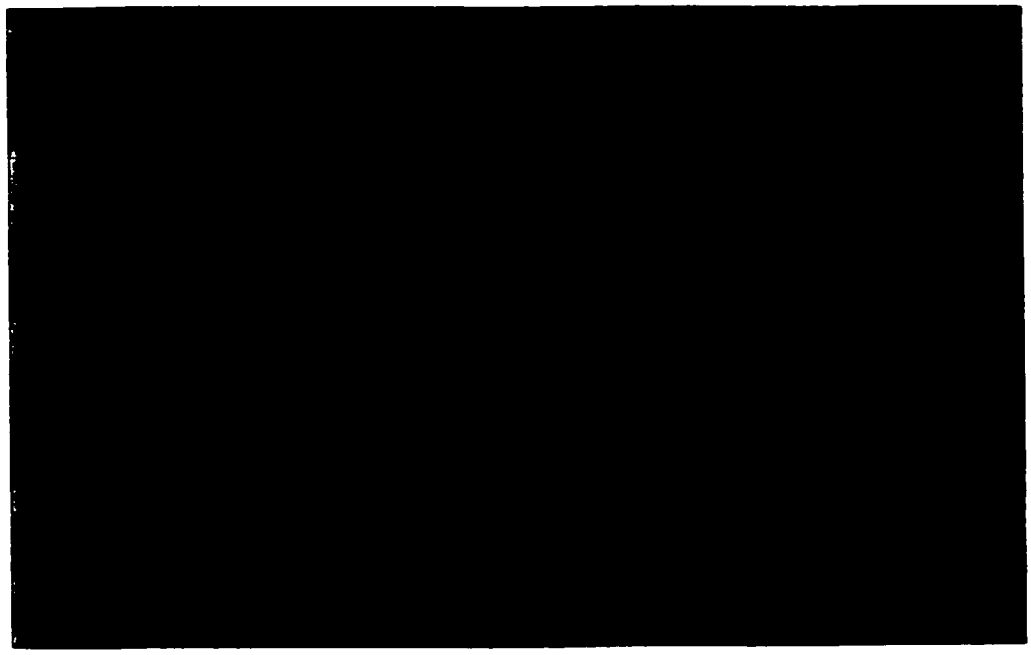


Figure 5.4.5 Immunohistochemical staining of BHV-1 sheep lung. The animals were challenged with Cooper strain by intra-tracheal injection. Samples were collected at day 2 p. i.. (A) Hemaoxylin and eosin staining revealed lung congestion and infiltration of neutrophils and alveolar macrophages . Normal sheep lung is demarcated by arrows. (B) Staining with a pool of mAb against BHV-1 revealed positive staining (Brown cells). In the staining procedure, positive control was a BHV-1 infected bovine lung tissue and the negative control was an irrelevant mAb.

5.5 Recombinant BHV-1 infection in sheep

To determine whether recombinant viruses expressing either IL-1β or IFN-γ, displayed altered pathogenicity or immune modulating activity, we infected three groups of 5 lambs each with either recombinant gC-/LacZ+, BHV/IL-1β or BHV-1/IFNγ viruses. Based on the evolutionary similarities between ovine and bovine, it was expected that both bovine cytokines would be active in the sheep. Ovine IL-1β and bovine IL-1β have a 95 % relative homology on the amino-acid sequence and ovine IFN-γ and bovine IFN-γ share a 96 % relative homology between the predicted amino-acid sequences (McInnes *et al.*, 1997). Nevertheless, since IFN-γ has been shown to be very species specific and show poor cross species activity, we tested bovine IFN-γ anti-viral activity by using sheep fibroblast in the standard VSV inhibition assay. The bovine IFN-γ was able to activate sheep fibroblast into an anti-viral state against VSV, confirming the evolutionary conservation of bovine IFN-γ in sheep (Data not shown).

For the BHV-1 infection model, lambs were infected with 1×10^8 PFU of gC-/LacZ+, BHV/IL-1β or BHV-1/IFNγ virus by aerolisation. Each group of 5 animals was housed in an individual isolation room throughout the course of the experiment. Clinical signs were monitored daily for the first 7 days after challenge. Blood was collected every second day for total and differential counts and for FACs analysis. After 4 weeks, the sheep were challenged with BHV-1 strain 108 to assess protection.

5.5.1 Clinical response

During the primary viral challenge, the clinical veterinarian observed that the

sheep had lesions consistent with an Orf virus infection. Since the majority of animals had already received the challenge dose, it was decided to continue with the experiment. During the post-challenge monitoring period, no group developed a fever (Figure 5.5.1A). Body temperatures were elevated, but not statistically significant for both gC-/LacZ+ and BHV/IL1β groups during the first two days p. i.. In contrast, the mean body temperature of the BHV-1/IFNγ group remained constant throughout the first seven days p. i.. Clinical symptoms were minor for all groups throughout the monitoring period (Figure 5.5.1B). The BHV-1/IFNγ group showed only mild clinical signs on day 3 p. i., and both gC-/LacZ+ and BHV/IL1β groups had detectable clinical signs on day 2 through day 5 p. i.. Peak clinical scores were recorded on day 3 p. i. for all groups. Overall, changes in body temperature and clinical scores were minor when animals were infected with gC⁻ recombinant viruses.

5.5.2 Viral Titer

Nasal secretions were collected daily for 8 days post challenge, using a cotton swab inserted into the right nostril. The opposite nostril was monitored for clinical signs of virus infection. Most animals shed virus for the first two days p. i. (Figure 5.5.2). Peak viral shedding was low in all groups; no animal achieved viral titers over 10,000 PFU/ml. There was no statistical difference among groups for either mean virus

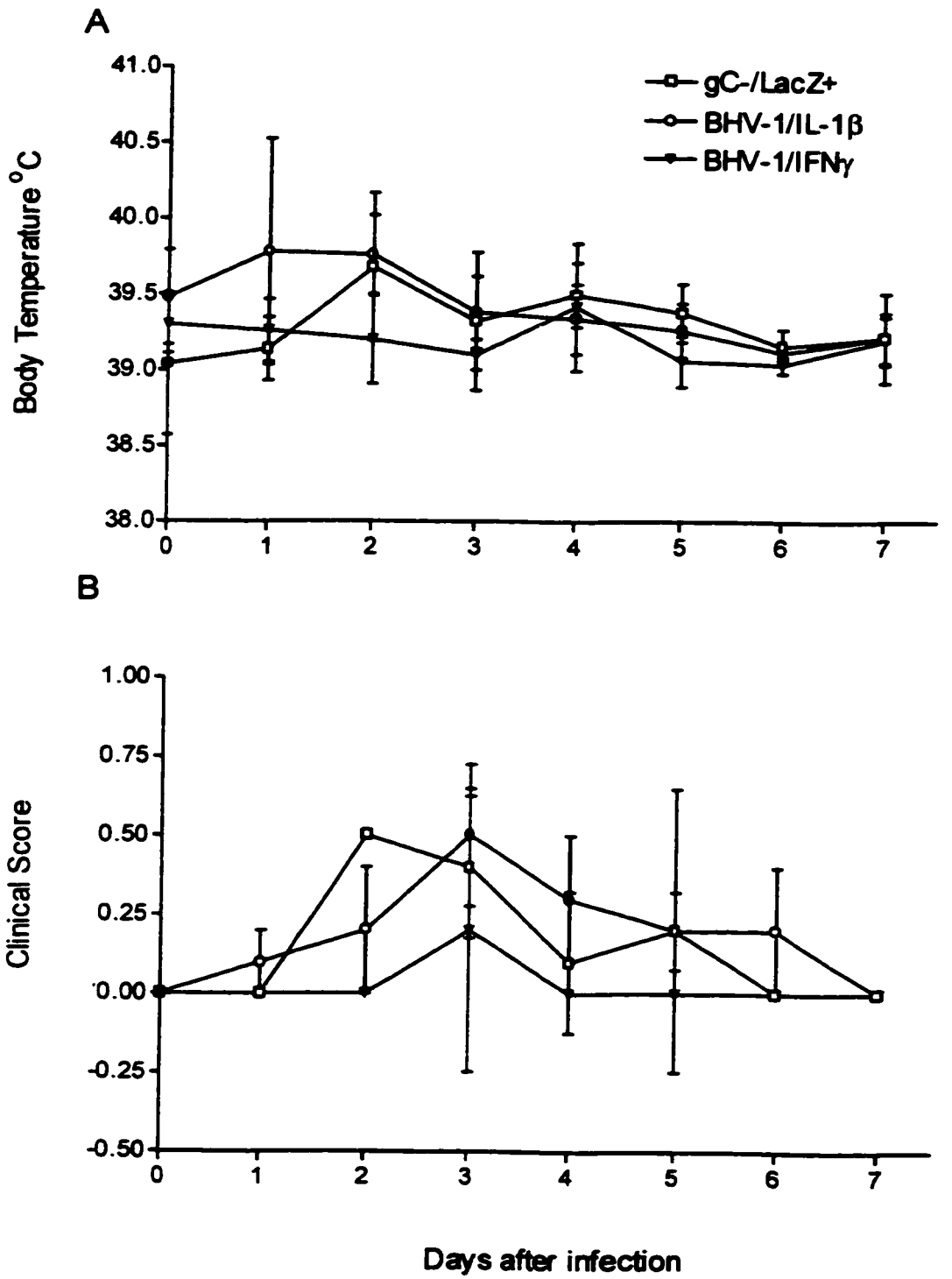


Figure 5.5.1 Clinical responses following recombinant BHV-1 challenge. (A) Rectal temperature (B) Clinical score. Lamb were challenged on day 0 with 1×10^8 PFU of recombinant gC-/LacZ+(circle), BHV-1/IL-1β (square) or BHV-1/IFNγ (triangle). Clinical monitoring was performed daily for 7 days p.i..

