Induction of interferon and interferon-induced antiviral effector genes following a primary bovine herpesvirus-1 (BHV-1) respiratory infection

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Abstract

In vitro investigations have identified a variety of mechanisms by which herpesviruses evade interferon-stimulated antiviral effector mechanisms. However, these immune evasion mechanisms have not been evaluated during a bovine herpesvirus-1 (BHV-1) infection. This study investigated the transcription and secretion of type I and II interferons (IFNs) and the transcription of IFN-stimulated genes (ISGs) during a primary BHV-1 infection of the upper respiratory tract (URT) in naive calves. IFN-α, -β and -γ transcription in nasal turbinates and protein levels in nasal secretions increased following infection. Increased IFN type I and II secretion was detected 3 days post-infection (p.i.) and IFN production increased in parallel with virus shedding. Expression of ISGs, including Mx1, OAS and BST-2, also increased significantly (P<0.05) in nasal turbinates on day 3 p.i. and elevated ISG expression persisted throughout the period of viral shedding. In contrast, RNAase L gene expression was not induced during the BHV-1 infection in the nasal turbinates, but was induced on day 10 p.i. in the trachea. In vitro studies confirmed that recombinant bovine (rBo)IFN-α, -β and -γ induced expression of Mx1, OAS and BST-2, but decreased RNAase L transcript in bovine epithelial cells. Relative to vesicular stomatitisvirus (VSV), BHV-1 was resistant to the antiviral activity of rBoIFN-α and −γ, but treatment of epithelial cells with 10 ng rBoIFN-β ml⁻¹ effected an 80% inhibition of BHV-1 replication and complete inhibition of VSV replication. These observations confirm that the transcription and translation of type I and II IFNs increase during BHV-1 infection, while the transcription of some ISGs is not inhibited.

INTRODUCTION

Interferons (IFNs) are a diverse family of proteins that function as an important component of the innate immune defence against viral infections. There are three major types of IFNs, types I, II and III, known to have antiviral activity. Type I bovine IFNs include nine subtypes of IFN-α and three subtypes of IFN-β [1]. Bovine type II IFN comprises only IFN-γ, encoded by a single gene [2], and type III bovine IFN is currently represented by IFN-A3 [3]. IFNs act in a biphasic manner, with increased IFN production activating IFN signalling pathways. IFN production is induced following detection of viral components by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs). Both type I and II IFNs are known to establish an ‘antiviral state’ when bound to appropriate cell-surface receptors prior to viral infection [4]. This antiviral state involves activation of interferon-stimulated genes (ISGs) and subsequent production of antiviral effector proteins, including double-stranded RNA-dependent protein kinase R (PKR), myxovirus resistance (Mx) proteins, oligoadenylate synthetase–RNAase L (OAS–RNAse L) and BST-2 or tetherin [5]. These antiviral effector proteins target different steps in the viral replication cycle to prevent both assembly and release of infectious virus particles [5]. It is not known, however, if both type I and II bovine IFNs activate similar ISGs and anti-viral effector proteins.

Bovine herpesvirus-1 (BHV-1) is a member of the subfamily Alphaherpesvirinae, characterized by lytic infection of epithelial cells at mucosal surfaces and the establishment of latent infections in peripheral sensory neurons [6]. BHV-1 was first described as the cause of a venereal disease in cattle known as Bläschenausschlag by Reisinger and Reimann [7]. A respiratory form of BHV-1 infection was later discovered in 1953 and described as infection of the upper respiratory tract (URT); this was named infectious bovine rhinotracheitis (IBR) [8]. Primary BHV-1 respiratory infections may last 10 to...
12 days and bovine nasal turbinates, pharyngeal tonsils and trachea are the primary sites of infection in the URT [9]. BHV-1 is a potent inducer of type I and II bovine IFNs [10-12] and there is evidence that BHV-1 evades both type I and II IFN-induced antiviral effector mechanisms. Pre-treatment of calves with recombinant bovine IFN-α did not significantly reduce virus shedding following an experimental BHV-1 challenge [10], and expression of bovine IFN-γ by a recombinant BHV-1 did not significantly reduce viral replication [12]. Previous studies have confirmed the induction of ISGs, such as OAS, in blood leukocytes following BHV-1 infection [11], but no studies have evaluated the expression of ISGs at the site of viral infection in the URT. Thus, it is not known whether local production of type I and II IFNs results in increased ISG expression at the site of viral infection and whether the onset of ISG expression coincides with a decline in viral replication.

