Differential infection efficiencies of peripheral lung and tracheal tissues in sheep infected with *Visna/maedi virus* via the respiratory tract

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The main routes of transmission of *Visna/maedi virus* (VMV), an ovine lentivirus, are thought to be through ingestion of infected colostrum and/or milk or through inhalation of respiratory secretions. Whereas oral transmission appears to be mediated via epithelial cells within the small intestine, the mechanism of virus uptake in the respiratory tract is unknown. In addition, it is not known whether infection is mediated by cell-associated or cell-free VMV, previous studies having not addressed this question. Intratracheal (i.t.) injection of VMV is known to be a highly efficient method of experimental infection, requiring as little as $10^{1}$ TCID$_{50}$ VMV for successful infection. However, using a tracheal organ culture system, we show here that ovine tracheal mucosa is relatively resistant to VMV, with detectable infection only seen after incubation with high titres of virus ($>10^{5}$ TCID$_{50}$ ml$^{-1}$). We also demonstrate that i.t. injection results in exposure of both trachea and the lower lung and that the time taken for viraemia and seroconversion to occur after lower lung instillation of VMV was significantly shorter than that observed for tracheal instillation of an identical titre of virus ($P=0.030$). This indicates that lower lung and not the trachea is a highly efficient site for VMV entry in vivo. Furthermore, cell-free virus was identified within the lung-lining fluid of naturally infected sheep for the first time. Together, these results suggest that respiratory transmission of VMV is mediated by inhalation of aerosols containing free VMV, with subsequent virus uptake in the lower lung.

INTRODUCTION

*Visna/maedi virus* (VMV) is a member of the lentivirus subgroup of retroviruses that causes interstitial pneumonia, encephalitis, arthritis and mastitis in sheep (Sigurdsson et al., 1952; Sigurdsson & Palsson, 1958). The main routes of transmission of VMV are thought to be through ingestion of infected colostrum and/or milk or through inhalation of respiratory secretions (Pepin et al., 1998; Blacklaws et al., 2004). Whereas oral transmission appears to be mediated via uptake of virus by small-intestinal epithelial cells (Preziuso et al., 2004), the mechanism of virus uptake in the respiratory tract is as yet unknown.

Recent studies have identified intratracheal (i.t.) inoculation as a highly efficient route of experimental infection. In one study, the minimum infectious dose of VMV for the i.t. route was found to be $10^{1}$ TCID$_{50}$ delivered in a 1 ml volume, whereas that for the intranasal route was $5 \times 10^{6}$ TCID$_{50}$ (Torsteinsdottir et al., 2003). This suggests that the lower respiratory tract and not the upper respiratory tract (nasal cavity and nasopharynx) is the major site of VMV entry during respiratory transmission. It is unclear exactly which parts of the respiratory tract are exposed by i.t. instillates, although certainly trachea and possibly lower lung (bronchioles and alveolar areas) may be involved. Comparisons between trachea and lower lung for infection efficiency have not been performed. Therefore, it is unclear whether the high sensitivity of the tracheal route for infection represents a unique feature of the tracheal mucosa or is due to exposure of lower lung, or both.

In addition to the lack of knowledge regarding the site of VMV uptake during respiratory transmission, the precise nature of the infectious agent is unclear. It has long been presumed that respiratory transmission of VMV is mediated by inhalation of free virus or cell-associated virus, most likely VMV-infected alveolar macrophages (AMs) (Pepin,...
et al., 1998; Blacklaws et al., 2004; Peterhans et al., 2004). However, whereas productively infected AMs have been identified in a number of studies of naturally infected sheep (Lujuan et al., 1994; Brodie et al., 1995; Gelmetti et al., 2000), to date cell-free VMV has not been identified conclusively within the lung-lining fluid of infected individuals. The nature of the infectious agent may have important implications regarding the sites of initial virus uptake, as cell-associated VMV would be preferentially deposited within the upper respiratory tract, whereas only smaller particles of <5 μm diameter, such as cell-free VMV particles, which have a diameter of approximately 100 nm (Lee et al., 1996), are likely to reach the lower lung (Zhang et al., 2000; Gordon & Read, 2002). Therefore, identification of free VMV would appear to be crucial for lower lung involvement to occur during natural respiratory transmission.

