Experimental African swine fever: apoptosis of lymphocytes and virus replication in other cells

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In order to determine the cause of cellular death of lymphocytes in pigs with acute African swine fever and the relationships between African swine fever virus (ASFV) and interstitial cells, ten pigs were inoculated with a highly virulent strain of ASFV (Malawi '83) and samples taken for ultrastructural study of hepatic and renal interstitial tissues. We demonstrated death by apoptosis of lymphocytes and virus replication in fibroblasts, smooth muscle cells and endothelial cells in the interstitial tissues of pigs inoculated with ASFV. From day 5 onwards, apoptotic lymphocytes and intense virus replication in hepatic interstitial macrophages and fibroblasts were observed. By day 7, apoptotic lymphocytes and virus replication in macrophages, interstitial capillary endothelial cells and fibroblasts in the kidney were observed. Virus replication was also seen in smooth muscle cells of hepatic and renal arterioles and venules.

Our results suggest that mononuclear phagocyte system (MPS) cell activation, and the resulting release of cytokines, could induce apoptosis of lymphocytes and virus replication in non-MPS cells.

The liver is a secondary site of virus replication in African swine fever virus (ASFV) infections; both the parenchyma and the interstitium contain mononuclear phagocyte system (MPS) cells (Flaks, 1971), traditionally considered to be target cells for this virus (Hess, 1971; Mebus, 1987). The kidney, however, is considered not to be an ASFV replication site, and does not contain MPS cells (Zollinger & Mihatsh, 1978). Both organs were used for the present study of virus action on MPS cells, lymphocytes and other interstitial cell populations, which was designed to further our knowledge of the biology of the virus and the pathogenesis of the disease.

The interstitial lesions observed in liver and kidney during acute African swine fever (ASF) are characterized by intense oedema, haemorrhages of varying intensity and a mononuclear cell infiltrate. Cell death of infiltrate cells, mainly macrophages and lymphocytes, is also reported to be a frequent feature of the disease (Konno et al., 1971; Mebus, 1987). MPS cell necrosis is related to virus replication (Sierra et al., 1989), but the cause of cell death in lymphocytes is unknown.

Ultrastructural studies have revealed in vivo virus replication in other cell types, including hepatocytes (Sierra et al., 1987), endothelial cells (Sierra et al., 1989; Gómez-Villamandos et al., 1995a), renal epithelial cells and glomerular mesangial cells (Gómez-Villamandos et al., 1995b). Replication in non-MPS cells during later stages of the disease has been attributed to MPS cell destruction at earlier stages (Fernandez et al., 1992).

Apoptosis, or programmed cell death, was first described by Kerr et al. (1972), but its significance was not fully realized until a few years ago. Cell suicide by apoptosis is due to DNA fragmentation and may be induced by several mechanisms (Ucker, 1991; Buja et al., 1993). Recent studies have shown that infections by both DNA and RNA viruses induce apoptosis of lymphocytes (Griebel et al., 1990; Ohno et al., 1993). Electron microscopy is currently considered to be one of the best means for evaluating specific modes of cell death (Wyllie et al., 1981; Collins et al., 1992); ultrastructural changes in cells undergoing apoptosis are highly specific, and enable apoptosis to be distinguished from cell necrosis (Arends & Wyllie, 1991; Collins et al., 1992; Buja et al., 1993).

This study describes apoptosis in infiltrate lymphocytes, and ASFV replication in non-MPS cell populations.
Fig. 1. (a) Liver 5 days p.i. Macrophage with severe CPE. ASFV replication (*) and virus budding (arrowhead) are indicated. Numerous apoptotic lymphocytes with different stages of condensation of chromatin (arrows) and rare surface blebbing (bold arrow) are visible. Note that the peripheral margination of chromatin caused by ASFV replication (macrophage) is morphologically different to margination by apoptosis (lymphocytes). An apoptotic lymphocyte (L) is shown being phagocytized by an activated macrophage. Bar marker represents 2 μm. Insert: L, normal lymphocyte. Bar marker represents 1.5 μm. (b) Kidney 7 days p.i. showing an ASFV replication site (*) in a macrophage. Mature virion (arrow). Bar marker represents 1 μm. (c) Kidney 7 days p.i. Severe fragmentation of the nucleus of an apoptotic lymphocyte. Note the presence of numerous aggregations of chromatin (arrows). The cytoplasm appears normal. Bar marker represents 500 nm.
in the hepatic and renal interstitium of pigs inoculated with a virulent ASFV isolate (Malawi '83). A mechanism for induction of apoptosis is proposed which is related to MPS cell activation and the resulting release of cytokines by these cells.

