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BRIEF COMMUNICATIONS

Experimental African Swine Fever: Evidence of the Virus in Interstitial Tissues of the Kidney

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Infection with African swine fever (ASF) virus causes death or leads to the carrier state. First described by Montgomery in 1921, ASF is a peracute or acute infectious disease characterized by a 100% mortality and by considerable circulatory and clotting mechanism changes in animals dying from the disease.[8] ASF virus replicates in the cells of the mononuclear phagocyte system and has a marked preference for certain lines of reticular cells;[7] replication has also been seen in hepatocytes.[9]

Hypotheses explaining the pathogenesis of bleeding and shock include a direct effect of the virus on endothelial cells,[6] a view that is supported by some immunofluorescence studies,[4] and replication in vitro in pig endothelial cells.[10] Endothelial cells taken from pigs 2 to 3 days post-infection and grown in vitro have been found to release infective virus over several days, which suggests that the virus replicates in vivo. No morphological changes can be detected at the light microscopic level,[1] but ultrastructural analysis of the endothelial cells reveals an increased phagocytic activity.[5]

Kidneys of pigs infected with ASF virus have congestion and hemorrhages, diffuse tubular necrosis, glomerular hyalinization, and moderate cell infiltration at the end of the acute form. Various authors[3,4,7] have reported positive immunofluorescence to ASF antigen in renal mononuclear cells of the intertubular spaces,[4] in isolated macrophages and glomeruli,[3] and in two animals that died.[4] The renal interstice, the narrow spaces between glomeruli and tubules, contains intertubular capillaries stemming from the division of the efferent vessels of the glomeruli. Two cell types have been described at this level: cells similar to fibroblasts and cells similar to lymphocytes. These cells, together with isolated macrophages, are enclosed by loose connective tissue with collagen microfibrillae. This paper describes findings in the renal interstice of pigs experimentally inoculated with the virulent isolate E70 and reports on the behavior of endothelial cells in the intertubular capillaries of the kidney in animals infected with ASF virus.

For this study, nine male large white cross-Landrace pigs were used; each weighed approximately 20 kg at the beginning of the experiment and was free from parasitic and infectious disease.

Animals were divided into three groups, each consisting of three animals. Two animals from each group received intramuscular inoculation of 5×10^5 HA50 of the highly virulent E70 isolate of the ASF virus (supplied by I.N.I.A. of Madrid) which replicates not only in the mononuclear phagocyte system but in hepatocytes also. The other animal was used as control. Animals were killed at 3, 5, and 7 days post-inoculation (dpi) by vascular perfusion following induction of deep anesthesia with azaperone.

Organs were fixed by perfusion in 2.5% glutaraldehyde in 0.1 M phosphate buffer; samples of renal parenchyma were embedded in paraffin, glycol-methacrylate, and araldite.

For the structural study, sections of various widths were taken, depending on the type of embedding. Staining techniques used were hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Feulgen, and toluidine blue. For ultrastructure, 60-nm sections embedded in araldite were stained with uranyl acetate and lead citrate.

The number of cells found in the interstitium and in the interior of capillaries of all nine animals employed was counted by the following method: a cortical and a medullar specimen were cut from each kidney (one per animal) and diced into small cubes. Six to 12 randomly picked cubes per kidney were embedded in araldite. Semithin sections were stained with toluidine blue and used to evaluate two cubes per kidney (one for the cortex and one for the medulla) with respect to their parenchymal content. Three technically acceptable sections per cube were selected, from which 12 consecutive fields of 90×90 nm (the quadrant of a standard copper grid) were used to count cells.

Three days after inoculation edema had separated the cellular and fibrillar elements of the interstice. Edema was still present at 5 dpi, becoming more intense at 7 dpi, particularly in those areas where there was some modification of endothelial cells.

No significant alterations were seen in the number or morphology of fibroblasts and fibrocytes. Lymphocyte morphology remained normal with no variation in number.