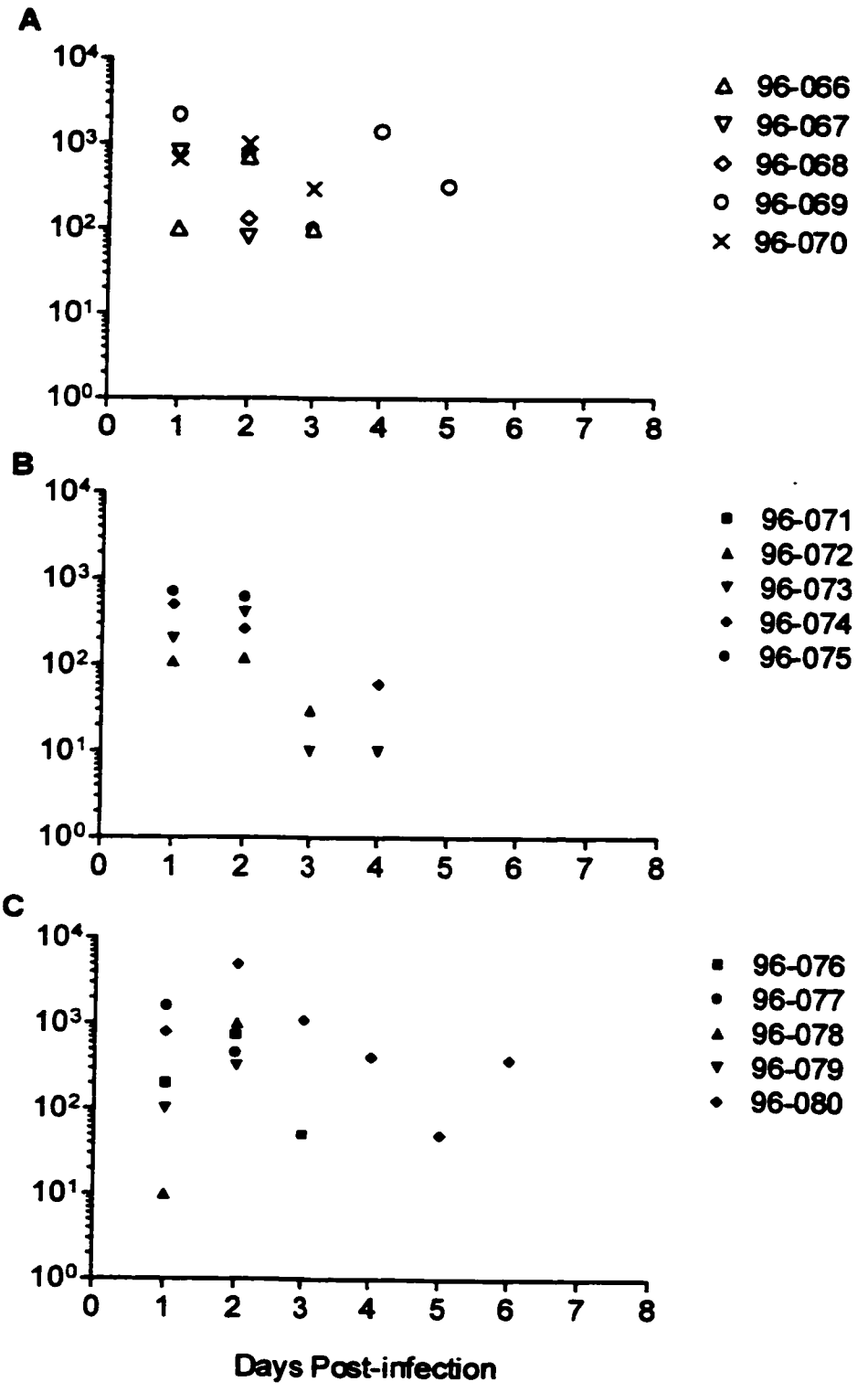


Figure 5.5.2 Virus shedding in nasal secretions. (A) gC-/LacZ+. (B) BHV1/IL1β. (C) BHV-1/IFNγ. Viral shedding was detected by swabbing the right nostril with a cotton applicator. The swab was placed into 1 ml of MEM and titrated in 24-well plates. Individual animals are identified on the right hand margins.

titer or the duration of viral shedding. The majority of animals had stopped shedding infectious virus by day 4 post-infection. Overall, the recombinant BHV-1 viruses appeared equally attenuated during the initial challenge.

5.5.3 Peripheral blood leukocyte population dynamics

Blood was isolated for total and differential counts during the first 8 days after infection (Figure 5.5.3). Similar to BHV-1 infection in cattle, there was a slight decrease in total WBC, lymphocytes and neutrophils (Griebel *et al.*, 1987). There were no statistical differences among groups, except on day 4 p. i. when the BHV-1/IL1β group had a significant decrease in total WBC ($P < 0.05$). As shown in Figures 5.5.4 and Figure 5.5.5, the FACs analysis revealed a similar decrease in lymphocyte subpopulations as reported for BHV-1 infection in cattle (Griebel *et al.*, 1987). However, in the sheep model, infection with recombinant BHV-1 viruses did significantly change the blood leukocyte population dynamics.

5.5.4 Humoral and cellular response

To determine whether recombinant viruses expressing IL-1β and IFN-γ could alter serum antibody titers or cell-mediated immune responses, gD-specific ELISA and proliferative responses were assayed. As shown in Figure 5.5.6, all three groups of lambs developed proliferative responses to truncated gD and U. V. irradiated virus. There were no significant differences in the proliferative responses.

A strong antibody response developed within the first four weeks after

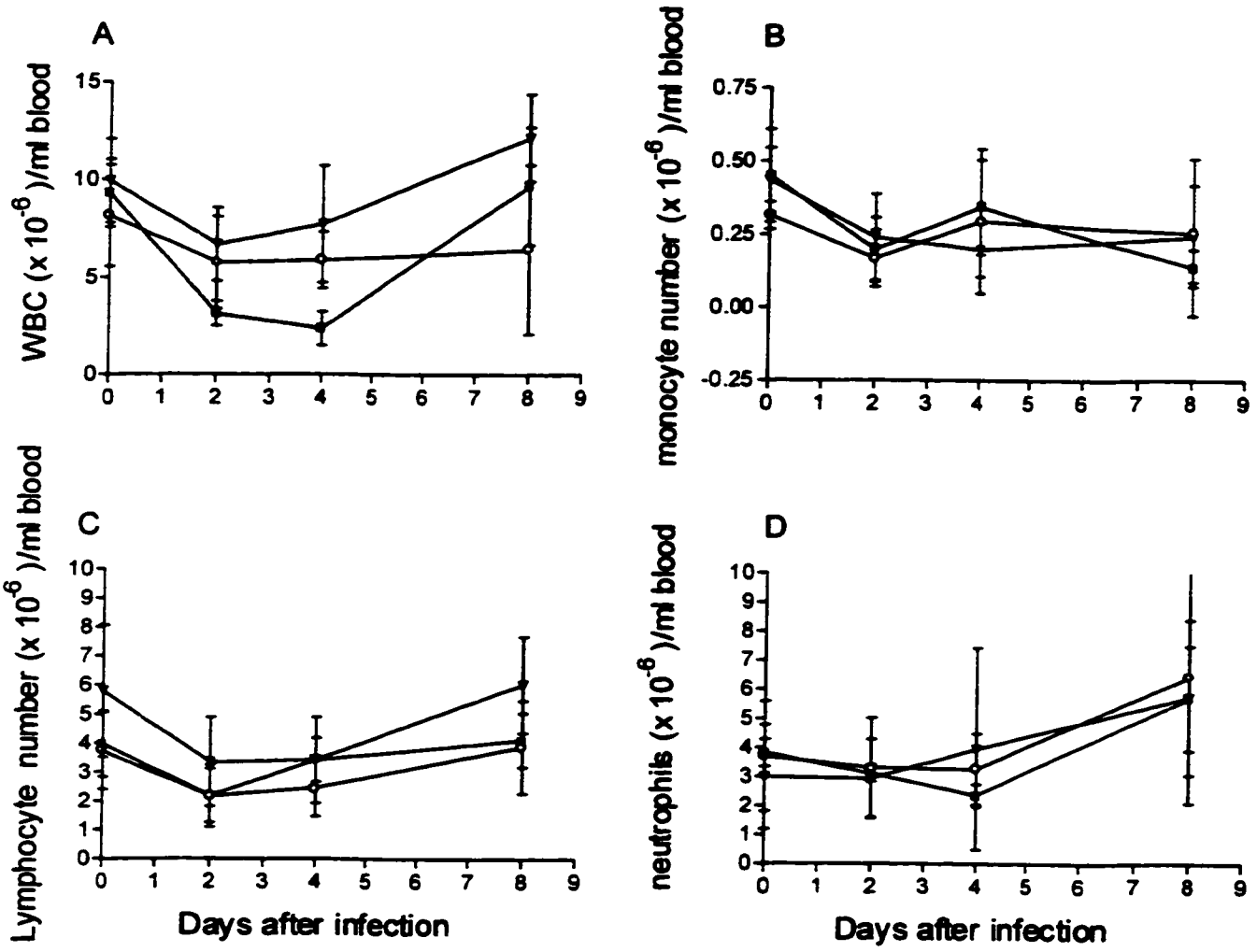


Figure 5.5.3 Changes in the hematologic profiles of recombinant BHV-1 infected lambs. Lambs were challenged on day 0 with recombinant gC-/LacZ+ (circle), BHV/IL-1β (square) and BHV-1/IFNγ (triangle). (A) Total white blood cell counts. (B) Monocytes. (C) Lymphocytes. (D) Neutrophils cells. Data are expressed as means ± one S.D..

