Cytokine and Toll-like receptor mRNAs in the nasal-associated lymphoid tissues in cattle during foot-and-mouth disease virus infection

Zhidong Zhang1*, John Bashiruddin1, Claudia Doel1, Jacquelyn Horsington1, Stephanie Durand1 and Soren Alexandersen2

1Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey, GU24 0NF, UK; 2Danish Institute for Food and Veterinary Research, Department of Virology, Lindholm, DK-4771, Kalvehave, Denmark.

Abstract

Introduction: A number of studies have shown the importance of tissues in the pharyngeal area in foot-and-mouth disease virus (FMDV) infection during acute disease and persistence. Materials and Methods: To explore characteristics of the local mucosal immune response in bovine nasal-associated lymphoid tissues (NALT) to FMDV infection during acute disease and persistence, a quantitative real-time RT-PCR assay was used to measure mRNA expression of bovine cytokines IFN-α, β and γ, IL-2, IL-1α, TNF-α, and Toll-like receptors (TLR)-3 and -4. The NALT from dorsal soft palate were collected from cattle at 7 dpi and from carriers and non-carriers at 64 dpi. Experimental infection was with FMDV type O UKG34/2001 isolate. Results: In comparison with uninfected animals, expression of IFN-α mRNA was significantly increased in NATLs during acute disease. An increased expression of IFN-γ and IL-1α mRNA was also observed but was much lower than IFN-α. There was a slight increase in mRNA expression of TNF-α and IL-2. During persistence, the level of IFN-α mRNA expression in carrier cattle was lower than non-carrier cattle. In contrast, the level of TNF-α mRNA expression in carrier cattle was much higher than in non-carrier cattle. For TLRs, an increased expression of TLR-4 but not TLR-3 in NALT during the acute stage of infection was observed when compared with uninfected animals. No differences in the expression of TRL-3 and-4 mRNA were detected in NALTs between carrier and non-carrier cattle. Conclusion: IFN-α mRNA was significantly up-regulated in bovine NALT in response to FMDV infection during the acute stage of disease. TNF-α mRNA was significantly up-regulated in bovine NALT during persistence.

Introduction

Foot and mouth disease virus (FMDV) is a member of the Picornaviridae which causes a highly contagious disease of cloven-hoofed animals and is one of the most economically important diseases of livestock worldwide (Alexandersen et al., 2003b). Importantly, persistent infection (so-called carrier stage) can occur following clinical or sub-clinical disease in either naive or vaccinated ruminants (reviewed in Alexandersen et al., 2002). A carrier is defined as an animal from which live virus can be recovered for longer than 28 days after exposure (Salt, 1993, Alexandersen et al., 2002). Persistent infection appears to occur only in a proportion of virus-exposed ruminants in which virus becomes undetectable eventually (Alexandersen et al., 2002). In contrast, pigs infected with FMDV do not become persistently infected. The significance of persistently infected animals (carriers) lies in their potential to act as a source of new outbreaks (Bengis et al., 1986, Dawe et al., 1994) and make control efforts even more costly. For example, during the 2001 UK outbreak, a key issue was whether or not to vaccinate. There were concerns that some animals would become subclinical carriers in which disease would be masked by vaccination and that this would adversely affect trade prospects.

The mechanism(s) by which FMDV persistence is established and maintained remains unclear. The acquisition of FMDV infection occurs most often by the inhalation of infectious aerosol and uptake of virus at oral/respiratory mucosal epithelial surfaces. A number of studies have shown the importance of the pharyngeal tissues in FMDV infection during acute disease and persistence (reviewed in Alexandersen et al., 2002, 2003b, Zhang and Alexandersen, 2004). The epithelial cells of the bovine pharyngeal region (soft palate and pharynx) have been identified as the site for FMDV persistence in ruminants (Zhang and Kitching, 2001). Recently, we have shown that the presence of viral RNA in tissues of the bovine dorsal soft palate correlated with the presence of infectious virus in oesophago-pharyngeal (OP) fluid samples (Zhang and Alexandersen, 2004) and also that the half-life of viral clearance in probang fluid samples is prolonged early on in animals that subsequently becomes carriers (Zhang et al., 2004). However the factors that control FMDV infectivity in the pharyngeal mucosa have not been described, although evidence suggests that the cell-mediated immunity may be involved in the clearance of persistent virus (Ilott et al., 1997, Childerstone et al., 1999). Improving our understanding of this will provide fundamental knowledge to help develop an effective
strategy for FMD control. This paper investigates characteristics of the local mucosal immune response in bovine nasal-associated lymphoid tissues (NALT) in the pharyngeal region to FMDV infection during acute and persistent stages in order to determine associations of FMDV-induced changes with viral persistence/clearance.

