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# Ultrastructural pathology of the bone marrow in pigs inoculated with a moderately virulent strain (DR'78) of African swine fever virus

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**Summary.** Interpretation of changes in bone marrow during infectious processes is quite complex. This paper reports bone marrow lesions observed in pigs inoculated with a moderately virulent ASF virus strain and studies their relationship to the pathogenesis of the disease. In this work, we have carried out the structural and ultrastructural study of the bone marrow of 14 Large White x Landrace pigs that were inoculatd by the intramuscular route with $10^5$ 50% hemodsorbing doses ($HAD_{50}$) of the Dominican Republic'78 ASF virus strain. The inoculated pigs were killed at 3, 5, 7, 9, 11, 13, 15 and 17 days postinjection. Analysis of cells and structures belonging to the two main bone-marrow compartments, the hematopoietic cells and the hematopoietic micro-environment, showed that after inoculation with a moderately virulent strain, the most significant changes occurred in macrophages and megakaryocytes, consisting in virus replication in these cell populations and apoptosis of megakaryocytes, related with the sudden and transitory thrombocytopenia detected in the subacute ASF.

**Key words:** Bone marrow, Virus, Pathology, Ultrastructure

## Introduction

Interpretation of changes in bone marrow during infectious processes is quite complex. Such changes can generally be classified quantitatively and qualitatively. Quantitative anomalies include hyperplasia and hypoplasia of different cell lines and require simultaneous peripheral-blood evaluations for proper identification. Qualitative alterations include morphological aberrations of precursor cells, and changes such as necrosis, macrophage proliferation and plasmocytosis (Janhl et al., 1960; Rebar and Lewis, 1976; Lennert and Hubner, 1984; Weiss, 1986; Rebar, 1993). Additionally, resultant extramedullar inflammation prompts release of colony-stimulating factors which increase leukocyte production, while peripheral platelet consumption triggers an increase in megakaryocytes in bone marrow (Burkhardt, 1971; Miller et al., 1974; Lewis and Rebar, 1979; Lennert and Hubner, 1984; Rebar, 1993).

In recent years a large number of viral agents have been related to bone marrow failure and suppression. These include parvoviruses, cytomegaloviruses, Epstein Barr virus, human immunodeficiency viruses and dengue virus. Bone marrow failure may be prompted by a number of mechanisms, including cytotoxicity and the infection of both stromal and hematopoietic cells. Most of these viral processes cause varying degrees of bone-marrow hyperplasia; thrombocytopenia is an almost constant finding (Rosenfeld and Young, 1991; Nakao et al., 1989).

African Swine Fever (ASF) is a hemorrhagic disease caused by a DNA virus and is considered an ideal model for *in vivo* study of virus-macrophage interaction (Martins and Leitao, 1994). ASF is characterised by blood-flow alterations in the form of diffuse hemorrhages, increased membrane permeability, instability in the circulatory system, disseminated intravascular coagulation (DIC) and thrombocytopenia (Villeda et al., 1993, 1995) .

This paper reports bone-marrow lesions observed in pigs inoculated with a moderately virulent ASF virus strain and studies their relationship to the pathogenesis of the disease.

## Materials and methods

In this work, we have carried out the structural and ultrastructural study of the bone marrow of 14 Large White x Landrace pigs that were inoculatd by the intramuscular route with $10^5$ 50% hemodsorbing doses (HAD50) of the Dominican Republic'78 ASF virus strain. The inoculated pigs were killed at 1, 3, 5, 7, 9, 11, 13, 15 and 17 days postinjection. This experiment was carried out in the Institute for Animal Health, Pirbright

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(U.K.).

Tissues were fixed by vascular perfusion with 2.5% glutaraldehyde in 0.1M phosphate buffer. Before perfusion, the animals were sedated with azaperone and anaesthetized. Samples from perfused animals were embedded in paraffin wax or Epon 812. Paraffin-wax sections were stained with haematoxylin and eosin and avidin-biotin-peroxidase complex techniques (antibody Factor VIII-related antigen). Thick sections were cut and stained with toluidine blue. For transmission electron microscopy, 50-nm sections were stained with uranyl acetate and lead citrate.

