Endothelial cell infection in vivo by equine infectious anaemia virus

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Equine infectious anaemia virus (EIAV) infection of horses is characterized clinically by recurrent episodes of fever, thrombocytopenia and anaemia. In vivo, the only site of virus replication that has been previously demonstrated for EIAV is the tissue macrophage. In this study, in situ hybridization for EIAV was combined with immunohistochemistry for cell-type-specific markers to identify infected endothelial cells. EIAV-infected endothelial cells and macrophages were detected in horses infected with either virulent wild-type or with weakly virulent tissue culture-adapted strains of EIAV. The role of endothelial cell infection in the pathogenesis of EIAV remains undefined, but could contribute to the development of thrombocytopenia. However, endothelial cell infection does not appear to be a determinant of virulence for EIAV.

Equine infectious anaemia (EIA) is a persistent lentiviral infection of horses that is characterized clinically by recurrent episodes of fever, thrombocytopenia and anaemia. These episodes tend to decrease in both frequency and intensity over time, and most horses become life-long subclinical carriers (Sellon et al., 1994). Thrombocytopenia is a salient feature of EIAV infection that is proportional to the level of viraemia (Clabough et al., 1991; Crawford et al., 1996; Tornquist et al., 1997). Infection with virulent strains of EIAV may result in acute, severe clinical disease with profound thrombocytopenia and haemorrhagic diathesis (Clabough et al., 1991; Crawford et al., 1996). Infection with less virulent strains typically results in mild to moderate disease without haemorrhage, and in many cases is entirely subclinical (Sellon et al., 1994).

Initially described by McGuire et al. (1971), and confirmed by Clabough-Sellon et al. (1992), the tissue macrophage remains the only recognized in vivo host cell for EIAV productive replication. These studies were performed on horses infected with the highly virulent Wyoming strain of EIAV (EIAV_Wyo). EIAV_Wyo and other wild-type EIAV strains are also highly macrophage-tropic in vitro, and can only be propagated in primary equine macrophage cultures (Carpenter & Chesebro, 1989; Kono & Yokomizo, 1968).

Most laboratory strains of EIAV have been derived from EIAV_Wyo but are considerably less virulent than the parent strain (Orrego et al., 1982). EIAV_Wyo was initially adapted in vitro to equine dental cells by Malquist et al. (1973). Variants of the Malquist strain (e.g. WSU5 strain; EIAV_WSU5), also replicate in vitro in other cell types of equine origin, including dental fibroblasts (Klevjer-Anderson et al., 1979; Malquist et al., 1973), kidney cells (O'Rourke et al., 1988) and endothelial cells (Maury et al., 1998) in addition to macrophages. The ability to replicate in non-macrophage cell types in vitro is retained with in vivo passage. However, the in vivo cellular tropism of the less virulent strains has not been examined.

Horses in this study were maintained and handled by methods approved by the Washington State University Institutional Animal Care and Use Committee. Three horses were experimentally infected by intravenous injection: two with EIAV_Wyo (1×10⁶ and 1×10⁴ horse infectious doses, respectively), and one with EIAV_WSU5 (1×10⁶ TCID₅₀). Rectal temperatures and the number of erythrocytes, platelets and leukocytes were monitored daily.

All three horses developed fever (>38.4 °C) and thrombocytopenia (<15 000 platelets/μl) (Crawford et al., 1996). The two horses infected with the virulent EIAV_Wyo strain developed severe clinical disease and were euthanized in extremis at days 15 and 21 post-infection. Persistent fever, beginning at days 7 and 10 post-infection, was accompanied by a progressive decline in platelets. Clinical signs referable to thrombocytopenia, including petechiation of oral mucous membranes and haemorrhagic enteritis, were observed in both horses during the terminal stages of disease when platelet counts were <30 000/μl.

