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Original article

Subcellular changes in the tonsils of pigs infected with acute African swine fever virus

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Summary — A study of the pathogenesis of acute African swine fever (ASF) was carried out in pigs inoculated with a highly virulent strain of ASF virus to determine the sequential development of the subcellular changes in a particular lymphoepithelial organ, the tonsil. The apoptosis of the lymphocytes and the inhibition of lymphocyte proliferation were the main changes that occurred in the tonsillar lymphoid structures. This may explain the early lymphopenia observed in acute ASF. Moreover, vascular changes, consisting of increased vascular permeability, activation of endothelial cells and loss of these cells, might have been the cause of the characteristic haemorrhages found in the lymphoid organs during this disease. Virus replication has been observed in the epithelial cells, fibroblasts and reticular cells beginning on day 5 post-infection. The activation of the endothelial cells, apoptosis of lymphocytes, decreased lymphocyte mitosis and virus replication in non-mononuclear phagocyte system (MPS) cells all occurred after an intense proliferation and activation of the tonsillar macrophages and coincide with virus replication, which occurs in the macrophages 5 days post infection.

african swine fever / tonsil / apoptosis / viral replication / ultrastructure

Résumé — **Modifications subcellulaires dans les amygdales de porcs infectés par une souche hautement virulente du virus de la peste porcine africaine.** Une étude de la pathogénie de la peste porcine africaine (PPA) a été menée sur des porcs inoculés avec une souche hautement virulente du virus PPA pour déterminer le développement séquentiel des changements

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subcellulaires dans un organe lymphoépithélial particulier, l'amygdale. L'apoptose des lymphocytes et l'inhibition de la prolifération des lymphocytes ont été le principal changement apparu dans les structures lymphoïdes amygdaliennes. Ceci peut expliquer la lymphopénie observée très tôt au cours de la PPA. De plus, les changements vasculaires consistant en une augmentation de la perméabilité vasculaire, une activation des cellules endothéliales et une perte de ces cellules pourraient être la cause des hémorragies caractéristiques trouvées dans les organes lymphoïdes au cours de cette maladie. La réplication du virus a été observée dans les cellules épithéliales, les fibroblastes et les cellules réticulaires à partir du cinquième jour après l'infection. L'activation des cellules endothéliales, l'apoptose des lymphocytes, la diminution de la mitose des lymphocytes et la réplication du virus dans les cellules du système phagocytaire non mononucléaire sont apparues après une intense prolifération et une activation des macrophages des amygdales et coïncide avec la réplication du virus, qui a lieu dans les macrophages le cinquième jour après infection.

peste porcine africaine / amygdale / apoptose / réplication virale / ultrastructure

INTRODUCTION

African swine fever (ASF) is caused by a lipoprotein-enveloped icosahedral DNA virus (Viñuela, 1987), and is perhaps the most controversial and interesting model for swine diseases in which the monocyte/macrophage is the main target cell for virus replication (Martins and Leitão, 1994). ASF is characterized by hemorrhaging (Colgrove et al, 1969; Edwards et al, 1984), disseminated intravascular coagulation (DIC) (Pan, 1987; Villeda et al, 1993), severe lymphopenia (De Tray and Scott, 1957; Gómez-Villamandos et al, 1995a) and the production of non-neutralizing antibodies (Martins and Leitão, 1994). The pathogenesis of the hemorrhaging is related to the stimulation of the endothelial cells and the subsequent loss of the endothelial lining. Virus replication takes place in these cells only during the final stages of the disease (Gómez-Villamandos et al, 1995b,c). The loss of the endothelial cells allows the platelets to adhere to the basement membrane (Gómez-Villamandos et al, 1995b) aggravating the DIC, which commences prior to the appearance of this lesion (Villeda et al, 1993; Gómez-Villamandos et al, 1995c).

