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Lesions of Experimental Equine Morbillivirus Pneumonia in Horses

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Abstract. Laboratory examinations of equine morbillivirus included experimental reproductions of the disease caused by the virus by transmission of mixed lung and spleen taken from two field equine cases into two horses and by inoculating tissue culture virus into a further two horses. The most distinctive gross lesions of the diseases that developed in three of the horses was that of pulmonary edema characterized by gelatinous distension of subpleural lymphatics. Histologically, the lesions in the lungs were those of serofibrinous alveolar edema, alveolar macrophages, hemorrhage, thrombosis of capillaries, and syncytial cells. Clearly defined vascular lesions in three horses that became clinically affected within 8 days of inoculation of virus included intramural hemorrhage, edema, and necrosis and syncytial cells in the endothelium of pulmonary vessels (~40–70 μm in diameter). Vascular lesions accompanied by parenchymal degeneration were also seen in the heart, kidney, brain, spleen, lymph node, and stomach. A fourth horse, which survived for 12 days, had detectable lesions only in the lungs, which were more chronic than those in the other three horses, a greater degree of cellular infiltration, and fewer well-defined vascular lesions. Sections stained by an indirect immunocytochemical method showed equine morbillivirus antigen was present in the vascular lesions and along alveolar walls. When endothelial cells were examined by electron microscope, cytoplasmic virus inclusion bodies containing filamentous structures were seen that reacted to an immunogold test to equine morbillivirus antigen. The presence of the syncytia in the small blood vessels in the lungs and other organs was interpreted as an important characteristic of the disease and consistent with a reaction to a morbillivirus.

Key words: Alveolar edema; equine morbillivirus: horses; human; syncytia; vasculitis.

In recent years, there has been a considerable increase in the number of animal species known to be infected by morbilliviruses. There has been an extension of the range of animal species affected by canine distemper[11] and newly recognized viruses such as phocine and porpoise distemper.[18,20] In late September 1994, an outbreak of severe respiratory disease occurred in horses, mainly on one property, in Brisbane, Australia. Over a short period, 14 horses died or were euthanatized, and one man died. Subsequent investigations showed that the cause of the disease was a previously unreported virus[19] now known as equine morbillivirus (EMV). A second outbreak has also been identified in which two horses and one human were affected.[13,23] In this report, we describe the light-microscopic, immunocytochemical, and some ultrastructural features of lesions observed in four horses experimentally infected with the virus during the investigations.

Materials and Methods

Homogenates of blood, spleen, and lung taken from two of the affected horses in the field outbreak were inoculated into two recipient horses (case Nos. 1 and 2), ~10 ml intravenously and 1 ml intranasally. Supernatant from virus-infected Vero cells containing 2×10^7 median tissue culture infective dose ($TCID_{50}$) EMV was administered to a further two horses, 5 ml intravenously and ~10 ml by intranasal aerosol (case Nos. 3 and 4). Necropsies were conducted from which a wide range of tissues were collected and fixed in 10% neutral-buffered formalin. Samples were also collected from a similar range of tissues for virus isolation and polymerase chain reaction from all four horses, which confirmed that EMV was present in each of the horses.[19] Formalin-fixed tissues were embedded in paraffin, cut at 5 μm, and stained routinely with hematoxylin and eosin (HE). In addition, sections were stained by an indirect immunofluorescent and immunoperoxidase stain for EMV by a method using antibody in the serum of a human convalescing from the disease.[19]

Homogenates (10% w/v) of lung from each of the experimental horses were prepared for negative-contrast immunoelectron microscopy (NCIEM). Homogenates were adsorbed onto carbon-coated 400-mesh filmed gold grids. The samples were then incubated with antibodies from convalescent human and horse sera,[19] labeled with 10 nm protein A-gold,[15] and stained with 2% phosphotungstic acid (pH 6.8). Necropsied lung tissue from all experimental horses was also processed for conventional electron microscopy and for postembedding immunoelectron microscopy (PEIEM).

Table 1. Summary of the clinical signs, gross pathology, and histopathology of the lung observed in four horses inoculated with extracts containing equine morbillivirus (EMV) from either field cases (F) or tissue culture (C). Clinical signs recorded were: incubation period (IP) in days postinoculation; maximum rectal temperature in C (Temp.), which was usually recorded on the day of or the day before death; maximum, near terminal, heart rate (HR); and whether there was death (D) or euthanasia (E). Gross lesions were dilated subpleural lymphatics (DL) and congestion (Con) while histological lesions were syncytial cells (SC), alveolar edema (AE), cellular infiltration into alveoli (CI), and the presence (Yes) or absence (No) of lesions in organs other than the pulmonary system (OO) (usually vasculitis and accompanying ischemia). Assessments were made on a comparative − to +++ basis of severity.

Case No./ Inoculum	D/E	Clinical Signs			Gross Pathology		Histopathology			
		IP	Temp.	HR	DL	Con	SC	AE	CI	OO
1, F	D	7	40.0	72	+++	+++	+	+++	++	Yes
2, F	E	11	41.2	60	−	−	+	−	+++	No
3, C	D	3	39.2	68	+++	+++	++	+++	+	Yes
4, C	E	4	39.0	74	+++	−	+++	+	+	Yes

For conventional electron microscopy, the tissues were fixed immediately upon removal from the horses in cacodylate- (0.1 M, pH 7.2, 320 mosmol/kg) buffered 2.5% (v/v) glutaraldyde (4 C, 1 hour) and cacodylate- (as above) buffered osmium tetroxide (1% v/v), dehydrated through graded alcohol (70–100%), and embedded in Spurr's resin. For PEIEM, samples were fixed in 0.1% (v/v) glutaraldehyde in cacodylate- (refer above) buffer (20 minutes, 4 C), processed for embedding into L. R. White resin, and incubated[15] using the above sera and protein A-gold as the electron-dense marker. All samples were examined in a Hitachi H600 scanning transmission electron microscope (Hitachi, Nakka Works, Mito, South Tokyo, Japan) at 75 kV.

