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Endothelial Cell Infection and Thrombosis in Paralysis Caused by Equid Herpesvirus-1: Equine Stroke

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With 8 Figures

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Summary

Eight mares were infected with equid herpesvirus-1 subtype 1 isolated from a case of equine paresis. In two mares killed at 4 d.p.i. immunofluorescence showed endothelial cell infection together with thrombosis in the rete arteriosus of the nasal mucosa and also in the spinal cord of one of these mares. Circulating platelet counts in the other six mares fell as early as 2 d.p.i. and remained depressed for seven days. Circulating immune complexes started to appear at 2 d.p.i., reached maximum levels at 10 d.p.i., but were undetectable at 28 d.p.i. Three of the six remaining mares developed varying degrees of inco-ordination at 8 and 9 d.p.i. In the two inco-ordinate mares that were killed at 9 and 10 d.p.i. the haemorrhages in the spinal cord and brain were associated with extensive endothelial cell fluorescence and thrombus formation. Clinical paresis coincided with an increase in circulating complement fixing and neutralising antibodies which in all six mares were higher against the subtype 2 isolate than subtype 1.

In five yearlings infected with a subtype 2 isolate of EHV-1 platelet counts remained normal and neither immune complexes nor viraemia, nor inco-ordination were detected.

Introduction

Inco-ordination was first associated with abortion induced by equid herpesvirus 1 (EHV-1) by MANNINGER (1949) and the virus was first isolated

from the central nervous system (CNS) of ataxic animals by SAXEGAARD (1966). After recorded outbreaks in North America and Europe (9, 12, 21) an outbreak in the UK (4) led to the isolation of a subtype 1 virus (16). The syndrome was reproduced experimentally in North America and U.K. (9, 12, 16) and all authors were agreed that the lesions in experimental and field cases were characterised by a focal vasculitis with associated oedema, haemorrhage and thrombosis, with the axonal and myelin degeneration being focal and secondary to these vascular changes (10, 13, 23). However only LITTLE and THORSEN (1976) isolated virus from the CNS and the other workers postulated that the lesion might be induced by immune complexes. Recent work with the UK isolate indicated that a paresis isolate of EHV-1 subtype 1 had a tropism for endothelial cells (17). The present work describes the pathogenesis of paresis in a group of mares and discusses the relevance of immune complexes and antibody in mediating these events.

Materials and Methods

Animals

Eight Welsh mares (1–8) between 6 and 18 or more years old were inoculated intranasally with 1.0 ml of a $10^{5.5}$ TCID 50/ml suspension of EHV-1 subtype 1. 1.5 ml of the same aliquot was inoculated subcutaneously in the neck. Two mares were killed 4 days after inoculation to investigate the distribution of virus in early infection; a further co-ordinate mare was killed at 9 d.p.i. in order to compare co-ordinate and ataxic animals. Nasopharyngeal swabs and leucocytes (5×10^6) were taken prior to inoculation and daily thereafter for 14 days. Sera for immune complex and antibody assays were obtained twice weekly until 28 d.p.i. Samples for platelet counts were taken daily over the first four days and thereafter at 3, and latterly at 7 day intervals. Daily rectal temperatures and observation at exercise were made.

A group of five yearling welsh ponies (1–5) of which three were fillies and two were colts, were monitored in a similar manner after inoculation intranasally with 1.0 ml of a 5.5 TCID 50/ml of a subtype 2 isolate of EHV-1.

Virus Isolates

The subtype 1 isolate (Ab 4) was recovered from leucocytes of a paraplegic gelding and after primary isolation in RK 13 cells it was passaged in equine embryonic kidney cells (EEK). The inoculum was 10th passage virus.

The subtype 2 isolate (2252) was at 15th passage from a nasal swab taken from an outbreak of respiratory disease in thoroughbreds (J. A. MUMFORD – personal communication).

Virus Isolation

Nasopharyngeal swabs, leucocytes, and tissues taken at post mortem were treated as previously described using low passage EEKs for isolation (17). Tissues included nasal mucosa, trachea, lung, tonsil, thymus, spleen, liver, kidney, duodenum, jejunum, ileum, salivary gland, thyroid, adrenal, conjunctiva, cornea, lachrymal gland, and the submandibular, retropharyngeal, bronchial and mesenteric lymph nodes, as well as cervical, thoracic and lumbar spinal cord, and samples of cerebral cortex and the cerebellar folia.