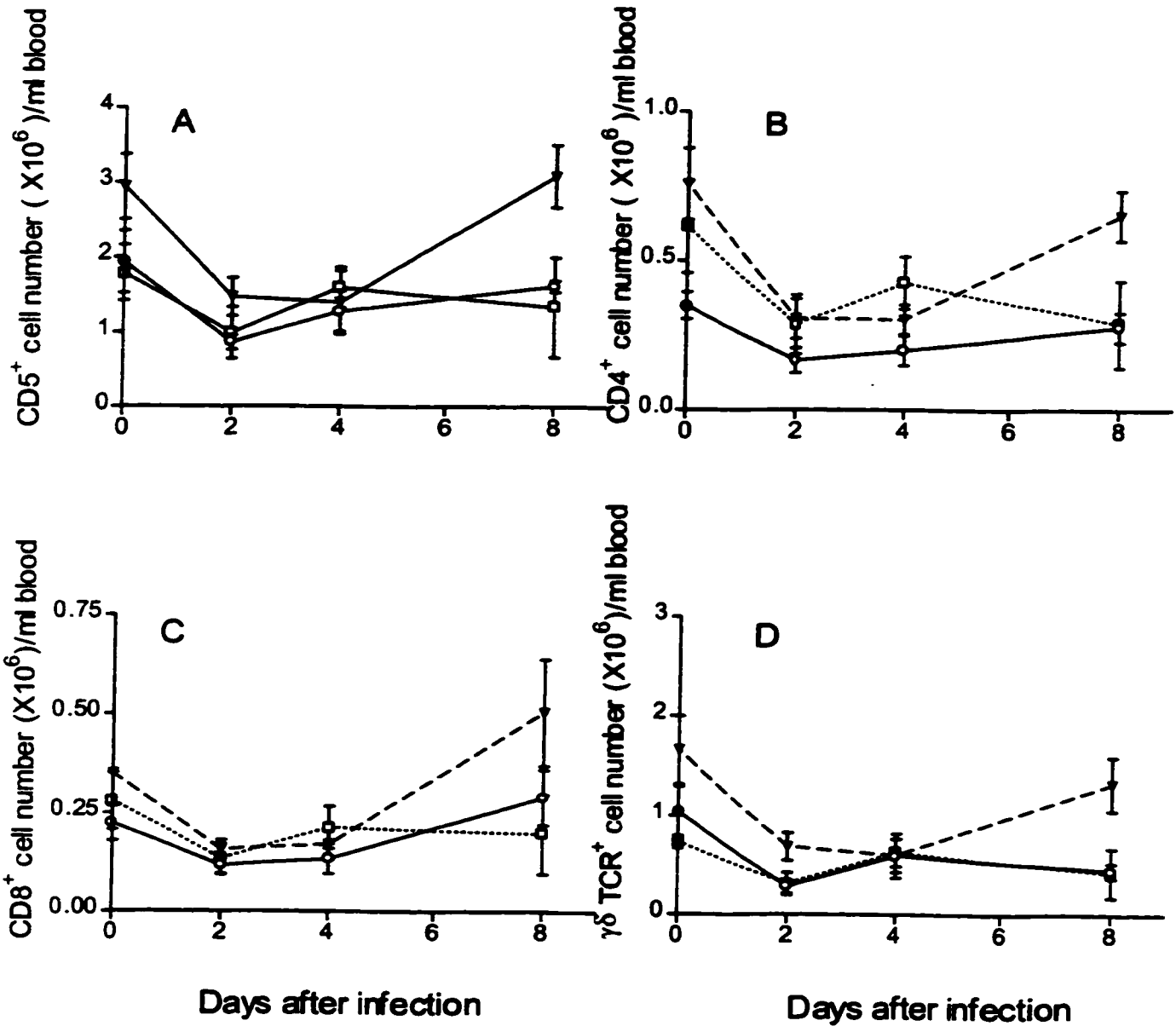


Figure 5.5.4 Changes in T lymphocyte subpopulations in peripheral blood. Flow cytometric analyse were performed following isolation of cells with a blood lysis method. Animals were challenged on day 0 with recombinant gC-/LacZ+ (circle), BHV/IL-1β (square) and BHV-1/IFNγ (triangle). Data are expressed as means ± one S.D..

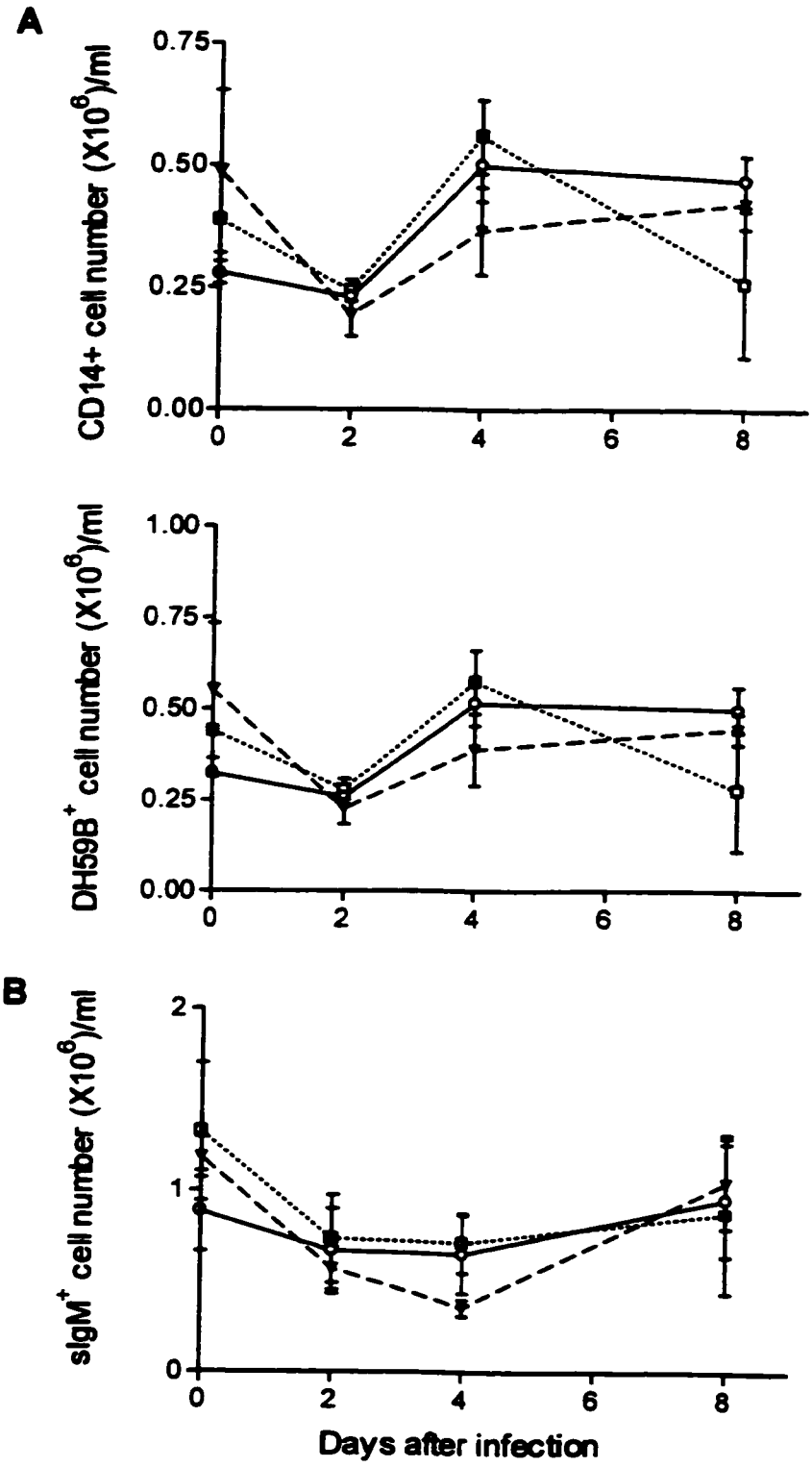


Figure 5.5.5 Changes in monocyte and surface IgM$^+$ B cells. (A) Monocytes (CD14$^+$; DH59B$^+$). (B) B cells surface IgM$^+$. Flow cytometric analysis was performed on blood mononuclear cells isolated with a blood lysis method. Animals were challenged on day 0 with recombinant gC-/LacZ+ (circle), BHV/IL-1β (square) and BHV-1/IFNγ (triangle). Results are expressed as means ± S.D..