Cross-bred pigs \((n = 10)\) of both sexes were used for this study; these pigs weighed approximately 30 kg at the beginning of the experiment and were free from infectious and parasitic diseases; they were fed with Prime Grover (SCATS Ltd) and housed in isolation rooms under controlled environmental conditions. Two pigs were used as an uninfected control. Eight pigs were inoculated, by the intramuscular route, with \(10^5\) 50% haem-adsorbing doses \((HAD_{50})\) of the Malawi '83 ASFV isolate. This isolate of ASFV is highly virulent and was derived from ticks \((Ornithodorus moubata)\) collected during an outbreak of ASF in Malawi in 1983 (Haresnapce, 1984). The animals did not show any changes in behaviour caused by the experimental conditions, and the clinical signs observed in these animals were characteristic of ASF. Animals were sacrificed in pairs at 1, 3, 5 and 7 days post-infection \((p.i.)\). This experiment was carried out in the Institute for Animal Health, Pirbright, UK in accordance with the Code of Practice for the Housing and Care of Animals used in Scientific Procedures.

Tissues were fixed by vascular perfusion with 2.5% glutaraldehyde in 0.1 M-phosphate buffer \((pH\ 7.4)\) at a pressure of 120 mm Hg. Prior to perfusion, animals were sedated with azaperone \((Stresnil;\ Jannsen Animal Health)\) and anaesthetized with thiopental-sodium \((Thiovet;\ Vet Limited)\). Samples from perfused animals were embedded in Epon 812 \((Fluka)\). For transmission electron microscopy, 50 nm sections of liver, kidney and lymphoid tissue were stained with uranyl acetate and lead citrate and observed with a Philips CM-10 transmission electron microscope.

Hepatic interstitial tissue contained an intense cellular infiltrate consisting of macrophages, lymphocytes, neutrophils and a small number of plasma cells from 5 days \(p.i.\) onwards. A similar inflammatory infiltrate was also observed in the kidney at this time, but was intense at 7 days \(p.i.\) only. Macrophages showed clear morphological evidence of cell activation; they were swollen, with a proliferation of lysozyme structures and phagocytosis of cell debris in evidence. Neutrophils were partially degranulated. Virus replication and CPE were observed in macrophages \((Fig.\ 1a,\ b)\). Replication sites were visible as organelle-free areas containing membranous structures and virions, hexagonal particles 175–190 nm in diameter, at various stages of maturity \((Fig.\ 1b)\). Some budding from infected cells was also observed, creating outlets for virus particles. The CPE caused by virus replication gave these cells a rounded appearance, with a rounded nucleus and peripheral margination of chromatin \((Fig.\ 1a,\ b)\). Macrophage necrosis was generally associated with replication phenomena, although this was not true for other elements of the cell infiltrate.

Cell death of lymphocytes was found to be due to apoptosis. Apoptotic lymphocytes showed condensation of chromatin, with occasional loss of the nuclear membrane, fragmentation of the nucleus and the presence of intracytoplasmic granular chromatin masses \((Fig.\ 1c)\); the cytoplasmic membrane remained intact, although at later stages chromatin debris was engulfed by a membrane unit to form apoptotic bodies. The cytoplasm of lymphocytes undergoing apoptosis was more electron-dense than that of normal lymphocytes and rare surface blebbing was visible. Apoptotic cells and apoptotic bodies were observed phagocytized by macrophages \((Fig.\ 1a)\). No signs of apoptosis were observed in lymphocytes of the liver and kidney of both control pigs and previously sacrificed animals.

In the hepatic and renal interstitium, cells with fusiform nuclei were observed interspersed among collagen fibres; these cells were attached to each other by long, thin cytoplasmic processes, joined together by membrane condensations. Their cytoplasm contained abundant dilated rough endoplasmic reticulum cisternae, with few or no secondary phagosomes. These characteristics enabled such cells to be identified as fibroblasts. In some cases ASFV replication sites with membranous structures, immature virions \((Fig.\ 2a)\) and mature virions \((Fig.\ 2b–d)\) were observed in the cytoplasm of these cells; peripheral margination of chromatin \((Fig.\ 2a)\) and vacuolization of the cytoplasm \((Fig.\ 2a,\ c)\) were also seen.

The interstitial capillaries of both organs carried activated monocytes, degranulated neutrophils including a proliferation of immature forms and lymphoid cells, some of which were undergoing apoptosis. CPE and virus replication sites were visible in renal interstitial capillary endothelial cells in the final stages of the disease \((Fig.\ 2e)\).