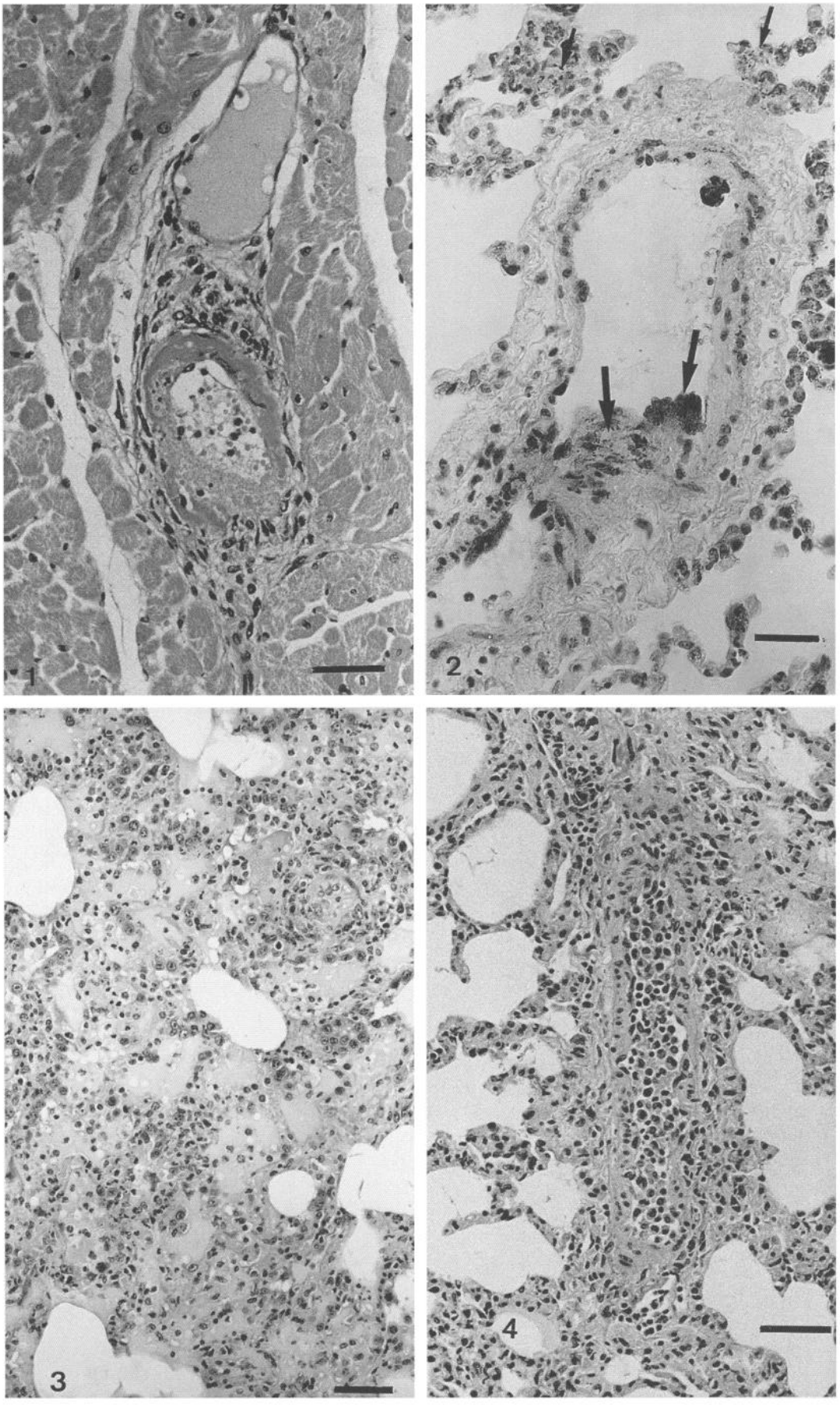
Results

Table 1 summarizes the clinical findings and observations of gross and histopathologic lesions in the four horses.

Case No. 1

Case No. 1 was a mare that died 8 days after being inoculated with homogenates of lung and spleen. On days 7 and 8 postinoculation, it had temperatures of 40.0 C and 39.0 C and heart rates of 44 and 72, respectively. For the last 2 days, its respiratory rate varied, and its breathing was occasionally labored. Its demeanor varied from somnolence to mild agitation, and it frequently looked behind at its flanks. Apart from occasional hemorrhages visible in other tissues and a very enlarged dark spleen, the only significant lesion at necropsy was bilateral dilation of pulmonary lymphatics visible superficially under the pleura as ribbons of gelatinous fluid up to ~2 cm in diameter. It was more marked and spread out in a deltalike manner, ventrally. The remaining lung was moist, heavy, and dark blue in color. There was no visible fluid in the bronchi or trachea.