The mechanism(s) by which BHV-1 may evade IFN antiviral activity has been investigated in vitro. BHV-1 immediate early protein, bovine ICP0 (bICP0), was reported to induce degradation of IRF-3, resulting in decreased IFN-β transcription [13]. Similarly, bICP0 may also inhibit intermediate kinases in the interferon-signalling pathway, including TANK-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKe), resulting in an inhibition of IFN-β promoter activity and decreased ISG expression [14]. Another in vitro study reported that an immediate early BHV-1 protein, bICP27, inhibits the activity of the promoters of bovine IFN-β, resulting in an inhibition of IFN-β transcription [15]. Other IFN evasion mechanisms have been identified for members of the Alphaherpesvirinae subfamily, such as herpes simplex-1 (HSV-1). HSV-1 encodes gene products that block IFN-α production and inhibit the antiviral activity of PKR through dephosphorylation of eIF-2 [16]. Varicella-zoster virus (VZV) also inhibits PKR through phosphorylation of eIF-2α [17]. Additionally, HSV-1 has been shown to block BST-2 function through virion host shut-off protein (VHS)-mediated degradation of BST-2 mRNA [18]. Thus, in vitro studies provide evidence that herpesviruses may inhibit transcription of IFN genes and inhibit IFN-induced antiviral effector proteins at both a transcriptional and post-transcriptional level. It has not been determined in vivo, however, whether BHV-1 evades the IFN response through an inhibition of IFN transcription or translation, or through an inhibition of ISGs at a pre- or post-transcriptional level.

In this paper, we confirm that BHV-1 infection induces transcription and secretion of both IFN-α and −γ in the URT, and for the first time also demonstrate the coincidental transcription and secretion of IFN-β. Production of both types I and II IFNs in the URT preceded peak levels of virus shedding in nasal secretions. To further test the hypothesis that BHV-1 evades IFN antiviral activity through an inhibition of ISG transcription, we also analysed ISG transcription in URT tissues. This analysis supports the conclusion that BHV-1 evasion of IFN, a key innate immune defence, does not occur through an inhibition of either the transcription or the translation of IFNs, or a transcriptional inhibition of ISGs.

RESULTS

Virus shedding persists after induction of IFN secretion

BHV-1 shedding in the nasal secretions of infected calves increased significantly (P=0.002) by day 3 p.i. to a mean value exceeding 1 x 10^6 p.f.u. ml^-1 of nasal secretion, and remained at this level until day 7 p.i. (Fig. 1a). Virus shedding then decreased approximately three logs on day 10 p.i. to a mean value of approximately 1000 p.f.u. ml^-1.

IHC staining of tissues collected from the URT with rabbit anti-BHV-1 antisera revealed a similar temporal pattern for virus replication (Fig. 1b). No visible staining for BHV-1 was observed in any of the tissues prior to BHV-1 infection (Fig. 1b). In contrast, nasopharyngeal tonsils from all animals (n=6) had visible focal staining of epithelial cell for BHV-1 on days 3 and 5 p.i., but detectable staining then decreased to approximately 70 % (4/6 animals) on day 7 p.i. and 50 % (3/6 animals) on day 10 p.i. A similar focal staining pattern was observed with nasal turbinates, but visible staining was only detected in tissue samples from all six animals on day 5 p.i.

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Fig. 1. Viral replication in the upper respiratory tract following a primary BHV-1 infection. (a) Shedding of infectious BHV-1 particles in nasal secretions. Virus titres were determined by plaque assay and the data presented are values for individual animals (n=6/time point). (b) BHV-1 infection of nasal turbinate, nasopharyngeal tonsil and tracheal mucosa was detected using polyclonal rabbit anti-BHV-1 sera. The data presented are the number of individual animals (n=6/time point) from which tissue samples had visible staining of BHV-1 antigens in mucosal epithelial cells.
within the nasal turbinates or trachea, but there was extensive loss of epithelium overlying the nasopharyngeal tonsils (data not shown). This observation is important when considering qRT-PCR results for tissue samples collected from nasal turbinates, since these samples included both BHV-1 infected and uninfected epithelial cells.

High levels of infectious BHV-1 in nasal secretions on day 3 p.i. coincided with a significant ($P<0.01$) increase in IFN-α secretion (Fig. 3a). Peak IFN-α levels were detected on day 5 p.i. IFN-α secretion declined significantly on day 7 p.i., even though virus shedding persisted at a high level. Thus, onset of IFN-α production in nasal secretions did not correlate with a decline in virus shedding and peak IFN-α levels did not coincide with a decline in virus production.

The commercial ELISA used to quantify bovine IFN-β in nasal secretions gave an optical density reading that was consistent with IFN-β being present prior to viral infection (Fig. 3b). There was, however, a significant ($P<0.05$) increase in IFN-β levels in nasal secretions on day 5 p.i. (Fig. 3b). A high level of BHV-1 shedding was, therefore, observed both before and after peak IFN-β production. Finally, a significant ($P<0.0001$) increase in IFN-γ production was detected in nasal secretions on days 3, 5 and 7 p.i. (Fig. 3c). Therefore, a high level of virus shedding persisted for at least 2 days after maximum levels of IFN-α, -β and -γ were detected in nasal secretions.