Pigs infected with ASF virus generally suffer from severe lymphopenia, which

occurs suddenly during the middle phase of acute ASF (De Tray and Scott, 1957; Gómez-Villamandos et al, 1995a). The pathogenic mechanism for this remains unknown. Morphological studies have shown that the destruction of lymphocytes in the cellular infiltrate of various organs is due to programmed cell death or apoptosis (Gómez-Villamandos et al, 1995d), a phenomenon not hitherto studied in lymphoid organs during ASF. Other changes in lymphocyte function occur during ASF, including inhibition of proliferation (Wardley, 1982; Canals et al, 1995) and natural killer cell activity (Mendoza et al, 1991), due in both cases to factors released by the infected cells (Wardley, 1982; Mendoza et al, 1991).

The tonsil is considered to be an independent lymphoepithelial organ, because it is not affected by the immune responses arising from elsewhere in the organism (Quiding-Järbrink et al, 1995). It is an important lymphoid structure composed of lymphatic nodules containing B-lymphocytes, diffuse lymphoid structures containing T-lymphocytes and a complex and highly developed capillary system (Scadding, 1990; Quiding-Järbrink et al, 1995). These features render it an interesting organ for the study of both the pathogenesis of vascular lesions and lymphocyte alterations

in pigs infected experimentally with ASF virus.

This paper reports the results of a morphological study of tonsils from swine experimentally infected with a highly virulent ASF virus isolate to determine the relationship, in space and time, between changes in macrophage and vascular lesions, lymphopenia and virus replication in non-MPS (MPS = mononuclear phagocyte system) cells.

MATERIALS AND METHODS

Animals and virus strain

Ten 2-month-old Large White x Landrace cross-bred pigs of both sexes were used for this study. The pigs weighed approximately 30 kg at the beginning of the experiment, and were free from infectious and parasitic diseases. They were fed with Prime Grower (Scats Ltd, Winchester, UK) and housed in environmentally controlled isolation rooms. Two pigs were used as uninfected controls. Eight pigs were inoculated, by the intramuscular route, with $10^{5.5}$ 50% haem-adsorbing doses (HAD₅₀) of the Malawi 1983 ASF-virus isolate (Haresnape, 1984). The animals did not show any changes in behaviour due to the experimental conditions, and the clinical signs later observed in these animals were characteristic of ASF. Rectal temperatures were recorded daily. The inoculated animals were sacrificed in pairs at 1, 3, 5 and 7 days post-inoculation (dpi). This experiment was carried out at 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.

Processing of specimens for light and electron microscopy

The 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. Before perfusion, the animals were sedated with

azaperone (Stresnil®) (Jannsen Animal Health, Belgium) and anaesthetized with thiopental-sodium (Thiovet®) (Vet Limited, UK). The samples from the perfused animals were embedded in paraffin wax or Epon 812 (Fluka, Buchs, Switzerland). Paraffin wax sections were stained with a modification of Gomori's silver-methenamine stain (Moser 1995). Thick sections (1 µm) were cut and stained with toluidine blue (1% aqueous solution). For transmission electron microscopy, 50 nm tonsil sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-10 transmission electron microscope.

Apoptotic and mitotic lymphocyte counts

Counts of apoptotic lymphocytes in the tonsillar lymphatic nodules and diffuse lymphoid tissue were made on paraffin-embedded sections 4 µm in diameter stained with a modification of Gomori's silver-methenamine stain. Sections were observed at 620 x (20 areas of $30 \times 10^3 \mu\text{m}^2$ per animal and lymphoid structure). The apoptotic cells were identified by the condensation and/or fragmentation of chromatin (Moser, 1995). The cells undergoing mitosis were counted on the same sections.

Data on the number of apoptotic and mitotic lymphocytes were analyzed by variance analysis and when a significant result was found, the means were compared by the Newman-Keuls test for multiple comparisons. Differences between counts were considered to be significant at $P < 0.05$.

RESULTS

Interfollicular interstitium and lamina propria

The first and most significant change taking place in these areas was macrophage activation at 3 dpi. Subsequent changes were increasingly indicative of hyperactivation.

