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Development of microscopic lesions in splenic cords of pigs infected with African swine fever virus

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Summary — Acute forms of African swine fever are characterized by hemorrhagic lesions in the lymphoid organs. This paper reports the evolution of lesions in the splenic cords of pigs inoculated with African swine fever (ASF) virus (strain Malawi’83). Ultrastructural examination of the splenic cords of the infected pigs revealed numerous macrophages attached to the muscle cells harboring virus replication center and cytopathic effects at 3 dpi (days post-infection). From 5 dpi, the splenic cords contained a large number of erythrocytes associated with abundant fibrin deposits, mainly arranged around the muscle cells, from which macrophages had disappeared. It is likely that the ASF virus replication, and consequent cytopathic effects, observed in the fixed macrophages of splenic cords, may be responsible for the fibrin deposition.

pig / virus / African swine fever / macrophage / spleen

Résumé — Évolution des lésions microscopiques dans les cordons spléniques de porcs infectés par le virus de la peste porcine africaine. Les formes aiguës de la peste porcine africaine se caractérisent par des lésions hémorragiques dans les organes lymphoïdes. Nous avons étudié l’évolution des lésions observées au niveau des cordons spléniques de porcs auxquels on avait inoculé le virus de la peste porcine africaine (souche Malawi’83). L’étude ultrastructurale des cordons spléniques des porcs infectés a révélé la présence de nombreux macrophages adhérant aux cellules musculaires avec réplication du virus et un effet cytopathique à 3 jours post-inoculation (jpi). À partir de 5 jpi les
INTRODUCTION

African swine fever (ASF) is caused by a large enveloped icosahedral DNA virus considered to belong to a separate family (Brown, 1986). Virus replication takes place in the cytoplasm, chiefly in cells belonging to the mononuclear phagocyte system (MPS) (Mebus, 1988), and is considered to play a key role in the pathogenesis of the disease (Mebus, 1988).

Acute forms of the disease are characterized by death within 6 or 7 dpi (days post-infection), and by the presence of hemorrhagic lesions, mainly in the lymphoid organs (Moulton and Coggins, 1968; Nunes Petisca and Martins Gonçalves, 1976). Macroscopic lesions of the spleen have been variously described as splenomegaly (Moulton and Coggins, 1968; Konno et al, 1972), hyperemic splenomegaly (Konno et al, 1972), or hemorrhagic splenomegaly (Nunes Petisca and Martins Gonçalves, 1976). Microscopic analysis reveals the retention of a large number of erythrocytes, together with abundant cellular debris (Moulton and Coggins, 1968; Colgrove et al, 1969; Konno et al, 1972; Mebus and Dardiri, 1979; Mebus, 1988) which for some authors results from the destruction of reticulin fibers (Moulton and Coggins, 1968; Konno et al, 1972; Nunes Petisca and Martins Gonçalves, 1976).

Immunohistochemical studies performed to identify the main cells involved in the pathogenesis of spleen lesions have revealed the presence of the viral antigen in macrophages located primarily in the red pulp, while the lymphoid structures are reported to be practically negative (Colgrove et al, 1969; Mebus, 1988; Minguez et al, 1988; Galo and Nunes Petisca, 1990; Fernández et al, 1992a, b; González-Juarrero et al, 1992; Pérez et al, 1994).

Although splenic cords are traditionally described as being composed of a network of reticular fibers totally engulfed by star-shaped reticular cells, smooth muscle fibers have also been reported in the pig spleen (Ramis et al, 1991; Ueda et al, 1991; Carrasco et al, 1995). The structure of porcine splenic cords has recently been described (Carrasco et al, 1995) as a network of smooth muscle cells surrounded by a new population of fixed macrophages sharing certain characteristics with pulmonary intravascular macrophages and Kupffer cells. The reticular cells in swine splenic cords are reported to be poorly developed, and are found only in the adventitia of venous sinuses, scattered along the splenic cords and, more rarely, adhering to muscle cells (Carrasco et al, 1995).

This paper describes the development of splenic lesions in acute ASF.