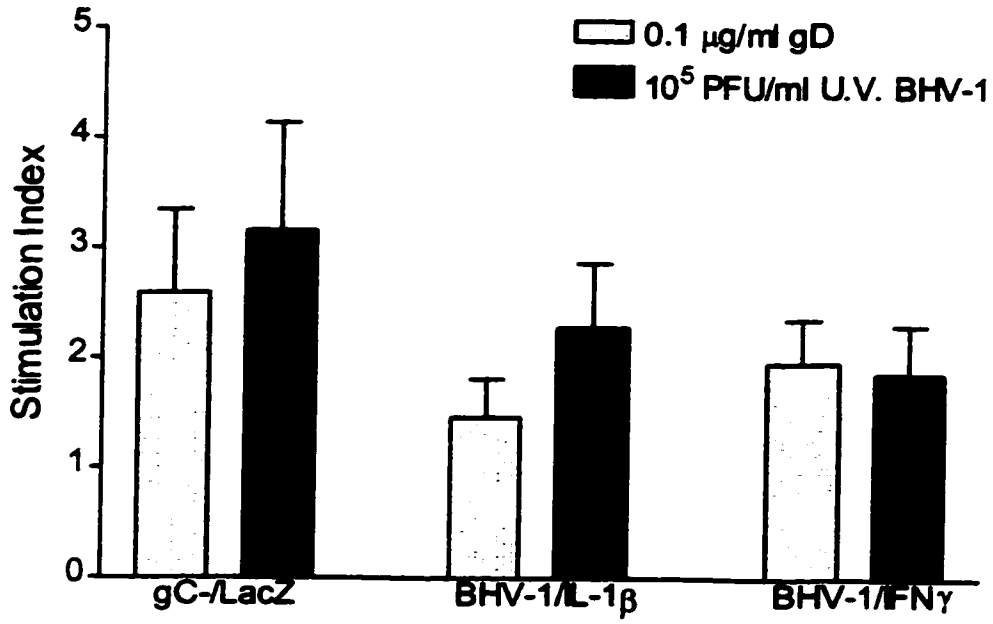


Figure 5.5.6 BHV-1 specific proliferative responses. PBMC were isolated 2 weeks after challenge and stimulated with either BHV-1 gD (0.1ug/ml) or whole irradiated BHV-1. Samples are expressed as mean ± one S.D..

primary infection (Figure 5.5.7) However, serum antibody titers were similar for all three groups. This suggested that the production of IL-1β and IFN-γ by recombinant BHV-1 did not influence the amplitude of the humoral immune response. Virus neutralizing antibody titers also developed after the initial challenge with a further increase in titer following a secondary challenge with BHV-1 Cooper strain on week 5 (Figure 5.5.8). Again, there was no differences among the experimental groups.

5.5.5 Assessing immune protection

Immune protection was assessed by challenging with 1×10^8 PFU of BHV-1 strain 108, 5 weeks after the recombinant virus challenge. The presence of an Orf virus infection may have further interfered with this BHV-1 challenge. As shown in Table 5.5.1, control animals, with an active Orf infection displayed much lower levels of viral shedding relative to the previous experiment with the Cooper strain (section 5.4). The majority of animals shed virus on day 2 but at extremely low titers and shedding was not detected after day 2 p. i.. All recombinant BHV-1 animals showed partial protection but animals from all three immunized groups shed virus on day 2 after challenge (Table 5.5.1).

Table 5.5.1 Strain 108 challenge. All sheep were challenge with 10^8 PFU of strain 108 on week 5. Viral secretions were monitored by nasal swabs.

Groups	Number of animals shedding virus (day 2): n=5	Mean virus titer
Naive	5	4.9×10^2
gC-/LacZ+	1	1.0×10^1
BHV-1/IL-1β	4	3.8×10^2
BHV-1/IFN-γ	3	2.1×10^2

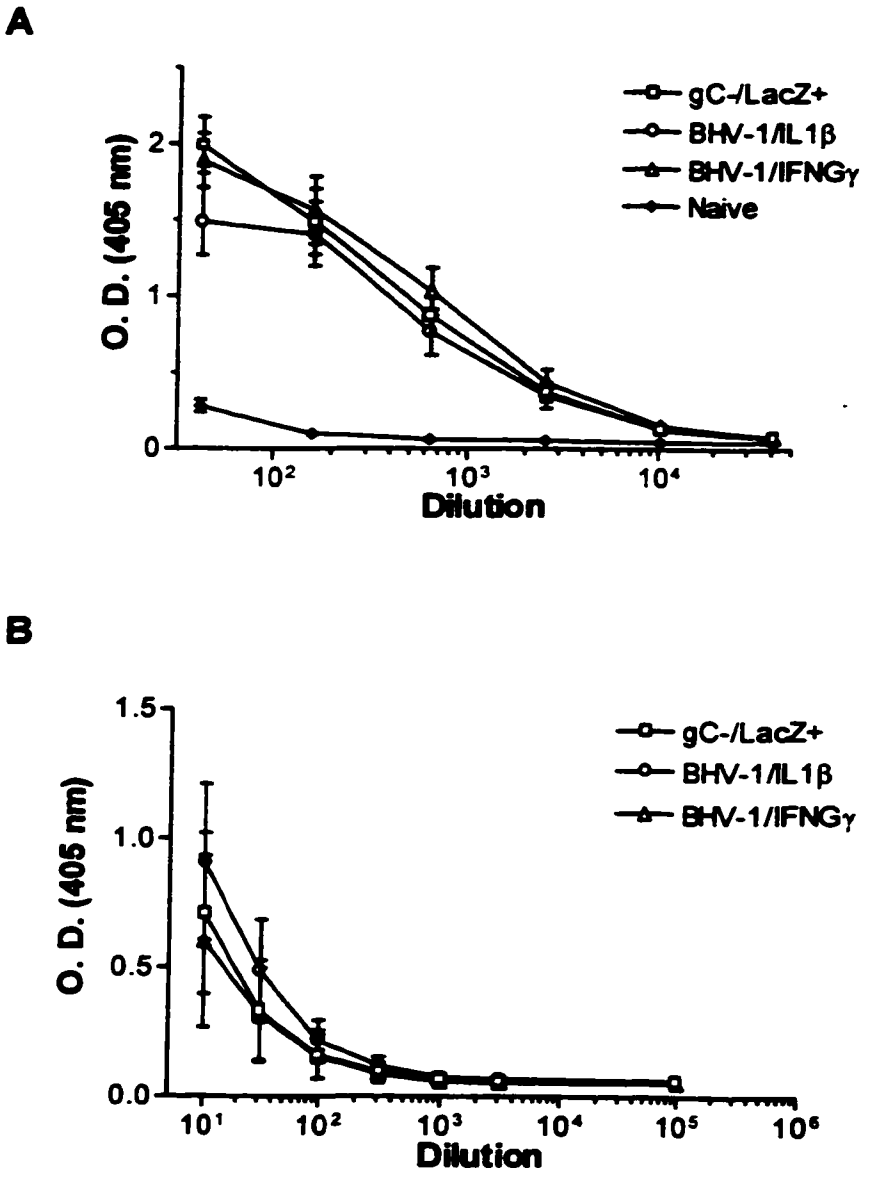


Figure 5.5.7 Serum antibody responses. (A) gD specific IgG response in serum 4 weeks after initial challenge is expressed as optical density. (B) gD specific IgG in nasal secretions. Groups were gC-/LacZ+ (square), BHV/IL-1β (circle) and BHV-1/IFNγ (triangle) and naïve animals (diamond). Data are presented as ± one S.D.

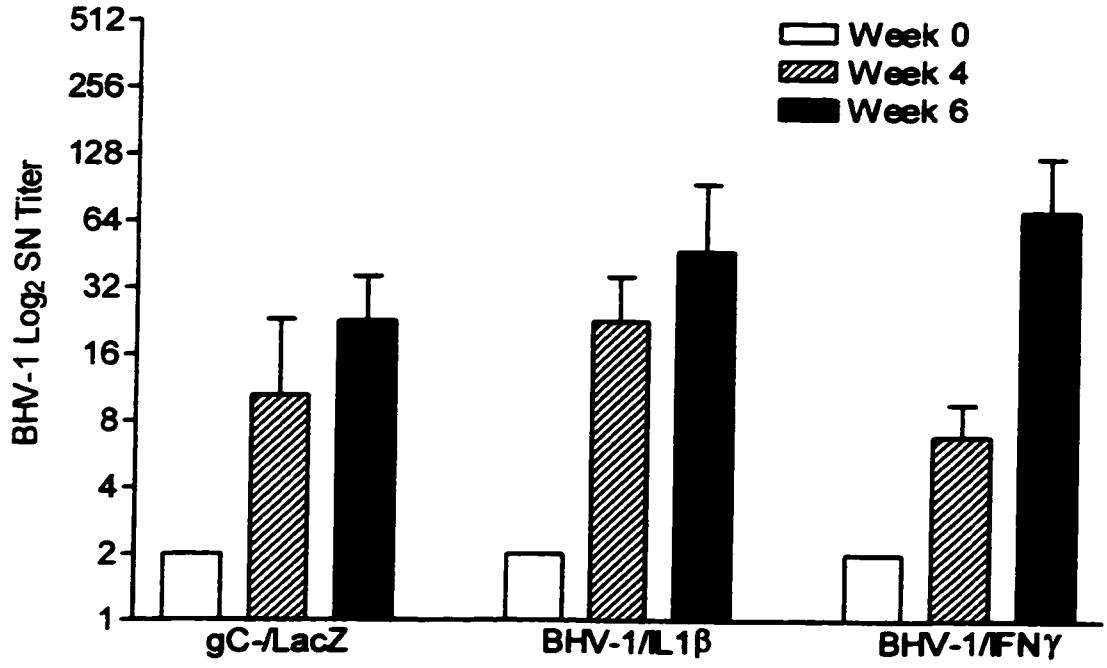


Figure 5.5.8 Serum neutralization titer. BHV-1 neutralizing titers are expressed as the reciprocal of the highest dilution which result in 50 % viral inhibition. Data are presented as ± one S.D.

6.0 DISCUSSION

6.1 Construction and expression of a BHV-1 vector expressing IL-1β

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 were promising, the systemic administration of rBoIL-1β required multiple injections which resulted in toxic side-effects when given at high doses (Reddy *et al.*, 1993; Godson *et al.*, 1995). The construction of a recombinant BHV-1, expressing boIL-1β cytokine had the potential to overcome these limitations by delivering the cytokine to the site of infection over an extended period of time. This might allow the cytokine to act within a local microenvironment, targeting cells involved in the induction of an immune response decreasing the chances of systemic side-effects.