Subcellular changes due to activation consisted of cell enlargement, rounding of cell outlines, proliferation of primary lysosomes and increased phagocytosis. These changes became more marked and hyperactivated macrophages were observed from 5 dpi onwards (fig 1). Virus replication and cytopathic effects were observed in the macrophages and a small number of fibroblasts from 5 dpi. Replication sites were visible as organelle-free areas containing membranous structures and virions (hexagonal particles 175–190 nm in diameter) at varying stages of maturity. Some virus budding from the infected cells was also observed. The cytopathic effect caused by virus replication gave these cells a rounded appearance, with a rounded nucleus and a peripheral margination of the chromatin.

For the first 3 dpi, the capillaries were empty or contained very few cell elements. However, by 5 dpi they contained moderate amounts of cell debris and a varying amount of fibrin networks, both of which contained interspersed virions. They also contained monocytes showing evidence of virus replication, neutrophils, which later blocked the lumina of some capillaries, erythrocytes and

platelets. At this stage, the lesions were indicative of increased vascular permeability and included perivascular edema (fig 1) of varying intensity and increased evidence of diapedesis in the blood cells, particularly the erythrocytes and monocytes, some of which contained virus replication sites and/or cytopathic effects (fig 2).

The most significant morphofunctional change encountered at 5 dpi was the activation of endothelial cells. This was characterized by cell enlargement, an increased number of micropinocytotic vesicles, proliferation of primary and secondary lysosomes and projection of pseudopodia which engulfed the cell debris, circulating cells and fibrin deposits (fig 3). These morphological changes in the endothelial cells were accompanied by a loss in the junctions between adjacent cells, which produced spaces of varying sizes. New junctions were formed, especially between pseudopodia. As a result of these endothelial alterations, the subendothelial space bounded by the endothelium and the basement membrane showed irregular dilatation and contained cell debris among which were virions, monocytes,

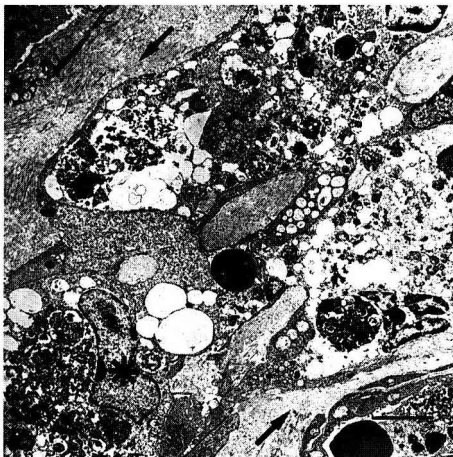


Fig 1. 5 dpi. Interstitium. Hyperactivate macrophages with abundant phagocytosed cell debris (*). Interstitial edema (arrows). Bar: 5 µm.



Fig 2. 5 dpi. Interstitium. Diapedesis of a monocyte (arrows) with an area free of cytoplasmic organoids (*) and cytoplasmic vacuolization characteristic of the cytopathic effects associated with ASF virus replication. Bar: 1.5 µm.

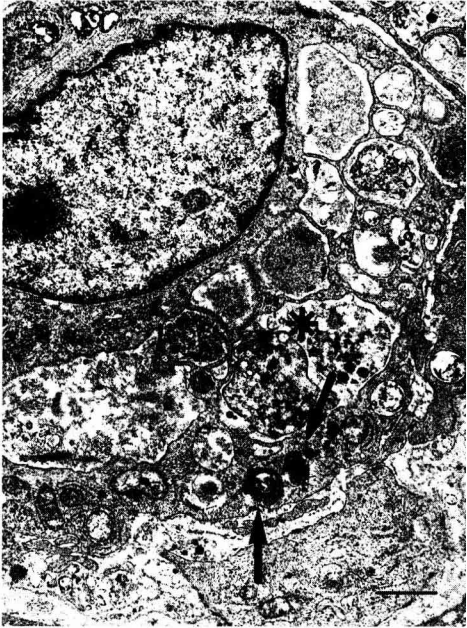


Fig 3. 5 dpi. Interstitium. Activated endothelial cell: the cell is swollen and has many primary and secondary lysosomes (arrows) and cell debris enveloped by cytoplasmic projections (*). Bar: 1 µm.