The aims of this study were twofold: firstly to identify the sites of the respiratory tract that are responsible for virus uptake during i.t. inoculation, and secondly to determine whether cell-free VMV occurred within the lung-lining fluid in naturally infected sheep. To assess the contribution of the trachea to initial virus uptake, a tracheal organ culture system was developed and subsequently used to determine the sensitivity of the tracheal mucosa to VMV ex vivo. In addition, patent blue, an inert protein-binding dye routinely employed for in vivo tracking studies (Hirsch et al., 1982), was used to identify areas of the respiratory tract exposed during i.t. inoculation, and the relative sensitivities of areas of the lung involved during i.t. inoculation were determined using a novel in vivo infection model. Finally, lung-lining fluid samples obtained from three naturally infected sheep were analysed for the presence of cell-free VMV. The results of this study will potentially identify sites of virus entry during natural respiratory transmission of VMV and clarify the nature of the infectious agent.

METHODS

Virus propagation and titration. Low-passage VMV strain EV1 (Sargan et al., 1991) was propagated routinely on ovine skin cells (OSCs) in DMEM supplemented with 10% FCS. Virus-containing culture medium was clarified by centrifugation for 10 min at 400 x g and subsequently filtered through a 0.2 μm syringe-driven filter (Nalgene).

Viruses were determined as described previously using OSCs as indicator cells (Ebrahimi et al., 2000). Titres were expressed as either tissue culture infectious dose (TCID50) for samples containing ≥20 TCID50 ml−1 or the percentage of wells with visible cytopathic effect (CPE) after incubation with neat sample for samples <20 TCID50 ml−1.

Ovine tracheal organ culture and infection with VMV. The procedure was based on that of Campbell et al. (1979) and Lin et al. (2001). Proximal trachea was removed aseptically from VMV-seronegative sheep immediately post-mortem and placed in cold PBS containing (ml−1) 100 U penicillin, 100 μg streptomycin, 4 μg amphotericin B and 10 μg gentamicin (expant-PBS). After washing three times in explant-PBS for 5 min each, tracheal tissue was cut into 6 mm diameter discs using a sterile punch biopsy needle (Kruuse A/S). Tracheal discs were placed with epithelium surface upward in 6-well plastic tissue culture dishes (Nunc) and allowed to stick down at room temperature for 5 min prior to addition of 5 ml DMEM supplemented with (ml−1) 100 U penicillin, 100 μg streptomycin, 4 μg amphotericin B, 10 μg gentamicin, 100 μg insulin and 2% Ulotroser-G (Ciphergen) (expant medium). Organ cultures were subsequently incubated at 37 °C with 5% CO2 in a humidified atmosphere. After 4 h, the medium was removed and each organ culture was incubated in 5 ml explant medium containing varying concentrations of VMV (105 to 106 TCID50 ml−1) for 2 h. Organ cultures were then washed five times in PBS and subsequently incubated in fresh explant medium for 7 days. Negative controls included harvesting organ cultures after the final PBS wash (day 0) and incubation with no virus (mock infection). Each dilution of virus was performed in duplicate for each source animal, and the experiment was repeated using tracheas obtained from six animals.

After 7 days, organ cultures were halved. DNA was extracted from one half using a DNeasy tissue kit (Qiagen) and samples were stored at −80 °C prior to PCR analysis for VMV provirus. The other half was snap-frozen in isopentane/dry ice. Tissue sections were cut to 6 μm thick, mounted on poly-l-lysine-coated slides (BDH) and stored at −80 °C prior to immunocytochemical (ICC) analysis for VMV capsid protein. Virus titrations were performed on samples of wash fluid from the last washing step after virus incubation and day 7 culture supernatants.

To assess organ culture viability, the strength of epithelial cilia beating in six preliminary organ cultures was assessed by direct visualization using a Fluovert inverted light microscope (Leica) and scored subjectively as either absent, weak, moderate or vigorous. In addition, day 3 (n=6) and day 7 (n=6) organ cultures were fixed in 10% buffered formalin, paraffin embedded, sectioned and stained with haematoxylin and eosin (H&E) to assess tissue morphology.