MATERIALS AND METHODS

Ten Large White x Landrace pigs of both sexes, weighing roughly 30 kg at the start of the experiment, were used for this study. Two pigs were used as controls, and the remainder were inoculated im with \(10^5\) HAD\(_{50}\) of ASF virus strain Malawi'83 (Haresnape, 1984), classified as highly virulent and hemadsorbent. The animals exhibited no behavioral changes due to the experimental conditions and developed symptoms characteristic of ASF (Colgrove et al, 1969; Wilkinson, 1989). The inoculated animals were slaughtered in pairs at 1, 3, 5 and 7 dpi. The experiment was performed at the Insti-
tute of Animal Health, Pirbright (UK), in accordance with the Practice Code for the Housing and Care of Animals used in Scientific Procedures.

The tissues were fixed by vascular perfusion using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), at a pressure of 120 mmHg. Prior to perfusion, the animals were tranquilized with azaperone (Stresnil, Janssen Animal Health, Belgium) and anesthetized with sodium thiopental (Thiovet, C-Vet, UK). The samples were embedded in paraffin and Epon 812 (Fluka, Buchs, Switzerland). For the ultrastructural analysis, 4 µm thick sections were cut and stained with hematoxylin and eosin. Thin sections (1 µm) were cut and stained with toluidine blue (in a 1% aqueous solution). For transmission electron microscopy, 50 nm samples were stained with uranyl acetate and lead citrate and viewed through a Philips CM-10 transmission electron microscope.

RESULTS

The examination of the splenic cords in the control and experimental animals sacrificed 1 dpi revealed the virtual absence of circulating blood cells due to perfusion. There remained an intertrabecular network of myocytes surrounded by macrophages. The few reticular cells observed were generally located on the adventitial aspect of the venous sinuses, scattered along the splenic cords and, more rarely, adhering to muscle cells. The only difference observed between controls and experimental animals slaughtered 1 dpi was that the macrophages in the splenic cords of the latter group presented an increase in both size and phagosome number.

The animals slaughtered 3 dpi exhibited hyperemic splenomegaly, despite the perfusion. The splenic cords contained large numbers of erythrocytes, some of them with viral particles attached to the membrane, and an increase in

Fig 1. 3 dpi. Macrophages associated with muscle cells containing virus replication centers (asterisk). (A) Macrophage maintaining its linear densities (arrows) Bar: 2 µm. Insert: detail of the linear densities. Bar: 1 µm. (B) Macrophage presenting a rounded profile and detaching from the muscle cells. Bar: 2 µm.
macrophage numbers. Most of these macrophages, which were observed both attached to the muscle fibers and free in the splenic cords, showed peripheral margination of their nuclear chromatin and a clear, rounded organelle-free area of cytoplasm containing complete and incomplete viral particles (fig 1). The macrophages containing virus replication centers, and in particular those attached to muscle cells, had fewer filopodia and projections enveloping muscle fibers, with occasional loss of linear densities in the membrane; they presented a rounded profile with progressive detachment from the muscle cell (fig 1B). In some areas of the splenic cords, myocytes were surrounded by cellular debris (fig 2) interspersed with occasional viral particles (fig 2A), fibrin deposits and degranulated platelets which presented pseudopodia in close contact with the collagen fibers which were surrounding the myocytes (fig 2B).

At 5 dpi, the animals exhibited intense hyperemic splenomegaly. The red pulp was completely filled with erythrocytes, fibrin deposits and cellular debris. The smooth muscle fibers were surrounded by abundant fibrin deposits and cellular debris (fig 3), among which degranulated platelets (fig 3A) and virions (fig 3B) were frequently observed. Macrophages attached to muscle cells were scarce, and contained virus replication centers.

At 7 dpi, the hyperemic splenomegaly remained intense. The red pulp was completely filled with large masses of cellular debris interspersed with erythrocytes, a few neutrophils, monocytes and fibrin bundles and enmeshed erythrocytes and cellular debris, among which viral particles were observed (fig 4).

DISCUSSION

The most significant finding in the splenic cords of pigs infected with a highly virulent strain was the virus replication in the macrophages attached...
to the network of muscle cells. These macrophages disappeared and were replaced by a large number of erythrocytes associated with abundant fibrin deposits.

