Apoptosis of thymocytes in experimental African Swine Fever virus infection

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Summary. This paper reports on the lesions occurred in the thymus in experimental acute African swine fever (ASF). Twenty-one pigs were inoculated with the highly virulent ASF virus (ASFV) isolate Spain-70. Animals were slaughtered from 1 to 7 days post infection (dpi). Three animals with similar features were used as controls. Thymus samples were fixed in 10% buffered formalin solution for histological and immunohistochemical study and in 2.5% glutaraldehyde for ultrastructural examination. For immunohistochemical study, the avidin-biotin-peroxidase complex (ABC) technique was used to demonstrate viral protein 73 and porcine myeloid-histiocyte antigen SWC3 using specific monoclonal antibodies. Cell apoptosis was evaluated by the TUNEL assay. Blood samples were taken daily from all pigs and were used for leukocyte counts. The results of this study showed a severe thymocyte apoptosis not related to the direct action of ASFV on these cells, but probably to a quantitative increase in macrophages in the thymus and their activation. A decrease in the percentage of blood lymphocytes was observed at the same time. No significant vascular changes were observed in the study. With these results we suggest that ASFV infection of the thymus does not seem to play a critical role in the acute disease. Although severe apoptosis was observed, animals died because of the severe lesions found in the other organs.

Key words: African Swine Fever (ASF), Virus, Thymus, Apoptosis

Introduction

African swine fever (ASF) is a fatal disease that affects animals of Suidae family (Wardley and Wilkinson, 1977). It is caused by the only virus that belongs to the recently created family Asfarviridae (van Regenmortel et al., 2000). The main target cell of ASF virus (ASFV) is the monocyte-macrophage (Mebus, 1988). Lymphoid depletion and haemorrhages in lymphoid organs are some of the most characteristic lesions of the disease (Wilkinson et al., 1981; Gómez-Villamandos et al., 1995b,c; Carrasco et al., 1996, 1997). This disease was first described in Kenya in 1921 (Montgomery, 1921), with outbreaks of acute disease showing high mortality and extensive haemorrhage of tissues. It was subsequently recognised in other countries of Africa where it continues to be an economically devastating disease (Oura et al., 1998).

The factors contributing to the development of the severe lesions in acute ASF are unknown (Oura et al., 1998). Acute ASF is characterised by lymphopenia and a state of immunodeficiency (Sánchez-Vizcaíno et al., 1981). The destruction of the lymphoid tissues was firstly ascribed to necrotic cell death (Konno et al., 1972; Mebus, 1987, 1988), but recently it was assumed to result from cell apoptosis (Gómez-Villamandos et al., 1995b; Carrasco et al., 1996; Oura et al., 1998).

Though thymus is a lymphoid organ and apoptosis of lymphocytes is one of the main lesions produced in ASF, the pathology of thymus and the possible involvement in the pathogenesis of ASF has not been thoroughly studied. Firstly, thymus was thought to be resistant to ASF infection, showing very light histopathological changes, even in the latter stages of the disease (Carnero et al., 1976). By in situ hybridisation techniques, a large number of cells with positive inclusions described as intracytoplasmic granules or inclusion bodies occupying a very big proportion of the cytoplasm were described (Galo and Nunes Petisca, 1990). In other virus infections of swine, a severe
Thymus atrophy has been described; for instance, Classical Swine fever (CSF) virus infection induces apoptosis of thymic lymphocytes (Sato et al., 2000; Sánchez-Cordón et al., 2002) and of lymphocytes in other localisations (Gómez Villamandos et al., 2003; Sánchez-Cordón et al., 2003).

This study report on the lesions produced in the thymus during acute ASF using structural, ultrastructural and immunohistochemical methods to show qualitative and quantitative changes in the cell populations of this organ.

**Material and methods**

**Animals, virus and experimental design**

Twenty four Large White x Landrace pigs, weighing roughly 30 kg and 3 months old at the start of the experiment, clinically healthy and free of antibodies against ASF virus, CSF virus, Aujeszky disease virus and porcine reproductive and respiratory syndrome were used for this study. The animals were housed in isolation at the Centro de Investigación en Sanidad Animal in Valdeolmos, Madrid, Spain. Twenty one pigs were inoculated by intramuscular route, with 10^5 HAD50 of the virulent Spain-70 isolate of ASFV (E-70). This experiment was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures (Directive 86/609/EEC).

