African swine fever: a disease characterized by apoptosis

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The cell tropism, organ distribution and resultant pathology of African swine fever were compared in domestic pigs infected with lethal (Malawi) and sublethal (Malta) isolates of African swine fever virus (ASFV). After infections with both isolates, ASFV was predominantly localized in cells of the mononuclear phagocytic system and was not observed in endothelial cells in lymphoid tissue. More severe tissue destruction and cell depletion, associated with high levels of infected macrophages, were seen in lymphoid tissues from domestic pigs infected with the virulent Malawi isolate compared to the less virulent Malta isolate of ASFV. The abundant lymphocyte death was caused by apoptosis and not necrosis. In the spleen, as early as 3 days post-infection (p.i.), many lymphocytes in the B and T cell areas of the white and red pulp were apoptotic. Apoptosis in the T cells of the periacinar lymphoid sheaths in the spleen, however, occurred later, at 5–7 days p.i. In lymph nodes apoptosis was observed in T lymphocytes as early as 4 days p.i. and extended to B lymphocytes in the follicles later in infection. In pigs recovered from infection with the sublethal Malta isolate, virus was found to persist in lymph nodes and tonsils for up to 48 days p.i. and was located in cells, surrounded by apoptotic lymphocytes, in the paracortex of lymph nodes up to 32 days p.i. Taken together, these observations suggest that apoptosis of uninfected lymphocytes was induced by cytokines or apoptotic mediators released from ASFV infected macrophages.

Introduction

African swine fever (ASF) is a highly contagious disease of domestic pigs and European wild boar (Sus scrofa) caused by an icosahedral, double-stranded DNA virus. The disease was first described in Kenya in 1921 (Montgomery, 1921), with reported outbreaks of peracute disease with a high mortality and extensive haemorrhage of tissues, and was subsequently recognized in other countries of Africa where it continues to be an economically devastating disease. In the late 1950s ASF appeared for the first time outside Africa, in the Iberian Peninsula. Depending on the infecting virus isolate, ASF causes syndromes ranging from peracute to chronic, and recovered pigs may become healthy carriers. At present there is no vaccine and the only effective control policy is based on identification and slaughter of infected pigs (Wilkinson, 1989).

The factors contributing to the development of the severe lesions in acute ASF are unknown. In the acute stage, ASF is characterized by lymphopenia and a state of immunodeficiency (Sanchez-Vizcaino et al., 1981). In addition to haemorrhage, one of the lesions most characteristic of acute ASF is an intense destruction of lymphoid tissues which, in the past, was described as necrotic cell death (Konno et al., 1972; Mebus, 1987, 1988). Due to the recent recognition of the apoptotic cell death pathway, much interest has been focused on distinguishing between apoptotic and necrotic cell death in ASF pathogenesis. On the basis of EM observations with tissue from virulent ASFV Malawi infected pigs, apoptosis has been observed in uninfected lymphocytes in lymph nodes and the hepatic and renal interstitium of liver and kidney tissues (Carrasco et al., 1996 b; Gomez-Villamandos et al., 1995). Also, in a recent study investigating apoptosis in tissues by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP end labelling technique (TUNEL), apoptosis of infected cells, as well as uninfected lymphocytes, was observed in lymph nodes of pigs infected with the virulent E70 Spanish isolate of ASFV (Ramiro-Ibanez et al., 1996).

It is intriguing that two open reading frames in the viral genome are homologous with proteins known to inhibit apoptosis. An ASFV gene has been identified which contains all known protein domains associated with bcl-2 activity (Afonso et al., 1996). Its protein product, p21, suppresses apoptotic cell death in a mammalian lymphoid cell line. A
second ASFV gene was found to be homologous to baculovirus iap genes, which encode proteins that inhibit apoptosis during virus infection (Chacon et al., 1995; Yanez et al., 1995). These two genes may promote survival of infected macrophages, resulting in a more efficient productive infection.