Megakaryocyte counts were performed on 4-$\mu$m sections of bone marrow from inoculated and control animals stained for Factor VIII-related antigen as has been recently described by Gómez-Villamandos et al. (1998).

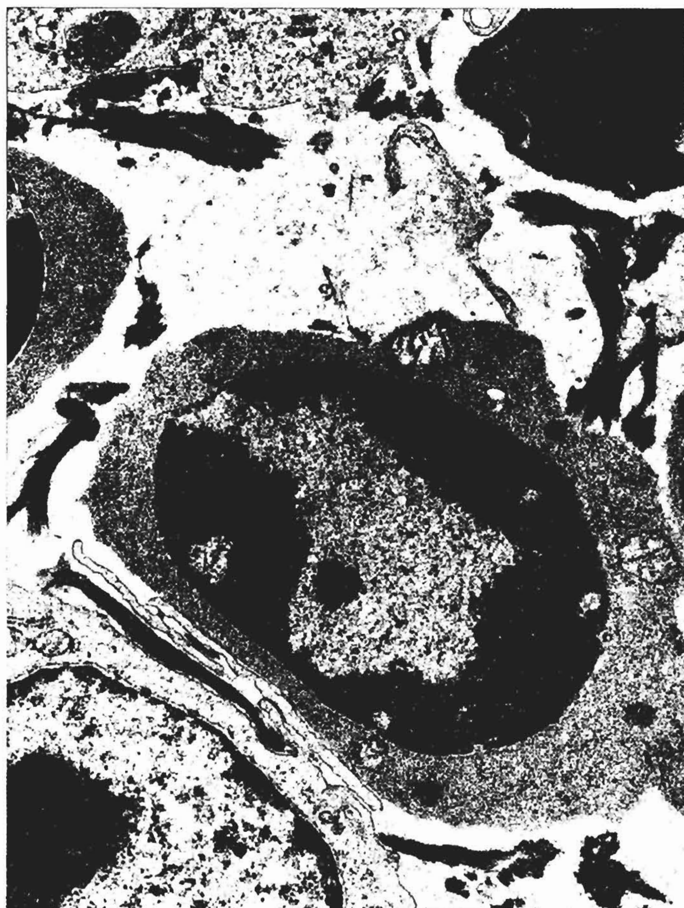
## Results

Bone marrow may be divided into two major compartments: the hematopoietic cells and the hematopoietic microenvironment, the latter consisting of macrophages, reticular cells, vascular structures and the interstitium.

### *Hematopoietic microenvironment*

In the interstitium, fibrin networks, cell debris and apoptotic bodies as result of apoptosis in lymphoid cells were observed among stromal and hematopoietic cells at 3 and 5 dpi (Fig. 1), and to a lesser extent at 7 days postinjection.

Macrophages rose in number from 3 days postinjection and much more intensely from 9 days postinjection onwards. These cells showed signs of phagocyte activation, which became progressively more acute during the course of the experiment: there was a considerable increase in cell size, moderate cytoplasmic vacuolisation, and primary and secondary lysosome proliferation and abundant phagosomes were observed (Fig. 2). The material captured by activated macrophages was highly heterogeneous: from 3 days postinjection, numerous macrophages showed evidence of