**Blood collection and leukocyte counts**

Blood samples were taken from control animals to obtain baseline values and from inoculated animals at 1-7 dpi. Considerable care was taken in the collection of the blood samples to avoid hemolysis and tissue contamination. Blood samples were obtained from the anterior cava vein with plastic syringes and disposable needles. Blood was mixed with 1% ethylene-diaminetetraacetic acid (9:1 ratio by volume) and diluted 1:200 with 1% ammonium oxalate in distilled water. A haemocytometer chamber was used for the leukocyte count. Percentage of the different leukocyte populations was determined using morphological features over 500 cells per animal, previously stained by May-Grunwald-Giemsa method.

**Tissue processing**

Three inoculated animals were randomly selected and slaughtered at 1, 2, 3, 4, 5, 6 and 7 dpi. The three remaining pigs were used as non-inoculated controls and slaughtered at 7 dpi. Animals were tranquillised with azaperone (Stresnil®; Janssen Animal Health, Belgium) and then painlessly slaughtered with a lethal dose of sodium thiopental (Thiovet®, C-Vet, England). Samples from thymus were taken and fixed in 10% buffered formalin (pH 7.2) for histopathological and immunohistochemical study, and for TUNEL technique. Also, samples of thymus were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for ultrastructural study.

After fixation, samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. For structural and immunohistochemical analysis, sections of 4 µm-thick were cut and stained with hematoxilin & eosin (HE) and processed for immunohistochemical techniques.

Samples for Transmission Electron Microscopy (TEM) were postfixed in 2% osmium tetroxide, dehydrated in acetone and embedded in Epon 812® (Fluka Chemie AG, Buchs, Switzerland). Sections of 50 nm were counterstained with uranyl acetate and lead citrate, and viewed under a Philips CM-10 transmission electron microscope.

**Immunohistochemical techniques**

The Avidin-biotin-peroxidase complex (ABC) technique was used for the immunohistochemical detection of viral protein 73 (vp73) of ASFV (Pérez et al., 1994) and porcine myeloid-histiocyte antigen SWC3 (Berndt et al., 2000). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in methanol for 30 minutes at room temperature. Antigen retrieval was developed by 0.1% pronase (Sigma Chemical Company, Poole, Dorset, U.K.) enzymatic digestion for 10 min (vp73) or microwave for 10 min (SWC3). Then, tissue sections were rinsed in phosphate buffered saline (PBS, pH 7.4, 0.01 M) and incubated with 10% normal goat serum (NGS) (Sigma) for 30 minutes at room temperature. The primary antibody was incubated overnight at 4 ºC diluted 1:10 in 10% NGS. A secondary goat anti-mouse Immunoglobulin G (Dako, Glostrup, Denmark) was used and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) diluted 0.035% in TRIS buffered saline (pH 7.6) with 0.01% hydrogen peroxide was applied for 1 minute as the chromogen. The slides were then counterstained with Mayer hematoxylin for 1 min, dehydrated and routinely mounted. Specific primary antibodies were replaced by PBS or normal mouse serum in tissue sections used as negative controls.

**TUNEL**

DNA fragmentation was detected in 4 µm-sections of paraffin-embedded tissues fixed in 10% buffered formalin. Tissue sections were deparaffinized, rehydrated, and treated for endogenous peroxidase activity quenching as described above. Sections were permeabilised by incubation in 20 µg/ml proteinase K in PBS for 20 min and washed twice for 5 min in PBS. The terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) method was used for the histochmical detection of apoptotic cells. The cells
were detected with a kit that utilises horseradish peroxidase (In Situ Cell Death Detection, POD®; Boehringer Mannheim, Indianapolis, USA); according the manufacturer’s directions. The colour reaction was developed and slides were counterstained with Mayer haematoxilin. Negative controls were always included in each series of sections assayed.

Cell counting and statistical analysis

Immunopositive cells against vp73, SWC3 and TUNEL were counted in 25 randomly selected, and high magnification graded fields of 0.20 mm² (medulla and cortex of section from all the thymus) per inoculated and non-inoculated animals.

The number of macrophages was counted with the sections immunolabelled with anti-SWC3. Results of macrophage numbers, and TUNEL and vp73 positive cells are expressed as mean of immunoreactive cells per mm². Data were examined by the analysis of variance, followed by t-paired Student test for mean comparisons.

Differences between control and inoculated animals were considered to be significant at p<0.05.

Results

Clinical findings and gross lesions

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. The control animals remained healthy and inoculated animals began to present unspecific signs from 2 dpi, consisting in pyrexia (39.5-41 °C), loss of appetite, lethargy and respiratory and gastrointestinal disorders.