Also relevant to the disease pathogenesis are the cellular tropism and persistence of the virus in vivo. The primary site of ASFV replication is in cells of the mononuclear phagocytic system (Pan, 1987; Fernandez et al., 1992a, b, c). Replication of ASFV has also been reported in other cell types such as endothelial cells (Wilkinson & Wardley, 1978; Fernandez et al., 1992a, b, c), megakaryocytes (Colgrove, 1968; Edwards et al., 1984), platelets (Neser et al., 1986; Gomez-Villamandos et al., 1996), neutrophils (Casal et al., 1984; Carrasco et al., 1996a) and hepatocytes (Sierra et al., 1987). However, infection of cells not of the mononuclear phagocytic system has only been observed in the latter stages of infection and is thus not thought to be central in the pathogenesis of the disease. Recent work suggests that lymphocytes are resistant to ASFV infection (Casal et al., 1984; Fernandez et al., 1992a, b, c; Perez et al., 1994; Gomez-Villamandos et al., 1995).

Few studies have been carried out to investigate the persistence of ASFV in recovered pigs. Persistence studies have revealed that viral DNA is detectable in peripheral blood mononuclear leukocytes at greater than 500 days p.i. by PCR assay, although infectious virus was not isolated from the same sample (Carrillo et al., 1994). This indicates that monocytes/macrophages may be persistently infected with ASFV. In this study, sites of virus persistence have been investigated using the sublethal Malta isolate of ASFV.

The location and phenotype of cells infected with the highly virulent Malawi isolate and the moderately virulent Malta isolate of ASFV were investigated by immunocytochemistry on infected tissues. The organ distribution of virus infected cells in tissues was quantified and the subsequent pathological changes identified in order to investigate if there is a link between the frequency of infected cells and the pathological changes seen in tissues taken from pigs infected with the two isolates. An improved understanding of the mechanisms and nature of cell depletion and the related factors that contribute to the development of the severe lesions which occur in acute ASFV infection will address the question of why domestic pigs survive infection with some isolates of ASFV but not others. These observations form a baseline for similar studies on the resistant natural host, the bushpig (accompanying paper: Oura et al., 1998a).

**Methods**

**Preparation of tissue specimens from ASFV infected pigs.** Twelve Large White × Landrace pigs weighing 30 kg were inoculated intramuscularly with the highly virulent haemadsorbing Malawi isolate (Haresnape et al., 1984) of ASFV (10^9 HAD₅₀) and the moderately virulent haemadsorbing Malta isolate (Wilkinson et al., 1978) of ASFV (10⁴ HAD₅₀) per pig. This isolate of ASFV causes 100% mortality in domestic pigs. One pig was used as the uninfected control and was sacrificed at the start of the experiment. One pig was sacrificed at 2 days p.i. and two pigs were sacrificed at 3, 4, 5, 6 and 7 days p.i. In a separate study, 16 Large White × Landrace pigs weighing 30 kg were inoculated intramuscularly with the moderately virulent and haemadsorbing wild type (HAD₅₀) isolates of virus in pigs. Tissues including liver, spleen, kidney, gastro-hepatic lymph node, mesenteric lymph node, parotid lymph node, submandibular lymph node, thymus, lung and tonsil were removed, rapidly frozen (liquid N₂) and stored at −80°C. Each tissue sample (5 µm) was cut and placed onto Superfrost Plus slides (BDH).

**Haemadsorption test.** Primary pig bone marrow cultures were used for the titration of infectious virus in blood and tissue. Cultures