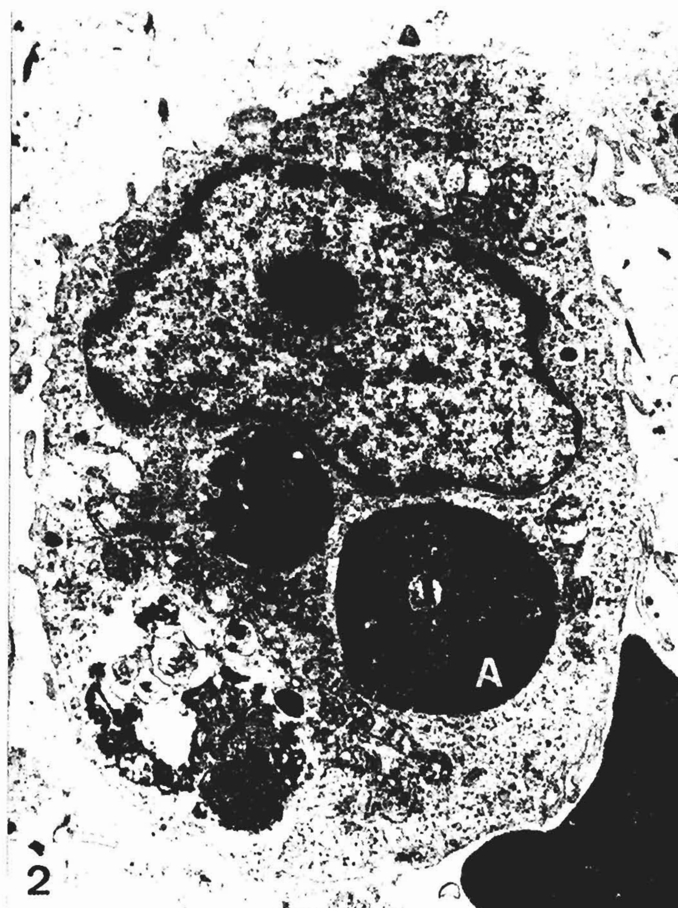



**Fig. 1.** 3 days postijection. Fibrin networks in the bone marrow interstitium. x 18,700

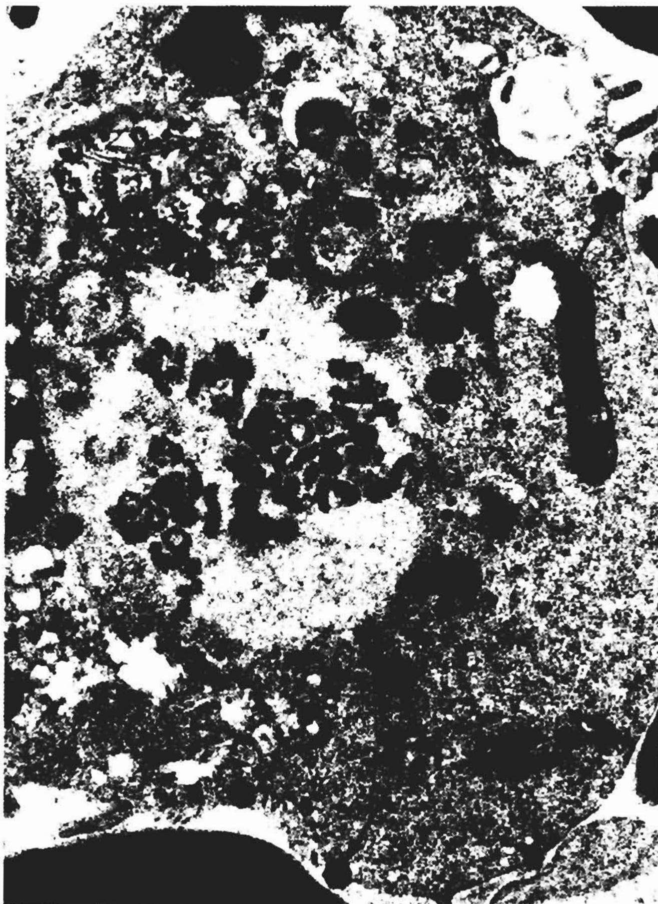
**Fig. 2.** 5 days postinjection. Macrophage with phagocyted cell debris and apoptotic body (A). x 10,800

erythrophagocytosis and contained intracytoplasmic electron-dense protein crystals. From 5 days postinjection onwards evident phagocytosis affected mainly, although not exclusively, lymphoid cells; affected cells had pyknotic nuclei or peripheral chromatin condensation and the highly electron-dense cytoplasm characteristic of cells undergoing apoptosis. Apoptotic bodies were also visible as rounded masses of chromatin surrounded by a small portion of cytoplasm and/or membrane (Fig. 2). These findings were observed with similar frequency throughout the progression of the disease.