IFN levels in nasal secretions may not correlate directly with cytokine production within tissues. Therefore, qRT-PCR analysis of IFN gene expression in nasal turbinates was completed to determine whether IFN protein levels in nasal secretions reflected host responses at the site of viral infection. There was marked inter-animal variability in IFN-α transcript abundance when analysing nasal turbinates collected prior to BHV-1 (Fig. 3d). Following BHV-1 infection, there was a consistent decrease in the median ΔCt values of IFN-α transcript, but this change was only significant ($P<0.05$) on day 7 p.i. While protein levels in nasal secretions did not directly mirror IFN-α transcript abundance in nasal turbinates, the qRT-PCR analysis confirmed that IFN-α transcript increased early after viral infection. Furthermore, peak IFN-α and -β transcript abundance and secretion both occurred during the period of peak virus shedding.

The qRT-PCR analysis also confirmed an early increase of IFN-β transcription following BHV-1 infection with significantly ($P<0.05$) increased transcript abundance on days 3, 5 and 7 p.i. (Fig. 3e). IFN-β gene transcript abundance then declined on day 10 p.i., but did not return to pre-infection levels. Thus, qRT-PCR analysis revealed an early and sustained increase in transcript abundance for IFN-β genes in BHV-1 infected tissue, but increased transcription of IFN-β genes was not closely associated with a decrease in virus shedding.

The abundance of IFN-γ gene transcript also varied widely among individual animals prior to BHV-1 infection. High
intra-group variance limited the capacity to detect significant changes in transcript abundance when analysing six animals/time point. There was, however, a consistent decrease in median $D_{CT}$ values for IFN-γ gene transcript on all days analysed following BHV-1 infection (Fig. 3f). This decrease in $D_{CT}$ values was maximal on day 5 p.i., coinciding with maximum IFN-γ protein levels in nasal secretions (Fig. 3c). Thus, qRT-PCR results were consistent with ELISA data (Fig. 3c).

Expression of antiviral effector genes in nasal turbinates

Monitoring expression of known antiviral effector genes provides an independent confirmation that biologically active levels of IFN were present in infected tissues. qRT-PCR analysis revealed a highly significant ($P<0.001$) increase in both OAS (Fig. 4a) and Mx1 (Fig. 4b) transcript within 3 days p.i., and this increase persisted throughout viral infection. A similar expression pattern was observed...
for BST-2 (Fig. 4c), with a significant (P<0.01) increase in BST-2 transcript on days 3, 5 and 7 p.i. and a decline in transcript abundance on day 10 p.i. In contrast, RNase L transcript abundance (Fig. 4d) was significantly (P<0.001) decreased on days 5 and 7 p.i., but approached pre-infection levels on days 10 p.i.

Expression of antiviral effector genes in trachea

Transcription of IFN-induced antiviral effector genes was also analysed for tracheal tissue samples. A significant (P<0.05) increase in OAS (Fig. 5a) and Mx1 (Fig. 5b) transcript was observed within 3 days p.i., with sustained increase throughout the period of viral infection. A significant increase in BST-2 transcript level was not detected until day 10 p.i. (Fig. 5c). As observed with nasal turbinates, there was a significant (P<0.05) decrease in RNase L transcript on days 3 to 7 p.i., but this was reversed on day 10 p.i. when transcript abundance was significantly (P<0.01) increased.

Thus, increased transcription of multiple ISGs was detected within 3 days p.i. for both nasal turbinates and trachea. While there were tissue-specific differences in ISG transcriptional responses, we consistently observed that the onset of increased ISG expression was not associated with a decline in virus shedding (Fig. 1a) or tissue infection (Fig. 1b).

IFN induction of antiviral effector genes in bovine epithelial cell qRT-PCR analysis revealed a significant induction of three of the four IFN-induced effector genes analysed in nasal turbinate samples following BHV-1 infection. Coincidental production of multiple IFN types (Fig. 3) suggested that more than one IFN type may be responsible for the observed increases in ISG transcript. There is limited information regarding bovine ISG responses to IFN-α [19], and little is known regarding the induction of ISGs by bovine IFN-β and -γ. Therefore, we investigated whether rBoIFN-α, -β and -γ have the capacity to induce expression of the four antiviral effector genes analysed following BHV-1 infection. Mx1, OAS and BST-2 transcription increased 10- to 100-fold within 4 h after bovine epithelial cells were exposed to rBoIFN-α, and remained elevated for 12 h (Fig. 6a). Similarly, Mx1, OAS and BST-2 transcription increased within 4 h after exposure to 100 ng ml⁻¹ of IFN-β, but transcription then decreased to basal levels by 6 h post-treatment (Fig. 6b). rBoIFN-γ also induced a sustained 10- to 100-fold increase of Mx1, OAS and BST-2 transcription throughout the 12 h period (Fig. 6c). Both rBoIFN-α and -γ induced increased RNase L transcription at 4 h post-
treatment (Fig. 6a, c), but RNAse L transcription was not altered by rBoIFN-β treatment (Fig. 6b). Thus, both type I and II rboIFNs have the capacity to induce the expression of multiple ISGs in bovine epithelial cells.