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**Fig. 1.** Morphometric analysis of ASFV infected cells in pig tissue. Paraffin embedded tissue sections (5 µm) from pigs 2–7 days p.i. with Malawi isolate (open bars) and 2, 4 and 7 days p.i. with Malta isolate (solid bars) were immunostained with anti-vp73 MAb 4H3. (A) Spleen, (B) submandibular lymph node, (C) tonsil. The mean number of infected cells per mm² tissue from 30 fields was calculated. Standard error is shown.
were prepared as described previously (Wilkinson et al., 1977). Cells were added to 96-well tissue culture grade microtitre plates (100 µl; 1 x 10⁶ cells per well). Growth medium (100 µl; Earl's saline, 15%, v/v, porcine serum buffered with 10 mM HEPES pH 7.4) with 0.5% (v/v) washed homologous red blood cells was then added to each well. Dilutions (10-fold) of the sample to be titrated were made in PBS plus 1% (v/v) adult bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and added in quadruplicate to plates and incubated at 37 °C. The plates were examined for haemadsorption during a 6 day period and the virus titre was estimated (Reed & Muench, 1935).

### Immunocytochemistry

Standard immunohistochemical techniques were carried out as described by Oura et al. (1996b), using the Vectastain ABC ELITE kit (Vector). Paraffin embedded sections were dewaxed in xylene and rehydrated through a series of alcohol dilutions. Sections were treated as described by Fernandez et al. (1992b), by incubating the slides in buffered citrate (0.01 M, pH 3.2) for 3 h at 37 °C. Sections were permeabilized in 0.05% trypsin (Sigma) at 37 °C for 15 min and washed, and non-specific binding was blocked with horse serum (1:100 in PBS). The primary antibody (undiluted hybridoma tissue culture supernatant) was incubated on the section for 30 min. Antibodies used were: the anti-vp73 MAb 4H3 (Cobbold et al., 1990), which was effective on paraffin sections, the anti-B cell MAB C35 (Saalmuller, 1996) and the anti-T cell MAB PPT3 (Yang et al., 1996), which were effective on cryosections. Endogenous peroxidase was quenched by incubating the sections for 30 min in ImmunoPure Peroxidase Suppresser (Pierce). A secondary antibody, goat anti-mouse antibody conjugated to biotin (1:100 in PBS) diluted in 2% normal swine serum with PBS was applied to the sections for 30 min. The sections were then washed and the ABC biotin/streptavidin/HRP mix (Vector ELITE ABC kit) was applied to the sections for 30 min followed by 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (Vector). Following colour development the sections were counterstained with haematoxylin (Vector), dehydrated and permanently mounted. Cryosections were fixed in ice-cold acetone for 10 min and no trypsin permeabilization step was necessary. All incubations were carried out at room temperature unless stated.

### Staining tissue sections for apoptosis by in situ terminal end-labelling

The TUNEL technique was used to label DNA strand breaks in apoptotic cells in tissue sections (Gavriliu et al., 1992). Paraffin embedded sections were dewaxed, rehydrated through alcohols and then washed. Endogenous peroxidase was completely quenched by incubating the sections for 30 min in ImmunoPure Peroxidase Suppresser (Pierce). The sections were incubated in Proteinase K (50 µg/ml) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 mM Tris–HCl pH 7.4) for 10 min at room temperature, washed and then covered in TdT buffer (30 M...
Fig. 2. For legend see facing page.
transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate pH 8.7) for 15 min at room temperature. The sections were then washed and incubated in blocking buffer (0.2% gelatin, 2% BSA in PBS) for 15 min at room temperature, rinsed in PBS, and covered with anti-fluorescein antibody conjugated with horseradish peroxidase (cell death detection kit, Boehringer Mannheim) at 37 °C for 30 min. After washing the slides, the substrate DAB (Vector) was added to the sections until optimum colour development was achieved. The sections were counterstained with haematoxylin (Vector), dehydrated and permanently mounted.

Results
Organ distribution of ASFV in pig tissues infected with Malta and Malawi isolates
Organ distribution of Malta and Malawi isolates of ASFV was determined by immunocytochemistry on paraffin embedded tissue sections immunostained with the anti-vp73 MAb 4H3 (Cobbold et al., 1996). The levels of virus replication in tissues throughout the acute stages of Malta and Malawi infections was measured by counting the number of infected cells per mm² of tissue. The mean number of infected cells and standard errors from 30 fields were measured. The data reported (Fig. 1, Table 1) are from single pigs, but very similar results (± 20%) were obtained with the duplicate animals. Initial virus replication was seen, with both isolates, at 2 days p.i. in the red pulp of the spleen. Virus replication then spread to other organs. Considerably higher levels of virus replication were seen in tissues infected with Malawi isolate compared to Malta isolate, throughout infection (Fig. 1, Table 1).