At 5 days postinjection, a moderate number of cells showed the cytopathic effect characteristic of ASF virus replication: on toluidine-blue-stained sections, cell size was considerably increased, with evidence of rounded nuclei, peripheral margination of chromatin, cytoplasmic vacuolisation and rounded areas of lower staining intensity, designated inclusion bodies. Ultrastructural analysis identified the latter as virus replication sites; these were perinuclear organelle-free cytoplasmic areas, containing membrane structures and hexagonal virions at differing stages of maturity, ranging from 175 to 190 nm in diameter (Fig. 3). Within these replication sites, there was evident proliferation of polyribosomes in close proximity to viral particles; in some cases, these had acquired a spiral or helix formation ("helicoidal polyribosomes") (Fig. 4). Signs of cytoplasmic vacuolisation observed structurally corresponded to dilation of the cytoplasmic vacuolar system, including rough endoplasmic reticulum cisternae, nuclear envelope and the Golgi complex. This evidence of viral replication was most marked at 5 days postinjection, decreasing thereafter to a low at 7 days postinjection, after which it was no longer observed. A small number of macrophages were also ingesting other macrophages harbouring viral replication.

The morphology of reticular cells and vascular sinus endothelial cells was similar to that of controls throughout the experiment, with no evidence of activation or viral replication.

Fibrin strands were moderately abundant in venous



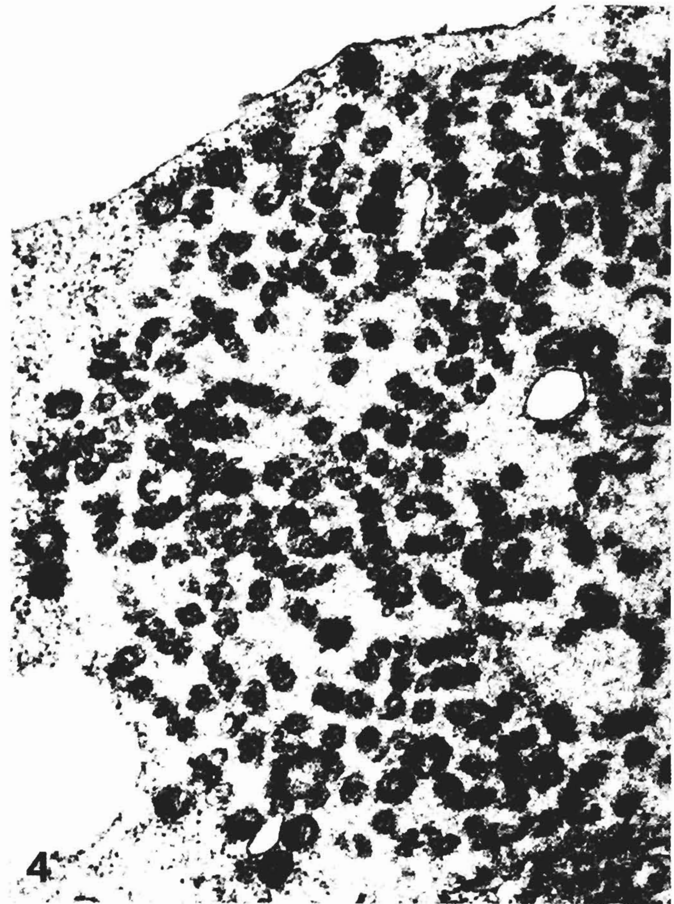



**Fig. 3.** 5 days postinjection. Macrophage with ASF virus replication site consisting of membranous structures and viral particles in different stages of maturity. Virus budding (arrow). x 16,600

**Fig. 4.** 5 days postinjection. Detail of an ASF virus replication site. Note the presence of helical polyribosomes. x 31,200