Intense cell infiltrate in the walls of a few hepatic and renal arterioles and venules produced vessel wall disruption. This cell filtrate consisted of a small number of lymphocytes, some undergoing apoptosis, and numerous macrophages, many of which displayed evident CPE and visible intracytoplasmic ASFV replication sites. Smooth muscle cells in these blood vessels contained ASFV replication sites, CPE and budding virus \((Fig.\ 3)\). These cells were identified by their spindle shape, the presence of organelles in perinuclear areas and, especially, by parallel and discontinuous bundles of thick and thin intracytoplasmic myofilaments, oriented along the long axis of the cell, with dense bodies scattered throughout
the cytoplasm and associated with the cell membrane. The plasma membrane had numerous caveolae and micropinocytic vesicles (Fig. 3). Abundant free virions, with and without envelopes, were observed in hepatic and renal interstitial samples (Fig. 2b).

This study shows that the cell death of lymphocytes in
hepatic and renal infiltrate in ASF is due to apoptosis. ASFV was also shown to replicate in two non-MPS cell populations: fibroblasts and smooth muscle fibres. ASFV replication in capillary endothelial cells of these animals has previously been reported by our team (Gómez-Villamandos et al., 1995a).

ASF is characterized by lymphopenia and a state of immunodeficiency (Sanchez-Vizcaino et al., 1981). The death of infiltrate lymphocytes in ASF, traditionally attributed to necrosis, is a lesion characteristic of the disease (Konno et al., 1971; Mebus, 1987); the cause of death is unknown, although the present study shows that...
it cannot be attributed to the direct action of the virus on these cells. Apoptosis of lymphocytes that is not associated with virus replication has been reported in bovine herpesvirus infection in vitro (Griebel et al., 1990).

The morphological characteristics of apoptosis include condensation of chromatin, vacuolization of the cytoplasm, compaction of cytoplasmic organelles, cell shrinkage and segmentation of the nucleus (Collins et al., 1992). Necrosis is characterized by uncontrolled swelling of the cell followed by rupture of the plasma membrane, and nuclear changes occur at the later stages (Buja et al., 1993). These ultrastructural characteristics allow apoptosis to be distinguished from necrosis. Evidence of apoptotic lymphocytes observed here from 5 days p.i. onwards, but absent from controls and from experimental animals at 1 and 3 days p.i., indicates that apoptosis of lymphocytes is associated with the disease.

Various authors have shown that different cytokines may induce apoptosis in a number of cell populations; a key role in induction has been attributed to TNF-α and interleukins (Hernández Caseles & Stutman, 1993), whose major involvement in the pathogenesis of haemorrhages in acute ASF has also been reported (Gómez-Villamandos et al., 1995a). TNF-α and interleukins may induce apoptosis in various cell populations (Piguet et al., 1990; Zychlinsky et al., 1991; Hernández & Stutman, 1993; McDevitt et al., 1993; Migliorati et al., 1993), including lymphocytes in acute viral infections, giving rise to states of immunodeficiency (Razvi & Welsh, 1993; Inoue et al., 1994), similar to that described for ASF (Sanchez-Vizcaíno et al., 1981). MPS cells are a major organic source of TNF-α and interleukins (Tracey & Cerami, 1992). As the present study shows, these cells proliferate and are activated in areas with apoptotic lymphocytes, and may release TNF-α. Moreover, the TNF-α liberated by macrophages/monocytes triggers the interleukin cascade (Tracey & Cerami, 1992). The interstitial presence of lymphoblasts and plasma cells from 5 days p.i. onwards, and the production of antibodies at the same stage (Wardley & Wilkinson, 1980), confirm the release of cytokines necessary for the cell-mediated and humoral immune response in these animals.

The abundant replication and necrosis observed in hepatic and renal interstitial macrophages give rise to a large number of free interstitial virions. This, coupled with the destruction of ASFV target cells (macrophages) in these areas, probably favours the infection of non-MPS cells during the later stages of the disease (Fernández et al., 1992). However, the observation here that virus replication in fibroblasts, smooth muscle cells and renal interstitial capillary endothelial cells occurs when viable MPS cells are still present (liver at 5 days p.i. and kidney at 7 days p.i.), together with the intense proliferation of macrophages, suggest the existence of another, more complex, mechanism of infection in non-MPS cells. This mechanism might be related to the activation of MPS cells and the chemical mediator released by them, which may induce the expression of the specific membrane receptor for ASFV, as is the case with hepatitis B virus (David & Reinke, 1987). The fact that fibroblasts and smooth muscle cells lack phagocytic capacity rules out the possibility that ASFV may have entered these cells together with phagocytosed cell debris, and supports the hypothesis of a specific membrane receptor for ASFV (Alcami et al., 1990), which must be present in all cells in which the virus replicates.

Replication of the virus in smooth muscle cells of arterioles and venules, and the presence of macrophages with replication sites in vessel walls, may possibly account for the detection of virus antigen in these vessels (Colgrove et al., 1969). These phenomena, like the observation of replication in endothelial cells, should not be considered the primary cause of haemorrhage, since the former are found at 5 days p.i. and replication is only observed at 7 days p.i. The vascular lesions described undoubtedly aggravate existing haemorrhages.

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References


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