Antibody Assay

Complement fixing antibodies were assayed as described previously (30).

Platelet Counts

1.0 ml of blood was collected in 100 i. u. of heparin. The sample was allowed to stand at room temperature for 20 minutes after which the platelets in the separated plasma were diluted 10^{-3} and counted in a Coulter Counter against a standard suspension of equivalent sized polystyrene particles.

Immune Complex Assay

The polyethylene glycol precipitation-complement consumption test (PEG-CC) devised by HARKISS and BROWN (1979) was adapted for measurement of circulating immune complexes (ICs). PEG precipitates were prepared from duplicate 250 µl serum samples by the sequential addition of 250 µl EDTA (40 mM) in PBS (pH 7.2) and 500 µl of 6 per cent w/v PEG in PBS. After incubation at 4° C for 16–18 hours the precipitates were pelleted by 1700 g centrifugation at 4° C for minutes, the supernatants carefully removed and the insoluble material washed a further three times in 3 per cent PEG-PBS. The final pellets were dissolved in 200 µl sucrose barbital buffer (SBB) (2) and an equal volume of fresh standard equine serum added as a source of complement. The resulting mixtures were incubated at 37° C/30 minutes, brought to 1.0 ml with SBB and 900 µl transferred to plastic cuvettes.

Residual complement was immediately assayed by the addition of 100 µl 0.5 per cent suspension of rabbit IgG (15) sensitised pig erythrocytes and lysis followed at one minute intervals in a Beckmann DU 8 spectrometer using a 600 nm wavelength and carrier temperature of 37° C. The measurement intervals were decreased to 21 seconds with maximum haemolytic rate (MHR) mixtures.

Controls consisted of a single batch of laboratory standard serum collected, stored and treated in the same way as test sera; mixtures containing normal serum and red cells only which were used to measure the MHR, and mixtures with red cells only to exclude spontaneous lysis. The inclusion of a laboratory standard serum acted as an internal standard and longitudinal control. Results were expressed as Percentage IC formation and calculated by the following formula:

IC formation $(\%) = [1-(\text{THR} \div \text{MHR})]-[1-(\text{SHR} \div \text{MHR})] \times 100$ where THR = haemolytic rate of test sera, SHR = haemolytic rate of the laboratory standard serum and MHR = maximum haemolytic rate.

Histology

Tissues as for virus isolation (17) were taken from the post mortem examination of five mares (2 ataxic, 3 normal locomotion). Having taken samples for virus isolation the spinal cord and brain were chilled at 4° C for an hour prior to serial blocking at 1 cm intervals to look for haemorrhages. At least 3 levels of lumbar, thoracic, and cervical spinal cord, and of cerebellum and cerebrum were taken for immunofluorescence and histological examination. Tissues were fixed in 10 per cent neutral buffered formalin. Selected sections were stained according to the Picro-Mallory technique for fibrin (9) in addition to routine haematoxylin and eosin.

Immunofluorescence

8 µ frozen sections of routine tissues, and multiple sections from CNS (see above) were fixed in acetone and stained with a hyperimmune anti-EHV-1 rabbit serum and an anti-rabbit IgG fluorescein conjugate as described previously (17).

Results

Clinical Observations

All of the eight mares infected with subtype 1 virus had temperatures $\geqq 39.0°$ C on at least one occasion while only two of the five yearlings infected with subtype 2 virus had similar increases. Nasal and/or conjunctival discharge was detected in four of the mares and in three of the yearlings between 1 and 5 d.p.i.

Eight days after inoculation mares 3 and 4 showed signs of hind leg incoordination when walking, particularly when turning. Mare 4 (M 4) and an asymptomatic mare (M 6) were killed for examination on day 9. Also on day 9 mare 2 became stiff in both hind legs and was unwilling to trot, by 10 d.p.i. she had lost the use of both hind legs and, although sensory reflexes were present, she could only sit in a dog position; she was killed at this stage. Mare 3 remained inco-ordinate when turning for six weeks after the development of ataxia and was kept in isolation. However eight weeks later her gait was normal again.

None of the yearlings infected with the subtype 2 virus showed any locomotor abnormality at any time.