**BHV-1 evasion of IFN antiviral activity**

The persistence of a high level of BHV-1 shedding (Fig. 1a) following significant induction of IFN-α, -β and -γ protein and gene expression in nasal turbinates (Fig. 3) suggested that BHV-1 effectively evades IFN-induced antiviral effector mechanisms. To address this possibility, a plaque inhibition assay was used to compare rBoIFN inhibition of BHV-1 replication relative to vesicular stomatitis virus (VSV), a virus well-documented to be sensitive to IFN inhibition [20]. A 50 % inhibition of VSV p.f.u. was observed when cells were pre-treated with 0.005 ng rBoIFN-α ml⁻¹ and complete inhibition of VSV replication was observed following treatment with 1 ng rBoIFN-α ml⁻¹ (Fig. 7a). In contrast, rBoIFN-α inhibition of the BHV-1 isolate 108 (Fig. 7a) and Cooper isolate (data not shown) only approached 50 % when cells were pre-treated with 10 000 ng rBoIFN-α ml⁻¹. This is an IFN-α concentration over 1000-fold higher than that detected in nasal secretions from infected animals (Fig. 3a). rBoIFN-β also displayed strong antiviral activity, with 50 % inhibition of VSV p.f.u. following pre-treatment of cells with 0.005 ng rBoIFN-β ml⁻¹ and complete inhibition of VSV replication with 10 ng rBoIFN-β ml⁻¹ (Fig. 7b). rBoIFN-β concentrations of 0.05 and 10 ng ml⁻¹ were able to effect 50 and 80 % inhibition, respectively, of BHV-1 108 (Fig. 7b) and Cooper isolates (data not shown). Thus, substantial inhibition of BHV-1 replication was observed with rBoIFN-β concentrations similar to those detected in nasal secretions on day 5 p.i. (Fig. 3b). rBoIFN-γ also displayed substantial antiviral activity, with 50 % inhibition of VSV p.f.u. when cells were pre-treated with 0.01 ng rBoIFN-γ ml⁻¹ and complete inhibition of VSV replication with 100 ng rBoIFN-γ ml⁻¹ (Fig. 7c). When comparing the concentration of rBoIFN required to completely inhibit VSV replication, rBoIFN-γ was 100-fold less active than rBoIFN-α and 10-fold less active than rBoIFN-β. Furthermore, the highest concentration of rBoIFN-γ tested in vitro, which was 500-fold higher than that detected in vivo (Fig. 3c), effected less than 30–40 % inhibition of BHV-1 isolate 108 (Fig. 7c).

**DISCUSSION**

IFNs provide a key innate defence against viral infections, but viral pathogens have developed a variety of strategies to prevent the induction of IFNs or block the activity of IFN-induced antiviral effector proteins. In *in vitro* studies have
identified numerous mechanisms by which viruses block IFN induction and signalling, or disrupt the activity of IFN-induced anti-viral effector proteins. Few studies have been completed, however, to determine whether these immune evasion strategies are relevant \textit{in vivo}, where multiple IFN types or subtypes can be activated simultaneously, and multiple viral transcripts and proteins are being expressed. BHV-1 provides an interesting example of an acute respiratory infection known to induce a robust response of both IFN-\(\alpha\) and -\(\gamma\) [11], but high levels of IFN secretion coincide with sustained viral replication (Figs 1 and 3). For the first time, we also demonstrated that a primary BHV-1 infection is associated with significant \((P<0.05)\) induction of IFN-\(\beta\) transcript (Fig. 3e) and protein (Fig. 3b) in the bovine URT. Furthermore, induction of both type I (IFN-\(\alpha\) and -\(\beta\)) and type II (IFN-\(\gamma\)) responses occurred early during infection, but viral replication persisted at a high level and for 10 days despite this multifaceted IFN response.