Cell tropism of ASFV in pig tissues infected with Malta and Malawi isolates
The cell tropism and organ distribution of ASFV in lymph node, spleen, liver, kidney, lung and tonsil were determined by immunocytochemistry (summarized in Table 1). Results show both the mean number of infected cells and the cell tropism of ASFV, in tissues 4 and 7 days p.i. with Malta and Malawi isolates. Both Malta and Malawi isolates infected predominantly cells of the mononuclear phagocytic system, and there were considerably higher levels of virus replication in tissues infected with Malawi isolate compared to Malta isolate. Direct examination of lymph nodes demonstrated virus in cells both spatially and morphologically consistent with macrophages. In the gastrohepatic lymph node of a pig, 7 days p.i. with Malawi, vp73 was localized in many extrafollicular cells (Fig. 2A). In contrast, throughout acute infection with Malta isolate, vp73 was seen in fewer extrafollicular cells (Fig. 2B).

There was, however, a different pattern of cell tropism between the two isolates (Malta and Malawi) in the lung and tonsil. In Malawi infected tonsil, both extrafollicular macrophages and cells located within the epithelium of tonsillar crypts were infected (Fig. 2C). In Malta infected tonsil, the majority of infected cells were located within the epithelium of tonsillar crypts with few ASFV positive cells elsewhere (Fig. 2D).

Two broad groups of macrophages occur in porcine lung. Pulmonary intravascular macrophages are located in the alveolar capillary wall, whereas alveolar macrophages are located in the air spaces. Throughout Malawi infection, pulmonary intravascular macrophages but not alveolar macrophages stained positive for vp73 (Fig. 2E). However, in Malta infection, alveolar macrophages were found to be vp73 positive (Fig. 2F).

Comparison of pathology of lymphoid tissue in pig tissues infected with Malta and Malawi isolates
Spleen and lymph node sections were examined morphologically by haematoxylin and eosin (HE) staining, by TUNEL staining for the presence of apoptotic cells, and by immunocytochemistry for the presence of ASFV infected cells, throughout the acute stages of Malawi and Malta infections. Higher levels of tissue destruction, apoptosis of lymphocytes and ASFV infected cells were seen in the acute stages of Malawi infection compared to the acute stages of Malta infection. In a direct comparison between Malawi and Malta infected tissues at 4 days p.i., similar spleen sections were stained with HE, TUNEL and anti-vp73 MAb 4H3, respectively. At 4 days p.i. with Malawi, the majority of lymphocytes in the white pulp were pyknotic, as were many cells in the red pulp which was haemorrhagic and depleted of cells (Fig. 3A). Interestingly, the lymphocytes making up the periarteriolar lymphoid sheath (PALS) surrounding the arteriole were not pyknotic. Staining similar spleen sections by the TUNEL technique revealed that the majority of cells in the white pulp were apoptotic, with the marked exception of the PALS. Also, many cells in the red pulp were apoptotic (Fig. 3B). Similar spleen sections stained with anti-vp73 MAb 4H3 revealed vp73 positive cells in the red pulp but not in the white pulp (Fig. 3C).

In contrast, similar spleen sections from Malta infected pigs (4 days p.i.) were stained with HE (Fig. 3D), TUNEL (Fig. 3E) and anti-vp73 MAb 4H3 (Fig. 3F). Very little tissue pathology was seen (Fig. 3D) and few apoptotic cells were observed in the white and red pulp (Fig. 3E). Only a limited number of
vp73 positive infected cells were seen localized only in the red pulp (Fig. 3F).