The horse infected with the low-virulence EIAV_WSU5 strain experienced an episode of mild fever and thrombocytopenia from days 9 to 21 post-infection, and thereafter recovered. A second episode ensued on day 27, and the horse was necropsied during clinical disease on day 29. No haemorrhage was noted at any time. As revealed by the presence of viral RNA in the serum, detected by RT–PCR as previously

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Fig. 1. (a) Photomicrograph of a macrophage (solid arrow) containing viral RNA in the spleen of a horse infected with EIAV<sub>Wyo</sub>. The cell is double-labelled by immunohistochemistry for lysozyme (brown-staining cells) and in situ hybridization for EIAV gag with the 35S-labelleld antisense probe (dark silver grains). An adjacent cell (open arrow), lining the lumen of a vessel (L), is positive for viral RNA but negative for lysozyme. Bar, 10 µm. (b, c) Photomicrographs of endothelial cells containing viral RNA in the spleen of a horse infected with EIAV<sub>Wyo</sub> (arrows). The cells are double-labelled by immunohistochemistry for von Willebrand’s factor (brown-staining cells) and in situ hybridization for EIAV gag with the 35S-labelled antisense probe (dark silver grains). Bars, 10 µm. (d) Photomicrograph of an adjacent section of the tissue in (b) and (c), labelled by immunohistochemistry for von Willebrand’s factor and in situ hybridization with a 35S-labelled sense probe for EIAV gag to demonstrate the level of nonspecific hybridization and background silver grain formation. Bar, 10 µm. Sections of spleen labelled by immunohistochemistry using normal rabbit IgG as the primary antibody did not contain significant nonspecific staining (data not shown).

described (Tornquist et al., 1997), all three horses became detectably viraemic at the onset of fever and thrombocytopenia (data not shown). Samples of bone marrow, heart, intestine, kidney, liver, lung, lymph nodes, spleen and thymus were collected at necropsy, fixed in 4% paraformaldehyde and embedded in paraffin.
Immunohistochemistry for cell-specific antigens was performed on deparaffinized sections as previously described (Oaks et al., 1998) using a [³²P]dUTP-labelled cRNA probe antisense to a 450 nucleotide gag segment of EIAV genomic RNA, and detected by autoradiography (NTB2 emulsion; Eastman Kodak) after 5 to 14 days exposure at −80 °C. In tissues that have not been subjected to conditions that denature DNA, this probe is specific for viral genomic RNA. Controls for specificity included hybridization with the complementary sense probe, that is specific for viral DNA, on nondenatured infected tissues, with the antisense probe on uninfected tissues, and predigestion with RNase to confirm that the target was RNA. Nonspecific hybridization was not observed in any of the controls.

In situ hybridization was performed on all tissues to determine the distribution and cellular tropism of EIAV during clinical disease. In situ hybridization for viral RNA was performed on deparaffinized sections as previously described (Oaks et al., 1998) using a [³²P]dUTP-labelled cRNA probe antisense to a 450 nucleotide gag segment of EIAV genomic RNA, and detected by autoradiography (NTB2 emulsion; Eastman Kodak) after 5 to 14 days exposure at −80 °C. In tissues that have not been subjected to conditions that denature DNA, this probe is specific for viral genomic RNA. Controls for specificity included hybridization with the complementary sense probe, that is specific for viral DNA, on nondenatured infected tissues, with the antisense probe on uninfected tissues, and predigestion with RNase to confirm that the target was RNA. Nonspecific hybridization was not observed in any of the controls.

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Immunohistochemistry for cell-specific antigens was performed first on sections of liver, spleen and intestine, and followed by in situ hybridization to identify the phenotype of cells expressing viral RNA. RNase degradation of the cRNA probe was prevented during in situ hybridization procedures by preparing all immunohistochemistry reagents in DEPC-treated water and buffers, and inclusion of heparin (4000 units/ml) in all antisera (Shapiro & Young, 1981). Deparaffinized sections were treated with 3 % hydrogen peroxide in methanol to inactivate endogenous peroxidase, rehydrated, and permeabilized with 0·1 % Pronase. Nonspecific protein binding was blocked by treatment with 5 % normal goat serum in 125 mM Tris, 350 mM NaCl and 0·05 % Triton-X. The following primary antibodies were used: rabbit anti-von Willebrand’s Factor (Dako A082, 1:1000) as a marker for endothelium (Sehested & Hou-Jensen, 1981), and rabbit anti-lysozyme (Dako A099, 1:300) as a marker for macrophages and granulocytes (Krugliak et al., 1986). Normal rabbit IgG fraction (Dako X903, 1:1000) was used as a negative control, and did not demonstrate any nonspecific antibody binding. Bound primary antibody was detected with biotinylated goat anti-rabbit immunoglobulin, an avidin–biotin–peroxidase complex (Vectastain Elite Rabbit kit, Vector Labs), and 3,3’,5,5’-diaminobenzidine (DAB) (Vector Labs). Following immunohistochemistry, in situ hybridization was performed as above.