Histologically, lesions were visible in the lung, lymph node, stomach, spleen, brain, and kidney. In the lungs, there was severe serofibrinous edema, especially in ventral portions. There were occasional fibrin thrombi in capillaries, which in some cases had neutrophils within the fibrin deposits. There were alveolar macrophages, some of which were pyknotic, and there were many hemorrhages, especially in ventral subpleural areas. The interlobular lymphatics were dilated with very pale-staining fluid and were sparsely populated with macrophages. Occasional syncytial cells were visible in the endothelium of small pulmonary vessels and along alveolar walls. Some pulmonary vessels that were probably small arteries and arterioles, ~40–70 μm in total diameter and with muscular walls, had tunica media with pyknotic nuclei and were raggedly distended with intramural hemorrhage or edema, and in some, there was fibrinoid necrosis. In the heart, there was degeneration of some vessels similar to those in the lungs accompanied by infiltration in and around by mononuclear leukocytes (Fig. 1). There was extensive single-cell necrosis, edema, syncytial cells, and some plasma cells in the lymph nodes. The spleen was congested and hemorrhagic, and there was extensive necrosis of red pulp characterized by loss of cells, pyknosis, and karorrhexis. In the kidney, some glomeruli were atrophied and had neutrophils and/or microthrombi and other protein depositions in the tufts and within the urinary spaces of Bowman's capsules. Renal tubules were dilated, and there was some edema, hemorrhage, and necrosis in arcuate arteries. There was ulceration and hemorrhage in the mucosa of the glandular stomach. Arterioles in the underlying lamina propria had swelling of the tunica media with pyknosis and some syncytial cells in the endothelium. Subarachnoid vessels were degenerated, and there were some foci of vacuolation in cerebral gray and white matter. Syncytial cells were visible in the endothelium of some blood vessels in the mediastinal and mesenteric lymph nodes, spleen, glomeruli, brain, and stomach. Occasional syncytia were also seen in the tunica media of some degenerating blood vessels.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In the lungs, there was extensive strong immunofluorescent and immunoperoxidase staining along alveolar walls and some endothelial linings of pulmonary arterioles and venules (Fig. 2), including some syncytial cells, but there was none in bronchi and within dilated lymphatics. Other tissues reacting included the endothelium of some subarachnoid and small cerebral vessels, some cells in glomerular and renal pelvis capillary endothelium, occasional endothelial cells in the lymph node but not the spleen, and in the lamina propria of the stomach, including a syncytial cell, and, to a much lesser extent, the gastric glandular epithelium.

Case No. 2

Case No. 2, a gelding inoculated with lung and spleen homogenate, became ill after 11 days and was euthanatized with a captive-bolt pistol on the 12th day postinoculation. Its temperature rose from day 8 postinoculation, to a peak of 41.2 C on day 11, then eased to 39.8 C on day 12. Its respiratory rate varied markedly, rising as high as 60 per hour over the last 4 days. It had shown some agitation over the last 3 days. The only visible gross lesions were in the lungs, which were a mottled yellowish light brown in color and were firmer than normal.

Histologically, the only visible lesions were in the lungs. There were numerous masses of mononuclear cells in the alveoli. These were quite variable in size and morphology (Fig. 3). Some groups of pneumocytes had basophilic cytoplasm, had become aligned, and were probably early manifestations of adenomatoid epithelialization. There were also some syncytial cells prominent along the alveoli and in a shrunken form in the endothelium of some medium-sized vessels. Some blood vessels had intraluminal masses of round cells with prominent nuclei (Fig. 4), apparently lying free and detached from the vessel walls. Most of these cells were mononuclear, but some were apparently binuclear, and, rarely, some resembled neutrophils. There was serofibrinous edema in ventral parts, and thrombi were seen in some vessels. Lymphatics were dilated, but to a lesser extent than the other three horses. They stained less for protein and contained slightly more macrophages. There were occasional foci of lymphocytes and of neutrophils.

Immunocytochemical staining revealed numerous discrete foci of strongly stained cells in the alveolar walls. These were in areas away from those with strong cellular reaction. Bronchioles were unaffected. There was no staining in other organs.

Case No. 3

Case No. 3 was a gray mare that died 4 days after inoculation with EMV in tissue culture. It had been depressed for the last 24 hours prior to its death. Its body temperature rose only as high as 39.2 C at 3 days postinoculation, while its heart rate was 46 at day 2 and 68 at day 3 postinoculation. Its respirations were sometimes labored, but its respiratory rate only rose to about 30. At necropsy, there was congestion and pulmonary edema with dilated ventral lymphatics similar to case No. 1.

Histologically, lesions were particularly marked in ventral portions in the lungs. These lesions consisted of congestion and proteinaceous edema, dilated lymphatics in ventral areas, and vessels with intramural edema and pyknosis and syncytial cells in blood vessel walls, especially the endothelium. In the spleen, there was congestion and hemorrhage, pyknosis, and atrophy, and some syncytial cells were visible within both vessel walls and macrophages in the splenic sinuses. There was some fibrinoid degeneration or intramural edema and hemorrhage in some arcuate arteries in the kidney and small (~15–30 μm) arteries in the muscle wall of the heart. In the brain, there were foci of vacuolation associated with some shrunken neurons, some syncytial cells in small blood vessels, and occasional extravascular neutrophils and lymphocytes. In the ce-

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Fig. 1. Heart from case No. 1, which developed severe pulmonary edema 7 days after inoculation with homogenates of blood, lung, and spleen, taken from two affected horses in the field outbreak of equine morbillivirus pneumonia. There is a small artery with tunica media that is raggedly distended with intramural edema and fibrinoid necrosis, and there is perivascular infiltration by mononuclear cells. HE. Bar = 38 μm.

Fig. 2. Lung from case No. 1 (see Fig. 1). Using an indirect immunoperoxidase test,[19] there are reactions to antibody in the serum taken from a human convalescing from equine morbillivirus pneumonia. The reactions (arrows) are in vascular endothelium of small blood vessels (including the syncytial cell) and alveolar walls. Hematoxylin counterstain. Bar = 43 μm.

