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Pathogenesis of Wild-Type and Leaderless Foot-and-Mouth Disease Virus in Cattle

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Four calves were experimentally infected via aerosol with foot-and-mouth disease virus. Two were infected with a wild-type virus derived from a full-length infectious clone (A12-IC), and two were infected with a clone-derived virus lacking the leader gene (A12-LLV2), with euthanasia and tissue collection at 24 and 72 h postexposure (hpe). Clinical disease was apparent only in the animal given A12-IC and euthanized at 72 hpe. In situ hybridization revealed that the animal infected with A12-IC and euthanized at 24 hpe had abundant viral nucleic acid in the lung, present in clusters of positive cells in the respiratory bronchiolar epithelium and associated subepithelial regions. At 72 hpe in the A12-IC-infected calf, viral nucleic acid in the lung was present in interstitial areas, and in addition, viral nucleic acid was detectable in epithelial tissues around histologically apparent vesicles. In animals infected with A12-LLV2, viral nucleic acid was detectable in the lung at both 24 and 72 hpe, but staining revealed a more localized distribution with less nucleic acid than was found in animals given A12-IC. Therefore, it appears that after aerosol exposure to A12-IC, early replication is in the region of the lung, with subsequent dissemination to distal sites. In comparison, the A12-LLV2 virus is much less widely disseminated in the lung at 24 hpe, with no lesions or virus detectable in secondary sites at 72 hpe. The greatly reduced pathogenicity of A12-LLV2 may make it an excellent candidate for a modified live viral vaccine.

Foot-and-mouth disease (FMD), caused by an *Aphthovirus* in the family *Picornaviridae*, is characterized by temporary and debilitating oral and pedal vesicles which make animals reluctant to eat or walk, resulting in a transient but drastic decline in production. The FMD virus (FMDV) is spread by aerosol and is extremely contagious. Because of the serious production problems caused and its highly contagious nature, FMD is considered to be the most economically important disease of livestock worldwide. Most countries which are free of FMD, including the United States, maintain rigid quarantine and import restrictions to prevent its introduction, so that they may participate actively in international trade of animals and animal products (10). Currently, the most effective control in slowing spread of the disease through livestock populations is slaughter of infected and exposed animals coupled with ring vaccination using a killed vaccine.

A live attenuated viral vaccine with no risk of reverting to virulence could aid in controlling an outbreak of FMD. Recently, a genetically altered variant of FMDV which is lacking the coding region for the leader (L) proteinase was developed (12). The L protein is a papain-like proteinase that autocatalytically cleaves itself from the polyprotein (2, 7, 9, 13, 15, 16) and cleaves p220, a subunit of the cap-binding protein complex, eIF-4F, involved in the initiation of translation at the 5′ end of most eukaryotic mRNAs (4, 8, 11). Cleavage of p220 correlates with the shutoff of cap-dependent host protein synthesis, but viral mRNA translation occurs by a cap-independent mechanism, and its translation is not affected (6). The leaderless virus was shown to be somewhat attenuated in the tissue culture lines tested and in suckling mice (12). The aim of this study was to examine this virus in the natural host and to compare its pathogenesis with that of the parent wild-type virus.

Of four Hereford calves, aged 9 to 12 months, two were infected by aerosol with 2×10^8 PFU of an infectious clone-derived virus (A12-IC) (14) and two were infected with 2×10^8 PFU of a virus lacking the L coding region (A12-LLV2) (12). The latter virus was constructed with the N-terminal Gly codon of VP4 positioned directly following the second (Lb) Met codon of the L protein (Fig. 1). Each virus was delivered in 1.0 ml of tissue culture medium via a nebulizer in an enclosed hood over a 15-min period. Animals were monitored clinically and euthanized at 24 and 72 h postexposure (hpe). There was no clinical disease detected in either of the animals exposed to A12-LLV2 or in the animal exposed to A12-IC for 24 h. However, at 72 hpe, the animal exposed to A12-IC was febrile (41.8°C) and salivating and had intact vesicles visible in the interdigital clefts of the left front and right rear hooves. Postmortem, the only lesion in the animal exposed to A12-IC and euthanized at 24 hpe was moderate edema of the mediastinal lymph nodes, while in the animal euthanized at 72 hpe, there were multiple vesicular lesions, in various stages of development, in three of four interdigital clefts and on the tongue. In addition, in this animal, pharyngeal tonsil and retropharyngeal, mediastinal, and tracheobronchial lymph nodes were moderately edematous with multifocal petechiation. Grossly, there were no abnormal findings in either of the animals infected with the A12-LLV2 virus.