Early in infection (2 days p.i.) with Malawi, low levels of apoptosis were observed in red pulp lymphocytes and no evidence of apoptosis was seen in the white pulp of the spleen. However, at 3 days p.i. a high degree of apoptosis was detected in the vast majority of the lymphocytes in the white pulp of the spleen, and high levels of apoptosis were seen in cells of the red pulp. Apoptosis of lymphocytes in the white and red pulp progressed up to 7 days p.i., at which stage the white pulp was mostly destroyed and the red pulp was severely depleted of cells. In contrast, in spleen from Malta infected pigs, low levels of apoptosis were seen in the red pulp at 2–4 days p.i., and no evidence of apoptosis was seen in the
white pulp. However, at 7 days p.i. moderate levels of apoptosis were seen in the red pulp, and some cells in the white pulp were apoptotic (data not shown).

In Malawi infected lymph nodes, apoptotic cells were first detected in the medullary and sinusoidal regions at 3 days p.i. (data not shown). Levels of apoptotic lymphocytes increased up to 7 days p.i. when an extensive degree of apoptosis was seen extending from the subcapsular region throughout the lymph node sinuses to the interfollicular areas and into the B cell follicles. Throughout infection with Malawi, the B cell follicles decreased in size and number. In contrast, in the acute stages of Malta infection some haemorrhagic congestion was seen, especially in the gastrohepatic lymph node, but low levels of apoptosis were seen at 2 and 4 days p.i. At 7 days p.i., however, high levels of apoptotic cells were seen in the medullary sinuses. These apoptotic cells were located in and around the medullary sinuses, and few apoptotic cells were observed in the cortex and B cell follicles. At 11 days p.i. levels of apoptosis in Malta infected lymph nodes reduced, reaching low levels at 14 days p.i. (data not shown).

In both spleen and lymph nodes, the degree of apoptosis of lymphocytes was directly related to the number of ASFV infected cells. The marked difference in numbers of ASFV infected cells and apoptosis of lymphocytes in Malawi and Malta infected lymphoid tissues may be important reasons why the Malawi isolate is lethal whereas the Malta isolate is sub-lethal.

Immunostaining of spleen cryosections from Malawi infected pigs was carried out with anti-porcine CD3 MAb PPT3 (Yang et al., 1996) and porcine CD21 MAb C35 (Saalmuller, 1996), which have been shown to specifically recognize porcine T and B cells, respectively. The aim was to identify the phenotype of apoptotic lymphocytes seen in the PALS, white pulp and red pulp of the spleen throughout infection with Malawi isolate. Serial cryosections of uninfected spleen were stained with anti-CD21 MAb C35 (Fig. 4E) and anti-CD3 MAb PPT3 (Fig. 4F). The central arteriole was surrounded by a PALS consisting of T cells. Adjacent to the PALS in the white pulp was a B cell rich area which also contained low numbers of T cells. The red pulp contained many T cells.

Fig. 3 (A, B) show that, at 4 days p.i., the majority of cells in the white pulp were apoptotic, with the exception of the PALS, and the red pulp was depleted of cells. In order to identify the phenotype of these apoptotic cells, serial spleen cryosections from pigs infected for 4 days with Malawi isolate were immunostained with anti-CD21 MAb C35 (Fig. 4C) and anti-CD3 MAb PPT3 (Fig. 4D). The apoptotic cells adjacent to the PALS in the white pulp consisted of both T and B lymphocytes; however, the B lymphocytes stained more weakly at 4 days p.i. (Fig. 4C) than in uninfected spleen (Fig. 4A), suggesting that CD21 was being lost from the cell surface during apoptosis. Interestingly, at 4 days p.i. very few T cells remained in the red pulp (Fig. 4D). At 7 days p.i. the majority of cells in the white pulp, including the PALS, were apoptotic and the red pulp was severely depleted of cells (data not shown). In order to identify the phenotype of the cells remaining in the spleen at 7 days p.i., serial spleen cryosections from pigs infected for 7 days with Malawi isolate were immunostained with anti-CD21 MAb C35 (Fig. 4E) and anti-CD3 MAb PPT3 (Fig. 4F). The remains of the white pulp consisted of weakly staining apoptotic B cells and a low numbers of T cells. Very few T lymphocytes remained in the red pulp.