Animals. For dye tracking studies, four adult greyface ewes were used. For VMV infection studies, 10 adult Suffolk-cross ewes were used. All sheep were commercially sourced. Prior to VMV infection studies, sheep were determined to be free from VMV provirus and seronegative for VMV-specific antibodies. All experimental procedures involving animals were approved by The University of Edinburgh’s Biological Services Ethical Review Committee and were performed under licence as required by the UK Animals (Scientific procedures) Act 1986.

I.t. inoculation. Sheep were sedated by intramuscular injection of xylazine hydrochloride (Rompun; Bayer) at a dose rate of approximately 0.5 mg kg−1. One millilitre volumes of patent blue dye (0.1% w/v in PBS) or cell-free virus (106 TCID50) were injected into the proximal third of the trachea via a 21 gauge needle, avoiding the tracheal cartilage. The neck was held in dorsoflexion for 1–2 min post-instillation. For patent blue dye tracking studies, sheep were allowed to recover in a pen with free access to food and water for 1 h post-instillation prior to euthanasia by intravenous injection of pentobarbitone (Euthatal; Rhône Mérieux) at a dose rate of 100 mg kg−1. Lungs were removed and the location of dye was recorded.

Differential exposure of trachea and peripheral lung. Delivery of inoculates to either trachea or lower lung was performed endoscopically under general anaesthesia. Induction of anaesthesia was achieved by intravenous administration of thiopentone sodium (Rhône Mérieux) at a dose rate of 20 mg (kg body weight)−1. Sheep were intubated and anaesthesia was maintained with gaseous halothane (2–3%) in oxygen and nitrous oxide under negative pressure ventilation.

For initial development of the differential exposure model, 1 ml volumes of patent blue dye (0.1% w/v in PBS) were instilled onto the
lateral aspect of the distal one-third of the trachea of two adult greyface ewes via a 21 gauge polyethylene catheter inserted into the lateral channel of a flexible fibre-optic bronchoscope (5.3 mm o.d.) (model FG-16X; Pentax). The position of the instillate was visualized at 15, 30, 45 and 60 min post-instillation. The sheep were then allowed to recover in a pen with free access to food and water and were standing within 15–20 min post-anaesthesia. After 1 h of recovery, sheep were euthanized, the lungs were removed and the location of patent blue dye was recorded.

For subsequent virus exposure experiments, 1 ml volumes containing 10^8 TCID_{50} cell-free VMV and 0.01 % patent blue dye (which had previously been shown not to affect virus titre) were instilled into either the distal trachea or the left cardiac lung lobe. Instillates were visualized for 1 h post-instillation as described above and sheep were subsequently allowed to recover. Sheep were bled weekly for the first 2 months and thereafter every 2 weeks until 6 months post-inoculation. Blood samples were analysed for the presence of VMV provirus and VMV-specific antibodies by PCR and ELISA, respectively, and the times to PCR positivity and seroconversion were recorded.

Isolation of peripheral blood mononuclear cells (PBMCs). Blood was collected into an EDTA-containing Vacutainer (Becton Dickinson) and PBMCs were isolated using a Ficoll-paque density gradient (GE Healthcare) according to the manufacturer’s instructions. Cells were pelleted by centrifugation at 100 g for 10 min and the resultant cell pellet was resuspended in 200 μl PBS. DNA was subsequently extracted using the DNeasy mini kit (Qiagen).

Detection of VMV provirus. VMV provirus was detected by semi-nested PCR (snPCR) using the first-round primer pair gag 1 and gag 5 and second-round primer pair gag 1 and gag 2 (primer sequences and positions within the VMV genome are shown in Supplementary Table S1 available in JGV Online). Reactions of 50 μl final volume contained 1.5 mM MgCl2, 0.2 mM dNTPs, 25 pmol each of sense and antisense primer and 2.5 U HotTaq (Biogene). For organ culture experiments, 100 ng template DNA was added to first-round reactions. For in vivo infection studies, 500 ng PBMC DNA was added to first-round reactions. A 3 μl aliquot from the first round of PCR was used in the second-round reaction.