Numerous tissues were collected in 10% formalin, processed to paraffin, and examined histologically and by in situ hybridization (ISH) (1). Histologically, the most striking findings were in the pedal and lingual tissues of the animal infected with A12-IC and euthanized at 72 hpe. Here there were vesicles, often multiple, in various stages of development. Also, several of the lymphoid tissues from this animal, notably retropharyngeal, mediastinal, and tracheobronchial lymph nodes, were markedly reactive and edematous. In the lungs of the

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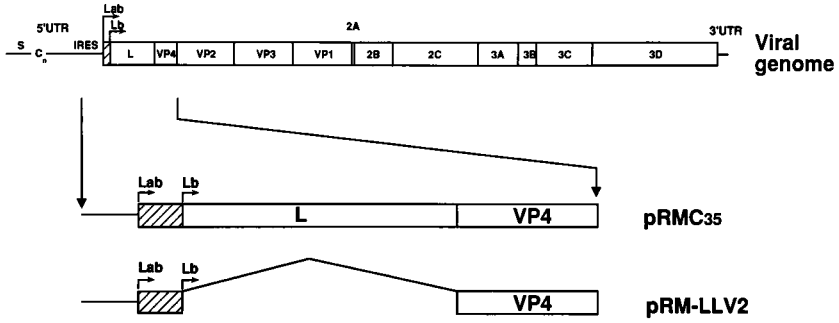

FIG. 1. Diagram of the FMDV genome and L-deleted genome. The viral open reading frame is boxed, and the shaded box corresponds to the 84-nucleotide region between the two in-frame initiation codons for Lab and Lb. Abbreviations: IRES, internal ribosome entry site; C_n, poly(C) tract; S, 5′ portion of the genome or small fragment; UTR, untranslated region.

animal exposed to A12-IC and euthanized at 24 hpe there was a generalized involvement of all respiratory bronchioles with increased cellularity of the adjacent alveolar septa, pavementing of macrophages in small blood vessels, edema, and foci of microhemorrhage (Fig. 2a). Similar changes were also present in the animal given A12-IC and euthanized at 72 hpe but were much more focal and confined in appearance. In contrast, in the animals given A12-LLV2, at 24 hpe, these affected respiratory bronchiolar foci were much harder to discern and define. At 72 hpe in the A12-LLV2-exposed animal, no pulmonary changes were discernible (Fig. 2b).

Tissues were examined by ISH using a digoxigenin-labeled 500-base negative-sense riboprobe corresponding to the 3D coding region (1), and the results are summarized in Table 1. In the animal given A12-IC and euthanized at 24 hpe, there was a multifocal but extensive ISH signal centered on the respiratory bronchioles (Fig. 3a), with staining evident in some epithelial cells but also prominent in subepithelial and interstitial sites. At 72 hpe, the staining was much more confined to focal areas of interstitial staining, often with associated edema (Fig. 3b). In contrast, in the animals given A12-LLV2, there was occasional single-interstitial-cell staining around respiratory bronchioles at 24 hpe (Fig. 3c), with rare instances of staining at 72 hpe (Fig. 3d).

In situ hybridization signals in epidermal sites and the oral epithelium were present only in the animal given A12-IC and euthanized at 72 hpe, with positive signal detected around all sites of epithelial lesion development as dense cytoplasmic staining (Fig. 4). In some tissues from this animal, notably the soft palate and tongue, clusters of positively staining cells were occasionally discernible in the upper stratum spinosum in the absence of any histologically apparent lesion. The only other tissues with positive signals from the animal given A12-IC and euthanized at 72 hpe were various lymphoid tissues, where the signal was weak and consisted of small clusters of positively staining cells in follicular areas and occasional single cells staining in perivascular locations. Pharyngeal tonsil and tracheobronchial lymph nodes had the greatest amounts of signal (Fig. 5). In the animal given A12-LLV2 and euthanized at 72 hpe, only the pharyngeal tonsil was positive, with a pattern similar to but much less intense than that of the animal exposed to wild-type virus and euthanized after the same time interval.