Gross Pathology

There were petechiae and ecchymoses on the nasal mucosa of all five mares but apart from the central nervous system (CNS) no other macroscopic abnormalities were detected. The CNS of the two mares killed at 4 d.p.i. and the asymptomatic mare killed at 9 d.p.i. had no CNS lesions, but several dark haemorrhages 2–8 mm in diameter were present in blocks of thoracic and lumbar spinal cord from mare 4 which had shown lateral deviation of the hock. The ataxic mare 2 had multiple haemorrhages of a smimilar size throughout the cervical and thoracic spinal cord in both grey and white matter; there were relatively fewer lesions in lumbar cord and brain; a seven month old foetus was present (none of the other mares were pregnant).

Virus Isolation

The recovery of virus from nasal swabs and leucocytes for the two groups of animals is recorded in Fig. 1. Nasal swabs yielded subtype 1 virus from 1–10 d.p.i. and leucocytes from 3–10 d.p.i. whereas subtype 2 virus was recovered only from nasal swabs between 1–9 d.p.i. It was rare for more than $1/10^5$ mononuclear leucocytes to yield virus. At post mortem examination of 24 tissues from each of the mares virus was isolated from the CNS of the most severely ataxic mare (M 2), but no virus was isolated from any tissues of the other abnormal mare (M 4) and only one or two tissues from the other three mares (see Table 1).

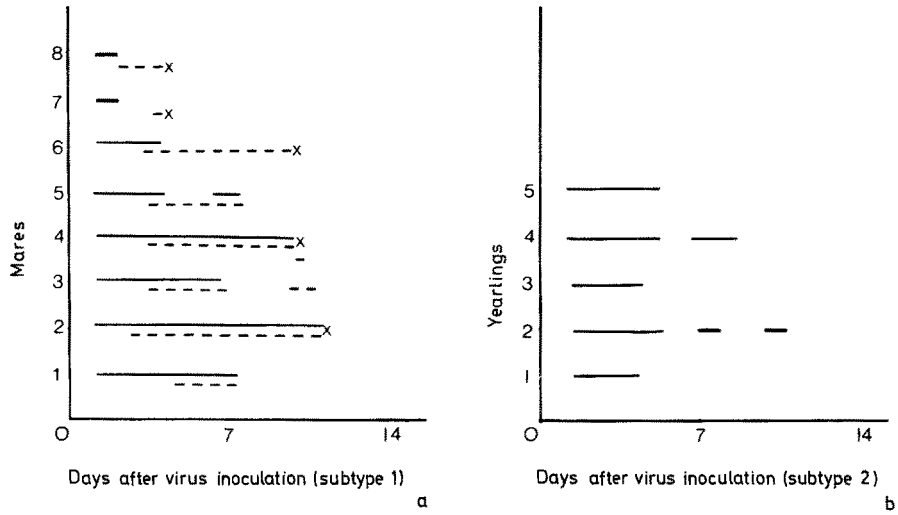

Fig. 1. Virus isolation from *(a)* eight mares inoculated with subtype 1-EHV 1 and *(b)* five yearlings inoculated with subtype 2-EHV 1. Viraemia (– – –) and nasal shedding (——) followed infection with subtype 1, but subtype 2 was restricted to the respiratory tract. *X* animal killed

Table 1. *Recovery of EHV1 from 24 tissues from each of 5 mares*

Mare	D.p.i.	Tissues yielding virus
2	10	Cerebrum, conjunctiva
4	9	None
6	9	Nasal mucosa, submandibular lymph node
7	4	Nasal mucosa
8	4	Nasal mucosa

Antibody

Changes in levels of CF antibody are recorded in Fig. 2. Neither group had existing CF antibody against the subtype 1 isolate but both had antibody against subtype 2. Although five of the six remaining mares had a 4-fold rise against the subtype 1 by 7 d.p.i., they also showed an equal or greater increase against subtype 2. In the yearlings the rise against the homologous subtype 2 had occurred in three out of five by 7 d.p.i. and in all five by day 14; but against the subtype 1 only 3/5 yearlings had shown fourfold rises by 21 d.p.i. The preinoculation virus neutralising (VN) antibody to subtype 2 virus was $\leqq 1:16$ in both groups of animals, and $\leqq 1:8$ against subtype 1. The increase in VN antibody in the mares was similar to that of CF antibody with levels being both higher and developing more rapidly against subtype 2 virus.