For the first round of PCR, the thermal cycling profile involved a 10 min pre-incubation at 95 °C followed by 15 cycles each consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 2 min and a final extension at 72 °C for 5 min. For reamplification, the number of cycles was increased to 35 and denaturation, annealing and extension times were 30 s, 30 s and 1 min, respectively.

Detection of VMV-specific serum antibodies. Serum samples were tested for the presence of virus-specific antibodies to the major core protein p25 of VMV and/or the viral transmembrane protein gp46 using a commercial ELISA kit (ELITEST-MVV/CAEV; Hyphen BioMed) (Saman et al., 1999). Absorbance values were measured using a Bio-Tek Microplate Autoreader (Bio-Tek Instruments).

Bronchoalveolar lavage (BAL) collection and processing. The lungs from three VMV-seropositive adult Rasa Aragonesa sheep with diffuse, severe VMV lesions were subjected to BAL as follows: a luer-tipped 50 ml syringe was wedged into selected segmental bronchi exhibiting gross VMV lesions and a single 40 ml aliquot of normal saline was used to lavage each lung segment. BAL samples were passed through sterile gauze into sterile 50 ml Falcon tubes on ice and subsequently centrifuged at 400 g for 7 min at 4 °C to separate out the cellular fraction. BAL supernatants were then passed immediately through a 0.2 μm syringe-driven filter (Nalgene) and stored at −80 °C prior to virus titration. Cytocentrifuge slides were prepared from the resultant cell pellets using a Cytospin 3 cytocentrifuge (Thermo-Shandon).

**ICC staining for VMV capsid protein p25.** Slides were fixed in 100 % methanol for 10 min at −20 °C and then incubated in 0.3 % hydrogen peroxide in PBS/0.5 % Tween 80 (PBS/T80) for 20 min at room temperature. After washing in PBS, slides were incubated in 10 % normal goat serum (NGS) in PBS/T80 for 1 h at room temperature prior to incubation with the monoclonal antibody VPM70 (anti-VMV p25, mouse IgG_{1}) (Reburn et al., 1992) diluted 1:2 in PBS/T80 containing 10 % NGS for 1 h at room temperature. Negative controls were provided by replacing the primary antibody with an identical dilution of isotype-matched control monoclonal antibody VPM53 (McOrist et al., 1989). After washing, secondary antibody (peroxidase-labelled polymer conjugated to goat antimouse immunoglobulins, EnVision Plus HRP system; Dako) was applied to sections for 30 min at room temperature. Antibody binding was detected by incubation with 3,3′-diaminobenzidine for 7.5 min at room temperature. Sections were counterstained with haematoxylin, dehydrated in graded alcohols, cleared in xylene and mounted in DPX mounting medium (Fischer Scientific). For cytocentrifuge preparations, 500 cells were examined and the number of VMV-positive cells was recorded.

**Statistical analysis.** Non-parametric statistical analysis was carried out using the Mann–Whitney procedure on Minitab version 14 for Microsoft Windows. Values of P<0.05 were determined to be significant.

**RESULTS**

**Assessment of tracheal organ culture viability**

To evaluate the tracheal organ culture methodology used in this study, assessment of epithelial ciliary activity and organ culture morphology was performed. Ciliary beating was present in all organ cultures assessed throughout the 7 day culture period and was either weak or moderate. Histopathological evaluation of organ cultures revealed preservation of a pseudostratified ciliated epithelium in both day 3 and day 7 organ cultures (data not shown). Some degenerative changes were observed in the submucosa at day 3 and day 7, including loss of structural integrity of the connective tissue layers, sloughing and karyorrhexis of the vascular endothelium and submucosal glandular epithelium and degeneration of the submucosal cell nuclei. Degenerative changes were more apparent in day 7 cultures.

**Infection of ovine tracheal organ cultures with VMV**

To determine whether tracheal organ cultures were infectable by VMV and the minimum infectious dose of virus required, tracheal organ cultures were incubated with varying titres of VMV strain EV1 and subjected to snPCR analysis for VMV provirus. A summary of the snPCR results is shown in Fig. 1(a). VMV gag proviral DNA was detected in 100 % of explants incubated with 10^6 TCID_{50} VMV ml^{-1} and in 66.6 % of explants incubated with 10^5 TCID_{50} VMV ml^{-1}. No proviral DNA was detected in any other organ culture incubated with virus. Day 0 and mock-infected explants were negative. A representative image of an agarose
gel of PCR products generated from organ cultures derived from one source sheep is shown in Fig. 1(b).