Materials and Methods

Ten standard Compton steers (Holsteins) 6-10 months of age were used. This included two uninfected animals used as controls. Animals for the infection studies were placed as pairs of two or four together in individual rooms in a biosecure animal building. Half of the animals in each room were selected at random and inoculated by subepidermo-lingual injection of approximately 0.5 ml of FMDV O UKG 34/2001 as described elsewhere (Alexandersen et al., 2003a, Zhang et al., 2004) while the other half of the animals were kept as direct contacts throughout the experiment. After inoculation, animals were monitored for clinical signs of disease and the rectal temperatures were recorded daily until 10 days after inoculation. Samples of OP-fluid and serum were collected before the start of the experiment (negative controls) and at intervals after infection. OP-fluid samples were collected using a probang cup (Sutmoller and Gaggero, 1965) and diluted in an equal volume of Eagle’s-HEPES medium (pH 7.2) containing 5% foetal calf serum (FCS) and stored at –80°C until required. Each OP-fluid sample was assayed on primary bovine thyroid (BTY) cells to determine the presence of infectious virus as described (Snowdon, 1966). RNA was extracted from OP fluid samples and serum with MagNA Pure LC NA Extraction Kit II (Roche, UK) as described previously (Zhang and Alexandersen, 2004). RNA was eluted in 50 µl of elution buffer and stored at –80°C until used. The level of viral RNA in samples was quantified by a quantitative RT-PCR assay as described previously (Zhang and Alexandersen, 2004).

NALT were collected at post-mortem from dorsal soft palate of cattle randomly selected and killed at the indicated time after infection and immediately put into RNAlater (Ambion, UK), then stored at –20°C until required. mRNA was extracted from tissues samples with MagNA Pure LC mRNA Extraction Kit II (Tissues) (Roche, UK) as described previously (Zhang and Alexandersen, 2004). mRNA was eluted in 50 µl of elution buffer and stored at –80°C until used.

mRNA expression for the cytokine IFN-α, β and γ, IL-1 α, and TNF-α were quantified using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, UK). RNA was reverse transcribed to cDNA with the TaqMan reverse transcriptase kit (Applied Biosystems, UK), followed by PCR amplification with the TaqMan Master Mix kit (Applied Biosystems, UK) in the presence of specific forward and reverse primers and fluorescently labelled probes specific for each gene of interest, using 1 µl of cDNA in a 25 µl final volume for 50 cycles. Similar analyses were also performed on mRNA of the housekeeping gene GAPDH on each sample. For each gene of interest, we confirmed that efficiency of amplification was comparable to that of GAPDH (data not shown), permitting calculations of the amounts of gene induction as described below.

mRNA expression for TLR 3 and 4 and IL-2 were quantified using the Master SYBR Green kit (Eurogenics, UK), using 1 µl of cDNA in a 25 µl final volume. Briefly, PCR was carried out in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) for 40 cycles. Amplification specificity was checked using melting curve following the manufacturer’s instructions. Similar analyses were also performed on mRNA of the housekeeping gene GAPDH on each sample. The efficiency of amplification was comparable to that of GAPDH (data not shown), and extents of gene induction were calculated as described below.

Quantitative RT-PCR data were analysed with the comparative Ct method (ΔΔCt). Briefly, first the difference (ΔCt) between the Ct values of the target and the normaliser: ΔCt = Ct (target) – Ct (normaliser) are calculated for each sample to be quantified. One of these samples is chosen as the reference for each comparison to be made. The comparative ΔΔCt calculation involves finding the difference between the sample ΔCt and the reference ΔCt. The last step in quantification is to transform these values to absolute values. The formula for this is: Comparative Expression Level = 2 ^-ΔΔCt. Data of cytokine expression were statistically evaluated for significance using the t-test (using MINITAB release 12.21 software). A P value < 0.05 was considered statistically significant.

Results

All animals inoculated with FMDV type O UKG 34/2001 developed signs of clinical disease within 1-2 days post inoculation (dpi) and in contact cattle around 3-5 days. Viral RNA and infectious virus in OP-fluid samples was positive by a quantitative RT-PCR assay and virus isolation assay on BTY cells from around 1 dpi (data not shown). Cattle were confirmed as carriers of FMDV by recovery of
infectious FMDV from OP-fluid samples collected at 28 dpi (Table 1). OP-fluid samples collected from two carriers at the time when the animals were killed was positive by virus isolation assay on BTY cells and the viral RNA loads was $5.2 \pm 0.7 \log_{10}$ copies/ml determined by a quantitative RT-PCR assay.