Fig. 3. Lung from case No. 2. This horse had been challenged in a similar manner to case No. 1 but survived for 12 days. There was diffuse interstitial pneumonia characterized by pulmonary edema and alveolar macrophages. The lesions in case No. 2 were more chronic compared with those in other cases in that there were more alveolar macrophages and fewer distinct vascular lesions. HE. Bar = 60 μm.

Fig. 4. Lung from case No. 2 (see Fig. 3). Some blood vessels have intraluminal masses of round cells with prominent nuclei in their lumens, interpreted as intravascular macrophages. HE. Bar = 54 μm.

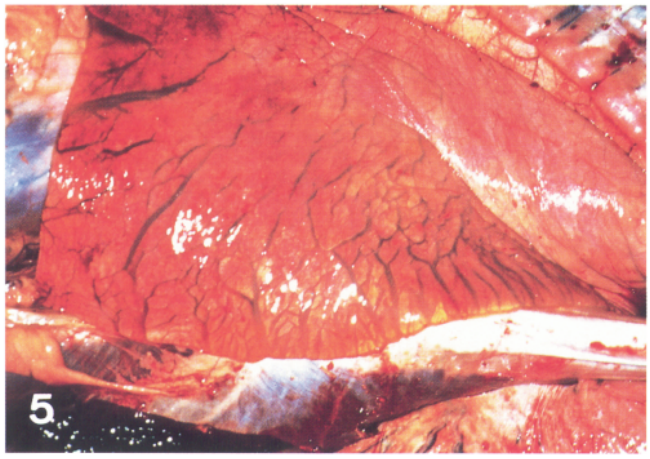

Fig. 5. Lung from case No. 4. This horse had been inoculated intravenously and intranasally with tissue-culture equine morbillivirus and became severely ill 4 days later. At necropsy, there was severe pulmonary edema characterized by gelatinous distension of lymphatics.

rebral sulci, meningeal vessels contained many mononuclear cells within their lumens.

Lung tissue stained by the immunoperoxidase method showed that cells reacted in more than half the alveolar walls as well as the walls of pulmonary arterioles and some venules, particularly along the endothelium. Bronchioles did not stain.

Case No. 4

Case No. 4 was a gray gelding euthanatized by head shot with a captive-bolt pistol at 5 days postinoculation. Its temperature only rose as high as 39 C at 4 days postinoculation when its heart rate was 55, which subsequently rose to 74 at 5 days. Its respiratory rate varied but rose at times over the last 2 days to >40 per minute. Prior to euthanasia, it had become very weak. Its gross postmortem appearance was similar to that of case Nos. 1 and 3 except that the color of the lungs was yellowish brown, as there was less congestion and cyanosis (Fig. 5).

Histologically, this horse was remarkable for the large number of syncytial cells in the endothelium of pulmonary vessels throughout the lungs (Fig. 6). Alveolar walls were thickened and there was some congestion and hemorrhage. Ventrally, lymphatics were dilated, and there was proteinaceous edema in the alveoli. Thromboses in capillaries and venules were also frequent ventrally. The lymph nodes were congested and hemorrhagic. There was some fibrinoid degeneration in the heart, and other vessels had hypertrophied endothelium. There was some renal tubular dilation, some glomerular pyknosis with thromboses, and some syncytia in the endothelium of arcuate and interlobular arteries. Syncytial cells, probably macrophages, were

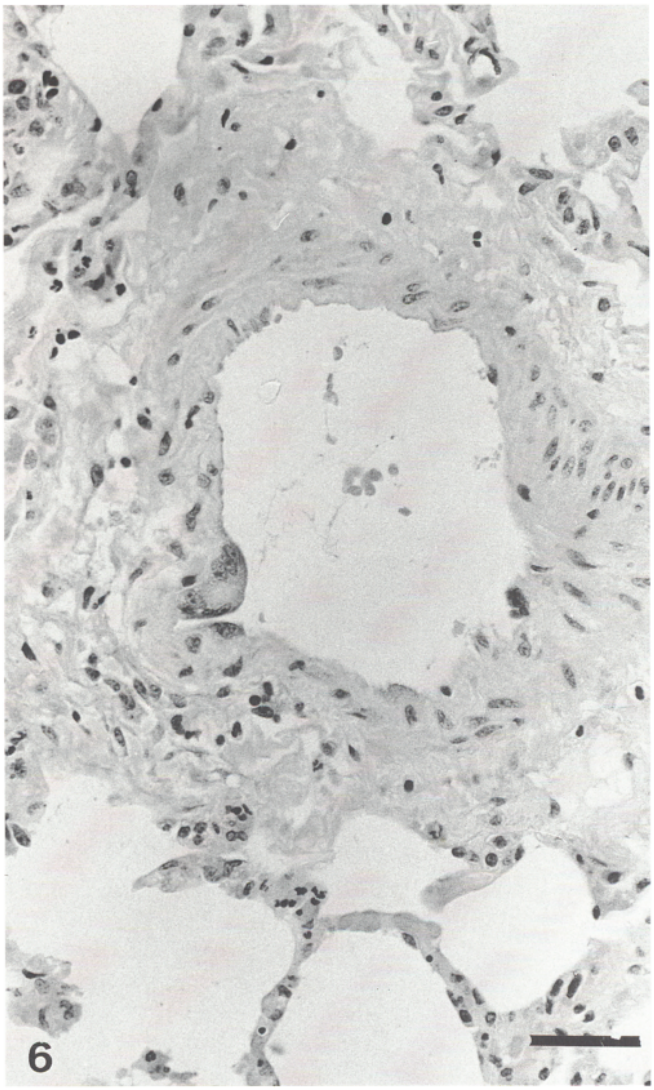

Fig. 6. Lung from case No. 4 (see Fig. 5). This was remarkable for the large number of bizarre syncytial cells in the endothelium of medium-sized vessels throughout the lungs. HE. Bar = 107 μm.

evident in the sinusoids of the spleen, which was very congested and/or hemorrhagic. There were foci of vacuolation in the neuropil of the cerebrum, loss of Purkinje cells, some extravascular lymphocytes, and fibrinoid degeneration or intramural edema and hemorrhage in subarachnoid arterioles.