The viral replication and cytopathic effects observed in the macrophages attached to the muscle cells shared the characteristics previously reported for other macrophage populations in ASF (Sierra et al., 1990; Carrasco et al., 1992; Gómez-Villamandos et al., 1995a, b). The cytopathic effects included peripheral margination of the chromatin, cytoplasmic vacuolization and, in pulmonary intravascular macrophages following the inoculation of a virulent ASF isolate, the swelling of the cells resulting in a loss of intercellular junctions (Sierra et al., 1990; Carrasco et al., 1992). In the spleen, the change in the shape and the loss of intercellular junctions in the macrophages attached to muscle cells resulted in the exposure of the collagen of the basal lamina of the muscle cells to blood plasma. This fact is responsible for the activation of the platelets and the fibrin deposition observed in these areas (Chen and Weiss, 1972; Carrasco et al., 1995). This activation of platelets and fibrin deposition around the muscle cells was more obvious when the macrophages were in necrosis due to virus replication.

The attached macrophages undergoing necrosis due to ASF virus replication probably correspond to the reticular cells described elsewhere as degenerating (Moulton and Coggins, 1968; Nunes Petisca and Martins Gonçalves, 1976; Mebus and Dardiri, 1979). The deposition of fibrin around the muscle cells from which macrophages had disappeared, and subsequently

**Fig 3.** 5 dpi. Smooth muscle cell (M) surrounded by abundant cell debris and a fibrin mesh. (A) Degranulated platelets can be seen among the fibrin mesh (P). Bar: 2 μm. (B) Viral particles can be observed among the cellular debris (arrows). Bar: 1 μm.

**Fig 4.** 7 dpi. Red pulp completely filled with cellular debris, erythrocytes and fibrin. Bar: 5 μm.
within splenic cords, may give rise to the accumulation of erythrocytes and the structural disorganization reported by many authors (Moulton and Coggins, 1968; Konno et al, 1972; Mebus and Dardiri, 1979; Mebus, 1988). Moreover, the appearance of fibrin in the splenic cords from 3 dpi onwards coincides with the appearance of the gross lesions most characteristic of the spleen in acute ASF, and described as hyperemic or hemorrhagic splenomegaly (Moulton and Coggins, 1968; Konno et al, 1972; Nunes Petisca and Martins Gonçalves, 1976; Mebus and Dardiri, 1979; Mebus, 1988).

Following the inoculation of the same ASF virus strain (Malawi’83), fibrin deposition has been reported in the liver from the earliest stage of the disease (3 dpi) (Gomez-Villamandos et al, 1995a). A characteristic feature of the liver is the presence of a macrophage population within the hepatic sinusoids. Similar findings are reported here for the spleen. Although the splenic macrophages are not located within blood vessels, the splenic cords are interposed in the bloodstream, and thus the intravascular deposition of fibrin may be related to virus replication in the intravascular macrophages (Gomez-Villamandos et al, 1995a). The possible relationship between replication in MPS cells and the deposition of fibrin (Gomez-Villamandos et al, 1995a, b) is also supported by the results obtained for the kidney following inoculation of the same ASF virus strain. There the fibrin is not detected until later stages of the infection (5 dpi), coinciding with the observation of monocytes supporting virus replication in renal capillaries (Gomez-Villamandos et al, 1995b). In the liver and kidney, the deposition of fibrin is related to an indirect mechanism. The virus replication in the macrophages is responsible for the activation of the endothelial cells (Gomez-Villamandos et al, 1995a, b) and this endothelial activation produces a disseminated intravascular coagulation process. In the spleen, the deposition of fibrin could be a direct consequence of virus replication, subsequent necrosis and loss of macrophages attached to muscle cells of splenic cords. This loss of macrophages exposes the basal lamina, which in turn exposes the muscle cells to the plasma blood. Platelet activation and fibrin generation around the muscle cells occur as a result. This direct mechanism in the spleen is supported by the absence of endothelial activation in the spleen. The necrosis of spleen macrophages may be related to an increase in fibrin monomers in the blood. As a result the main function of the spleen, blood clearance, is impaired.

In this paper we described the splenic lesions related to the fixed macrophages of splenic cords. A pathogenic mechanism was proposed; the cytopathic effects and necrosis of these cells might have been responsible for the deposition of fibrin in the splenic cords, the subsequent retention of a large number of erythrocytes in the spleen and the appearance of considerable amounts of cellular debris. These mechanisms may be present in other species in which these macrophages are present.

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