At 3 dpi the number of interstitial macrophages was slightly increased, reaching that of fibroblasts. Macrophages were large and contained numerous cytoplasmic organelles, chiefly rough endoplasmic reticulum, mitochondria, and primary and secondary lysosomes. No changes were recorded in the size or number of macrophages between 3 and 5 dpi. At 7 dpi, however, macrophages were more numerous, over twice the number of fibroblasts, and presented a variable morphology. Thus, 55% of macrophages were similar in aspect to those seen 3 and 5 dpi; 19% contained viral replication sites, and the rest (26%) consisted of cells with margination of chromatin, cells at various stages of necrosis, and masses of cell debris which may (9.8%) or may not (16.2%) contain

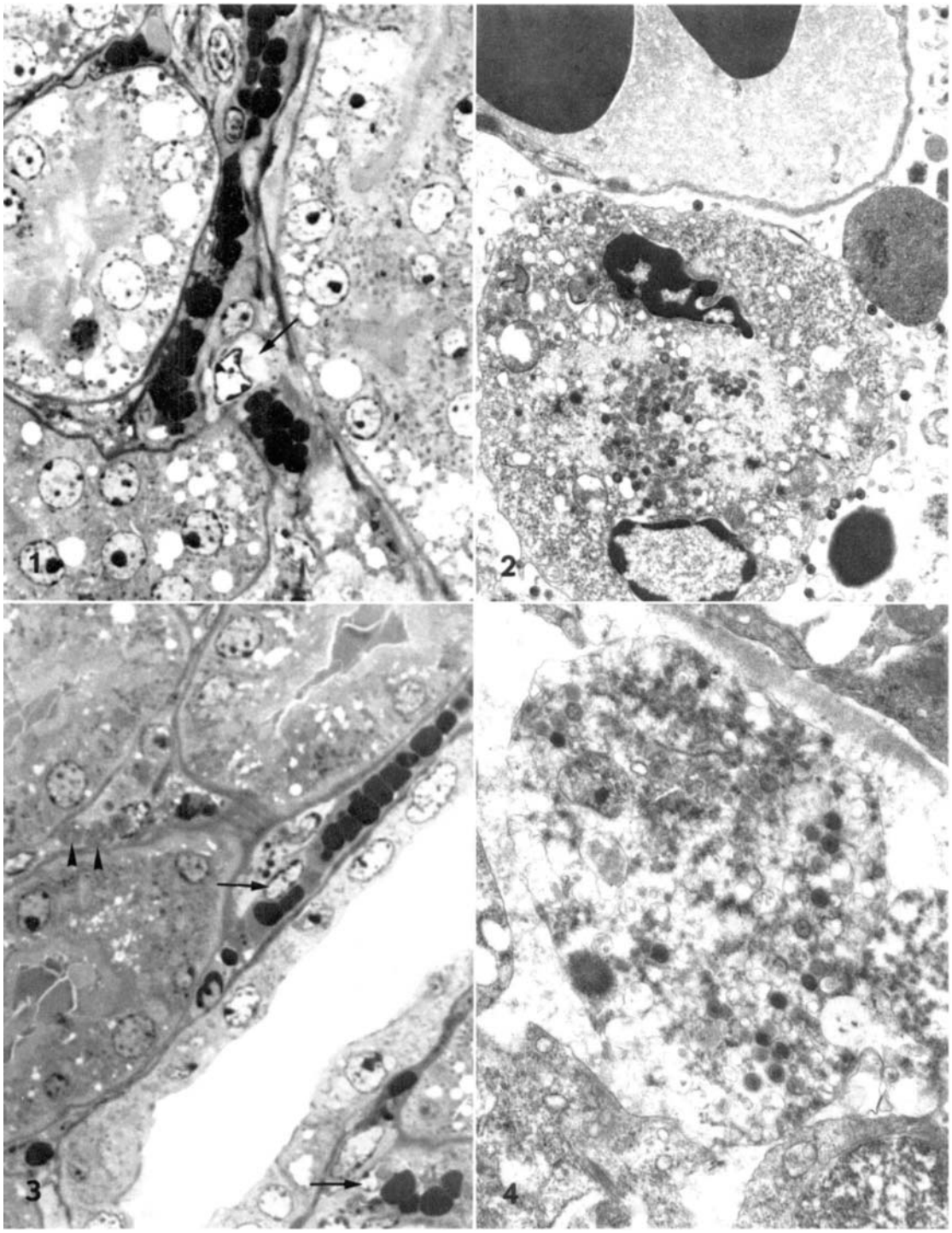

Fig. 1. Interstitial macrophage with a replication site (arrow). Intense vascular congestion, 7 days post-inoculation. Toluidine blue.

Fig. 2. Interstitial macrophage, virus replication site, several budding viral particles. Erythrocytes appear both inside and outside of blood vessels, 7 days post-inoculation.

Fig. 3. Vascular congestion and parietal hyperchromatosis of endothelial cells (arrows), necrotic areas with some rounded masses (arrowheads), 7 days post-inoculation. Toluidine blue.

Fig. 4. Membranous cytoplasmic enclave with cell debris and mature viral particles, 7 days post-inoculation.