In horses infected with either EIAV<sub>Wyo</sub> or EIAV<sub>WSU</sub>, cells containing viral RNA were identified in all tissues examined. The location, morphology and co-labelling with lysozyme identified these cells as primarily tissue macrophages (Fig. 1a), consistent with previous reports (Clabough-Sellon et al., 1992; McGuire et al., 1971). In addition, cells lining the lumen of blood vessels with the morphology of endothelial cells were also positive for viral RNA in these same horses (Figs 1a and 2). The presence of von Willebrand’s factor confirmed the identity of these cells as endothelium (Fig. 1b, c). Megakaryocytes, which also contain von Willebrand’s factor, were differentiated from endothelial cells by their highly characteristic morphology. The presence of infected endothelial cells was confirmed by co-labelling in liver, spleen and intestine, and by morphology alone in all other tissues examined. Although the ratio of infected macrophages to infected endothelial cells was not calculated, macrophages clearly were the predominant infected cell type. Hybridization of probe within the endothelial cell cytoplasm suggested true infection, in contrast to the labelling of the luminal surface that would be expected with adherent plasma virions. While vascular endothelial cell infection was widespread, and there was laboratory evidence of disseminated intravascular coagulation in the horses infected with EIAV<sub>Wyo</sub>, overt vascular damage or vasculitis were not histologically evident in any of the horses.

The data from this study identify the vascular endothelial cell as a new in vivo host cell for EIAV. Because lentiviral gag RNA is expressed only as part of the full-length transcript, or genomic RNA, its presence suggests productive virus replication (Pomerantz et al., 1990); thus EIAV-infected endothelial cells are most likely also sites of productive virus replication. In addition, Maury et al. (1998) have recently demonstrated the ability of some strains of EIAV to replicate in equine endothelial cells in vitro. However, the importance of infected endothelial cells to the pathogenesis or persistence of EIAV remains to be determined.

Although EIAV<sub>Wyo</sub> and EIAV<sub>WSU</sub> differ in their tropism for cell types in which they can replicate in vitro, this does not appear to be the case in vivo. Both viruses infected both macrophages and endothelial cells whereas infection of other types of cells such as fibroblasts or epithelium was not apparent. The primary difference between these virus strains was in the number of infected cells, with decreased total...
numbers of viral RNAexpressing cells for EIAV<sub>WSU3</sub> relative to EIAV<sub>WSU</sub>, as previously reported (Oaks et al., 1998). Thus, qualitative differences in tropism do not appear to account for the observed differences in virulence between these two viruses. However, as the assay used in this study detects only viral genomic RNA, the presence of cells with latent infections or restricted replication cannot be excluded for either strain. Disparity between <em>in vivo</em> and <em>in vitro</em> tropism has been noted for HIV-1 (Gartner & Popovic, 1990; Massari et al., 1990), and our finding for EIAV reinforces the caveat that conclusions about tropism based on <em>in vitro</em> data need to be interpreted with caution.

The basis for the attenuated virulence of tissue culture-adapted strains of EIAV remains to be determined, but may reside in the viral long terminal repeats which influence the amount of virus replication and thus the number of target cells that may be infected. Differences between the long terminal repeats in horses with subclinical infections (and decreased levels of virus replication) and clinical disease have been previously reported (Maury et al., 1997).

Endothelial cell infection could contribute to EIAV-associated thrombocytopenia. The pathogenesis of thrombocytopenia in EIAV infection is multifactorial, with both deficits in platelet production and increased consumption or sequestration of circulating platelets (Crawford et al., 1996). Immune-mediated enhancement of platelet consumption is consistent with the previously shown increased levels of platelet-bound immunoglobulins (Clabough et al., 1991). However, shortened platelet life-spans in severe combined immunodeficient horses indicates that non-immunological mechanism(s) enhancing consumption or sequestration are also present (Crawford et al., 1996). Although vascular lesions were not readily apparent in these horses histologically, subtle damage or activation of endothelial cells may promote platelet adherence and aggregation leading to thrombocytopenia, as has been proposed for HIV-1 (Cosgriff, 1989; del Arco et al., 1993).

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