The recombinant BHV/IL1β virus expressed the boIL-1β gene from the gC locus, utilising the gC promoter. Glycoprotein C is a late class gene. gC transcription is detectable being at 5 hrs p. i. followed by a sharp increase at 6 hrs which coincided with DNA synthesis (Seal *et al.*, 1992). The kinetics of boIL-1β mRNA expression followed the same pattern as gC transcription (Figure 5.1.3). A boIL-1β protein was detected in BHV/IL-1β infected cells with a molecular weight similar to the predicted 17-18 kDa of the mature form of IL-1β (Figure 5.1.5) (Maliszewski *et al.*, 1988).

Therefore, the inserted IL-1β gene was functional in terms of transcription and translation of the protein. The bioassay results confirmed that the boIL-1β protein expressed in BHV/IL-1β infected cells was biologically active. The level of boIL-1β expression at 24 h and 36 h was calculated to be approximately 160 units ml^{-1} and 320 units ml^{-1}, respectively. Similar levels of boIL-1β activity were detected in both the cellular fraction and supernatant fraction (data not shown). Although IL-1 activity was not detected in the supernatant at 12 h p. i., the kinetics of mRNA expression suggested that boIL-1β protein was produced in small amounts after 6 hours.

The boIL-1β expressed by BHV/IL1β was both cell-associated and secreted into the medium (Figure 5.1.5). However, a significant proportion of boIL-1β remained cell associated. Immunofluorescence showed that the majority of the cell-associated IL-1 was located in the cytoplasm (Figure 5.1.4). In this regard, expression of boIL-1β by the recombinant virus differed from normal IL-1β expression, since the 17 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 IL-1β converting enzyme (ICE) to generate the mature biologically active 17 kDa cytokine (Kostura *et al*, 1989; Black *et al.*,1989; Thornberry *et al.*, 1992; Cerretti *et al.*, 1992). The actual mechanism of IL-1β transport out of the cell is unknown but may be linked with ICE cleavage of the precursor protein via a non-classical transport pathway (Howard *et al.*, 1995). Our observation that much of the recombinant boIL-1β remained in the cytoplasm was unexpected since the mature sequence of the boIL-1β gene was fused to the gC signal peptide sequence (Figure 5.1.1B). We expected the

boIL-1β protein to be secreted since the construction of a gC fusion protein, lacking the gC transmembrane and cytoplasmic domain regions was efficiently secreted from infected cells (Liang *et al.*, 1993).

IL-1β retention in the cytoplasm may have resulted from an incomplete interaction with the ER/Gogli complex or possibly the presence of other factors such as ICE that were interacting with the gC-/IL-1β protein preventing effective secretion. Recently, a retroviral vector containing the IL-1β gene was constructed using a signal sequence from the IL-1 receptor antagonist. Fibroblasts transduced with this vector effectively secreted IL-1 (Wingren *et al.*, 1996). This observation confirms that IL-1β can be effectively secreted by constructing a signal sequence in front of the mature sequence of IL-1β. Another factor that may influence the efficiency of IL-1β secretion from BHV-1 vector infected cells may be the replication of BHV-1 itself and its effects on cellular transcription and translation. In addition, IL-1β accumulation in the cytoplasm may reflect a high level of IL-1β production under the influence of the strong gC promoter. This could result in an apparent accumulation of intracellular IL-1β that is unable to access an already fully utilized ER/Golgi apparatus. However, based on the analysis of IL-1β bioactivity in 24 hour p. i. culture supernatants, there is a substantial amount of IL-1β secretion (figure 5.1.6). Thus, secreted IL-1β should be able to interact with surrounding immune cells.

In vitro growth experiments indicated that the production of boIL-1β was not toxic to cells and did not affect viral growth. The growth kinetic curve showed that growth of recombinant BHV/IL1β was comparable to a gC$^-$ mutant (Liang *et al.* 1991).

This particular observation was important since it indicated that it was possible to compare both BHV/IL1β and gC⁻ negative mutants *in vivo*, without concerns regarding the immunological consequences of different levels of viral replication. We concluded that it would be possible to investigate the immunological effects of local boIL-1β production during a primary BHV-1 infection.

In conclusion, we constructed a recombinant BHV-1 vector expressing a biologically active boIL-1β protein. The recombinant IL-1β was both cell-associated and secreted into the medium and the expression of boIL-1β in infected cells did not affect the *in vitro* growth of the virus. We speculated that the expression of IL-1β, at the site of infection, may result in a direct or indirect stimulation of infected cells by the cytokine. The next step in our investigation of the recombinant BHV-1 vector was to determine the potential of the cytokine expressed by this vector to attenuate and modulate BHV-1 infection.

6.2 The construction and characterization of a BHV-1 vector expressing IFN-γ

The recombinant BHV-1/IFNγ virus had the expected configuration within the gC locus (Figure 5.2.2). The sequence of mature boIFN-γ was fused to the gC signal sequence. The purpose of this was to generate a protein that could be processed through the Golgi/ER pathway and secreted from the cell. The protein was glycosylated at both N-linked glycosylation sites, confirming that the signal sequence targeted the proteins to the ER/Golgi apparatus (Rinderknecht *et al.*, 1984; Cerriti *et al.*, 1986). Glycosylation digestion experiments confirmed that the N-linked glycans contained complex glycans, which was further evidence that the protein passed through

the ER/Gogli apparatus before being released into the medium (Figure 5.2.3.). Similar to the BHV/IL1β virus, recombinant BHV-1/IFNγ virus infection resulted in a diffuse pattern of cytoplasmic staining for IFN-γ (data not shown). This cytoplasmic staining suggested that recombinant IFN-γ was also produced at a level that exceeded the processing capacity of the ER/Golgi apparatus.

The bioactivity of IFN-γ was confirmed with both a viral inhibition assay and by the specific up-regulation of MHC class II expression on alveolar macrophages (Figures 5.2.4. and 5.2.5). Since the recombinant protein produced in BHV-1/IFNγ infected cells possessed both of these biological functions, we concluded that boIFNγ was folded properly to dimerize and could bind the IFN receptor (Bach *et al.*, 1997). IFN-γ activity was detected as soon as 12 h p. i. in both the cellular and supernatant fractions of BHV-1/IFNγ infected cells. The specificity of this biological activity was confirmed by blocking these effects with neutralizing anti-IFN-γ antibodies. The anti-IFN-γ antibodies did not completely inhibit MHC induction to background levels (43% versus 30.3%). This incomplete neutralization of IFN-γ may be explained by the specificity of the antibodies. The antisera was produced against rboIFN-γ expressed in E. coli (Dr. Dale Godson, personal communication).

A growth kinetic curve confirmed that recombinant BHV-1/IFNγ was able to replicate *in vitro* and this replication was comparable to gC⁻ virus at a m.o.i. of 5 (Figure 5.2.6) (Liang *et al.*, 1991). Again, 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. IFN-γ has anti-viral activity and it was possible that

production of recombinant IFN-γ could affect viral growth or plaque size development during replication in MDBK cells. However, no significant decrease in viral production or plaque development was observed even following infection at a low m. o. i. (Data not shown). Similar observations were reported for an HSV vector constructed to express IFN-α from a RSV LTR promoter (Mester *et al.*, 1995). The KOS derived vector did not show significant inhibition of viral replication in murine L cells, even at low m.o.i.. Also, two other viral constructs (Vaccinia virus and Simian Immunodefiency Virus –SIV) expressing IFN-γ were reported to replicate in tissue culture in a manner similar to wild-type virus (Kohonen-Corish *et al.*, 1989; Giavedoni *et al.*, 1996). Thus, only exposure of cells to recombinant virus supernatant prior to infection could inhibit viral replication .

These observations highlight the kinetics of viral inhibition by interferon. A specific time interval is required for the induction and expression of the intracellular proteins that inhibit viral replication. Viral replication initiated prior to the induction of these anti-viral proteins is not inhibited to the same extent as viral replication in cells pre-treated with interferon prior to infection. This raises an important question regarding the effectiveness of using a late promoter, like gC. The use of an early promoter could change the kinetics of IFN expression and may increase the potential for viral inhibition. In the HSV example, Mester *et al.*, (1995) used a constitutive RSV LTR promoter to express murine IFN-α. IFN-α mRNA was detected as early as 1.5 hours p. i. and achieved maximal expression by 12 hours. However, the use of the early promoter did not inhibit *in vitro* HSV replication when tested in various cell lines and

at multiplicity of infections ranging form 5 to 0.013 (Mester et al., 1995). This observation suggests that regardless of the promoter used, the time required to induce an anti-viral state limits the therapeutic value of IFN to the time prior to initiating viral replication. This is especially true for herpesviruses. In co-incubation experiments, cells were exposed to different levels of IFN-α and infected at the same time with BHV-1. In these experiments, IFN-α had low anti-viral activity even at 10,000 units/ml (Gillespie *et al.*, 1985).

The *in vitro* effects of pre-treating cells for 16 hours with recombinant IFN-α and IFN-γ confirmed that BHV-1 is resistant to low levels of IFN but viral replication can be inhibited at high concentrations (1000 units/ml) (Figure 5.2.6). This observation is consistent with previous experiments that investigated the effects of IFN-α on total viral yield from fibroblasts or alveolar macrophages. The present experiments confirmed that VSV was inhibited at low IFN concentrations but inhibition of BHV-1 replication required a two log increase in IFN concentration (1000 units/ml) to induce significant anti-viral effects (Babiuk *et al*, 1985; Bielefeldt Ohmann *et al.*, 1984). In general, it is known that herpesviruses are more resistant to interferon inhibition when compared with VSV (Rasmussen and Farley, 1975; Fulton and Root, 1978). The mechanism for herpesvirus resistance to IFN is not known.