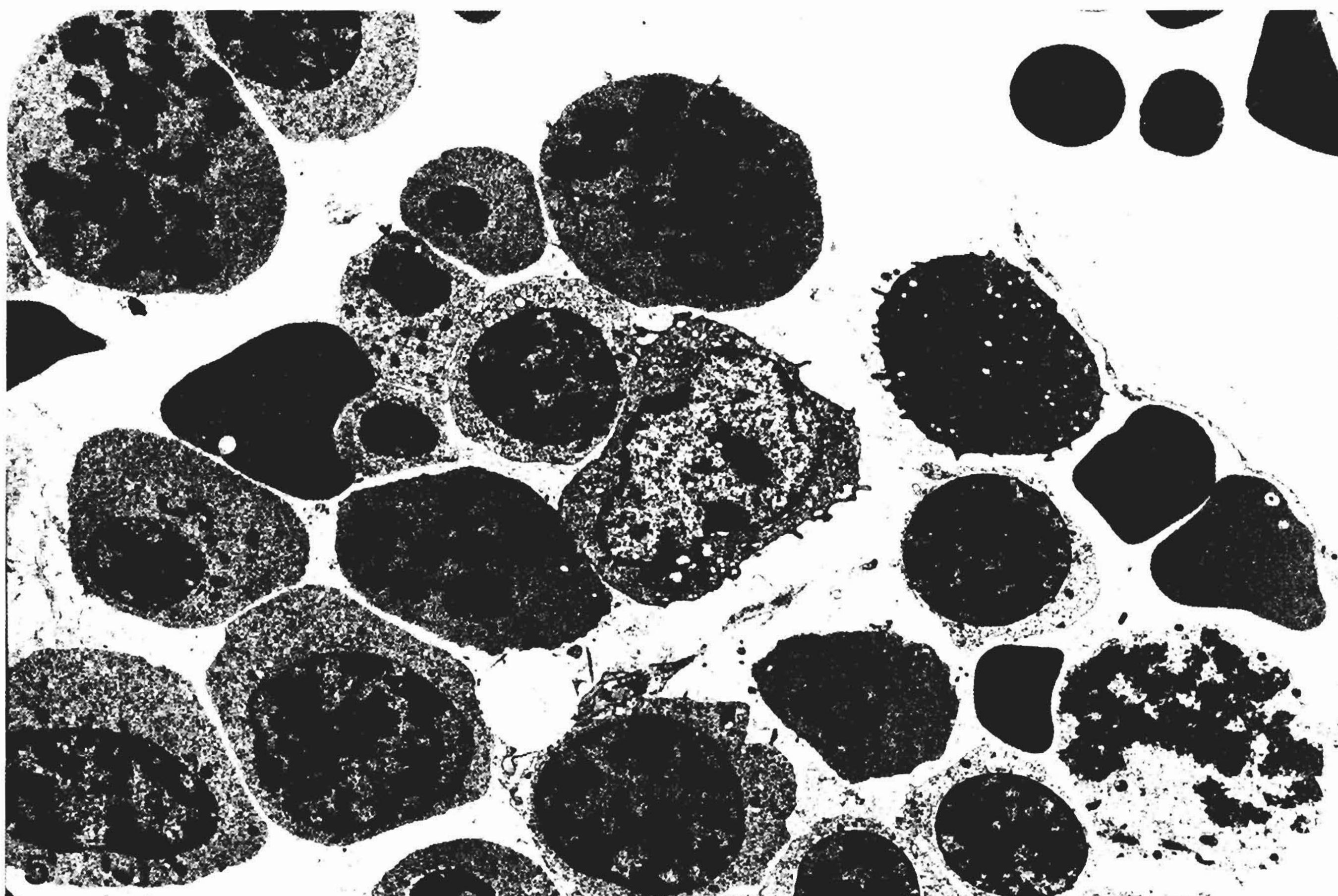


**Fig. 5.** 5 days postinjection. Haematopoietic focus with abundant mitosis. x 5,200

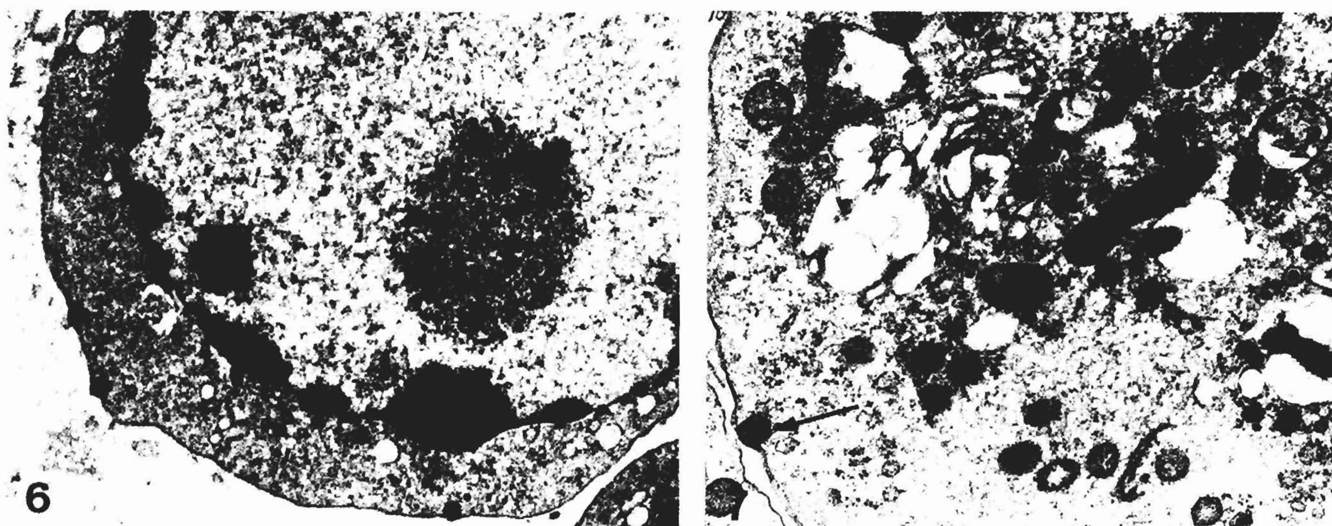



**Fig. 6.** 9 days postinjection. Lymphoid cells with intracytoplasmic mature ASF virus. x 14,520

**Fig. 7.** 5 days postinjection. Neutrophilic myelocytes with ASF virus replication site (*) and virus budding (arrow). x 21,100

sinus lumina 3 and 5 days postinjection, but had disappeared by 7 das postinjection. Intraluminal blood cells showed no significant morphological alterations, although there was evidence of monocyte activation, with phagocytosis of erythrocytes, necrotic or apoptotic cells and cell debris; virus replication was observed in blood cells at the same time as in macrophages. Circulating platelets showed signs of activation from 3 days postinjection. These changes have previously been described elsewhere (Gómez-Villamandos et al., 1996).

### *Hematopoietic cells*

Increased mitosis was observed in erythroid and myeloid cells from 5 days postinjection onwards (Fig. 5), while endomitosis was visible in megakaryocytes from 11 days postinjection.