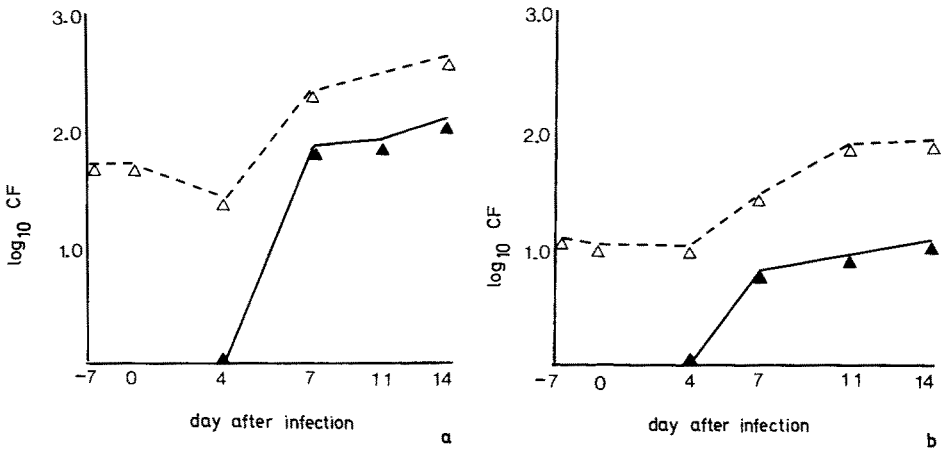

Fig. 2. Increase in CF titre ($\log_{10}$) in *(a)* eight mares infected with subtype 1 EHV 1, and *(b)* five yearlings infected with subtype 2 virus. CF antibody was measured against both subtype 1 virus (——) and subtype 2 virus (– – –) in each case. In the mares the subtype 1, given in the presence of subtype 2 antibody, evoked higher levels of antibody directed against the subtype 2 virus than against homologous virus

Platelets

Platelet counts are recorded in Fig. 3. In the mares the level of circulating platelets fell as early as 2 d.p.i. and remained depressed until 9 d.p.i.; thereafter they rapidly returned to normal levels. Numbers in the subtype 2 infected yearlings remained within the normal range throughout infection.

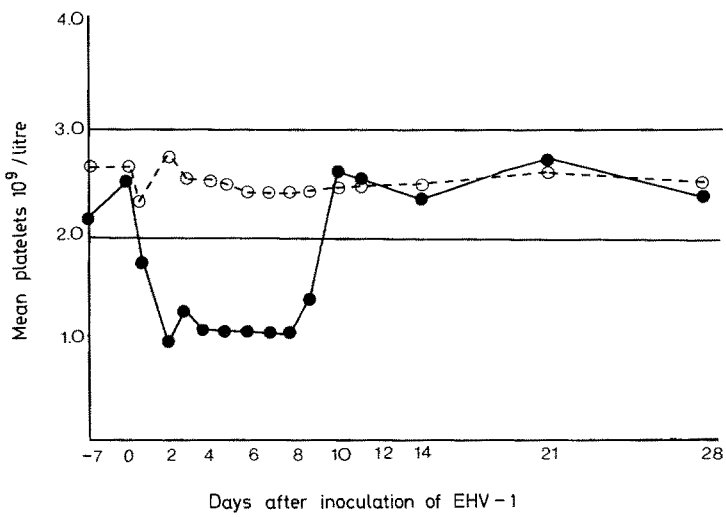

Fig. 3. Mean levels of circulating platelets in a group of six mares infected with EHV 1 subtype 1 (——) compared with those in five yearlings infected with subtype 2 virus (– – –). Platelet numbers were depressed between 1–9 d.p.i. with subtype 1 virus but remained within normal parameters after subtype 2 infection

Circulating Immune Complexes

Fig. 4 records immune complex formation after experimental infection with subtype 1 or subtype 2 virus. Circulating ICs were not detected during the course of experimental infection with subtype 2 virus. Conversely, subtype 1 infected animals began to show evidence of circulating ICs within 2 days of infection; the levels reached peaks at 4 and 10 days but gradually fell away between 10 and 28 d.p.i. Of interest are the final samples from mares 2, 4 and 6; mare 6 was killed as a control at 8 d.p.i. whereas mares 2 and 4 were killed after clinical indications of paresis.