In the lung, immunocytochemical staining of more than half of the alveolar walls was seen, as well as vascular endothelium of arterioles and some venules, including syncytial cells. Bronchi and bronchioles were occasionally stained, as were occasional subarachnoid and other small vessels in the brain.

Electron microscopy

Lung homogenates from the experimental horses were examined by NCIEM. Free-lying viral nucleo-

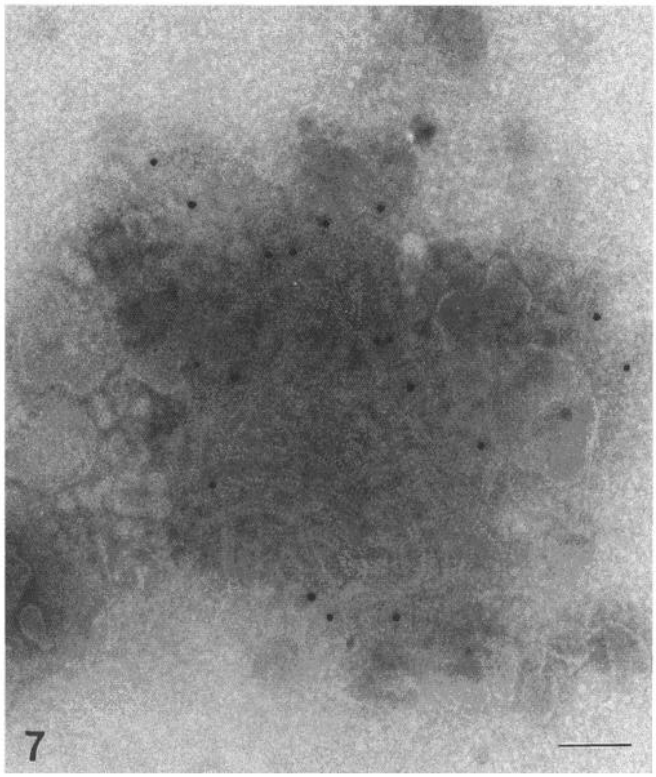

Fig. 7. Transmission electron micrograph of a lung homogenate from case No. 1. The herringbone appearance of the nucleocapsids are apparent. The nucleocapsids were incubated with convalescent human serum and 10 nm of protein A-gold. The sample was stained with phosphotungstic acid (pH 6.8). Bar = 100 nm.

capsids, but no whole virus particles, were observed. The herringbone appearance and 18-nm diameter of these structures were consistent with those described from viruses belonging to family Paramyxoviridae. Upon incubation with either convalescent human or horse serum, the nucleocapsids were specifically gold-labeled, indicating that the serums collected from convalescent humans and horses contained antibodies to viral nucleocapsids identified with the diseased lungs of the experimental clinical horses (Fig. 7).

The ultrastructural examination of lungs from experimental horses showed the presence of a diffuse proteinaceous material within most of the alveolar sacs or cavities. Within the alveolar walls, multinucleate cells were also observed. These cells were not frequent, but they were a characteristic feature of all lung tissue examined. The cells were juxtaposed to the circulating blood cells and were therefore designated endothelial cells (Fig. 8). The nuclei of these cells were either marginated (Fig. 9) or contained numerous areas of electron-dense material consistent with clumping of nuclear chromatin (Fig. 8). In the syncytial cells, there was disorganization of the cytoplasm, which varied from slight to severe swelling of mitochondria and dilution of the cytosol. Endothelial cells examined from the lung tissue of case No. 2 possessed swollen mitochondria with degenerating cristae, vacuolation, advanced dilution of the cytosol, and, as was characteristic of all cases, cytoplasmic viral inclusion bodies (Fig. 8). Within these viral inclusion bodies, filamentous structures were observed (Fig. 9), the diameter of which approximated 18 nm. When analogous sections embedded in L. R. White resin were incubated with either convalescent human or horse sera and protein A-gold, the viral inclusion bodies were specifically gold labeled (Fig. 10). While viral inclusion bodies were characteristic of all multinucleate endothelial cells, viruses were observed infrequently. The viruses were enveloped and located between the basement membrane of an endothelial cell and the adjacent basal lamina.

Discussion

The four horses described in this paper were shown to be affected by an interstitial pneumonia. The pneumonia and lesions in other organs were confirmed by immunocytochemistry, electron microscopy, and immune electron microscopy to be associated in situ with the virus isolated in the field outbreak, the newly named equine morbillivirus. In case Nos. 3 and 4, which had been inoculated with virus grown in tissue culture, the appearance of severe interstitial pneumonia confirmed that the virus itself could produce the same basic changes seen in horses inoculated with specimens derived from field cases (case Nos. 1 and 2) and also the changes seen in the field cases themselves (P. J. Ketterer, P. T. Hooper, and W. R. Kelly, unpublished). The most distinct features were the presence of alveolar exudates (serofibrinous and hemorrhagic) and the virtual absence of lesions in the large and small airways. These features are indicative of an interstitial pneumonia as defined by Dungworth.[9] Interstitial pneumonia could be associated with a variety of infectious, toxic, and allergic agents, some of which are other morbilliviruses, canine distemper in a variety of terrestial animals,[1] phocine, and dolphin distemper,[18,20] and peste des petits ruminants.[3] There is a variation with equine morbillivirus in that the virus seems to have a greater tropism for vascular tissues.