Table 1. Clinical profiles and recovery of FMDV in the OP-fluid samples of experimentally infected animals

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>dpi</th>
<th>Infection route</th>
<th>Status</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>UD34</td>
<td>64</td>
<td>Inoculated</td>
<td>Noncarrier</td>
<td>P</td>
</tr>
<tr>
<td>UD35</td>
<td>7</td>
<td>Inoculated</td>
<td>Acute</td>
<td>P</td>
</tr>
<tr>
<td>UD36</td>
<td>7</td>
<td>Contact</td>
<td>Acute</td>
<td>N</td>
</tr>
<tr>
<td>UD37</td>
<td>64</td>
<td>Contact</td>
<td>Noncarrier</td>
<td>N</td>
</tr>
<tr>
<td>UD40</td>
<td>64</td>
<td>Inoculated</td>
<td>Carrier</td>
<td>P</td>
</tr>
<tr>
<td>UD41</td>
<td>64</td>
<td>Inoculated</td>
<td>Carrier</td>
<td>P</td>
</tr>
<tr>
<td>UD42</td>
<td>64</td>
<td>Contact</td>
<td>Noncarrier</td>
<td>N</td>
</tr>
<tr>
<td>UD43</td>
<td>64</td>
<td>Contact</td>
<td>Noncarrier</td>
<td>N</td>
</tr>
</tbody>
</table>

*a days post inoculation. For direct contact animals, it is defined as days after contact.
*b infection were by subepidermo-lingual injection or by contact.
*c a carrier is defined as an animal from which live virus can be recovered for at least 28 days after exposure.
*d OP-fluid samples collected at the day the animals were killed were assayed on BTY cells to determine the presence of infectious virus (IV).

The presence of viral RNA in tissues of the bovine dorsal soft palate has been demonstrated (Zhang and Alexandersen, 2004). But the factors that control FMDV infectivity in the pharyngeal mucosa are unclear. Therefore, profile of the mucosal cytokines IFN-α, β and γ, IL-2, IL-1α, TNF-α and TLR-3 and -4 at mRNA levels in NALT tissues collected from dorsal soft palate of FMDV-infected cattle were analysed by a quantitative RT-PCR assay. As shown in Figure 1, at 7 dpi (n=2) expression of IFN-α mRNA was significantly increased (100000 times increase) in NALT. The increased expression of IFN-γ (6 ± 6 times increase), IFN-β (6 ±2 times increase) and IL-1α mRNA (4 ±1 times increase) was also observed but at a much lower extent than IFN-α. There was a slight increase in mRNA expression of TNF-α and IL-2 but at a lower extent than IL-1α. During persistence, the level of TNF-α mRNA expression in carrier cattle (1100 ± 65 times increase) was significantly higher than in non-carrier cattle (1 ± 0.7 times increase; $P = 0.027$). The level of IFN-β mRNA expression (26 ±3 times increase) in carrier cattle was also higher than non-carrier cattle (18 ±3. times increase) but there were no significant differences ($P = 0.26$). In contrast, the level of IL-1α mRNA expression (4 ±3 times increase) in carrier cattle was lower than non-carrier cattle (6 ±1 times increase). The level of IFN-α mRNA expression in carrier cattle was also lower than non-carrier cattle but there was no significant differences ($P = 0.92$).

The major innate recognition system for microbial invaders in vertebrates is now thought to be the Toll-like receptor (TLR) family (Beutler et al., 2003). Therefore, TLR-3 and-4 response in NALT during infection was also studied. As shown in Figure 3, an increased expression of TLR-4 (5 ± 3 times increase) but not TLR-3 in NALT during the acute stage of infection was observed when compared with uninfected animals. During persistence no increased production of TRL-3 was observed in NALT from both carrier and non-carrier cattle. For TLR-4 mRNA an increased expression of TLR-4 from both carriers and non-carriers was observed when compared with uninfected animals but there was no significant difference between them ($P = 0.7$).