Fig 4. 7 dpi. Interstitium. Loss of endothelial cells and disorganization of basement membrane (arrows) in an interstitial capillary with fibrin networks (F) and circulating cell debris. Enveloped virion (arrowhead). Bar: 1 µm.

some containing virus replication sites, and erythrocytes (microhaemorrhage). Activated macrophages and macrophages containing virus replication sites were frequently observed in the vicinity of the affected capillaries.

In the final stage of the disease, these endothelial changes became more pronounced. The most significant findings were loss of endothelium and the passage of large amounts of cell debris, fibrin and circulating cells into the perivascular space (fig 4). No virus replication or cytopathic effects were observed in the endothelial cells of these vessels.

Lymphoid follicles and perifollicular lymphoid tissue

The main subcellular change observed in the lymphoid structures was the apoptosis of the

lymphocytes. In the electron micrographs it was possible to see the different stages of the apoptotic process in the lymphocytes, with dramatic changes in both the nuclear and cytosolic structures, which consisted of the aggregation of the chromatin into dense, sharply delineated masses and a breakdown of the nuclear envelope, cytoplasmic condensation, development of broad surface protuberances and cytoplasmic vacuoles. Continuation of the apoptotic process was characterized by cell and nuclear fragmentation and apoptotic bodies (figs 5 and 6).

There was a statistically significant increase in the number of apoptotic lymphocytes from 5 dpi onwards ($P < 0.001$) and the apoptotic lymphocytes were more frequent in the diffuse lymphoid tissue. Extensive apoptosis, together with the necrosis of other cell populations, made it impossible to count

the number of affected lymphocytes at 7 dpi (fig 7). Following the observation of an increase in the number of mitoses in the lymphoid structures, the mitotic cell counts showed a slight but significant increase ($P < 0.05$) at 3 dpi, and declined thereafter (fig 8).

Macrophages in the lymphoid structures were more numerous than in control animals and showed signs of activation from 3 dpi. By 5 dpi, they were numerous, considerably enlarged and rounded and contained abundant evidence of phagocytosed cell debris

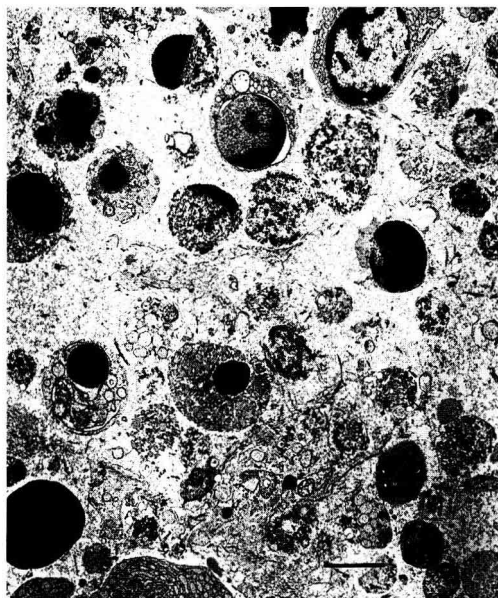


Fig 5. 5 dpi. Perifollicular lymphoid tissue. Severe edema and numerous lymphocytes in different stages of apoptosis. Bar: 2.5 μm .

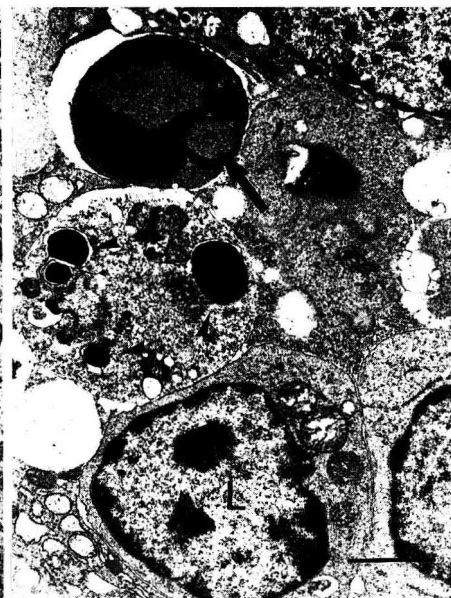


Fig 6. 5 dpi. Lymphoid follicles. Normal lymphocytes (L) and apoptotic lymphocytes with condensation of chromatin (arrow) and fragmentation of chromatin (arrowheads). Bar: 2.5 μm .