In case Nos. 1, 3, and 4, the vascular changes clearly identified with virus by immunocytochemistry, electron microscopy, and immune electron microscopy may have been because they were at an earlier stage of the disease. The intravenous route of challenge may have been a factor, but subsequent experiments with cats demonstrated the same vascular lesions regardless of the route of challenge.[14] The virus therefore has a distinct preference for vascular tissues and so differs

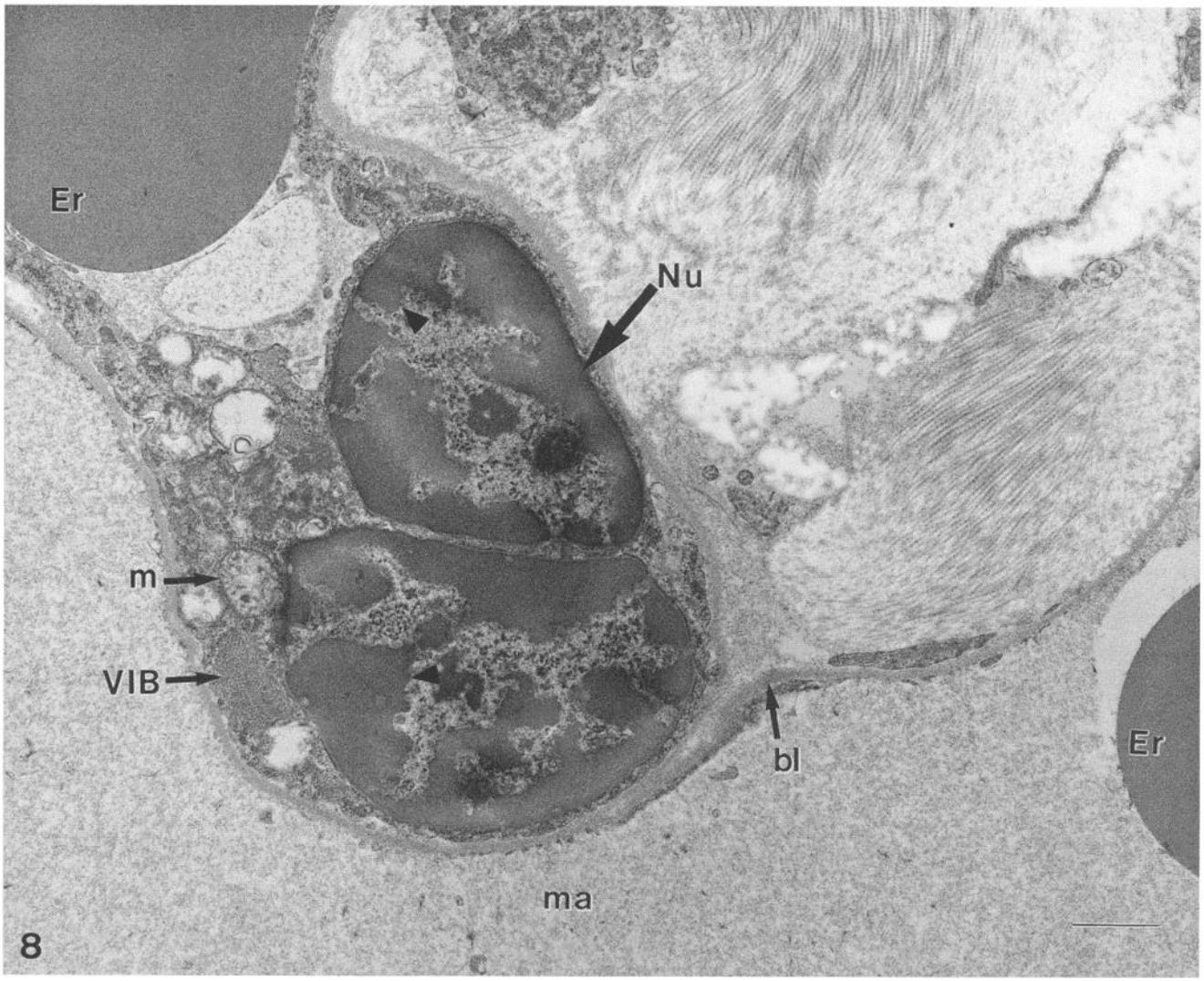

Fig. 8. Transmission electron micrograph of an ultrathin section of lung from case No. 2. A multinucleate cell (Nu) can be seen juxtapositioned to a capillary. The cell contains a virus inclusion body (VIB), nuclei with clumped chromatin (arrowhead), and degenerating mitochondria (m). Other abbreviations are for erythrocyte (Er), proteinaceous material within alveolar sac (ma), and basal lamina (bl). Bar = 1 μm.