In conclusion, at 4 days p.i. with Malawi isolate, the majority of the T lymphocytes in the red pulp had died by apoptosis and many of the cells (B and T lymphocytes) in the white pulp were apoptotic. However, at 4 days p.i. the T cells in the PALS were not apoptotic. At 7 days p.i., very few T and B lymphocytes remained in the spleen.

**Persistence of ASFV in pigs recovered from Malta infection**

Defining the nature and location of the cells which have a role in persistence was addressed in the following study. Domestic pigs were allowed to recover from ASFV Malta infection. Tissues and blood were removed from the pigs at a series of times p.i., titrated for virus by haemadsorption, and investigated for sites of virus replication by immunocytochemistry. Virus titres in the blood, spleen, submandibular lymph node and tonsil are compared (Fig. 5). Maximum blood titres of 8·3 log10 HAD50/ml and tissue titres of 6·8 log10 HAD50/g were reached by 4–7 days p.i., after which time virus was slowly cleared from the blood and spleen where it reached undetectable levels by 48 days p.i. In lymph nodes and tonsil, however, virus persisted at levels of up to 4 log10 HAD50/g tissue up to 48 days p.i. This indicates that the Malta isolate is able to persist in recovered pigs in lymph nodes and tonsil.

Immunocytochemical studies showed, surprisingly, that severely ill pigs, between 14 and 18 days p.i., had very low levels of virus replication in the tissues examined. Very low levels of virus replication were detected in tissues examined from pigs in terminal stages of ASFV Malta infection (14–18 days p.i.). In recovered pigs at 26 and 32 days p.i. small pockets of virus replication were observed in paracortical areas of lymph nodes. Indeed, at 32 days p.i. very few foci of infected cells were seen in all the lymph nodes examined. The pockets containing ASFV positive cells also contained cells with pyknotic nuclei undergoing apoptosis (Fig. 6). This demonstrates that ASFV is able to persist in cells, probably macrophages, in the paracortex of lymph nodes. The fact that the ASFV infected cells are surrounded by apoptotic lymphocytes even at 32 days p.i. enforces the theory that ASFV infected macrophages are, through the release of cytokines or apoptotic mediators, causing lymphocytes in close proximity to enter apoptosis. Pigs sacrificed at 41 and 48 days p.i. showed no evidence of virus replication in any of the tissues examined and no evidence of apoptosis with TUNEL (data not shown).
Fig. 4. Immunocytochemical localization of T cells and B cells in spleen cryosections from pigs infected with Malawi isolate of ASFV. Spleen serial cryosections were immunostained with anti-T cell MAb PPT3 or anti-B cell MAb C35. Signal was visualized with DAB (brown) and sections were counterstained with haematoxylin. (A) Uninfected spleen stained with anti-B cell MAb C35 (× 80), (B) uninfected spleen stained with anti-T cell MAb PPT3 (× 80), (C) spleen, 4 days p.i. with Malawi, stained with anti-B cell MAb C35 (× 160), (D) spleen, 4 days p.i. with Malawi, stained with anti-T cell MAb PPT3 (× 160), (E) spleen, 7 days p.i. with Malawi, stained with anti-B cell MAb C35 (× 160), (F) spleen, 7 days p.i. with Malawi, stained with anti-T cell MAb PPT3 (× 160). P, periarteriolar lymphoid sheath; R, red pulp; W, white pulp.
African swine fever: an apoptotic disease

Discussion

The pathogenesis of ASF was investigated by a systematic comparative study of the virus tropism, organ distribution and apoptotic cell loss in pigs infected with the highly virulent 100% fatal Malawi isolate and the moderately virulent 50% fatal Malta isolate of ASFV. In comparing cell tropism and tissue distribution of the virus, as well the resulting pathology, the aim was to gain an understanding of why some isolates of ASFV are highly virulent whereas others are moderately virulent or avirulent. The organ distribution of virus was significantly different in the acute stages of infection with the two isolates. Many more cells were infected in the acute stage of Malawi compared to Malta infection, and the higher level of ASFV replication was associated with more severe pathological damage and, in particular, higher levels of apoptosis in lymphoid tissues.