Previous \textit{in vitro} observations led to the conclusion that BHV-1 evaded IFN-\(\beta\) antiviral activity by inhibiting IFN-\(\beta\) transcription [13, 15] and the transcription of ISGs [14].
Our in vivo observations do not support this conclusion, since IFN-β transcription was significantly (P<0.05) increased in infected tissues, beginning as early as day 3 p.i. (Fig. 3e). Although increased gene transcription may not result in increased protein production, ELISA results (Fig. 3b) confirmed that bovine IFN-β production increased significantly (P<0.05) following BHV-1 infection. The contradictory results from in vitro studies [13, 15] and our in vivo study may be explained by several factors. The results obtained by Saira et al. [13] may be unique to the cell lines used and may not reflect the capacity of mucosal epithelial cells to respond to BHV-1 infection. We also designed qRT-PCR primers to detect transcripts from the three known bovine IFN-β genes (Fig. S1, available in the online Supplementary Material) and the transcriptional response we detected may represent transcripts from multiple IFN-β genes. Further studies may be warranted to determine whether BICP0 is equally effective in inhibiting the promoter activity of all bovine IFN-β genes. Finally, mucosal tissues in the URT represent a complex cell population (Fig. 3) with the potential to recruit immune cells. IFN transcript detected with RNA extracted from nasal turbinates and tracheal samples represents the collective response of both infected and uninfected cells (Fig. 3d–f). Other herpesvirus proteins, such as HSV-1 glycoprotein D, function as a potent activator of IFN responses in macrophages [21]. Thus, the IFN response detected at a tissue level may represent the response of a variety of cells to different viral proteins. The current observations highlight the importance of completing in vivo studies to confirm whether the IFN evasion mechanisms identified in cultured cells or implicated by studies in an aberrant host [22] adequately explain viral immune evasion in the natural host.

Other respiratory viruses, such as influenza, have been reported to induce less than 0.15 ng IFN-α ml⁻¹ in nasal secretions [23], a concentration 5- to 10-fold lower than we detected following BHV-1 infection (Fig. 3a). Previous in vivo and in vitro investigations indicated that BHV-1 replication was not inhibited by either rBoIFN-α [10] or -γ [12]. There was, however, no previous analysis of ISG expression in URT tissues following BHV-1 infection to determine whether local IFN production activated local antiviral defence mechanisms. The current investigation confirmed that BHV-1 evades the antiviral activity of both type I and II bovine IFNs at a concentration that effectively induces transcription of key antiviral effector genes (Fig. 6). This IFN evasion was a conserved trait among the plaque-purified BHV-1 isolates 108 and Cooper (data not shown), and in vitro studies support the previous conclusion that BHV-1 replication is not inhibited by either IFN-α or -γ (Fig. 7a, c). While IFN-β appeared to be a relatively weak inducer of antiviral effector gene transcription (Fig. 6b), it was the most potent inhibitor of BHV-1 replication (Fig. 7b). This in vitro observation suggests that IFN-β might play a role in inhibiting BHV-1 replication in vivo. IFN-β may reduce the magnitude of BHV-1 replication, but the early post-infection induction of IFN-β transcription and secretion suggests that it may have a limited effect on the duration of virus shedding (Fig. 3). A broader analysis of ISGs induced by bovine IFN-β is necessary to determine whether antiviral effector mechanism(s), other than those analysed in the present study, play a role in limiting BHV-1 replication.

Previous studies of ISG expression following BHV-1 infection focused on OAS activity in blood leukocytes [24]. Subsequent studies confirmed that rBoIFN-α specifically induces OAS activity in lung macrophages, while elevated OAS activity persisted in blood leukocytes after rBoIFN-α was no longer detectable in serum [25]. Current in vitro studies confirmed that rBoIFN-α is a potent inducer of OAS and other ISGs in bovine epithelial cells, while IFN-α was at least 10-fold more effective in activating sustained expression of ISGs than rBoIFN-γ (Fig. 6). This observation is consistent with the data reported by Liu et al. [26] and the proposal by Marié et al. [27] that IFN-α can activate a positive feedback loop to amplify expression of a delayed set of IFN-α genes. rBoIFN-β also significantly increased the expression of OAS, Mx1 and BST-2 by 100- to 1000-fold, but this induction of ISG expression was transient.

Expression of Mx1 and OAS genes in nasal turbinate and trachea increased significantly (P<0.05) within 3 days p.i. and elevated ISG expression persisted throughout the period of viral shedding. It is not possible to conclude which IFNs were responsible for increased ISG expression in tissues, since our in vitro analyses confirmed that all three rBoIFNs can induce antiviral effector gene expression in bovine epithelial cells (Fig. 6). This in vitro analysis also questions whether the ISGs analysed in the current study actually play a role in restricting BHV-1 replication. However, for a closely related herpesvirus, HSV-1, it has been reported that Mx1 and BST-2 can restrict HSV-1 replication [18, 28]. Both rBoIFN-α and -γ had strong antiviral activity against VSV replication and induced sustained ISG expression, but had limited capacity to inhibit BHV-1 replication. Based on the plaque inhibition assay results, the peak IFNα and -γ levels detected in nasal secretions (Fig. 3) would have the capacity to inhibit less than 20% of BHV-1 replication. Thus, in vitro results support the conclusion that IFN-α and -γ have limited capacity to directly inhibit BHV-1 replication, despite their capacity to induce antiviral effector genes. In contrast, IFN-β was a more potent inhibitor of BHV-1 replication in vitro at concentrations observed in vivo (Fig. 3). Thus, the early induction of IFN-β following BHV-1 infection may indicate that it plays an important role in limiting the magnitude of BHV-1 replication.