from other morbilliviruses such as measles[7] and distemper[6] viruses that locate in vascular endothelium during their tissue localization but do not appear to cause specific vascular disease. The tropism of the virus more closely resembles that of hantavirus,[28] and the pulmonary edema resembles that of African horse sickness.[21] In early cases, the vascular lesions are very distinct and vary from edema and hemorrhage of vessel walls or fibrinoid necrosis with pyknosis to numerous giant cells. The vascular degeneration resembles that of equine viral arteritis, but the latter disease has a greater predilection for organs in the abdominal cavity.[22] A particular lesion of interest in the experimental horses was the syncytial cell. Syncytial cells have been described as characteristic of morbillivirus infections.[16] Examples have been seen in distemper,[25] rinderpest,[2] and measles,[10] peste des petits ruminants,[5,24] phocine distemper,[12,17] and dolphin distemper.[8,26] The presence of syncytia in the endothelium and occasionally the tunica media of the blood vessels confirmed the vascular tropism of this virus. In more advanced lesions, there could be greater destruction of alveolar walls and less obvious vascular changes. These would then progress to the more chronic cases with the appearance of alveolar macrophages, which might be quite bizarre in appearance, as well as intravascular macrophages as seen in case No. 2. The intravascular macrophages were probably similar to those seen by Budiarso and Rikihisa[4] in the lungs of Bali cattle infected with Jembrana disease and may be important in the removal of particulates.[27]

There were some marked differences in these ex-

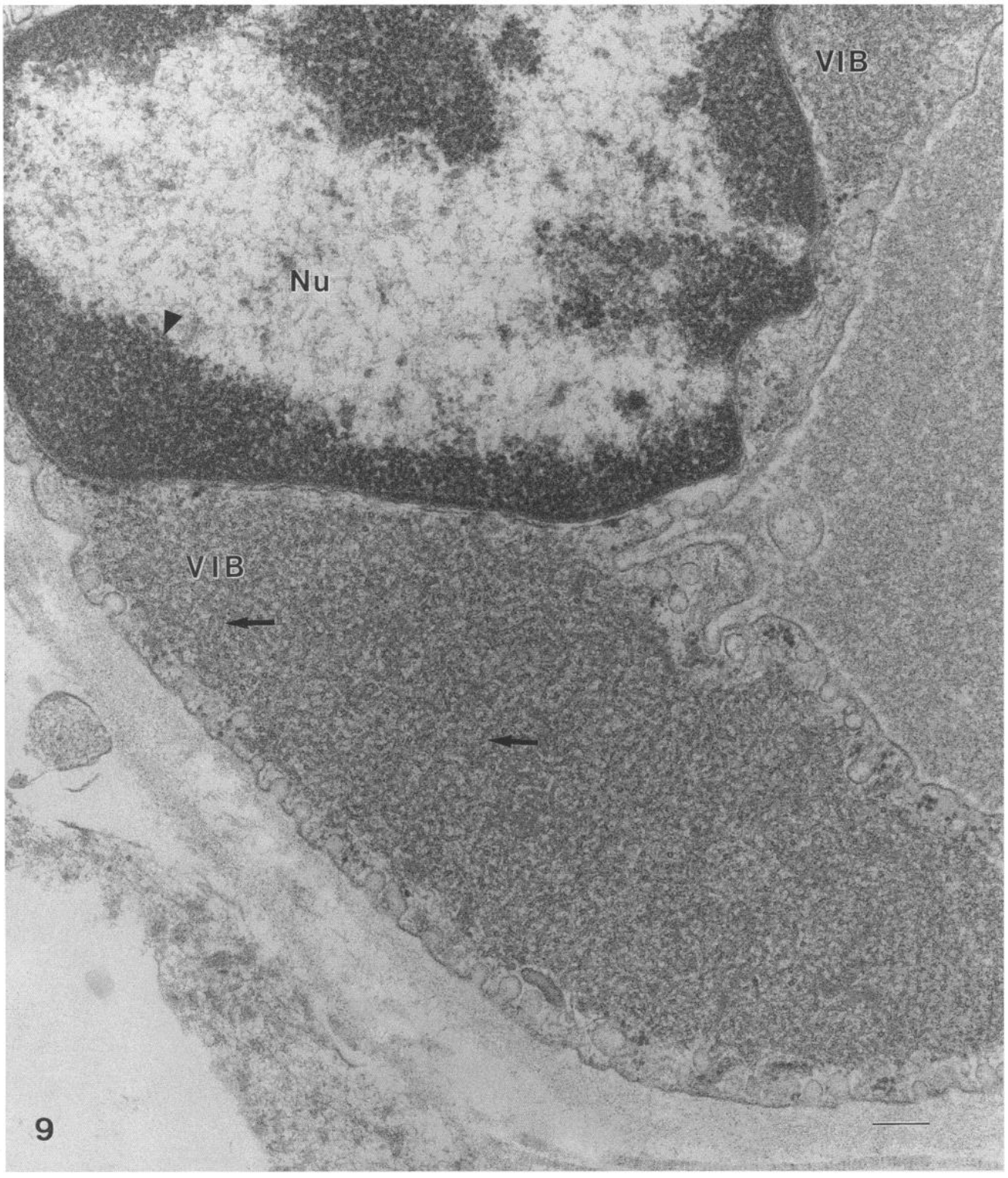

Fig. 9. Transmission electron micrograph of an ultrathin section of lung from case No. 1. The micrograph shows the cytoplasm of an infected endothelial cell containing two virus inclusion bodies (VIB). The inclusion bodies can be seen to consist of aggregates of filamentous nucleocapsids (arrows). The chromatin (arrowheads) of the nucleus (Nu) is marginated. The section was double stained with lead citrate and uranyl acetate. Bar = 200 nm.