Although BHV-1 is more resistant to type I and II interferons relative to VSV, biologically relevant levels of interferon can still affect viral replication *in vivo*. During experimental BHV-1 infections, interferon levels in nasal secretions increase to over 1000 units/ml on day 3 p. i. and remain elevated for at least 4 to 5 days p. i.. (Babiuk *et*

al., 1985; Bielefeldt Ohmann and Babiuk, 1985a). The decline in IFN levels at the end of this period coincides with a decrease in clinical signs and viral shedding. Also, during this period, calves may be more resistant to secondary viral challenge (Todd *et al.*, 1972; Cummins and Rosenquist, 1980). It is known that herpesviruses are inducers of interferon and are more resistant to interferon relative to other viruses. Because of this, it has been suggested that the decline in IFN levels on day 4 p. i. is an indirect reflection of decreased viral replication. However, the *in vitro* inhibition of BHV-1 replication by incubation with IFN-α and IFN-γ suggests that at IFN concentrations approaching 1000 units/ml it is possible to inhibit BHV-1 replication (Figure 5.2.6). This *in vitro* experiment indicated that at biologically relevant levels it is possible for IFN to inhibit BHV-1 replication. Thus, decreased viral replication in animals may be partly due to the anti-viral activity of endogenous interferon.

In summary, we constructed a BHV-1 recombinant virus that expressed biologically active IFN-γ. This biological activity was evident as both an anti-viral activity and the ability to up-regulate MHC Class II expression on alveolar macrophages. Alveolar macrophages play an important role in the control of respiratory infections (Bielefeldt Ohmann and Babiuk, 1986; Brown *et al.*, 1988) and a BHV-1 vector that produces IFNγ may activate AM. Thus, this recombinant virus may be a safer vaccine with less risk of a secondary bacterial infections.

6.3 Recombinant BHV-1/IFNγ cattle challenge.

We investigated the possibility that recombinant BHV-1/IFNγ could modulate immune responses. For these experiments, BHV-1 naive cattle divided into three groups. One group was infected with recombinant BHV-1/IFN-γ virus, one group was infected with gC-/LacZ+ BHV-1 and the third group was infected with wild-type Cooper virus.

After the primary infection, animals in all three groups had elevated rectal temperatures and displayed clinical signs of infection. Mean body temperatures were similar among all groups during the period of viral shedding (Figure 5.3.1) and there was no significant differences in clinical symptoms. Recombinant boIFN-γ injected intravenously at a dose exceeding >0.1 μg/kg induced a fever response in cattle (Roth and Frank, 1989; Chiang *et al.*, 1990). The delivery of IFN-γ by our recombinant BHV-1 vector may not have this systemic activity since IFN–γ was released at the site of infection. In addition, the level of acute phase protein, haptoglobin, was similar in the serum of all three groups. This provided further evidence that IFN-γ was acting at a local level and did not induce a systemic effect. In the bovine respiratory disease model, elevated haptoglobin is associated with severity and onset of bacterial infection (Godson *et al.*, 1996).

The expression of IFN-γ by the recombinant BHV-1 vector also had no detectable effect on peripheral blood cell dynamics. Changes in peripheral leukocyte subpopulations at day 3 p. i. included an overall decrease in T lymphocyte and monocyte populations (Figure 5.3.3). This observation is consistent with previous

observation made during an acute BHV-1 infection (Griebel *et al.*, 1987).

The production of IFN-γ by the recombinant virus appeared to have no attenuating effect on viral growth *in vivo*. Both gC-/LacZ+ and recombinant BHV-1/IFNγ deleted mutants had similar viral shedding patterns, but significantly lower levels than that observed with wild-type Cooper infected calves (Figure 5.3.2). These *in vivo* results are consistent with the *in vitro* growth kinetics curve results. In general, glycoprotein deleted BHV-1 mutants (gC, gG, gI and gE) show a significant reduction in viral shedding relative to wild-type parental virus (Liang *et al.*, 1992; Kaashoek *et al.*, 1994; Kaashoek *et al.*, 1998). This decrease in recombinant viral shedding, compared with wild-type Cooper virus, is probably a reflection of the importance of these glycoprotein in viral attachment and penetration (Roizman and Sears, 1996). It was shown that the gC$^-$ mutants have a lower cell binding efficiency and a lower penetration rate than wild-type Cooper virus (Liang *et al.*, 1991b)

In two previous reports of viruses expressing IFN-γ, both recombinant vaccinia and simian immunodeficiency virus (SIV) displayed reduced viral growth when compared with the control gene deleted strain (Kohonen-Corish *et al.*, 1990; Giavedoni *et al.*, 1997). One question that arises from our study is why didn't BHV-1, expressing IFN-γ, also exhibit attenuated phenotype? One possible explanation may be that unique aspects of vaccinia and SIV lifecycles made this strategy successful. Unlike a BHV-1 respiratory infection which infects primarily nasal epithelial, trachea and lung lobes, the vaccinia virus infects many different tissues including ovaries, lungs, spleen and brain (Karupiah *et al.*, 1990b; Sambhi *et al.*, 1991). Therefore, the amount of vaccinia

replication is high and subsequently the amount of IFN cytokine produced may also be much greater. Infection by vaccinia vectors expressing IL-2 and IL-4 can result in clinical symptoms that mimic what is seen when administering large amounts of recombinant cytokine (Bembridge *et al.*, 1998). In general, retrovirus such as SIV, infect a high number of lymphocytes both in the blood and lymph nodes and interact with follicular dendritic cells (Wood, 1990; Fauci *et al.*, 1996). This particular function would be an asset when expressing recombinant cytokines since expression could be targeted to the lymph nodes and follicular dendritic cells, possibly increasing the potential for immune modulation to occur. In addition, these virus may have different sensitivity to the anti-viral effects of IFN-γ.

A second explanation for the lack of immune modulation by BHV-1/IFNγ may be the levels of production of endogenous IFN-γ during a BHV-1 infection. Endogenous IFN-γ production may be sufficient to control viral replication and up-regulate immune cells. IFN-γ was detected by ELISA in nasal secretions collected from all 3 groups (Figure 5.3.5). BHV-1 challenged calves produced 2000 pg/ml on day 4 p. i. (approximately 6000 U/ml based on 3.0×10^6 units/mg standard) and over 100 pg/ml on day 6 p. i.. 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 interferon in nasal secretions (Babiuk *et al.*, 1985; Bielefeldt Ohmann and Babiuk, 1985). In these studies, the amount of interferon was assayed by inhibition of VSV replication. Therefore, the contribution of each type of interferon to this anti-viral

activity was not assessed. Traditionally, interferon in nasal secretions has been assumed to be IFN-α (Straub and Ahl, 1976). The present finding that high levels of IFN-γ are present in nasal secretions suggests that infiltrating NK and T cells may be important sources of IFN-γ within the nasal cavity during a primary infection. The availability of an ELISA for both bovine α/β and IFN-γ would help quantify the relative contribution of each type of IFN.

In general, IFN-γ is thought to play an important role in the clearance of herpesvirus and other acute viral infections (Campos *et al.*, 1989; Klavinskis *et al.*, 1989; Leist *et al.*, 1989; Karupiah *et al.*, 1990; Lucin *et al.*, 1992; Schijns *et al.*, 1994; Smith *et al.*, 1994; Bouley *et al.*, 1995; Yu *et al.*, 1996; Milligan and Bernstein, 1997; Geigor *et al.*, 1997). For herpesviruses, three different methods have been used to assess the involvement of IFN-γ. First, the production of the cytokine at the site of infection has been investigated. During HSV-2 infections, IFN-γ could be detected in vaginal secretions within 2 days and peaked at day 5 p. i. . This was followed by a decrease in virus growth and resolution of infection (Milligan and Berstein, 1997). Secondly, the addition of anti-IFNγ mAb has shown that IFN-γ derived from either CD4+ or CD8+ T cells, plays an important part in the effector phase of acute HSV cutaneous infection (Smith *et al.*, 1994). Finally, IFN-γ gene knock-out mice that were injected intracutaneously (Yu *et al.*, 1996) or intravitreally with HSV are more susceptible to HSV infection and display decreased viral clearance and lower survival rate (Cantin *et al.*, 1995; Bouley *et al.*, 1995). When infected at a low dose of HSV, IFN-γ -/- mice had increased lesion severity and increased viral persistence (Bouley *et*

al., 1995) but eventually virus can be cleared (Geiger *et al.*, 1997). This suggests that other factors can compensate for the lack of IFN-γ. The primary role of IFN-γ during herpesvirus infection may be one of immune regulation rather than a direct anti-viral role (Geiger *et al.*, 1997). Our results are consistent with this view, since the production of recombinant IFN-γ did not significantly inhibit viral growth during a primary infection.

Recombinant BHV-1/IFNγ virus did not induce significant differences in the serum IgG or mucosal IgA production following a primary infection. In addition, serum neutralization titers were similar for both BHV-1 recombinant viruses. In vaccine formulations, IFN-γ has been shown to enhance the antibody response (Nakamura *et al.*, 1984; Heath and Playfair, 1992; Lowenthal *et al.*, 1998; Vandenbroeck *et al.*, 1998). However, viral vectors expressing IFN-γ do not enhance the antibody titers (Kohonen-Corish *et al.*, 1990; Leong *et al.*, 1994; Giavedoni *et al.*, 1997). Furthermore, following dexamethasone reactivation of latent virus both serum IgG and virus neutralization titers showed a similar increase for all groups. Interestingly, after dexamethasone treatment, there was a significantly smaller increase in nasal IgA levels for our recombinant BHV-1/IFN-γ group (Figure 5.3.7). 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 (Kohonen-Corish *et al.*, 1990). Similarly, intranasal immunization with a fowlpox virus co-expressing influenza hemagglutinin and IFN-γ, produced low antibody responses but no difference in the CTL responses (Leong *et al.*, 1994). In addition, boosting with the

same virus did not enhance mucosal IgA secretion, which stayed below the detection limit of the assay (Leong *et al.*, 1994). Our results with dexamethasone reactivation may parallel this finding. However, unlike the fowlpox vector, we were able to detect an increase in nasal IgA after a primary infection.