In vitro studies confirmed that recombinant bovine rBoIFN-α and -γ induce sustained expression of Mx1, OAS, and BST-2, but only transient RNase L transcription in bovine epithelial cells (Fig. 6). A significant (P<0.01) decrease in RNase L transcript was observed in the nasal turbinate (Fig. 4d) and trachea (Fig. 5d) following BHV-1 infection. These results cannot be interpreted as evidence that BHV-1 infection inhibited expression of this ISG if IFNs only induce transient increases in RNase L transcript.
Furthermore, the pattern of ISG expression observed may be tissue-specific, since there was significantly (P<0.01) increased RNAse L transcript in the trachea on day 10 p.i. (Fig. 5d). RNAse L is potentially an important antiviral effector for limiting BHV-1 replication, since it degrades single-stranded mRNAs and inhibits the translation of viral proteins [29]. However, while IFN-β was a potent inhibitor of BHV-1 replication in vitro, it did not induce increased expression of RNAse L in bovine epithelial cells (Fig. 6b). Therefore, the present study provides no evidence to indicate that RNAse L may be an important antiviral effector protein for limiting BHV-1 replication.

BHV-1 evasion of IFN antiviral activity, especially for IFN-α and -γ, raises questions regarding the potential value of this multifaceted IFN response for the host. The induction of this potent innate immune defence may not play a major role in limiting the duration of viral replication; however, IFN-α not only activates antiviral effector genes (Fig. 5), but it also activates NK cells to produce IFN-γ [30]. IFN-γ may, then, have indirect antiviral effects through the induction of chemokines, such as CXCL10 and CXCL9, also known as monokines induced by IFN-γ (MIG). CXCL10 may amplify NK cell recruitment and MIG may recruit T-cells to the site of viral infection [31]. Thus, elevated IFN-γ levels at the site of viral infection may enhance the recruitment of both innate immune cells and virus-specific effector T-cells. NK cells were recruited to the lung following BHV-1 infection and these cells had an enhanced capacity to kill BHV-1-infected cells [32]. An analysis of immune cells recruited to the site of BHV-1 infection in the URT may clarify whether the IFN response indirectly benefits the host by enhancing viral clearance through the recruitment and activation of immune cells.

In conclusion, the present study confirmed that type I and II rBoIFNs have the capacity to induce the expression of several antiviral effector genes in bovine epithelial cells. At physiological concentrations, however, only rBoIFN-β was able to effect greater than 50 % inhibition of BHV-1 replication. In vivo studies confirmed that the transcription and secretion of both type I and II IFNs occurs within 3 days after a primary BHV-1 infection, and this IFN response is associated with a significantly (P<0.05) increased expression of multiple antiviral effector genes in the URT. However, viral replication in the URT continued for 10 days despite strong induction of both type I and II IFN, and the onset of ISG expression early after viral infection. In vivo data support the conclusion that BHV-1 evasion of the IFN response does not appear to occur through an inhibition of either IFN gene expression or ISG transcription.

METHODS

Animals

Female and castrated-male, 5- to 6-month-old, crossbred (Angus × Hereford) suckling calves (n=30) were purchased from a single commercial herd. Calves were identified as seronegative for BHV-1 by screening with a recombinant truncated glycoprotein D (tgD) antibody-capture ELISA [33]. For this study, calves were weaned, transported to the VIDO research facility, and adapted for 2 weeks to a diet of free-choice hay and 0.5 kg oats/day. Calves were housed in a single pen. The average mass of the calves was 232 kg, with body mass ranging between 174 and 242 kg. Tissue samples were collected from the nasal turbinates and trachea of six animals on days 0 (pre-infection), 3, 5, 7 and 10 post-infection (p.i.). All experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care, and the experimental protocol was approved by the University of Saskatchewan Animal Care Committee.