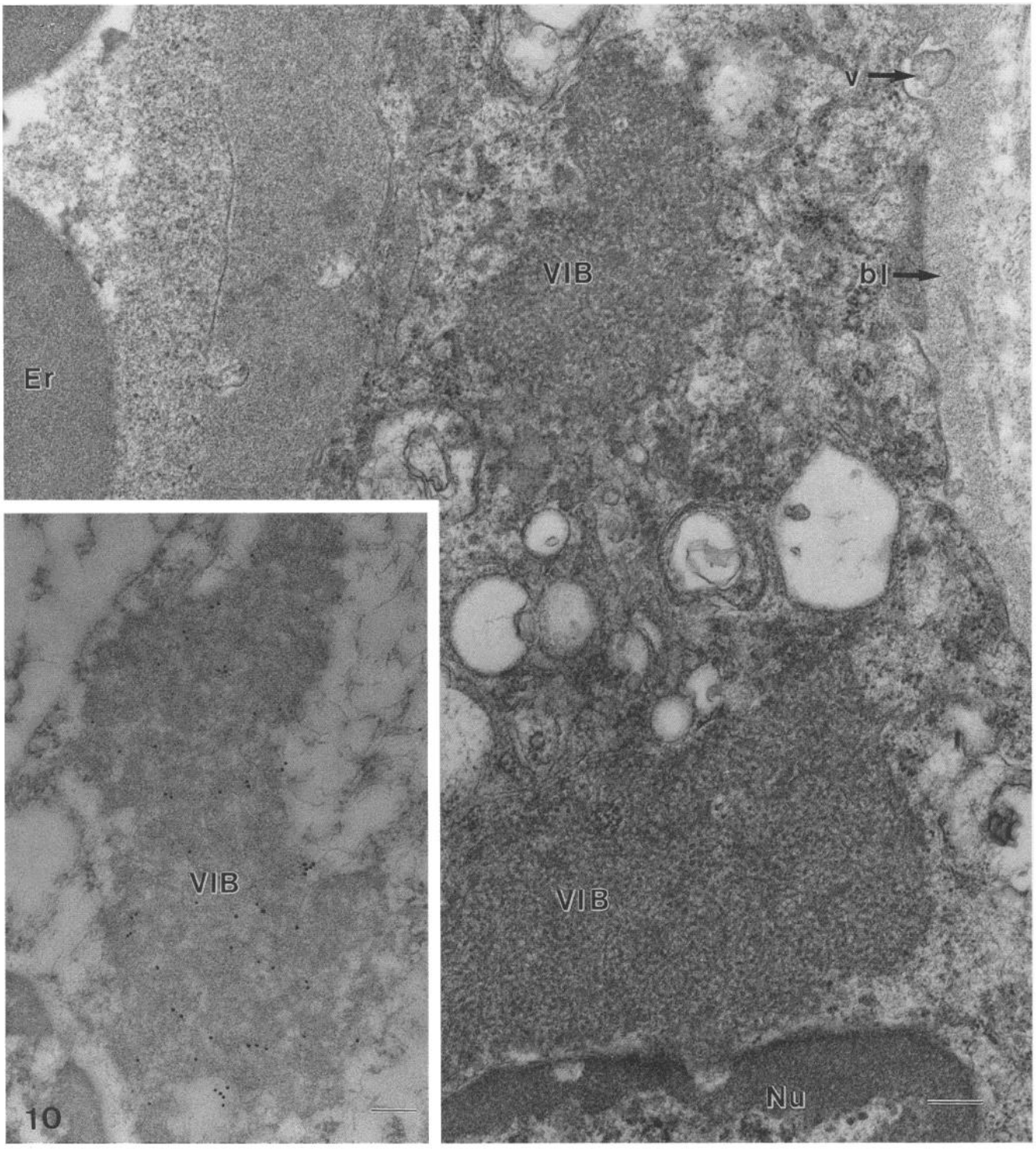

Fig. 10. Transmission electron micrograph of an ultrathin section of the lung from case No. 3. An enveloped virus (v) can be seen adjacent to the basement membrane of an endothelial cell and internal to the basal lamina (bl) of the alveolar wall. Other abbreviations are for erythrocyte (Er), virus inclusion body (VIB), and nucleus (Nu). The insert is a gold-labeled section embedded in L. R. White resin, labeled with convalescent human serum, and protein A-gold. All bars = 200 nm.

perimental cases and the horses seen in the field outbreak of disease. In the field cases, histopathologically visible lesions were mostly only seen in the lungs (P. J. Ketterer, P. T. Hooper, and W. R. Kelly, unpublished), whereas the experimental cases had a broad spectrum of organs affected. This may be a reflection of the routes of challenge, the quantities of virus, and the stage of the disease. At the time of the investigations, the characteristics and cause of the disease were entirely unknown. It was therefore important to determine as soon as possible if there was a transmissible agent so that the severe high-dose intravenous route was used as well as the more natural intranasal challenge, possibly provoking a greater spectrum of affected organs. However, later experiments with cats showed that the distribution of lesions could be independent of the route of challenge.[13]

Another variation from the field cases was the profound frothy discharge evident in the upper respiratory tract (P. J. Ketterer, P. T. Hooper, and W. R. Kelly, unpublished).[19] This was absent in all four experimental cases and may reflect variations in the environment, in some of the treatments provided to the field cases, or possibly longer times of lesion development in the field cases. Likewise, there was a variable intensity of immunocytochemical staining (P. J. Ketterer, P. T. Hooper, and W. R. Kelly, unpublished) of the field cases compared to the strong staining in experimental horses, which may have reflected a longer duration of development of lesion and subsequent loss of detectable antigen in the tissues.

One feature that seems to differ from other morbillivirus diseases was the apparent absence of histologically recognizable intranuclear or intracytoplasmic inclusion bodies. Groups of viral particles consistent with intracytoplasmic inclusion bodies were visible at the electron microscope level but were not seen clearly as inclusion bodies at the light microscope level.

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