To assess the metabolic activity of organ cultures at 7 days, 7-day-old organ cultures were infected with $10^6$ TCID$_{50}$ VMV ml$^{-1}$ as described above and provirus production was assessed by snPCR at 2 h ($n=4$) or 24 h ($n=4$) post-infection. VMV provirus was detected in all 24 h organ cultures but not in the 2 h cultures (data not shown), indicating de novo provirus production and, subsequently, that 7-day-old organ cultures were still metabolically active.

To assess productive virus replication in tracheal organ cultures, virus titration of organ culture supernatants and ICC for VMV capsid protein was also performed. ICC failed to detect VMV capsid protein in all organ cultures. A positive control sample consisting of in vitro-infected OSCs was run in parallel with all samples and was consistently positive. Positive virus titres were found in both last wash (day 0) and day 7 supernatants of organ cultures incubated with $10^6$ TCID$_{50}$ VMV ml$^{-1}$. However, no significant difference in titre was seen between day 0 and day 7 time points (Fig. 1c), suggesting that virus detected at day 7 was most likely to be carry-over from input virus. All other supernatants tested were negative.

**Tracking of i.t. inoculates**

To evaluate the lung distribution of fluid introduced via i.t. inoculation, patent blue dye was instilled into the tracheas of two adult greyface ewes by i.t. injection. Representative images of the distribution of dye 1 h post-inoculation are shown in Fig. 2. The majority of dye was located in the trachea and left cardiac lung lobes of both sheep. In one sheep, a small amount of dye was also present in the right cardiac lung lobe. Dye was located throughout the whole length of the trachea, including the region proximal to the injection site. The dye in the trachea was primarily located within the mucus layer overlying the tracheal mucosa. In lung lobes, dye staining exhibited a patchy distribution and was present in both smaller airways and lung parenchyma.

**Development of a differential lung exposure model**

To assess the relative sensitivities of trachea and lower lung to VMV, a differential in vivo exposure model was created to allow exclusive exposure of the trachea to 1 ml instillations without involving the peripheral lung. One millilitre of patent blue dye was introduced endoscopically onto the lateral aspect of the distal one-third of the trachea of two adult ewes and the location was recorded up to 2 h post-instillation. Representative images from one tracheal exposure experiment are shown in Fig. 3. Dye pooled in the ventral aspect of the distal one-third of the trachea just proximal to the entrance to the right apical lung lobe immediately post-instillation. During the 1 h of anaesthesia, dye moved proximally up the trachea towards the larynx. By 45 min post-instillation, dye was present at the distal end of the endotracheal tube. At post-mortem, dye was located at the proximal end of the trachea and within the larynx. A small amount of dye was present within the oral cavity. No dye was located in lower trachea or within any lung lobe, indicating good exposure of the trachea without any detectable exposure of the peripheral lung.
Comparison between tracheal and peripheral lung exposure to VMV

To compare the relative sensitivities of trachea and lower lung to VMV, seronegative adult sheep were inoculated under general anaesthesia with 1 ml VMV strain EV1 containing $10^6$ TCID$_{50}$ either directly into the left cardiac lung lobe ($n=4$) or into the trachea using the tracheal exposure model developed previously ($n=4$). Virus inoculates were spiked with 0.01% patent blue dye to allow direct visualization of virus instillations by endoscopy. In addition, the trachea from one sheep from the tracheal exposure group was traumatized with a 21 gauge needle to account for tracheal trauma resulting from i.t. injection.

Lower lung instillations remained within the left cardiac lung lobe throughout anaesthesia. Tracheal instillations pooled at the level of the distal trachea and moved proximally up the trachea towards the larynx at 45 min post-instillation. Two further sheep were inoculated with $10^6$ TCID$_{50}$ VMV strain EV1 to allow direct comparison with tracheal and lower lung exposures. Weekly blood samples were subsequently analysed for the presence of viraemia and seroconversion by PCR and ELISA, respectively.