Experimental infection and sample collection

All calves were aerosol-challenged with BHV-1 isolate 108 (5×10⁷ p.f.u./animal) on experiment day 0 as previously described [11]. A clinical veterinarian, blinded to treatment group, examined the calves daily and recorded their body mass and temperature. Nasal swabs for the analysis of virus shedding and nasal secretions for the analysis of IFN were collected from all animals prior to BHV-1 challenge and on days 1, 3, 5, 7 and 10 p.i. Nasal secretions for quantifying virus shedding were collected with sterile cotton swabs, which were then immersed in 1 ml of Eagle’s minimum essential medium (MEM) and transported on ice to the laboratory. Swabs were stored at −20 °C prior to performing viral plaque assays to quantify infectious BHV-1 particles. Nasal secretions for assaying IFN secretion were collected by inserting a cotton tampon into the ventral meatus of the nostril for 20 min. The absorbed fluid was expressed from the tampon and stored at −80 °C until analysed by ELISA. For tissue collection, cohorts of six calves were euthanized with an intravenous injection of Euthanyl (240 mg ml⁻¹; Bimeda-MTC) on days 0, 3, 5, 7, and 10 p.i., immediately following the collection of nasal swabs for virus and nasal secretions for IFN analysis. Tissue samples were collected within 15–20 min after euthanasia and three replicate samples were immediately placed in RNAlater or 10 % buffered formalin. Tissue samples from nasal turbinates were collected 10–12 cm from the external nares and tracheal mucosa was collected from the mid-trachea region of all calves. Formalin-fixed tissues were used for histology and immunohistochemical detection of BHV-1. Tissues fixed with RNAlater were stored at −80 °C until RNA was extracted for qRT-PCR analysis of transcripts from IFN genes and ISGs.

Enzyme-linked immunosorbent assay (ELISA)

Calves were screened for BHV-1-specific serum antibodies using an antibody-capture ELISA as previously described [34]. Briefly, recombinant BHV-1 tgD was used to coat ELISA plates for antibody capture following serial dilution of serum samples. Calves with serum antibody titres of less than 1/100 were considered seronegative. IFN-α and -γ concentrations in nasal secretions were quantified by ELISA as previously described [12]. The IFN-α ELISA used in this study employed a capture monoclonal antibody raised
against the BoIFN-α1 protein expressed from the cloned sequence of the BoIFN-α1 gene shown in Fig. S2 [35]. IFN-β concentrations in nasal secretions were determined using a commercial bovine IFN-β ELISA kit (Biomatik), following the manufacturer’s instructions.

Cells and viruses
A bovine mucosal epithelial cell line was established from fetal bovine intestine [34] and Madin–Darby bovine kidney (MDBK) cells were purchased from ATCC (ATCC no. CCL22). The mucosal epithelial cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 5 % FBS, 5 µg ml⁻¹ of Apo-transferin (Sigma), 0.025 µg ml⁻¹ of epidermal growth factor (EGF) (Sigma), 10 µg ml⁻¹ of insulin (Sigma) and 50 µg ml⁻¹ gentamicin (Gibco) per 500 ml DMEM. MDBK cells were cultured as previously described [36]. The BHV-1 isolates used were 108 (Animal Disease Research Institute, Lethbridge, AB, Canada) and Cooper strain (ATCC no. VR864). Vesicular stomatitis virus (ATCC no. VR158) was used as a positive control when quantifying the antiviral activity of recombinant bovine (rBo) IFN-α, -β and -γ.

BHV-1 plaque assay
MDBK cells were used to perform a plaque assay to quantify infectious BHV-1 shed in nasal secretions as described previously [36, 37].

Immunohistochemical (IHC) staining of bovine tissues for BHV-1 proteins
Tissues from the nasal turbinates, pharyngeal tonsils and trachea were collected from six animals euthanized prior to BHV-1 infection and six animals on days 3, 5, 7 and 10 p.i. These animals were from the same experiment as that used for monitoring virus shedding, IFN secretion, and collection of tissues for RNA extraction. Tissues were fixed in 10 % buffered formalin (HARLECO) prior to embedding in paraffin and 5 µm tissue sections were cut, treated for antigen recovery, and then stained with polyclonal rabbit anti-BHV-1 antisera [Prairie Diagnostic Services (PDS), Saskatoon, SK, Canada].

RNA extraction
The TRlzol method (Invitrogen), as described previously [38], was used to extract total RNA from nasal turbinates and trachea samples stabilized in RNAlater. RNA was extracted from 10⁶ cultured mucosal epithelial cells seeded per well in CellBind six-well plates (Corning). Cells were stimulated with 100 ng ml⁻¹ of either rBoIFN-α, -β, or -γ for the indicated time intervals (Fig. 5). At each time point, culture supernatant was removed and 1 ml of TRlzol was added to each well; this was gently pipetted up and down to lyse the cells and the lysate was then stored at −20 °C until the RNA was extracted. RNA extraction was performed as described previously [38]. RNA quality and total RNA were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with an RNA integrity number (RIN) of 7 or higher were used to prepare cDNA.

cDNA synthesis and quantitative real-time PCR (qRT-PCR)
Total RNA (1 µg) was reverse-transcribed using qScript cDNA SuperMix (Quanta Biosciences), following the manufacturer’s protocol. cDNA samples were diluted in DNAse/RNase free water for use as a template in qRT-PCR reactions.

qRT-PCR primer design and validation
Primer pairs for ISG transcripts (Table 1) were designed using DNAMAN software (Lynnon Corp), and primers for IFN-α, -β and -γ transcripts were designed using the Clone

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence*</th>
<th>Product size (bp)†</th>
<th>Primer efficiency‡</th>
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<td>RV</td>
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<tr>
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</table>