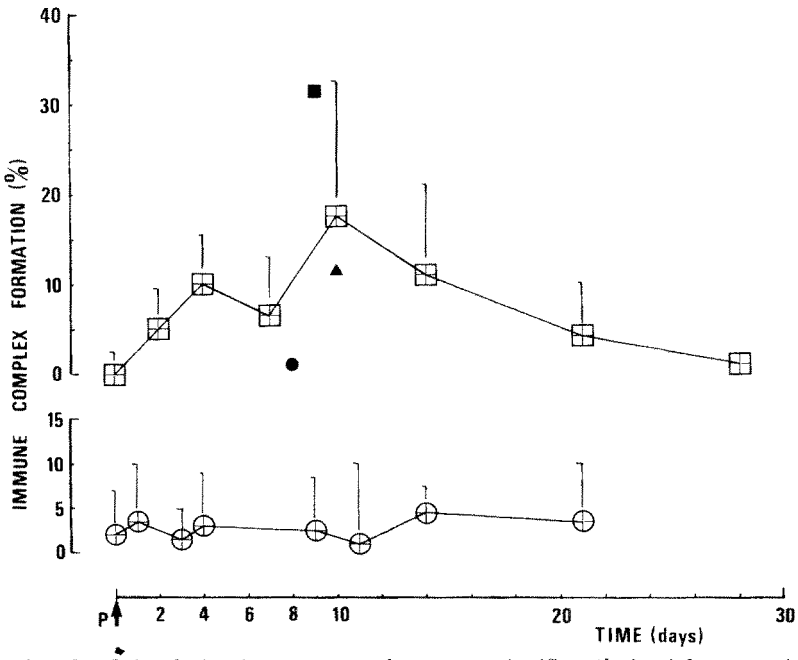

Fig. 4. Levels of circulating immune complexes rose significantly in eight mares infected with subtype 1 EHV 1 (□) but remained static in the 5 yearlings infected with subtype 2 EHV 1 (○). Mares 2 (▲) and 4 (■) were ataxic and had higher levels than the coordinate mare 6 (●)

Immunofluorescence

In the nasal mucosa of all of the five mares that were killed (i. e. two at 4 d.p.i. and three at 9–10 d.p.i.), virus was detected in the epithelium, in the endothelial cells of the rete ateriosus, and in migrating or intravascular mononuclear cells. Fluorescing endothelial cells occurred either as isolated cells or as plaque like formations and thrombi developed at the site of these plaques (Fig. 5). Not all infected endothelial cells were associated with thrombi, but thrombi were not seen without infected endothelial cells.

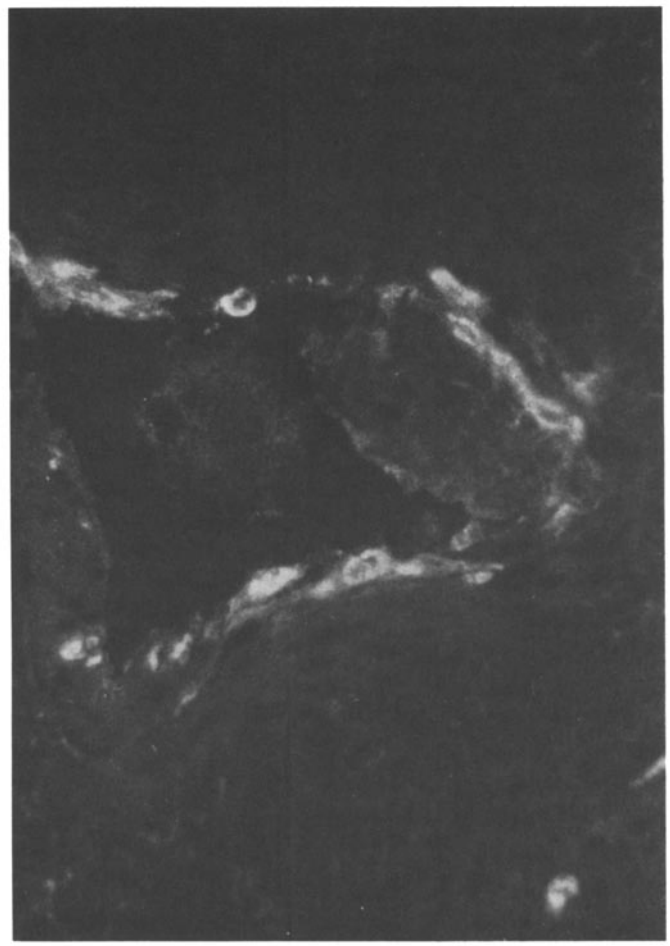

Fig. 5. Section of nasal mucosa from a mare killed 4 d.p.i. with subtype 1 EHV 1. Immunofluorescence shows that the virus is localised in plaques and isolated endothelial cells. A thrombus is already forming over the lateral plaque of infected cells (×1040)

Fluorescing mononuclear cells were lymphocytes or monocytes and were seen trapped in the fibrin network or in free circulation (Fig. 6).