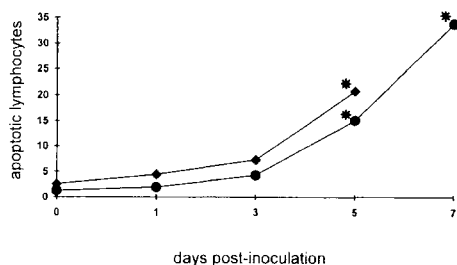


Fig 7. Number of apoptotic lymphocytes in lymphatic nodules (●) and diffuse lymphatic tissue (◆). Data are shown as mean values/area of $30 \times 10^3 \mu\text{m}^2$. Significant differences: * $P < 0.001$.

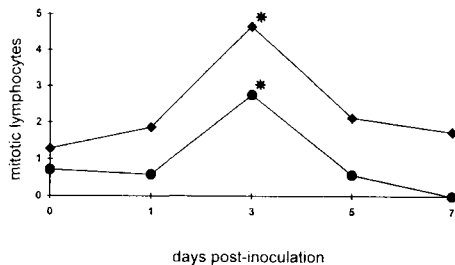


Fig 8. Number of mitoses in lymphatic nodules (●) and diffuse lymphatic tissue (◆). Data are shown as mean values/area of $3 \times 10^3 \mu\text{m}^2$. Significant differences: * $P < 0.05$.

(hyperactivated macrophages), which included apoptotic bodies and apoptotic lymphocytes (fig 9). Virus replication was observed from 5 dpi in a large number of macrophages (fig 10) and in a much smaller number of reticular cells in the follicles and associated lymphoid structures. Replication was observed in both the activated and hyperactivated macrophages. Macrophage activation and replication were more widespread in the macrophages of the diffuse lymphoid tissue, largely because the macrophage population was greater than in lymphatic nodules. No evidence of virus replication or cytopathic effects were observed in the follicular dendritic cells.

The ultrastructural changes in the lymphoid structure capillaries were similar in timing and intensity to those described in the interstitium and the lamina propria.

Tonsillar epithelium

In the control and experimental pigs slaughtered at 1 and 3 dpi, the tonsillar epithelium contained a moderate amount of infiltrate composed of macrophages, lymphocytes and

a small number of other cell elements. Bacteria were observed in the tonsillar lumen but were not observed invading either the epithelium or deeper structures.

At 5 dpi there was a considerable increase in the number of cells infiltrating the epithelium and deforming some epithelial areas in which the epithelial cells were compressed by abundant macrophages, lymphocytes, neutrophils and cell debris. The macrophages showed morphological changes that are characteristic of activation and contained virus replication sites and severe cytopathic effects (fig 11). Macrophages with replication sites were invading the epithelium from the lamina propria. Some lymphocytes were undergoing apoptosis, and apoptotic bodies were being phagocytosed by the macrophages (fig 11). A small number of epithelial cells, clearly identified by the presence of abundant highly developed desmosomes, also contained virus replication sites with cytopathic effects and virion budding (fig 12). Epithelial cells in which ASF virus replication was observed were mainly found in the basal layer. Virions, both with and without envelopes, were observed among the epithelial cells and intraepithelial cell infiltrate.

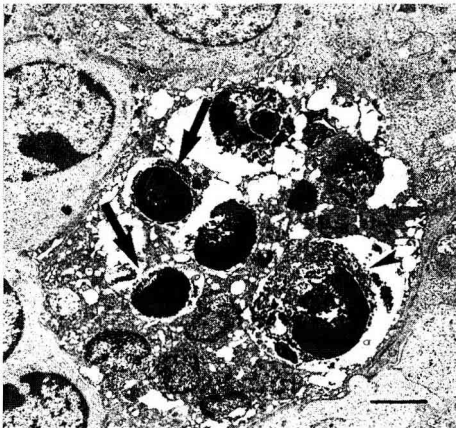


Fig 9. 5 dpi. Lymphoid follicle. Hyperactivated macrophages with abundant apoptotic bodies (arrows) and an apoptotic lymphocyte (arrowhead). Bar: 2 μ m.