Macroscopic lesions were typical of ASFV from 3 dpi and consisted mainly of haemorrhagic splenomegaly and haemorrhagic lymphadenitis in gastrohepatic and renal lymph nodes. Some animals killed at 5 dpi onwards showed petechiae and/or oedema in different localisations: gall bladder, urine bladder, lung and

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**Fig. 1.** Total white blood cells expressed in 10⁶/ml (A). Relative percentage of blood lymphocytes (B), neutrophils (C) and monocytes (D) based on observation of 500 total cells counted. A significant decrease in total leukocytes is observed from 3 dpi except on 4 dpi, accompanied by a decrease in the percentage of blood lymphocytes from 4 dpi onwards and an increase in the percentage of neutrophils and a decrease in the percentage of monocytes from 3 dpi onwards. *p<0.05
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Fig. 2. Histology (HE) of thymus from a control animal (A) and from an inoculated animal killed at 6 dpi (B). Severe lymphopaenia is observed in the inoculated animal (a “starry-sky” appearance observed) in comparison with the control. x 20

Fig. 3. Apoptosis of thymocytes in animals killed at 6 dpi observed by TEM (A x 6,600 and B x 2,200) and TUNEL (C x 20). Condensation and fragmentation of the nuclear chromatin is easily observed in thymocytes (A,B) and apoptotic thymocytes with DNA fragmentation are also observed by TUNEL (C). Number of apoptotic thymocytes observed by TUNEL (D); this number increases significantly from 4 dpi in the cortex and medulla. *p<0.05.
kidney.

**Leukocyte counts**

Total leukocytes in blood decreased significantly from 3 dpi onwards except on 4 dpi, when the difference from control animals was not statistically significant (Fig. 1). Leukopenia was a consequence of the decrease in the lymphocyte number and percentage. Neutrophils showed a relative increase from 3 dpi, along with an increase in the rate of immature neutrophils. Total number as well as percentage of monocytes decreased from 3 dpi (Fig. 1). Basophils and eosinophils did not show any significant changes (data not shown).

**Histopathological and ultrastructural changes**

**Apoptosis**

Animals killed at 1-2 dpi did not show any structural or ultrastructural changes. Apoptosis phenomena were observed mainly in cortex, and with less intensity in medulla, these being features similar to those observed in control animals.

The thymus did not suffer any change till 3 dpi, when a lymphoid depletion was observed, firstly in the medulla. Lymphoid depletion appeared in cortex at 4 dpi, and was increased in severity throughout the experiment (Fig. 2). Lymphoid depletion coincided with an increased in the number of apoptotic cells identified as shown by electron microscopy and TUNEL observations (Fig. 3).

Thymic cortical tissue of inoculated animals contained lymphocytes showing marked condensation of the chromatin and dilatation of the nuclear membranes, but the few cytoplasmic organelles present and the cytoplasmic membrane itself remained intact (Fig. 3). The nucleus was occasionally fragmented and cytoplasm contained granular clumps of dense chromatin surrounded by a nuclear envelope (Fig. 3). These changes were occasionally observed in the non-inoculated animals.

Some larger macrophages, with abundant cytoplasm containing phagocyted cell debris (“tingible-body” macrophages) were identified from 3 dpi, predominantly in cortex. These processes gave rise to the so-called “starry-sky” appearance, which worsened over the following days, leading to an intense depletion of the

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**Fig. 4.** Intense depletion of the thymic cortex (HE) at 6 dpi (A, x 40). Immunohistochemical detection of vp73 of ASFV in thymic cortex and medulla at 5 dpi (B, x 63), where infected macrophages are shown (arrows). Counts of vp73- positive cells (C); infected macrophages were observed from 3 dpi onwards. *p <0.05.
thymic cortex at 6-7 dpi (Fig. 4). Ultrastructurally, numerous macrophages were observed with phagocytosed apoptotic bodies from 3 dpi, the number of the observations of this phenomenon being higher with the advance of the disease. No significant vascular changes were noted in the course of the disease except for a light perivascular infiltrate of mononuclear cells in vessels from the cortex and capsule from 5 dpi.

**Viral infection**

No cells stained positive for vp73 were found in non-inoculated animals. Viral antigen was detected from 3 dpi and the number of positively stained cells increased significantly from 5 dpi onwards (Fig. 4). The immune reaction was observed mainly in the medulla and occasionally in the cortex and subcapsular areas from 4 dpi. The first cells to show signs of virus infection from 3 dpi were mostly macrophages, and occasionally reticuloepithelial cells.