Of the two mares killed at 4 d.p.i., fluorescing endothelial cells were also seen in the submandibular lymph node of mare 8 and isolated cells were also seen in the capillaries of the cervical spinal cord. In mare 2 and mare 4 with locomotor disorders, more extensive endothelial infection was present in the spinal cord either in meningeal arteriolar or capillary endothelium both as plaques or as isolated cells and with or without thrombi (Fig. 7). Arteriolar involvement also occurred in the lung and spleen of mare 4. In the asymptomatic mare killed at 9 d.p.i. (M 6) fluorescing cells were restricted to the nasal mucosa.

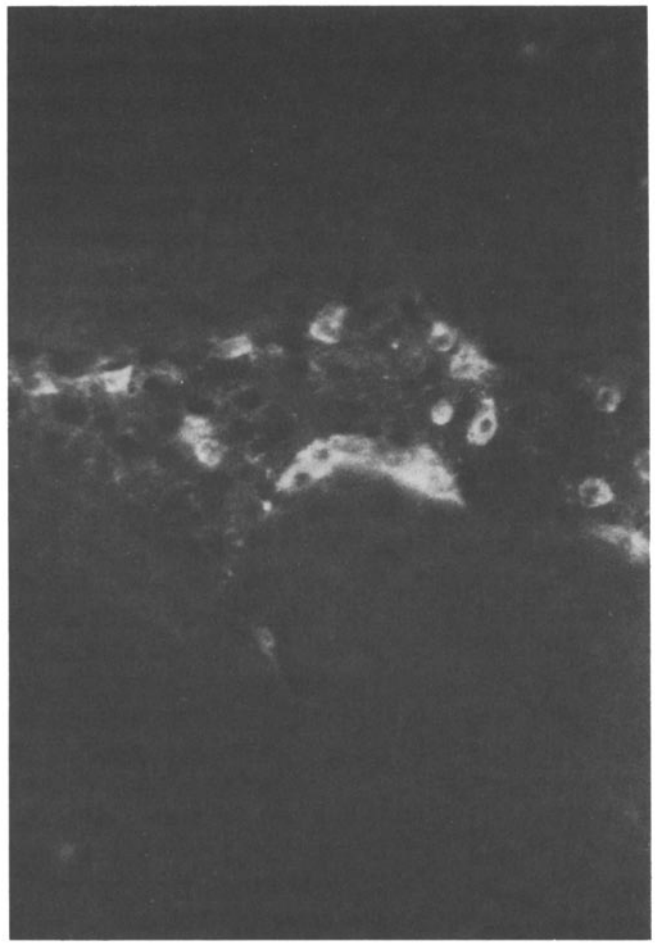

Fig. 6. In addition to infected endothelial cells both monocytes and lymphocytes show fluorescence with a rabbit α EHV-1 serum. As well as free circulating infected leucocytes there are two cells in the aggregation of a thrombus. Arteriole in the nasal mucosa of an ataxic mare 10 d.p.i. (× 1040)

Histopathology

While intranuclear inclusion bodies were unequivocally present in the epithelium of the nasal mucosa it was difficult to be sure by light microscopy that eosinophilic structures in endothelial cells were viral inclusions. The first indication of endothelial cell infection either in the nasal mucosa or CNS was the clustering of neutrophils seen in mare 8 at 4 d.p.i. This was rapidly followed by platelet aggregation and the deposition of fibrin which was most clearly demonstrated in Picro-Mallory stained sections (Fig. 7). Thrombi were clearly formed in the vessels of the nasal mucosa as early as 4 d.p.i. The