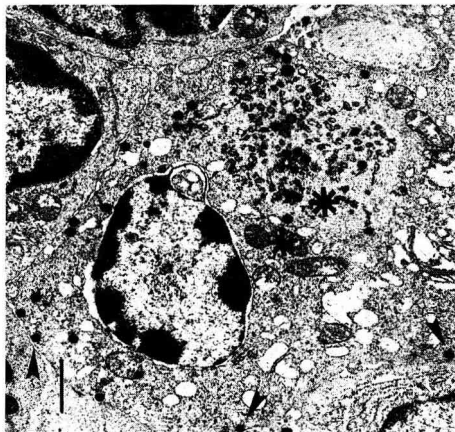


Fig 10. 5 dpi. Lymphoid follicle. Macrophage with ASF-virus replication site (*) and virus budding (arrowheads). Bar: 1 μ m.



Fig 11. 5 dpi. Epithelium. Intense cellular infiltration in the epithelium with numerous hyperactivate macrophages (arrows) some of which contain ASF virus replication site and cytopathic effects (*). Phagocytosed apoptotic bodies (small arrows). Bar: 5 μ m.

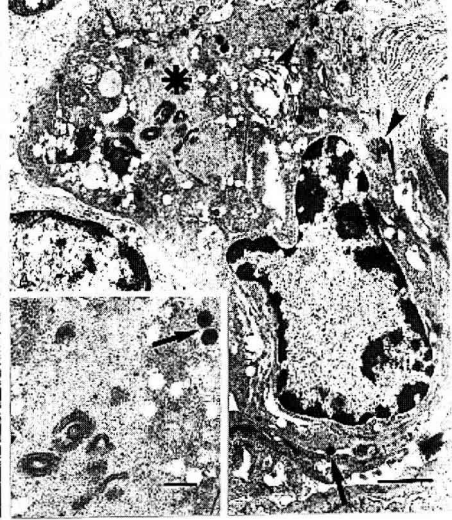


Fig 12. 5 dpi. Epithelium. Basal epithelial cell with ASF virus replication site (*) and virus budding (arrow). Desmosomes (arrowheads). Bar: 1 μ m. Insert: detail of ASF virus replication site with membranous structures and virions (arrows). Bar: 500 nm.

DISCUSSION

The results presented here show that the apoptosis of the lymphocytes, phagocytic activation of the endothelial cells and virus replication in non-MPS cells (fibroblasts, reticular cells and epithelial cells) all take place after the intense proliferation and activation of macrophages in the different tonsillar structures. This was seen 3 days after the pigs were infected with a highly virulent strain of ASF virus and in fact coincided with virus replication in the macrophages at 5 dpi. It was at this stage that the clinical signs became the most severe; the fever increased and the highest viremia titers were recorded (Colgrove et al, 1969; Viñuela et al, 1993).

The morphological changes affecting the macrophages from 3 dpi onwards are the proliferation of primary lysosomes, increased phagocytosis and micropinocytic vesicles and cell enlargement which is characteristic of

activation (Adams, 1992). The activation of the macrophages involves an increase in the synthesis and release of monokines, soluble factors and metabolites of arachidonate including prostaglandin E_2 (PGE_2), elevated chemotaxis and phagocytosis. This latter ceases to be evident when the macrophage becomes hyperactivated (Adams, 1992). The present study showed that during the middle phase of the disease there was an infiltration of macrophages. These cells were activated and became infected by the virus. The changes induced by activation, however, did not hinder virus replication, which in turn did not hinder phagocytosis. These findings concur with recent *in vitro* studies which show that the virus infects and replicates in macrophages without inhibiting their phagocytic activity (McCullough, 1995), although a previous paper has reported an impairment of antibody mediated phagocytosis in these cells (Martins et al, 1987–1988). Thus, the decline of

phagocytosis by macrophages in pigs infected with ASF virus (Sánchez-Vizcaino et al, 1981) would not appear to be due to virus replication but to the excess of material phagocytosed by these cells and their becoming hyperactive. Only in the final phase of the disease, at 7 dpi, did a large number of macrophages undergo necrosis. Further findings indicative of macrophage activation in ASF is the significant increase in PGE_2 (Anderson et al, 1987) and an increase in swine leukocyte antigen expression (González et al, 1989) at 3–5 dpi in animals inoculated with virulent isolates.

