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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 only can 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 dermal cells by Malmquist *et al.* (1973). Variants of the Malmquist strain (e.g. WSU5 strain; $EIAV_{WSU5}$), also replicate *in vitro* in other cell types of equine origin, including dermal fibroblasts (Klevjer-Anderson *et al.*, 1979; Malmquist *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 $EIAV_{Wyo}$ (1×10^6 and 1×10^1 horse infectious doses, respectively), and one with $EIAV_{WSU5}$ (1×10^6 $TCID_{50}$). Rectal temperatures and the number of erythrocytes, platelets and leukocytes were monitored daily.

All three horses developed fever ($> 38{\cdot}4$ °C) and thrombocytopenia [< 151000 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 $< 30000/\mu l$.

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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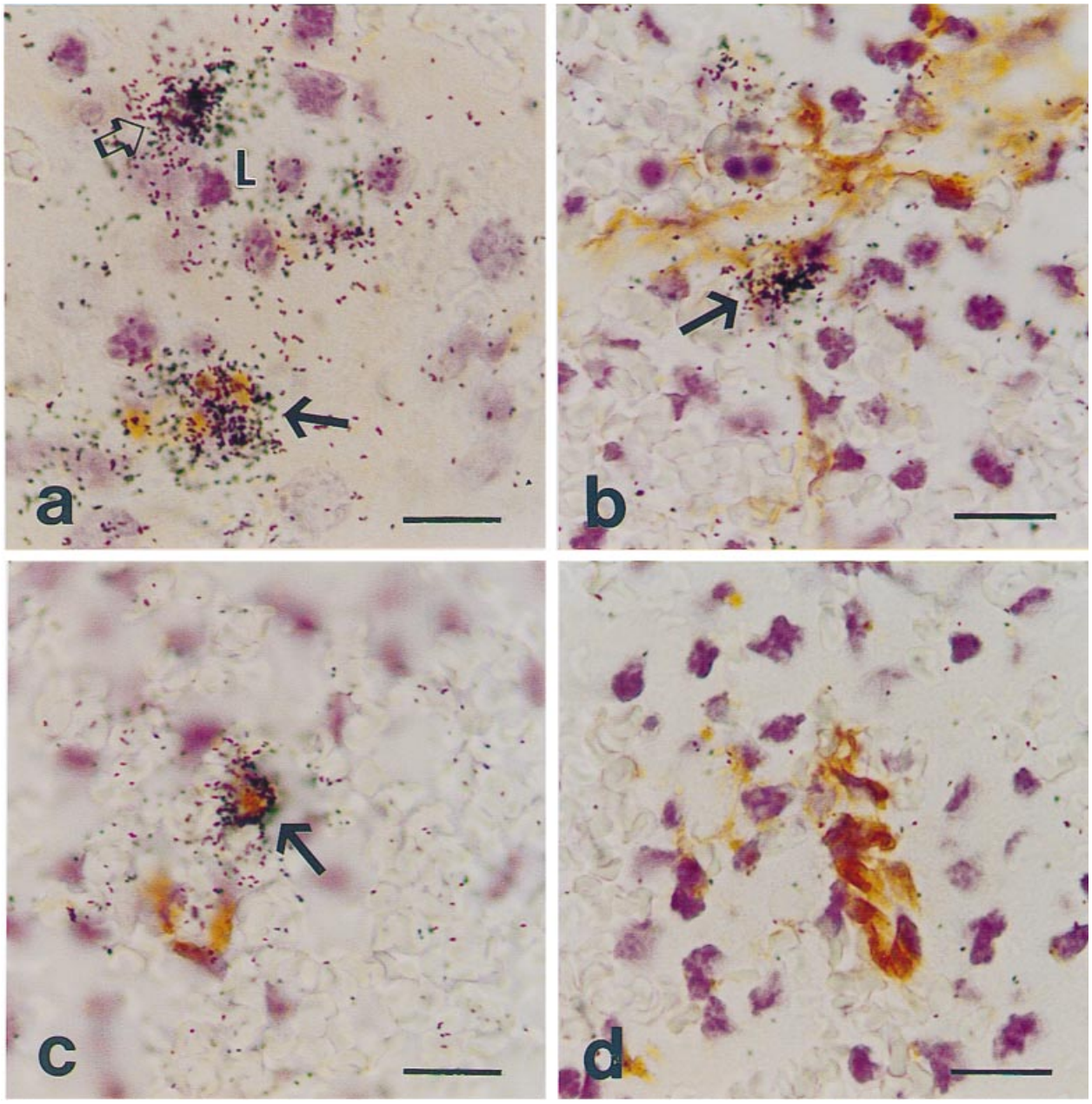

Fig. 1. (*a*) Photomicrograph of a macrophage (solid arrow) containing viral RNA in the spleen of a horse infected with $EIAV_{Wyo}$. The cell is double-labelled by immunohistochemistry for lysozyme (brown-staining cells) and *in situ* hybridization for EIAV *gag* with the ^{35}S-labelled antisense probe (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 $EIAV_{Wyo}$ (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 ^{35}S-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 ^{35}S-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.

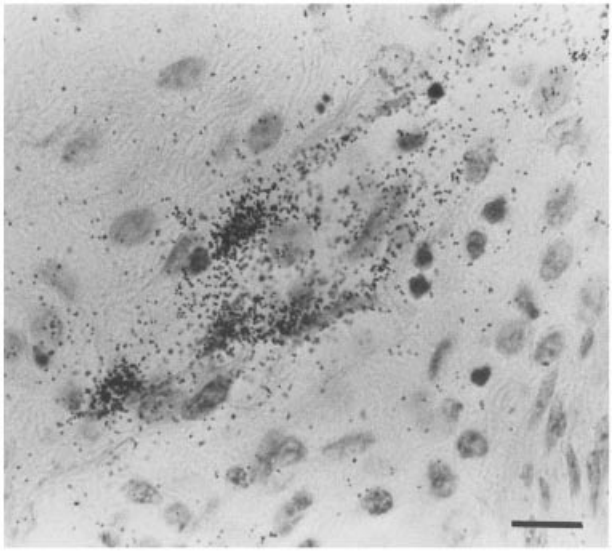

Fig. 2. Photomicrograph of viral RNA-containing endothelial cells from the colon of a horse infected with $EIAV_{Wyo}$. Cells are labelled by *in situ* hybridization for EIAV *gag* with the ^{35}S-labelled antisense probe (dark silver grains). Bar, 10 μm.

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 [^{35}S]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.

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 $EIAV_{Wyo}$ or $EIAV_{WSU5}$, 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. 1*a*), 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 1*a* and 2). The presence of von Willebrand's factor confirmed the identity of these cells as endothelium (Fig. 1*b*, *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 $EIAV_{Wyo}$, 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 $EIAV_{Wyo}$ and $EIAV_{WSU5}$ 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 RNA-expressing cells for $EIAV_{WSU5}$ relative to $EIAV_{Wyo}$ 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 *in vivo* and *in vitro* 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 *in vitro* 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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