Weekly PCR results are summarized in Fig. 4(a). I.t. injection resulted in viraemia at 3–4 weeks post-infection. Lung instillation resulted in viraemia at 2–3 weeks post-infection. Tracheal instillation resulted in viraemia at 8–24 weeks post-instillation, significantly delayed compared with lung instillation ($P=0.030$). Results of ELISA analysis are summarized in Fig. 4(b). Seroconversion occurred at 4–6 weeks post-infection for i.t. injection, 2–8 weeks post-infection for lung instillation and 12–24 weeks post-infection for tracheal instillation. The time to seroconversion was significantly greater for tracheal instillation compared with lower lung instillation ($P=0.030$). Traumatizing the tracheal epithelium prior to tracheal instillation did not decrease the time taken for viraemia or seroconversion to occur.

Identification of cell-free VMV within lung-lining fluid of naturally infected sheep

Cellular and cell-free fractions of post-mortem BAL samples obtained from three naturally infected sheep were analysed for the presence of VMV using a combination of virus titration and ICC. Within bronchoalveolar cell (BAC) populations, VMV-positive cells were identified with typical AM morphology (Fig. 5a). Virus was detected in all cell-free BAL supernatant samples, with co-cultivated OSCs exhibiting syncytial formation typical of VMV CPE within 7 days (Fig. 5b). The presence of VMV within these multinucleate cells was confirmed by ICC staining for VMV capsid protein (Fig. 5c, d), which revealed widespread staining throughout the syncytia.

The results of BAL supernatant virus titrations and VMV-positive cell counts are shown in Table 1. Virus titres within BAL supernatants ranged from $1.1 \times 10^2$ to $1.4 \times 10^3$ TCID$_{50}$ [ml BAL fluid (BALF)]$^{-1}$. Assuming the BAL procedure resulted in a dilution factor of approximately 1:100 (McGorum et al., 1993), these titres equate to $1.1 \times 10^4$ to $1.4 \times 10^5$ TCID$_{50}$ (ml lung-lining fluid)$^{-1}$. VMV capsid protein was detected in 1.4–4.4% of BACs. There was no direct correlation between the percentage of positive BACs and virus titre in the BALF, although the lowest virus titre was associated with the lowest percentage of positive BACs.
DISCUSSION

Tracheal organ cultures have been used in many ex vivo experimental models to examine the initial interactions between the host and various viruses and bacteria (Campbell et al., 1979; Dhinakar Raj & Jones, 1996; Rutman et al., 1998; Lund et al., 2001; Lin et al., 2001; Anderton et al., 2004). Organ cultures offer the advantage that both epithelial and interstitial cells are present in their normal anatomical arrangement, allowing realistic interactions between the cells in each compartment. In the case of in vitro infection with VMV, most cell types isolated and grown in primary cell culture have been shown to support productive VMV replication, unlike the situation in vivo, in which virus replication is highly restricted (Haase et al., 1977; Gendelman et al., 1985; Brodie et al., 1995). This loss of the normal restricted replication pattern as a result of in vitro culturing is a significant problem when trying to study normal in vivo VMV behaviour and, to date, in vitro studies of VMV infection have concentrated on primary cell cultures (Leroux et al., 1995; Craig et al., 1997; Lerondelle et al., 1999). Using an organ culture system, it was hoped that the observed VMV replication pattern more truly represented the situation in vivo.

In this study, it was shown that ovine tracheal organ cultures were capable of supporting VMV replication up to the level of proviral synthesis. No evidence of productive virus infection was found, with no release of virions into the culture medium and no viral protein detected using ICC. The permissive life cycle of the virus in vitro is usually complete within 3 days (Brahic et al., 1981; Haase et al., 1982; Vigne et al., 1987), and maintenance of organ cultures for 7 days post-infection prior to analysis was thought to allow sufficient time for productive VMV infection to take place. This suggests that VMV replication in tracheal organ cultures is restricted at some level between proviral synthesis and translation of structural viral proteins, thus mimicking a typical in vivo VMV replication pattern (Gendelman et al., 1985; Brodie et al., 1995). This is of interest, since organ cultures are without an adaptive immune system, implying that the restricted replication observed is not due to specific anti-VMV immune responses. It is possible that infected cells are not in the correct state of maturation or activation, as productive VMV replication is known to be closely linked to the maturation/activation state of the host cell (Gendelman et al., 1986). The restricted replication observed could result from a suboptimal environment for virus replication due to reduced cell viability. However, 7-day-old cultures were still capable of de novo provirus production and therefore were still metabolically active.