Apoptosis of lymphocytes has been reported as a cause of lymphopenia in a number of virus diseases (Ohno et al, 1993; Razvi and Welsh, 1993). The data in this paper show that there is an increase in the number of apoptotic lymphocytes in tonsillar lymphoid structures, which may be the cause of the lymphopenia observed in these animals (Gómez-Villamandos et al, 1995a). "Nuclear chromatin condensed in lymphoid cells" has been described in the lymph nodes and spleen of animals with acute ASF (Konno et al, 1971; Mínguez et al, 1988). Although the authors of these reports do not refer to this phenomenon as apoptosis, the morphology of the nucleus that they describe is similar to that described in this study in apoptotic lymphocytes, suggesting that massive apoptosis of lymphocytes may also take place in these other organs. Moreover, the inhibition of lymphocyte proliferation, morphologically demonstrated in the present study, would aggravate lymphopenia, and it is probably aggravated in the final phase of the disease by the destruction of the lymphoid structures, spleen, lymph nodes and tonsils, as a result of vascular lesions.

A number of different pathogenic mechanisms have been proposed for the programmed cell death of lymphocytes in virus diseases and may be directly or indirectly related to infection of the cell. Activated monocytes–macrophages, and/or macrophages

containing virus replication sites, may synthesize and release monokines which would trigger the apoptosis of the lymphocytes (Razvi and Welsh, 1993). The fact that apoptosis is more widespread in diffuse lymphoid tissue, where activated macrophages or macrophages containing virus replication sites are more numerous, would tend to support this hypothesis. Virus replication in lymphocytes as a possible pathogenic mechanisms of apoptosis of lymphocytes must be ruled out in ASF, since although the virus may infect the lymphocytes (Carrasco et al, 1996), it does not replicate in them. Moreover, the ASF virus contains a gene that may prevent apoptosis in infected cells (Neilan et al, 1993) which could account for the epithelial hyperplasia associated with virus replication (Gómez-Villamandos et al, 1995e).

The subcellular changes in vascular structures reported here are similar to those observed in the capillaries of non-lymphoid organs (Gómez-Villamandos et al, 1995b), and account both for the haemorrhages taking place in ASF and for the biochemical changes (Edwards et al, 1984) indicative of endothelial lesion that are detected following inoculation of highly virulent virus isolates. The main change in the endothelial cells of the capillaries in the tonsil is their phagocytic activation, which is usually associated with the action of chemical mediators (Cavender et al, 1989). In the present study, activated macrophages and/or macrophages containing virus replication sites were observed in the vicinity of damaged vessels. Activation of the endothelial cells would be responsible for the increase in tissue plasminogen activator detected in these animals from 4–5 dpi (Villeda et al, 1995). This increase is indicative of excessive activation of the fibrinolytic system and this, together with the vasodilatory role of PGE_2 (Anderson et al, 1987) could prompt a significant aggravation of the hemorrhages caused by the endothelial lesions described here. We did not observe viral replication in the endothelial cells in the tonsils. The viral antigens detected in the wall

of the small vessels of this organ (Colgrove et al, 1969; Pan, 1987) would be associated with subendothelial cellular debris containing virions and with diapedesis of monocytes with viral replication.

Viral antigens have been detected in tonsillar epithelium using a variety of immunohistochemical techniques (Colgrove et al, 1969; Pérez et al, 1994). The results obtained in the present study showed that the antigen presence in tonsillar epithelium was a result of virus replication in the intraepithelial macrophages and, to a lesser extent, the epithelial cells. Replication in the latter cells, fibroblasts and lymphoid follicle reticular cells was scarce and, while valuable for the study of the biology of the virus, is not thought to be of major significance in terms of the pathogenesis of the disease.

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