Interestingly, pigs dying of Malta infection between 14 and 18 days p.i. showed low levels of virus replication in the tissues examined. This agrees with an EM study carried out by Gomez-Villamandos et al. (1996) on tissues from pigs infected with the moderately virulent Dominican Republic isolate of ASFV, in which no evidence of ASFV replication in tissues was seen from 9–17 days p.i.

For many years it has been assumed by veterinary pathologists that the haemorrhagic pathology and endothelial cell activation reported in ASFV infections were direct results of virus infection of endothelial cells. This theory was supported by Wilkinson & Wardley (1978), who reported infection of endothelial cells by ASFV in vitro. In other studies, infection of endothelial cells in vivo in the liver and kidney was demonstrated in the terminal stages of infection with highly virulent isolates of ASFV (Fernandez et al., 1992a, b). In our and other studies (Fernandez et al., 1992a, b; Perez et al., 1994; Gomez-Villamandos et al., 1997) no significant infection of endothelial cells has been reported in lymphoid tissue (lymph nodes, spleen and tonsil) where the most obvious haemorrhagic pathology is observed. Thus the infection of endothelial cells may not be the primary cause of haemorrhage within the lymphoid system. If this is the case, then an indirect mechanism such as an aberrant cytokine profile may be responsible for the haemorrhagic pathology.
The question of persistence of ASFV in recovered pigs is critical to the development of control strategies, but is poorly understood. Defining the nature and location of the cells which have a role in persistence is addressed in this study. Domestic pigs were allowed to recover from ASFV Malta infections. Tissues were removed from the pigs at a series of time points p.i., titrated for virus by haemadsorption and investigated for sites of virus replication by immunocytochemistry. Virus titrations revealed that ASFV is cleared slowly from the blood, spleen, liver and kidney, reaching a steady rate, undetectable levels at 48 days p.i. However, virus titres remained high for longer periods of time in lymph nodes and tonsil which retained up to 5 log_{10} HAD_{50} of virus per g tissue up to 48 days p.i. This indicates that ASFV was able to persist preferentially in cells in lymph nodes and tonsil.

The alternative approach of tissue localization through the detection of viral protein by immunocytochemistry, however, revealed only few infected cells, in small pockets in the paracortex of lymph nodes, up to 32 days p.i. These pockets of virus replication were associated with apoptotic cells, which strengthens the argument that ASFV infected cells are indirectly signalling surrounding lymphocytes to enter apoptosis. At 41 and 48 days p.i., no virus replication was detected in lymph nodes by immunocytochemistry, although virus titres remained high. This could be due to the relative insensitivity of the technique or, alternatively, it is possible that at later stages of infection the virus, detected by haemadsorption, was adsorbed to the surface of red blood cells and no replication in macrophages was occurring.

Infection of pigs with highly virulent isolates of ASFV resulted in a rapid and drastic loss of lymphoid tissue through apoptosis (Ramiro-Ibanez et al., 1996). Similarly, in Malawi infected spleen, the majority of cells in the white pulp were apoptotic as early as 3 days p.i., with the marked exception of the cells surrounding the arteriole in the PALS. By 7 days p.i. all the white pulp including the PALS was completely destroyed, with only apoptotic bodies and cell debris remaining. There were also high levels of apoptosis as early as 2 days p.i. in T cells of the red pulp, which by 7 days p.i. was severely depleted of cells. Similarly, during Malawi infection apoptosis was initially seen in lymph nodes at 3 days p.i., principally in the medullary and cortical sinuses. Later, at 4–5 days p.i., broad bands of apoptotic cells were seen in the T cell areas of the paracortex, perhaps significantly in association with many ASFV infected macrophages. Finally, in the latter stages of infection (6–7 days p.i.) there was considerable apoptosis within the B cell follicles. Therefore, although the degenerative pathology developed later in lymph nodes than spleen, apoptosis of T cells preceded that of B cells in both spleen and lymph node. There is, however, an interesting exception in the spleen, in which the T cells of the PALS are the last lymphocyte population to enter apoptosis. As T cells are essential to all immune responses, their elimination through apoptosis would severely compromise the immune response of the pig to the virus, as of course would the later loss of follicular B cells.