Detectable infection was seen only in organ cultures incubated with high titres of virus ($\geq 10^5$ TCID$_{50}$ ml$^{-1}$). The 2 h contact time between virus and trachea in these studies was long relative to the rate of binding of VMV to its cellular receptor(s), which has been estimated to occur within 15 min in vitro (Kennedy-Stoskopf & Narayan,

Fig. 3. Representative images from one tracheal exposure experiment. One millilitre patent blue dye was deposited into the distal trachea under general anaesthesia via an endoscope. After 1 h of anaesthesia and 1 h of recovery, sheep were euthanized. (a) Deposition of dye onto the lateral aspect of the distal tracheal wall via a catheter (C) inserted into the lateral channel of an endoscope. (b) Dye pooled immediately in the ventral aspect of the distal trachea, just proximal to the entrance to the right apical lung lobe (RA). (c) At 45 min post-instillation, dye was present at the distal end of the endotracheal tube (ET). (d–g) At post-mortem, no dye was visible in any lung lobe (d) and was located at the proximal end of the trachea (arrow) and within the larynx (e). No dye was present at the entrances to the right main bronchus (RMB), left main bronchus (LMB) or right apical lung lobe (RA) (f). Sectioning of the left cardiac lung lobe revealed the lobe to be free of dye (g).

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compared with tracheal exposure instillation is a highly efficient route of infection with VMV.

lower lung exposure, and not tracheal exposure, is the reason for the high efficiency of infection observed for i.t. inoculation with VMV.

The relative inefficiency of VMV uptake by the trachea in vivo may be explained firstly by an inherent insensitivity of the tracheal mucosal cells to VMV, as suggested by the results of tracheal organ culture experiments performed in this study, and secondly by the action of innate mucosal defence mechanisms. For example, tracheal virus inoculations were transported efficiently from the distal trachea to the larynx by the mucociliary escalator within 45 min, resulting in only limited contact time between VMV and the tracheal mucosa. Given that infection of tracheal organ cultures was inefficient despite 2 h continuous exposure to VMV of identical titre and strain, it is likely that mucociliary escalator function plays an additional role in limiting VMV uptake in vivo.

The reasons for the high efficiency of lower lung instillation of cell-free VMV may be twofold. Firstly, there are abundant populations of a number of potential target cell types for VMV in the lower lung. These include respiratory tract dendritic cells, AMs, interstitial macrophages and bronchial epithelial cells, all of which have been implicated in VMV infection in vivo (Gendelman et al., 1985; Staskus et al., 1991; Brodie et al., 1995; Ryan et al., 2000; Carrozza et al., 2003).

Secondly, pulmonary clearance of inhaled particles and pathogens in the lower lung is primarily mediated via phagocytic uptake by AMs (Lehnert, 1992; Gordon & Read, 2002). It can therefore be seen that innate defence mechanisms of the lower lung may actually result in enhanced uptake of VMV, as interaction between AMs, a natural target cell, and free virus within the lower lung is likely to result in virus infection of AMs rather than virus inactivation. Thus, pulmonary clearance mechanisms in the lower lung would increase rather than reduce virus uptake.

Indeed, the intracellular bacterium Legionella pneumophila has been shown to utilize AM uptake to facilitate colonization of the lung (Yamamoto et al., 1994). Instillation of VMV into a lung lobe and subsequent analysis of AM populations for evidence of infection at an early (pre-lesional) time point would be required to confirm initial uptake of VMV by AMs.