Differences in the level of initial BHV-1 virus replication between reactivation and a primary infection may account for this difference in nasal IgA modulation. A primary infection should be initiated with numerous cells being infected and a rapid increase in virus replication until host factors control viral growth. In contrast, viral reactivation starts with a small number of infected cells that gradually increase to a detectable level within the environment of an active host immune response. Additional experiments would have to be done to determine if the lack of an amnestic mucosal IgA response was directly effected by IFN-γ release from the BHV-1/IFNγ vector. Nasal secretions obtained by nasal tampons often exhibit high background levels of antibody activity and have considerable variation within a group. Since the experimental groups were small (n = 3), it would be prudent to not over-interpret the present results.

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 (Figure 5.3.8 and Figure 5.3.9). One week after primary infection, these T cell responses were similar for all the experimental groups. This indicated that the lack of glycoprotein C does not result in a detectable difference in the induction of an immune response and, the production of recombinant IFN-γ did not

have detectable effects on T cell responses. The ability of gC⁻ mutants to retain immunogenicity was confirmed by their ability to induce an antibody response (Liang *et al.*, 1992), as well as T cell proliferative responses and NK-like responses (Denis *et al.*, 1996). Interestingly, a comparison of various glycoprotein deletion mutants, including gC, gE, gI and gG, showed that gC⁻ mutants induced the highest proliferative responses and the highest level of non-MHC restricted cytotoxicity (Denis *et al.*, 1996). It has been hypothesized that either gC⁻ mutants retain sufficient immunogenecity or that gC- mutants replicate better than parental BHV-1, which then generates more antigen (Denis *et al.*, 1996). However, our comparison of infectious viral titer in cotton tampons and nasal swabs suggests that gC⁻ mutants are more easily neutralized by components of nasal secretions when released in the nasal cavity (Figure 5.4.1). The difference between isolation methods was dramatic for both gC⁻ mutants tested. Therefore, the increased immunogenicity of gC⁻ mutants may be due to higher amounts of inactived gC virus being transported to the lymph nodes. This may result in greater immune activation.

The actual mechanism by which gC⁻ mutant virus is neutralized by nasal components is not known. One explanation may be gC's role in binding complement. Glycoprotein C from HSV, PRV, BHV-1 and equine herpesvirus can bind the third component of complement (C3) (Huemer *et al.*, 1995). It is possible that gC⁻ mutants lack the ability to evade complement neutralisation both directly or through antibody mediated pathways. Our results are the first *in vivo* evidence that gC may have an important immune evasion function in the nasal cavity during a primary infection. We did not analyze differences between collection methods after dexamethasone re-

activation. However, all gC⁻ mutant infected animals shed virus that was detected by using a nasal swab. In contrast, when cottons tampons were used in a previous experiment, no virus was detected following dexamethasone reactivation of gC-/LacZ+ from calves using cotton tampons (Liang *et al.*, 1992). To confirm that nasal secretions could neutralize BHV-1, mucus from the nasal cavity of naive animals was added to virus for one hour before titration. This experiment showed that components in naive nasal secretions could inhibit replication of both Cooper and gC-/LacZ+ viruses. The addition neutralization seen in gC⁻ virus during *in vivo* replication could be theoretically a result of the lack of binding to complement components by gC glycoprotein. This may result in an inability to evade virus neutralization by the alternative pathway of complement or evade complement receptor activated macrophages neutralization (Huemer *et al.*, 1995; Lubinski, *et al.*, 1998).

Dexamethasone treatment reactivated both Cooper and gC⁻ recombinant viruses from a latent state (Figure 5.3.11). Reactivation was associated with clinical signs and viral shedding. Overall, the level of virus shedding was similar for both control gC-/LacZ+ and the recombinant BHV-1/IFNγ virus. The level of viral shedding was also consistent with the viral titers observed during primary challenge. Both recombinant gC⁻ viruses shed 1-2 logs less infectious virus than wild type Cooper virus.

We confirmed that latent BHV-1/IFNγ recombinant virus retained a functional IFN-γ gene. Virus was isolated from a nasal swab sample after reactivation with dexamethasone and cultured *in vitro*. BHV-1/IFNγ virus produced a functional IFN-γ protein that retained by both anti-viral activity and the ability to up-regulate MHC II

expression (Figure 5.3.12). This confirmed the stability of the recombinant herpesvirus vector. Other viral vectors do not always display the same stability. When a SIV vector infected macaque monkeys, there was a deletion of the IFN-γ gene within 12 weeks p. i. (Giavedoni *et al.*, 1997). The use of a SIV vector to express IFN-γ showed that retrovirus vectors tended to be unstable and lose genetic material from their genome when it does not confer an evolutionary advantage (Giavedoni *et al.*, 1997).

In conclusion, we demonstrated that recombinant BHV-1 virus expressing IFN-γ does not cause adverse clinical effects and the vector was stable throughout the experiment period. Both gC⁻ recombinant viruses (BHV-1/IFNγ and gC-/LacZ+) induced detectable immune responses but the expression of exogenous IFN did not attenuate viral growth or modulate 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. The production of IFN-γ in the nasal cavity appears to be a natural part of BHV-1 pathogenesis. Finally, the expression of recombinant IFN-γ had no detectable effect on viral latency or re-activation of the latent virus. Thus BHV-1 may provide a stable viral vector but the expression of IFN-γ had no detectable effect on viral pathogenesis or the host immune response.

6.2 Sheep model for BHV-1 infections

To develop a less expensive model for testing recombinant vaccines, we investigated the possibility of using sheep as a model for recombinant BHV-1 infections. The year-round availability of naive lambs and the lower cost to purchase and house animals made this a practical approach. Other small animal models have

been tested for BHV-1 replication, especially cotton rats with BHV-1 strain 108 (Papp, Z, Thesis, U of S, 1997). However. preliminary studies with gC⁻ BHV-1 infection of cotton rats showed poor viral replication and shedding after intranasal challenge (data not shown). In addition, to ensure the activity of exogenous IFN-γ and IL-1β, we chose a closely related ruminant species. Previous analysis of cytokine homology have shown that ovine IL-1β has a 95% amino-acid homology with bovine IL-1β and ovine IFN-γ shares a 96% amino acid relative homology with bovine IFN-γ (McInnes et al., 1997). Furthermore, studies investigating the host range and possible natural reservoirs for BHV-1 have shown that BHV-1 can infected both goats and sheep in a farm situation (Trueblood *et al.*, 1978; Brako *et al.*, 1984; Lehmkuhl and Cutlip, 1985; Whetstone and Evermann, 1988).

After aerosol challenge of sheep with wild-type Cooper virus and gC-/lacZ+ BHV-1, clinical signs were much milder than those observed in cattle (Table 5.4.1) (Yates *et al.*, 1982; Wyler *et al.*, 1989). In cattle, classical clinical symptoms include pyrexia with nasal discharge, coughing, mucosal hyperaemia and lymphopenia (Yates, 1982). However, not all these symptoms may be present during experimental challenge. Clinical symptoms can range from severe to mild depending on the host immune status, the route of infection and BHV-1 strain and dose (Frank *et al.*, 1977; Shroder and Easterday, 1968; Yates, 1982). In our sheep infection, Cooper BHV-1 strain exhibited the highest clinical signs and temperature variation, especially with the higher challenge dose (4×10^8). Recent experimental infections of lambs investigating the epizooty of BHV-1 infection, showed that infection with NDDB strain of BHV-1

induced fever and clinical symptoms similar to an experimental infection of calves (Guliani *et al.*, 1995). Thus, different BHV-1 strains may induce clinical responses in sheep that are similar to those of cattle.

In the sheep model, Cooper virus also grew better than the gC-/LacZ+ BHV-1. This resulted in a 1-2 log higher titer of virus in nasal secretions. This difference reflected what we observed with cattle. Virus was isolated 24 h p. i. and viral shedding lasted for 6 to 7 days with peak shedding on day 2 p. i.. In experimentally infected calves, virus shedding tends to peak on days 4-5 post-infection and lasts 10-14 days p.i. (Jericho and Darcel, 1978; Gibbs and Rweyemamu, 1977). However, variation in the initial challenge dose and BHV-1 strain may have influenced the duration of viral shedding in the infected sheep.

The serum antibody response induced by the different BHV-1 strains correlated with the initial dose and level of viral replication. Lambs challenged with a higher dose of virus consistently developed higher antibody titers than lambs challenged with a lower dose (Figure 5.4.3). Furthermore, sheep infected with Cooper virus produced a higher antibody response than sheep infected with the same dose of gC$^-$ virus. Although the sheep model may not be a good model to study clinical responses, it was possible to compare the immunogenicity of different vaccine candidates.

The histological lesions detected after BHV-1 infection consisted of multiple foci of pulmonary necrosis that contained nuclear debris and an infiltration consisting of neutrophils and macrophages. These pulmonary lesions were similar to those reported for cattle (Jericho and Darcel, 1978; Yates *et al.*, 1983) and immunohistochemical staining for BHV-1 antigen confirmed that the lesions contained viral protein.

Pathological changes of congestion and infiltration of mononuclear cells resulting in lung consolidation of the lung were previously for BHV-1 experimentally infected sheep (Guliani *et al.*, 1994).