The pathogenesis of FMD, in particular the identification of the initial site of replication, has been the focus of numerous investigations which have implicated lung, pharyngeal area, or epithelial target tissues as possible sites of early replication (2, 3, 17). In the present study, it appears that the lung, specifically the area of the respiratory bronchioles, is the initial site of replication. In the animal given wild-type virus and euthanized at 24 hpe, there was extensive nucleic acid staining in a segmentally diffuse pattern, with many cells in the vicinity of a respiratory bronchiole replicating virus, suggesting that inhaled virus initially replicated in respiratory bronchiolar epithelium and then spread locally. The resulting pattern was one of multifocal yet extensive areas of viral synthesis. The presence of

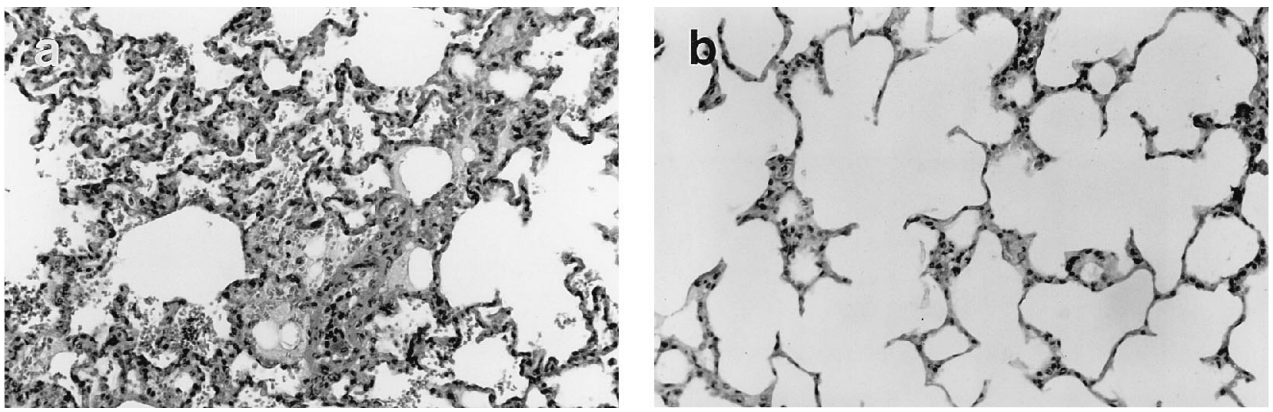

FIG. 2. Histopathologic sections (hematoxylin and eosin staining) of lung tissue from infected calves. (a) Interstitial areas surrounding respiratory bronchioles of the lungs from the animal euthanized 24 hpe to A12-IC are expanded by acute inflammatory cells and edema. (b) Interstitial areas from the lung of the animal euthanized 72 hpe to A12-LLV2 are quiescent and essentially normal.

TABLE 1. Results of ISH on various tissues from animals infected with A12-IC and A12-LLV2 viruses

Tissue (no. of samples/animal)	Result[a] for animal infected with:			
	A12-IC, euthanized:		A12-LLV2, euthanized:	
	24 hpe	72 hpe	24 hpe	72 hpe
Lung (4)	+++	++	+	+
Tongue (2)	−	++	−	−
Soft palate	−	++	−	−
Hard palate	−	−	−	−
Interdigital cleft (4)	−	+++ (3 of 4)	−	−
Coronary band (4)	−	−	−	−
Heel bulb (4)	−	+++ (1 of 4)	−	−
Teat	−	−	−	−
Mediastinal lymph node	−	−	NA	−
Tracheobronchial lymph node	−	+	−	−
Pharyngeal tonsil	−	+	−	±
Palatine tonsil	−	+	−	−
Retropharyngeal lymph node	−	+	−	−
Prescapular lymph node	−	+	−	−

[a] +, limited amount of staining; ++, moderate staining; +++, extensive staining; NA, not available; ±, very limited amount of staining.

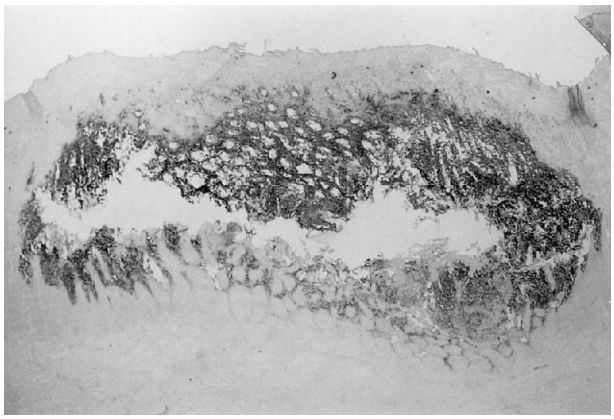

FIG. 4. Detection of FMDV RNA surrounding a vesicle by ISH. There is an extensive positive signal in epithelial cells surrounding a histologically apparent vesicle in the right hind interdigital cleft of the animal euthanized 72 hpe to A12-IC.