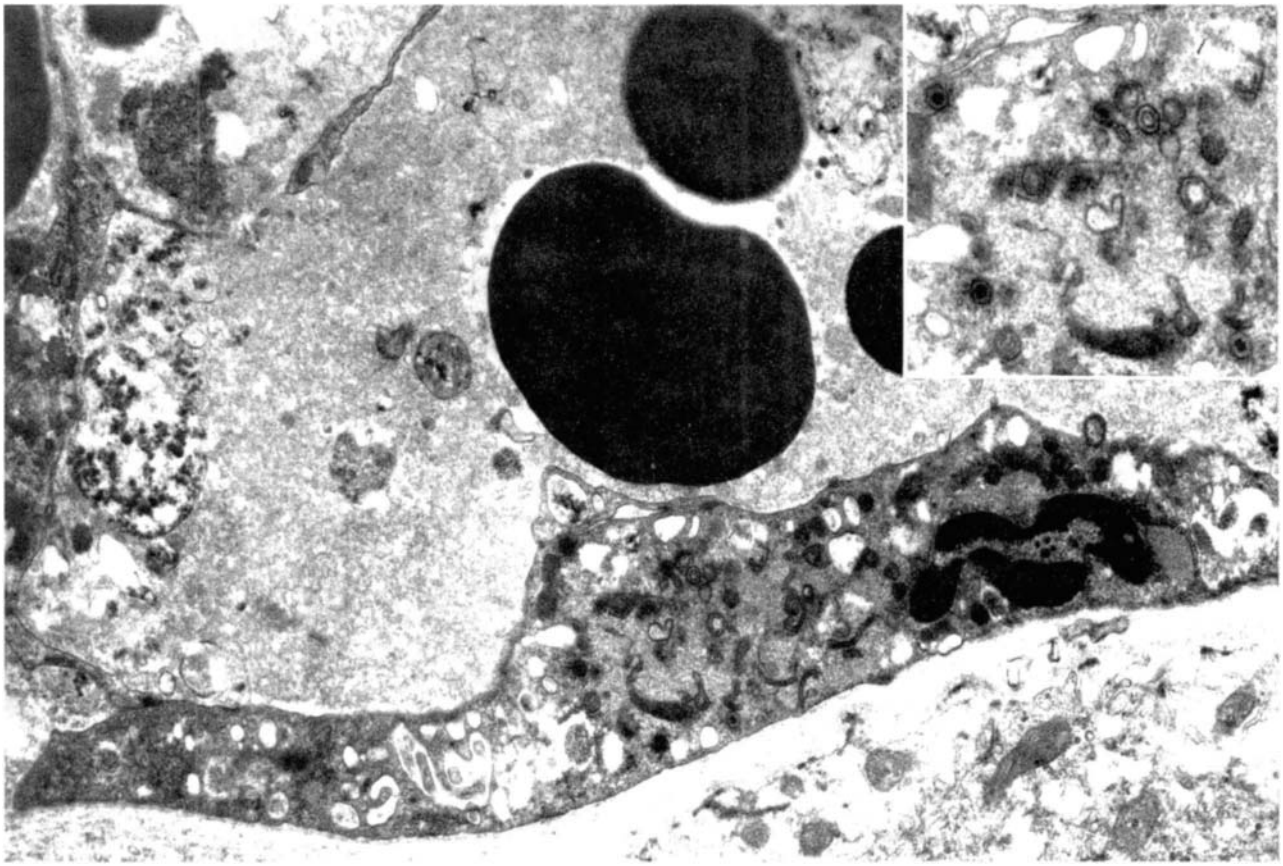

Fig. 5. Endothelial cell with virus replication site and several mature viral particles. Cell junctions between two endothelial cells. Inset: Same virus replication site and mature viral particles at higher magnification, 7 days post-inoculation.

virus. In short, macrophages and cell debris containing viral replication sites or virions accounted for 28.8% of all macrophages.

Interstitial macrophages not containing virus were similar to those seen 3 and 5 dpi, while macrophages containing viral replication sites (inclusions) had a large cytoplasm (Fig. 1) in which the substantial organelle-free area contained viral particles at different stages of maturity, together with replication-related structures and virus budding images. Golgi complex and mitochondria were seen around these organelle-free areas (Fig. 2).

Necrotic cells were numerous, accounting for approximately one-third of the macrophages (Fig. 3). These cells appeared to be infected, while others had "beret-shaped" nuclei with marginal displacement of chromatin; still others had no nucleus. The most striking feature, however, was the presence of large cytoplasmic vacuoles containing membranous structures which were generally oval, roughly 200 nm in size, and of variable electron density. Virus replication sites were seen in some of these cells, while others were found to contain organelle-free areas possibly reflecting a previous viral replication site. Mature viral particles were seen in other cells.