Preliminary challenge experiments using only aerosolized BHV-1 did not induce detectable BHV-1 lesions in the lung of lambs (data not shown). However, virus was isolated from tracheal samples containing epithelial cells of the upper respiratory tract, which is the primary site of BHV-1 infection in cattle (Gibbs and Rweyemamu, 1977). In contrast, pulmonary lesions are highly variable and may not always be evident during BHV-1 infection (Yates, 1982). The amount of lung replication may depend on the host's immunological status and the dose and strain of virus (Lupton and Reed, 1980). The present investigation indicated that sheep may also exhibit variable pulmonary lesions during BHV-1 infection.

Immune protection in the lamb model was assessed by challenging the previously infected sheep with BHV-1 strain 108. Following a primary infection, all sheep showed partial protection against a BHV-1 challenge. Viral shedding was decreased in both the level and duration of viral shedding. Animals receiving a high dose of Cooper during the primary immunization were completely protected against a secondary challenge. The naive control group had peak viral shedding at day 2 post-infection and viral shedding persisting for 6-7 days.

Therefore, based on this preliminary experiment, we concluded that sheep could serve as a alternative animals model for BHV-1 infection. Clinical symptoms were inconsistent with only mild hyperaemia of the nasal cavity following the high titer challenge with Cooper virus. However, the lamb model appeared useful for evaluating

the immunogenicity of recombinant BHV-1.

6.3 Recombinant virus sheep challenge model.

To assess whether cytokine expression could modulate BHV-1 infections in sheep, we infected three groups of five animals with either recombinant BHV-1/IFNγ, BHV-1/IL1β and control gC-/LacZ+. Similar to the results in cattle, the administration of recombinant BHV-1 to sheep resulted in no adverse clinical effects relative to the control virus. Recombinant boIL-1β can induce fever (Reddy *et al.*, 1990; Godson *et al.*, 1995), so we expected that the expression of this pro-inflammatory cytokine might result in increased inflammation or fever.

However, viral shedding was brief when compared to the previous gC-/LacZ+ infection of sheep. Reduced viral shedding may have resulted from a concurrent Orf virus infection and this may have limited the production of recombinant cytokine. The majority of viral shedding occurred on days 1 and 2 p. i., with no significant difference between recombinant viruses expressing cytokine and the control gC-/LacZ+ BHV-1 virus.

Orf virus or scabbymouth virus is a DNA parapoxvirus that replicates in epidermal cells and lasts 4-6 weeks during a primary infection (Mckeever *et al.*, 1988). Pox viruses have developed numerous evasion strategies to counter the host's immune response (Gooding, 1992). Similar to vaccinia virus, Orf encodes virulence factors which may inhibit the host immune response (Haig *et al.*, 1996). Theoretically, Orf co-infection with herpesvirus may have limited BHV-1 replication because of the active immune response against Orf virus. Interferon, GM-CSF and TNF-α production can be

detected in lesions during a primary Orf infection (Haig *et al.*, 1996).

A comparison of BHV-1 shedding among groups showed no differences in titer or duration. This was consistent with results in cattle. Overall, the cattle and sheep experiments confirmed that the expression of IFN-γ did not attenuate a primary herpesvirus infection. Several explanations for this observation were outlined in the discussion for the cattle experiments. The primary reason for no apparent modulation may be that insufficient levels of recombinant IFN-γ were produced to alter viral replication or clinical signs.

The analysis of total and differential WBC counts revealed a significant decrease in WBC on day 4 p.i. for the recombinant BHV-1/IL1β group. This decrease in cell number was caused by a decreased number of neutrophils in blood (Figure 5.5.3). However, this neutropenia contrasts with the neutrophilia reported following the injection of recombinant IL-1β into cattle (Godson *et al.*, 1995; Van Kessel *et al.*, 1996). The neutropenia observed in our study was not statistically significant, but may be caused by low levels of IL-1β produced by the recombinant virus. IL-1 can enhance endothelial cell expression of adhesion molecules and in this way may alter neutrophil migration into the tissue (Dinarello, 1992). However, whether the observed neutropenia was associated with increased cell trafficking from the blood into the lung and upper respiratory tract remains to be determined.

Our analysis of gD specific proliferative responses showed a similar but limited response from all groups (Figure 5. 5. 6.). All groups also developed an antibody response but there were no differences in the IgG titers in either serum or nasal

secretions. These results in sheep were consistent with our previous finding with recombinant BHV-1infection of cattle. These results are also similar to those reported for SIV viruses expressing either IL-2 or IFNγ, where no significant enhancement of antibody titer or CTL activity was observed. Only, when SIV expression of IFN-γ reduced viral growth in peripheral lymphocytes was there a significant decrease in the antibody titer. A similar correlation between decreased viral growth and reduced antibody titers was reported for vaccinia virus constructs (Kohonen-Corish *et al.*, 1990).

To assess immune protection in the 3 experimental groups, sheep were challenged with a BHV-1 strain 108. However, little viral shedding was detected during this challenge, even in the naive control group. The level of viral shedding by the recombinant virus groups suggested partial immune protection. The gC-/LacZ+ BHV-1 group showed that best overall protection with only 1 out 5 animals shedding detectable virus (Table 5.5.1). This observation confirmed that the gC$^-$ vector could generate a good immune response against BHV-1. The concurrent Orf infection may have also diminished the duration of viral shedding in the challenged animals.

6.4 General conclusions and future applications

Changing the immune response to a more effective and beneficial outcome has been the goal when developing strategies to modulate the immune response. Our understanding of what constitutes an appropriate and beneficial immune response has been defined by various methods, including both direct investigation of immune responses against pathogens and investigating the effects of modulating the immune response by different techniques. The construction and characterization of BHV-1 virus vectors that express cytokines may aid in the development of new vaccine strategies against lethal pathogens. This type of novel research has wide latitude in both theory and practice within the veterinarian field. With the available models, investigations can be conducted in both the laboratory and the host.

The conclusions of this thesis will be helpful for the future design of experiments that seek to engineer pathogens that express immune modulators. Also, this investigation contributed to our understanding of BHV-1 biology and pathogenesis. The finding of this thesis can be summarised in the following major points:

(1) The first goal of this project was to construct a BHV-1 vector expressing bovine cytokines inserted into the gC locus. This objective was predicated on previous research that investigated both the immunology and virology of BHV-1 (Fitzpatrick *et al.*, 1989; Liang *et al.*,1992 ; Campos *et al.*, 1994). The construction of recombinant BHV-1 vectors was problematic due to the lack of a positive selection system. Our initial screening method involved classical antibody based 'Black plaque methods' which relies on recombination frequency and large multiple plate screening searches.

We refined this selection method by using a reverse selection of the gC-/LacZ+ expressing mutant that gives a blue background from the lacZ reaction to contrast with a white positive recombinant plaque. This altered selection process simplified the identification of recombinant virus.

(2) It was shown that two cytokines could be expressed from the gC locus without affecting *in vitro* viral growth. By generating a signal sequence after the gC promoter, cytokines were effectively secreted from infected cells. However, the late kinetics of the gC promoter may not always be ideal for the expression of cytokines since the time required for activation of the immune response or host cells may exceed the time required to induce an effective antiviral response. Moreover, the deleted glycoprotein C may have unique immune and biological functions that may limit the usefulness of this vector. The gC protein plays a role in both viral attachment and penetration, as well as, a possible role in immune evasion.

(3) BHV-1 is a stable vector, even during latency and after recrudescence. Throughout the cattle infection experiment, BHV-1/IFNγ retained its capacity to produce biological active IFN-γ.

(4) In experiments with both cattle and sheep, there was neither detectable attenuation of viral infection or significant immune modulation. For BHV-1, IFN-γ has been shown to play an important role in the generation of an effective immune response (Campos *et al.*, 1992). By constructing a BHV-1 vector expressing IFN-γ, we assumed that a positive immunomodulation should result either by direct anti-viral mechanisms or through up-regulation of the immune system. Our investigation of

possible immune modulation focused on cellular and humoral immune response during a primary infection. This approach included both systemic and local mucosal responses such as IgA and IFN-γ secretion. It was not previously appreciated that BHV-1 is a potent inducer of endogenous IFN-γ. Future investigations may be more revealing if they focus on mucosal responses within the lung. The ability to perform repetitive lung lavages on infected calves would allow for an analysis of the phenotype and function of alveolar cell populations during an infection. (Dr. Keith West, personnel communications).

(5) The ability to detect immunomodulation may require more sensitive models, since both recombinant viruses exhibited only limited modulation. BHV-1/IL1β induced a transient decreased in WBC due to decreased neutrophils in the blood. BHV-1/IFNγ infection was only associated with the lack of IgA response after reactivation with dexamethasone. These limited changes in immune responses may indicate a low level of recombinant cytokine expression within the targeted sites. Successful viral vectors appear to either target into lymph nodes or produce high quantities of cytokine. The BHV-1–*Pausteurella hemolytica* infection model may reveal the potential of recombinant BHV-1 vector to activate innate effector functions and enhance the ability of macrophages and neutrophils to clear secondary bacterial infections. Furthermore, BHV-1 infection of colostrum deprived newborn calves may be a more sensitive model to detect possible immune modulation activity by recombinant BHV-1 vectors (Mechor *et al.*, 1987).

(6) BHV-1 infected sheep generated a BHV-1 specific immune response. Recombinant infections of both animal models (lambs/calves) revealed similar results. The initial investigation of sheep as an alternative model for BHV-1 infection showed that for certain vaccine approaches, sheep may be useful evaluating the immunogenicity of vaccines i.e. DNA vaccines.

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