greatly expanded and reactive pulmonary lymph nodes with evidence of virus by ISH, at 72 hpe, in the tracheobronchial lymph nodes further indicates that the lung may be the primary site of viral replication. At 72 hpe there was also virus in lymphoid tissue from the upper respiratory tract and pharynx, i.e., retropharyngeal lymph node and pharyngeal and palatine tonsils. Presumably, the source of this staining may be draining of virus from secondary replication in the tongue and soft palate, but the staining may also be due to pulmonary cells draining the lung via the muco-ciliary escalator and passing over the pharynx.

The results from this study can be compared with previous

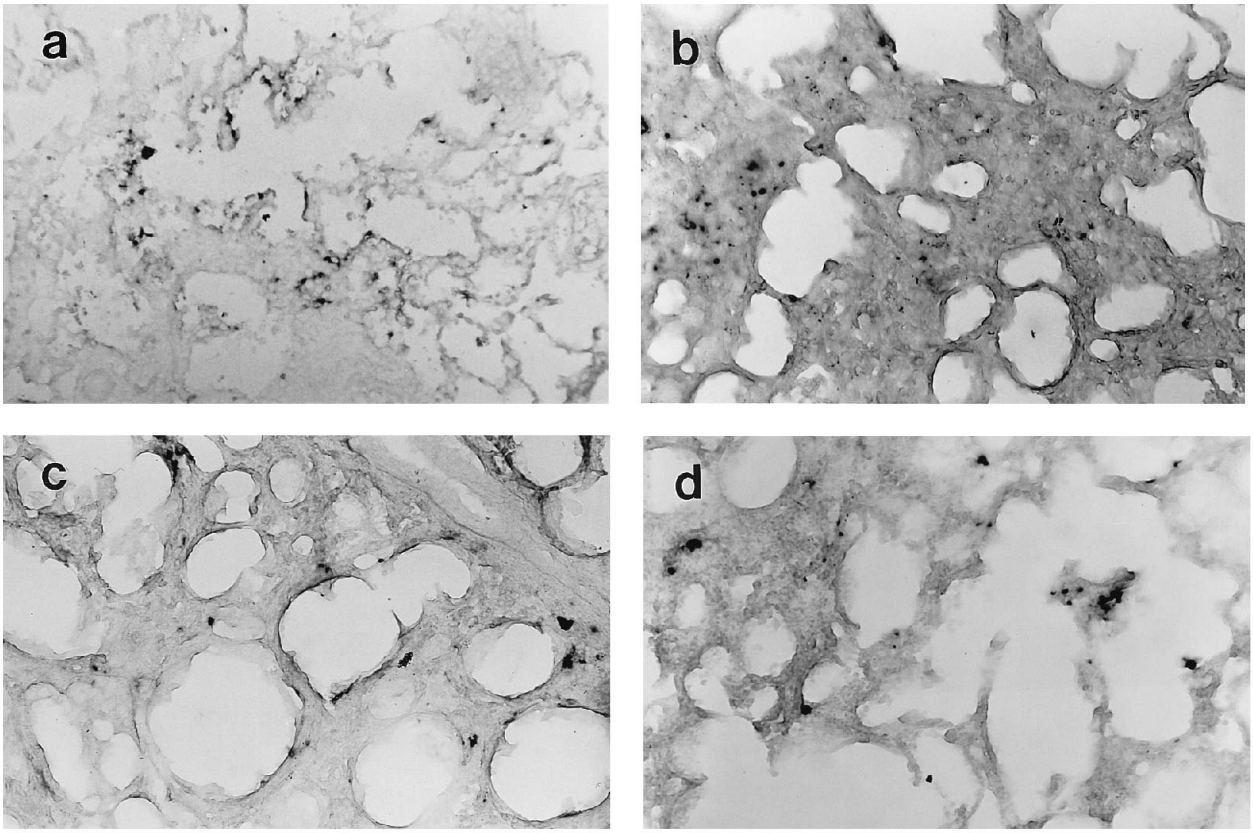

FIG. 3. Detection of FMDV RNA in pulmonary tissue by in situ hybridization. (a) There is an extensive positive signal within respiratory bronchiolar epithelial cells and also within some interstitial cells in the lung of the animal exposed to A12-IC 24 h previously. (b) Localized interstitial signal and edema are evident in the lung of the animal euthanized 72 hpe to A12-IC. (c) Very limited positive signal in the area of respiratory bronchioles of the lung of the animal euthanized 24 hpe to A12-LLV2. (d) Limited positive signal in the interstitium of the lung is present in the animal euthanized 72 hpe to A12-LLV2.