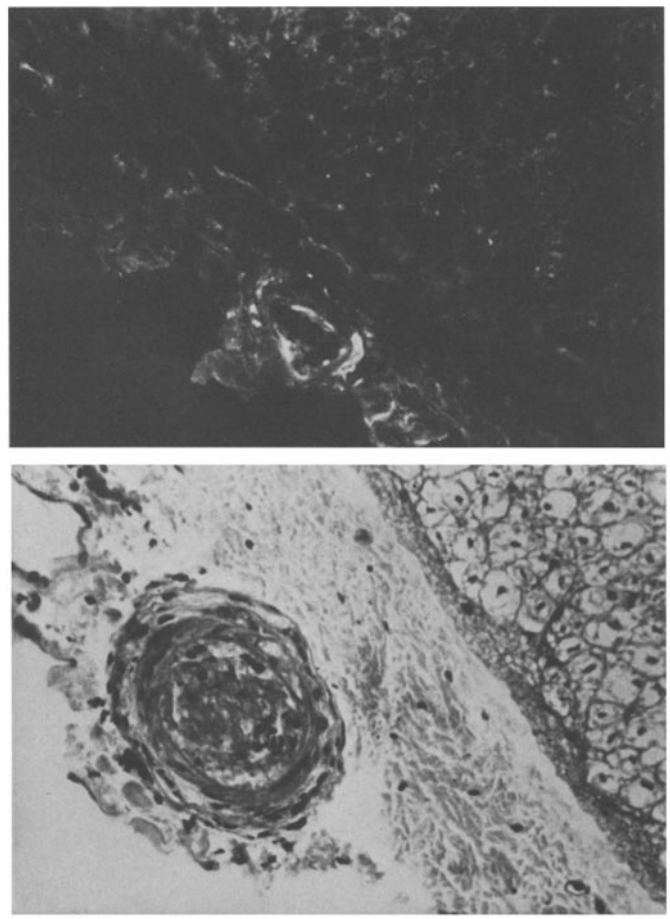

Fig. 7. Equivalent sections of a meningeal arteriole in the lumbar spinal cord showing both endothelial cell fluorescence using an EHV 1 antiserum and an occluding thrombus by conventional microscopy (picro-mallory stain). Sections from ataxic mare 2, 10 d.p.i. (× 640)

thrombi in the 2 mares with ataxia (M 2 and M 4) varied in size with some virtually occluding arterioles and including rows of mononuclear cells between layers of fibrin, while other thrombi were plaque like and scarcely projected into the lumen of the vessel. In the CNS arterioles were the most common site for thrombi but capillaries and venules were also affected. Vascular changes in the CNS involved both grey and white matter and the leptomeninges. Around the areas of haemorrhage, particularly in the ataxic mare number 2, there were sharply demarcated focal areas of axonal swelling and ballooning of the myelin sheath (Fig. 8) or chromatolysis of neurones. Petechiae were seen at all sites where endothelial infection was recorded while oedema was most noticeable in the CNS and lung.