The high sensitivity of the lower lung to cell-free VMV suggests that uptake of virus at this site may play a role in natural respiratory transmission. As cell-free virus was identified in the lung-lining fluid of naturally infected sheep in this study, it is possible that coughing by VMV-infected}

**Fig. 4.** Comparison of the efficiencies of i.t., tracheal and lower lung inoculation routes for VMV. Sheep were inoculated with 10^6 TCID<sub>50</sub> VMV strain EV1 via i.t. injection (i.t.), endoscopic instillation into trachea (Trachea) or endoscopic instillation into the left cardiac lung lobe (Lung). The trachea of one sheep in the tracheal instillation group (denoted by a large circle) was traumatized using a 21 gauge needle prior to virus instillation. The time taken for viraemia (detected by snPCR) (a) and seroconversion (detected by ELISA) (b) to occur was recorded for each route. Horizontal lines indicate mean values. Asterisks denote significance in the Mann–Whitney test.

1986). Taken together, these results indicate that uptake of VMV by ovine tracheal organ cultures is inefficient. Furthermore, as processing of tracheal organ cultures prior to virus incubation is likely to compromise a number of innate immune defence mechanisms severely, including loss of mucus and antimicrobial peptides from the epithelial surface and disruption of the mucociliary escalator (Zhang et al., 2000; Welsh & Mason, 2001), these results suggest an inherent insensitivity of tracheal cell populations to VMV.

It has been shown that i.t. inoculation in sheep results in exposure of both trachea and all parts of the lower lung to the instillation. Using a differential exposure model for lower lung and trachea, it was demonstrated that lower lung instillation is a highly efficient route of infection with VMV compared with tracheal exposure in vivo. The time taken for viraemia and seroconversion to occur after lower lung instillation was similar to that observed for i.t. inoculation. In addition, trauma to the tracheal epithelium prior to tracheal exposure to virus did not appear to increase the efficiency of virus infection. Therefore, it is concluded that
animals may generate free-virus-containing aerosols that are small enough to reach the lower lung. Indeed, in a study of cough-generated aerosols in humans, the majority of aerosolized particles were found to be between 0.65 and 3.3 μm in diameter (Fennelly et al., 2004), which would be small enough in theory to reach the lower regions of the lung. Therefore, inhalation of aerosols containing cell-free VMV into the lower lung may be an important mechanism of respiratory transmission in the natural situation. In addition, as aerosol transmission would require close contact between infected and non-infected animals, this may partly explain the recent observation that VMV transmission appears to be minimal in extensively reared flocks (Leginagoikoa et al., 2006).

In conclusion, this study has demonstrated that the tracheal mucosa is relatively resistant to infection and that the high efficiency of infection via the i.t. route results from lower lung exposure. The identification of cell-free VMV within the lung-lining fluid of naturally infected sheep, together with the high sensitivity of the lower lung to VMV, suggests that inhalation of aerosols containing free virus particles and subsequent exposure of the lower lung may play a significant role in natural respiratory transmission of VMV. Identification of cell types within the lower lung that are involved in initial virus uptake is required to elucidate the exact mechanism of virus entry in the lower lung.

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REFERENCES


Table 1. BAC immunohistochemistry for VMV and free-virus titrations of corresponding BALF from three naturally infected sheep

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<th>Sheep</th>
<th>VMV-positive BAC (%)</th>
<th>Free virus titre (TCID50 ml−1)</th>
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<td>N44-05</td>
<td>1.8</td>
<td>(1.4 \times 10^3)</td>
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<td>1.4</td>
<td>(1.1 \times 10^2)</td>
</tr>
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<td>705-02</td>
<td>4.4</td>
<td>(6.2 \times 10^2)</td>
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Fig. 5. Representative images from a naturally VMV-infected sheep demonstrating the presence of VMV within BAL cells and cell-free BAL supernatant. (a) ICC staining of BAL cells for VMV capsid protein. A VMV-positive cell with AM morphology is indicated by the arrow (bar, 20 μm). (b) Giemsa stain of OSCs co-cultivated with cell-free BAL supernatant at 7 days. Many multinucleate cells are present (arrowheads) (bar, 50 μm). (c–d) Cytocentrifuged multinucleate OSCs co-cultivated with cell-free BAL supernatant. (c) ICC staining for VMV capsid protein demonstrating positive staining throughout two multinucleate cells (arrows) (bar, 20 μm). (d) Isotype-matched negative control. The arrow indicates a multinucleate cell free of staining (bar, 20 μm).