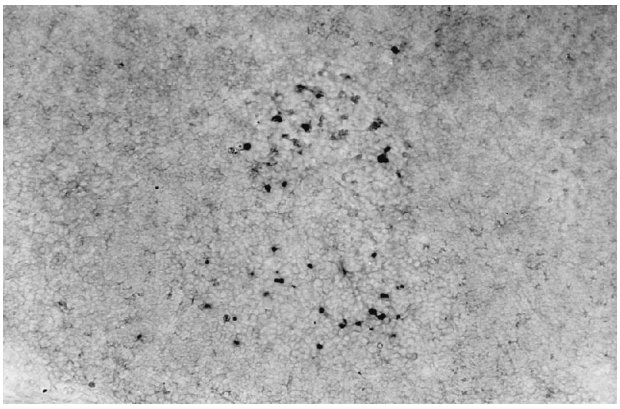

FIG. 5. Detection of FMDV RNA in a lymph node by ISH. Positive signals can be seen scattered within a localized area resembling a follicle in the tracheobronchial lymph node of the animal euthanized 72 hpe to A12-IC.

work concerning the initial site of viral replication. Sutmoller and McVicar (17) separated the upper and lower respiratory tracts by tracheostomy and, after instilling virus in the lung, subsequently recovered virus from the pharynx, concluding that the lung is the primary site of replication with hematogenous seeding of other sites, including the pharynx. These findings are in agreement with the present study using ISH. Infecting animals directly into the lung via a tracheotomy, Burrows et al. (3) found that viral titers from the pharynx were higher than those from the lung, leading them to assume that the lung may not be a primary site of initial viral growth. The discrepancy with our results may be due to sampling technique. As infected cells in the lung die and move up the muco-ciliary escalator, they could become concentrated in the mucus of the pharynx and produce misleading results. Brown et al. (2), using a biotin-labeled probe on frozen sections from cattle infected with a highly virulent FMDV Asia 1, found virus in pedal epithelial sites as early as 6 h after aerosol exposure, with the conclusion that after inhalation, virus moves very rapidly to sites of lesion predilection. The virus used in the present study, A12-IC, is considerably less virulent and slower to produce disease than the Asia 1 virus used in the earlier study, so perhaps the results of the two studies are not mutually exclusive.

Although we did not rigorously identify the cells containing virus in the subepithelial and interstitial sites around the respiratory bronchiole, morphologic evidence suggests that these were macrophage-type cells, perhaps tissue macrophages or pulmonary intravascular macrophages. Cells of the macrophage lineage have been implicated before in FMD pathogenesis. Furthermore, it is thought that Langerhans cells, epidermal antigen-presenting cells of macrophage origin, are responsible for transport of FMDV into epidermal sites (2, 5). Also, virus has been seen in alveolar macrophages (2).

In the present study, using the natural host and route of exposure, the leaderless virus was avirulent relative to wild-type virus. By ISH, there were distinct differences in viral replication patterns, especially noticeable in the lung, which appears to be the primary site of viral entry and proliferation after natural infection. In the A12-LLV2-infected animals, sites of viral replication in the lung were much more localized, indicating that cell-to-cell spread was either reduced or not able to occur. Also, there appeared to be no spread to secondary epithelial sites in the A12-LLV2-infected animal.

The cleavage of p220 by the L proteinase of FMDV correlates with the shutoff of host protein synthesis. This action increases the viral mRNA's access to the host's translation machinery and blocks antiviral responses requiring cap-dependent de novo protein synthesis, thus enhancing viral replication and spread to neighboring cells. It may be that lack of the L proteinase delays translation and replication of A12-LLV2 and allows cap-dependent translation of host factors responsible for an antiviral response, resulting in reduced spread of virus and the attenuation of virulence. Preliminary studies with primary lamb kidney cells indicate that in contrast to wild-type virus, A12-LLV2 is unable to spread to neighboring cells. Furthermore, ongoing bovine experiments suggest that the leaderless virus is also significantly attenuated by the subcutaneous inoculation route and able to induce an immune response. Consequently, this A12-LLV2 virus may be an excellent candidate for vaccine development.

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