At 7 dpi, abundant membranous cytoplasmic enclaves were seen (Fig. 3). These were more or less spherical in shape and contained numerous organelles, mature viral particles, and a large number of membranous structures; these appeared either free or within large vacuoles, similar in size to the viral particles, and had a homogeneous content of varying electron density. Their size was variable and they were generally arranged in small clusters in close proximity to each other (Fig. 4). These enclaves may represent cell fragments from macrophages which have undergone viral replication and necrosis.

Intertubular capillaries were totally dilated as a result of perfusion, in both control and inoculated animals. They were virtually empty except for animals sacrificed 7 dpi, in which erythrocytes made up 93.4% of the capillary content (Figs. 1, 3). The remainder consisted of macrophages or monocytes, some of which contained viral replication sites, neutrophils, and occasional lymphocytes. Membranous cell debris was seen in the vascular lumen, sometimes large but more often small, and occasionally had a visible membrane. Some of these membranous structures contained mature or immature viral particles, and various were seen both free and adhering to erythrocytes.

Endothelial cell features were generally uniform in each of the three batches inoculated, with numerous micropinocytic vesicles that may account for the edema in the intertubular spaces. At 7 dpi, however, some endothelial cells were enlarged with mitochondrial swelling, and erythrocytes were seen out of the vessels.

Small interstitial areas were found to contain marked edema and necrosis of cell elements in the area. In such cases endothelial cells showed clear cell swelling and marginal displacement of chromatin (Fig. 3). The endothelial plasma membrane contained a small number of slender, elongated protrusions into the vascular lumen, sometimes reaching the opposite endothelial cell and producing a bridge effect. These endothelial cells (Fig. 5) contained some areas devoid of cytoplasmic organoids with viral particles at different stages of maturity as well as mature viral particles throughout the cytoplasm and close to the plasma membrane. Such endothelial cells with viral replication sites were found inside the capillaries that belonged to those areas with strong vascular modifications adjacent to the mentioned areas.

The immunofluorescence to ASF virus seen in interstitial tissue in the final stages of the disease[4] has been confirmed by our observation of virions and viral replication in interstitial spaces at 7 dpi exclusively. Replication sites were seen in macrophages, as reported by Bool et al.[3] and Colgrave et al.[4] Numerous anuclear cell fragments with abundant viral particles, which probably were the remains of necrotic macrophages, might also represent the anuclear fluorescent masses seen by some authors[3] not only in the intertubular tissue but inside glomerular and intertubular blood vessels too.

The number of nucleated cells in the capillaries and the number of inclusions found on these cells were clearly lower in the kidneys than in other organs subjected to similar treatment, such as the lung (Carrasco, personal communication) and the liver (Gómez-Villamandos, personal communication).

Colgrove et al.[4] reported immunofluorescence to ASF virus in blood vessels during late stages of infection (6 dpi). We have seen replication of ASF virus strain E70 in endothelial cells of the renal interstitial capillaries in animals killed 7 dpi. This replication was found in reduced areas (two out of ten tissue blocks from the cortex) where it may affect not only a single endothelial cell but also various endothelial cells within either the same capillary or adjacent capillaries. In these areas, edema, degeneration, and necrosis, probably due to the action of the virus on the endothelial cells, were seen in interstitial cells.

Replication takes place in the endothelial cell, since it not only presents an organelle-free area and viral particles at different stages of maturity, but also has a cytopathic effect with margination of chromatin and cellular swelling. Thus, the hypothesis that viral replication does not produce morphological changes on endothelial cells is incorrect.[1] Moreover, the endothelial cell itself becomes a virus-releasing cell, as evidenced by the mature viral particles seen in areas close to the plasma membrane.

We believe that the replication of the ASF virus in endothelial cells and its cytopathic effect on these cells are responsible for the petechiae found in pigs with acute ASF infection. This interpretation is in agreement with the assertions of Maurer et al.,[6] to which we add a strong morphological basis. However, the role of other mechanisms in the production of hemorrhages at levels where no cytopathic effect on endothelial cells can be seen, or when less virulent isolates are involved cannot be completely ruled out. Thus, an increased PGE_2 release from macrophages (which would promote platelet aggregation and also increase the tendency for edema to occur) in the later stages of the disease has been detected with both virulent and less virulent strains of the virus.[2]

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