Discussion

The experiments described here present a quantitative analysis of the levels of cytokine and TLR mRNA in NALT tissues of cattle experimentally infected with FMDV O UKG 34/2001. Data from a single experiment provide evidence that a difference exists between carrier and non-carriers. In exploring the mucosal cytokine profile at mRNA levels in FMDV-infected cattle, we found the most obvious differences in the expressions of TNF-α, exhibiting a significant increase in carrier cattle. This may be due to the presence of viral RNA in pharyngeal region of carriers (Zhang and Alexandersen, 2004), which may represent a continued stimulation of the cells producing the cytokine. It has been
shown that TNF are involved in the polyclonal expansion of B cells in human retrovirus infection (Higuchi et al., 1997), as well as in chronic lymphocytic leukaemia (CLL) (Waage and Espevik, 1994). Therefore significant increase in expression of TNF in carriers may explain in part why high level of IgA was detected in OP fluid and saliva samples in carriers (Archetti et al., 1995). It is obviously important to work out how this cytokine influences FMD virus replication in the pharyngeal region and to find out where the TNF-α is coming from and whether it has any direct role in local protection.

A significant elevation of IFN-α mRNA was seen in NALT. An increased expression of IFN-γ was also observed but at a much lower extent than IFN-α. It has been hypothesised that the initiation of FMDV persistence is correlated with the amount of interferon produced in the cells. FMDV strains modified by passage in alternate hosts or repeated passage in cell cultures have reduced virulence in cattle and, in contrast to more virulent wild-virus, will induce the production of IFN (Sellers, 1963) with a correlation between lack of virulence in cattle and increased IFN production (Sellers and Mowat, 1968). The ability of FMDV to form plaques in cell culture is apparently correlated with suppression of IFN-α and β (Chinsangaram et al., 1999). Recent evidence has shown IFN-γ, to be a potent inhibitor of FMD virus in persistently infected cells.

**Figure 1.** Cytokine response in NALT during infection. NALT were collected at 7 and 64 dpi from infected animals with FMDV UKG34/2001 and then cytokine mRNAs were studied by a real-time RT-PCR. Data represent mean ± SD from at least two animals at each time point. Bars represent standard error of the mean.
infected bovine epithelial cells in vitro (Zhang et al., 2002). Other studies have shown that IFN-α and β inhibit FMDV replication (Chinsangaram et al., 2001). These evidences suggest that one mechanism controlling infection in vivo might be mediated through the inhibitory effect induced by IFNs. IFN-γ is produced primarily by natural killer cells and sub-populations of T-cells during the innate and adaptive phases of a response against viral infection. Agents that promote T-cell activation should induce IFN-γ synthesis, and it is known that double-stranded RNA, gram-positive bacterial components and endotoxins stimulate IFN-γ, often following the release of IL-12. Virus persists in pharyngeal tissues in carrier cattle (Zhang and Alexandersen, 2004). Double-stranded RNA would be a likely by-product of FMD replication, which could theoretically act as a stimulus for IFN-γ. The significance of this finding needs further study and it will be important to find out where the IFN is coming from and whether it has any direct role in local protection. Further studies are also required to conclusively investigate the shift of cytokine expression pattern during acute to persistent stages and in animals that clear the infection compared to carrier animals.

Recent evidences suggest that TLRs and their signalling pathways involve type I IFN induction in response to virus-specific molecular patterns (Doyle et al., 2003). In this study, an increased expression of TLR-4 was observed in NALT during FMDV infection, which may indicate a role in inducing expression of type I IFN mRNA in response to FMDV infection. In contrast, TLR-3 mRNA expression was not affected by FMDV infection, which may indicate that TLR3 may be not required for the generation of effective antiviral responses to FMDV infection, although it has been assumed that TLR-3 is likely to play an important role in host defence against virus infections (Doyle et al., 2003, Harte et al., 2003). The concept is also supported by a recent study showing that the absence of TLR3 does not alter either viral pathogenesis or impair host’s generation of adaptive antiviral responses to these viruses (Edelmann et al., 2004). However, it dose not rule out that the physiological amount of viral dsRNA naturally made during viral infection were sufficient to stimulate through TLR-3 a biological meaningful response to affect the outcome of infection. Further studies are needed to find out if TLRs has important role in local protection.

Conclusions

IFN-α mRNA was significantly up-regulated in bovine NALT in response to FMDV infection during the acute stage of disease. TNF-α mRNA was significantly up-regulated in bovine NALT during persistence.

Recommendations

Mucosal cellular immune responses may have an important role in controlling FMDV persistence. More work is required at mRNA and proteins levels so that virus and host effects during infection can be elucidated at mucosal surface.

Acknowledgements

We thank Bev Standing and Malcolm Turner for their assistance with the handling and management of experimental animals. This work was supported by the Department for Environment, Food and Rural Affairs (DEFRA), UK, Contract 2920 and EU Contract QLK2-2002-01719.
Reference