The observation that apoptosis of T cells precedes that of B cells in Malawi isolate infection is in agreement with previous ultrastructural studies (Carrasco et al., 1996b). In contrast, the virulent Spanish E70 isolate resulted in more intense apoptosis in B cell areas than T cell areas of lymph nodes (Ramiro-Ibanez et al., 1997), a discrepancy that stresses the importance of comparative studies of virus pathogenesis. Thus, in this work similar studies have been carried out in pigs infected with the highly pathogenic Malawi and the less pathogenic Malta ASFV isolates. In Malta infected lymphoid tissue there were greatly reduced numbers of ASFV infected cells and a considerably decreased apoptotic loss of lymphocytes than at similar time-points p.i. with Malawi isolate. This results in a less compromised immune system than in Malawi infection and therefore, perhaps as a result, 50% of the pigs are able to survive the infection.

The mechanism of lymphocyte apoptosis induced by ASFV infected macrophages is not defined, but release of cytokines such as TNF-α is one possibility. Cells of the mononuclear phagocytic system produce large quantities of TNF-α (Tracey & Cerami, 1992). Therefore, if the level of apoptosis depends on the amounts of cytokines released, and this in turn depends on the number of ASFV infected macrophages, this may explain the greater apoptotic pathology of highly virulent viruses such as Malawi compared with the less virulent isolates such as Malta. The fact that ASFV encodes a biologically active homologue of IκB (Powell et al., 1996) may be relevant. Although this ‘host evasion gene’ clearly does not protect the domestic pig, it may function more efficiently in the naturally resistant wild hosts of ASFV, the warthog and the bushpig. Other explanations for virus induced apoptosis include: (1) products, other than cytokines, released from infected macrophages; (2) loss of anti-apoptotic macrophage factors necessary for lymphocyte survival due to virus infection; (3) impaired phagocytosis of apoptotic cells by infected macrophages; (4) compromised follicular dendritic cells and/or interdigitating dendritic cells, resulting in selection of lymphocytes for cell death rather than for continued survival; and (5) induction of both FAS and active FAS ligand on the surface of infected lymphocytes, resulting in activation induced apoptosis. Alternatively, the lower levels of replication of Malta in tissues may result in diminished apoptosis.

ASFV is not unique in causing apoptosis, which has also been described in murine and avian influenza (Mori et al., 1995; Hinshaw et al., 1994). ‘By-stander’ apoptosis of uninfected lymphocytes has also been reported for other viruses, including human immunodeficiency virus (Lu et al., 1994), chicken anaemia virus (Jeurissen et al., 1992), infectious bursal disease virus (Vasconcelos & Lam, 1994), bovine herpesvirus type 1 (Griebel et al., 1990), herpes simplex virus type 1 (Ito et al., 1997) and murine cytomegalovirus (Koga et al., 1994).

The observation that indirect factors resulting from ASFV
infection of macrophages may cause apoptosis in surrounding lymphocytes may be advantageous for the survival of the virus. Controlled apoptosis in lymphocytes will result in a diminished immune response enabling the virus infected cells to evade the immune system for a sufficient time to produce a productive infection. This may have important implications for persistence, especially in the reservoir hosts (bushpig and warthog). The high levels of apoptosis of lymphocytes, on the other hand, as seen in domestic pigs infected with virulent isolates of ASFV, may contribute to the lethal pathology.

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References


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