*Sequence of forward (FWD) and reverse (RV) primers.
†Size of amplified PCR product.
‡Efficiency of product amplification.
Manager 9.0 program (Sci-Ed Software). Primers for IFN-α, -β and -γ transcripts were designed to validate IFN-α, -β and -γ protein levels detected by ELISA. The BoIFN-α1 [35] nucleotide sequence was identified to be 99% homologous (Fig. S2a) to BoIFN-α-D, and specific primers were designed for BoIFN-α-D. Sequences have been reported for eight subtypes of bovine IFN-α and three subtypes of IFN-β [1]. Despite high homology among the IFN-β subtypes (Fig. S1b), single nucleotide polymorphisms (SNPs) exist among the subtypes. To detect transcripts from all IFN-β subtypes, primer pairs designed for IFN-β genes were designed with IUPAC nucleotides, whereby 50% of the primer(s) contains one nucleotide, while the other 50% contains a different nucleotide at the same position (Table 1). Primer specificity for individual IFN genes was confirmed through the generation of a single peak in the melting curve and the detection of a single band following gel electrophoresis of PCR products. Additionally, the IFNα and -β PCR products were cloned in the PCR 2.1 vector using the TA cloning kit (Invi-

trogen). Cloned PCR products were sequenced with a CEQ 200XL DNA analysis system (Beckman Coulter) to confirm product identity and size. Sequencing of cloned IFN-β PCR products, IFN-β clones A and B, identified the products as IFN-β2 and IFN-β1 (Fig. S2c,d), respectively, confirming that the primers identified multiple IFN-β subtypes. One BoIFN-α-D PCR product was cloned and sequenced and confirmed to be 99% homologous to BoIFN-α-D (Fig. S2b). Primer amplification efficiency was also determined by performing a standard curve as described previously [39].

**qRT-PCR analysis of ISG and IFN-α, IFN-β and -γ gene expression**

Gene expression data for individual samples were normalized relative to β-actin, which was previously validated as a suitable reference gene in bovine mucosal epithelial cells [40]. We also confirmed that expression of the reference gene did not change significantly over time. qRT-PCR was performed using the PerfeCTa SYBR Green FastMix for iQ (Quanta BioScience). Briefly, 9 µl of Perfecta SYBR green master mix (2x), 3 µl of the primer pair at 3.3 µM and 3 µl cDNA template were added to a 15 µl final volume. The reaction was performed in a BioRad iCycler iQ PCR detection system using the following programme: 1 cycle at 95 °C for 30 s, 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. After the cycling, the temperature was increased, starting from 56 °C, at a rate of 1 °C every 10 s to build a melting curve (40 times). Amplification data obtained for individual genes were expressed as threshold cycle (Ct) and subtracted from the Ct of the β-actin reference gene to obtain ΔCt (ΔCt= Ct gene – Ct β-actin). The fold change was calculated using the formula

Fold change = 2−ΔΔCt.

ΔCt was calculated by subtracting the ΔCt value of the control (untreated or uninfected sample) from the time-matched ΔCt value for the experimental group (treated or infected) [41].

**Interferon plaque-inhibition assay**

Monolayers of mucosal epithelial cells were prepared by seeding 12-well CellBind plates (VWR) with 2.5×10⁴ cells/well. Cultures were 70–80% confluent within 3 h of seeding and duplicate wells were then treated with IFN-α, -β and -γ doses ranging from 0.01 to 10 000 ng ml⁻¹. Duplicate wells with no IFN treatment were used as positive controls for virus plaque-forming units (p.f.u.). Confluent bovine mucosal epithelial cell cultures were incubated with the indicated type and concentration of interferon for 12 h before the medium was aspirated. Fifty p.f.u. BHV-1 (108) or VSV in 0.5 ml serum-free DMEM were added to each well for 1 h before the monolayer was overlaid with bovine anti-BHV-1 antisera for BHV-1-infected cultures or 1% methylcellulose in complete media for VSV-infected cultures. Cultures were then incubated at 37 °C for 40–45 h before 80% methanol and 0.5% crystal violet were used to fix and stain cells. Viral plaques were enumerated under an inverted microscope.

**Statistical analysis**

Data were analysed using GraphPad software (version 7.0a). Virus shedding by infected calves was analysed by using a two-way ANOVA to analyse the effect of days post-infection on BHV-1 shedding. A Mann–Whitney test was used to compare the levels of IFNα, -β or -γ detected in nasal secretions and nasal turbinate tissues following BHV-1 infection relative to uninfected tissues from day 0. The Student t-test was used to compare IFN inhibition of BHV-1 and VSV viral plaques. A Mann–Whitney test was used to analyse changes in the expression level of individual ISGs in nasal turbinate and tracheal tissues relative to the initial time point.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