Viral infection was also confirmed at ultrastructural level. Infected cells were found to contain typical ASFV replication sites and showed a cytopathogenic effect, consisting of nucleus rounding, peripheral margination of chromatin and development of cytoplasmic vacuoles. Elongated membranous structures and viral particles of 175-195 nm in diameter, some of them with an electrondense nucleoid, were observed in these replication sites (data not shown).

**Discussion**

The thymus has not been sufficiently studied in African swine fever and it is not considered as a main viral replication organ (Galo and Nunes Petisca, 1990), even though it is a lymphoid organ, and the viral replication and lesions in these organs along with a lymphopaenia, are essential features in ASF (Sánchez-Vizcaíno et al., 1981; Gómez-Villamandos et al., 1995a,c; Carrasco et al., 1996, 1997). In this work, we have demonstrated progressive changes in the thymus taken from swine inoculated with a virulent strain of ASFV (isolate E-70) from 2-3 dpi, with lymphoid depletion, macrophage infiltration and viral replication in these cells.

Acute ASF is characterised by lymphopaenia

![Fig. 5](image-url)
(Sánchez-Vizcaíno et al., 1981; Wardley et al., 1983) with destruction of lymphoid tissue and widespread bleeding (Maurer et al., 1958; Mebus, 1988; Gómez-Villamandos et al., 1995c). In the course of the experiment, there was a marked increase in the intensity and extent of lymphoid changes in thymus, as was observed in other lymphoid organs of pigs inoculated with virulent isolates of ASFV (Carrasco et al., 1996, 1997; Gómez-Villamandos et al., 1997). In this study, lymphopaenia detected in peripheral blood coincided with a significant increase in lymphocyte death by apoptosis in thymus, coinciding with apoptosis in other lymphoid organs (Carrasco et al., 1996), contributing to the lethal pathology of ASF (Oura et al., 1998).

Apoptosis is a mechanism involved in cell death and biological renewal of tissues (Kerr et al., 1972; Majno and Joris, 1995). Subcellular changes observed in thymocytes, with condensation of nuclear chromatin and the formation of apoptotic bodies due to fragmentation of nuclear DNA, are indications of apoptosis (Gavrieli et al., 1992; Kerr et al., 1995; Gómez-Villamandos et al., 2001). Viral replication in lymphocytes could be a cause of apoptosis in ASF, but this phenomena has not been observed (Casal et al., 1984; Mínguez et al., 1988; Gómez-Villamandos et al., 1995a, 1997; Carrasco et al., 1996) macrophages being the target cells for ASF virus infection in the thymus. The virus may reach the thymus by monocyte with viral replication and erythrocytes and lymphocytes containing viral particles, which might cross through the blood-thymus barrier of the medulla. ASF virus was observed in macrophages from 3 dpi onwards, mainly in the medulla of the thymus, where the blood thymus barrier is weaker than in the cortex.

The number of macrophages increased significantly from 3 dpi in the medulla and from 4 dpi in the cortex. Signs of phagocytic and of secretory activation was observed in a large number of macrophages from 3 dpi onwards. Some of these macrophages showed signs of viral infection, a close relation existing between these changes and apoptosis in lymphocytes. The mechanism of lymphocyte apoptosis induced by ASFV infected macrophages is not defined, but release of cytokines is one possibility (Gómez-Villamandos et al., 1995a, Carrasco et al., 1996: Oura et al., 1998). In vitro, ASFV infection of macrophages induces the production of TNF-α is able to induce apoptosis in non-infected lymphocytes. Other cytokines such as IL-1B, IL-2 and IL-6 may also induce apoptosis in different cell populations (Hernández Casellez and Stutman, 1993; McDevitt et al., 1993; Migliorati et al., 1993; Saldeen, 2000), and the role of these cytokines has been described in other viral diseases leading to immunosuppression (Peters et al., 1989; Ohno et al., 1993; Razvi and Welsh, 1993; Haagmans et al., 1994; Inoue et al., 1994; Rosenzweig et al., 2000; Sánchez-Cordón et al., 2002). The secretion of these cytokines can also induce the chemotactic recruitment of circulating monocytes in the thymus.

Extensive vascular changes are also a feature of acute ASF (Gómez-Villamandos et al., 1997) associated to endothelial damage by phagocytic endothelial activation non related with viral replication in these cells (Gómez-Villamandos et al., 1995c, 1997), and in vitro studies (Vallée et al., 2001) have suggested that apoptosis could play a role in endothelial death. The lack of vascular changes in the thymus may be due to the late and limited spreading of ASFV in this organ. At the same time, the animal presents severe vascular lesions in other organs (Gómez-Villamandos et al., 1995a-c; Carrasco et al., 1996) in which viral replication in macrophages is abundant.

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References


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