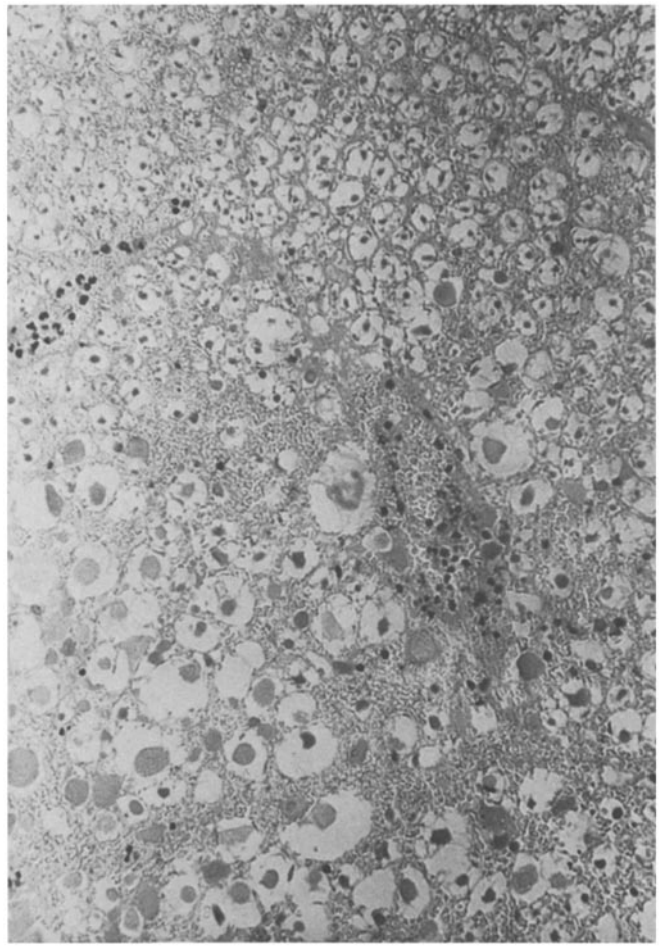

Fig. 8. Haemorrhage from a ruptured capillary in the ventral white matter of the lumbar spinal cord is associated with focal ischaemic swelling of axons and ballooning of myelin sheaths. Section from the ataxic mare 2, 10 d.p.i. (× 640)

Discussion

The subtype 2 isolate of EHV-1 behaved as described elsewhere (3) in being confined to the respiratory tract and not initiating a viraemia. The paresis induced by the subtype 1 isolate was a sequel to a vasculitis as described by other authors (10, 13, 23). However previous workers have frequently failed to recover EHV-1 from the CNS of experimental or field cases (10, 23) and have postulated that the lesion is mediated by immune complexes rather than a sequel to a lytic infection. In the present work recovery of virus from the CNS at post mortem examinations was low (1/3) but immunofluorescence indicated that endothelial tropism of the virus was the initiating

and persisting feature. Infected endothelial plaques and occasional thrombi were seen in the nasal mucosa of both mares killed at 4 d.p.i. The decrease in circulating platelets as early as 2 d.p.i. confirms that thrombosis was an early event in the pathogenesis of this syndrome. The immune complexes, detectable in serum as early as 2 d.p.i., may have contributed to coagulation and platelet aggregation via an interaction with phagocytes at endothelial sites of virus release (25, 31). In this there may be similarities to Dengue Haemorrhagic Fever (DHF) (26) where ICs have been considered responsible for complement activation, thrombocytopaenia and intravascular coagulation (1). However, in EHV-1 the action of ICs would seem to be secondary and localised since lesions did not occur in vessels where endothelial cells did not support viral replication, nor in organs such as the kidney.

Inco-ordination correlated with the peak of ICs, an increase in CF antibody, and a return towards normal platelet levels; indicating that the pathological changes had already occurred. The increased circulating antibody may have accentuated cell lysis, but all mares had preinfection VN antibody and this existing subtype 2 antibody may have been cardinal to early IC formation, cell lysis and thrombosis. The significance of a subtype 1 "paresis" isolate establishing infection in the presence of and evoking the memory of a recent subtype 2 response is interesting to evaluate. The nature of the ICs particularly needs further investigation. Again there are analogies with DHF of humans where more recent work indicates that the vascular injury develops if subtype 2 infection follows a recent subtype 1, 3 or 4 but not in homologous infections (5, 27). Both lymphocytes and monocytes are infected by EHV-1 subtype 1 (29) and this was confirmed by virus isolation and by immunofluorescence in the current experiment. There can be little doubt that their role in disseminating infection is critical; whether or not a subtype 2 antibody "enhances" infection of leucocytes and endothelial cells as in the haemorrhagic form of Dengue Virus infections (6, 7, 20) has yet to be determined. What is likely is that the high levels of circulating antibody in EHV-1 paresis and the endothelial distribution of virus may account for the difficulty in isolating virus from tissues at necropsy.

The endothelial distribution of virus in the CNS and the subsequent thrombosis, oedema, haemorrhage and ischaemic degeneration of neurones and nerve fibres is in contrast to the more common neurotropism of herpesvirus infections in other species (24). However a comparable vasculitis associated with HSV infection of endothelial cells has been described in an infant (22) with the vessels of most tissues being infected and the outcome fatal. No reason for the predilection of EHV-1 for the endothelium of nasal mucosa and CNS has been established, but a generalised case in a foal with this subtype 1 EHV-1 virus has also been observed (17). Further analogous isolated examples of vasculitis and thrombosis have also been associated with Human cytomegalovirus (11, 18). The equine work therefore serves to con-

firm that a vasculitis resulting from an endotheliotropism is another manifestation of herpesvirus infection and elaborates some of the events which are part of the pathogenesis of the syndrome.

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