The most significant changes in hematopoietic cells were the appearance of viral replication in different cell populations and a large number of apoptotic cells.

Free virions were observed within the cytoplasm of lymphoid cells (Fig. 6), but no replication sites were observed.

Viral replication was observed in promonocytes, neutrophilic myelocytes (Fig. 7), megakaryocytes (Fig. 8) and megakaryoblasts at 5 days postinjection; characteristics were similar to those described previously in macrophages.

In lymphoid cells, apoptosis observed at 3 and 5 days postinjection was far more marked than in controls.

**Table 1.** Number of megakaryocytes per area and per cent of apoptotic megakaryocytes of bone marrow inoculated with DR'78 ASF virus strain.

| | CONTROL | 1 dpi | 3 dpi | 5 dpi | 7 dpi | 9 dpi | 11 dpi | 13 dpi | 15 dpi | 17 dpi |
|---|---|---|---|---|---|---|---|---|---|---|
| MKs/area | 35.33 | 36.21 | 33.33 | 20.00* | 9.528* | 12.24* | 17.35* | 22.28* | 42.76* | 33.33 |
| Apop. MK (%) | 1 | 1 | 2 | 9* | 28* | 21* | 5* | 1.5 | 2 | 2 |

* P < 0.05 (each value vs control). dpi: days postinjection

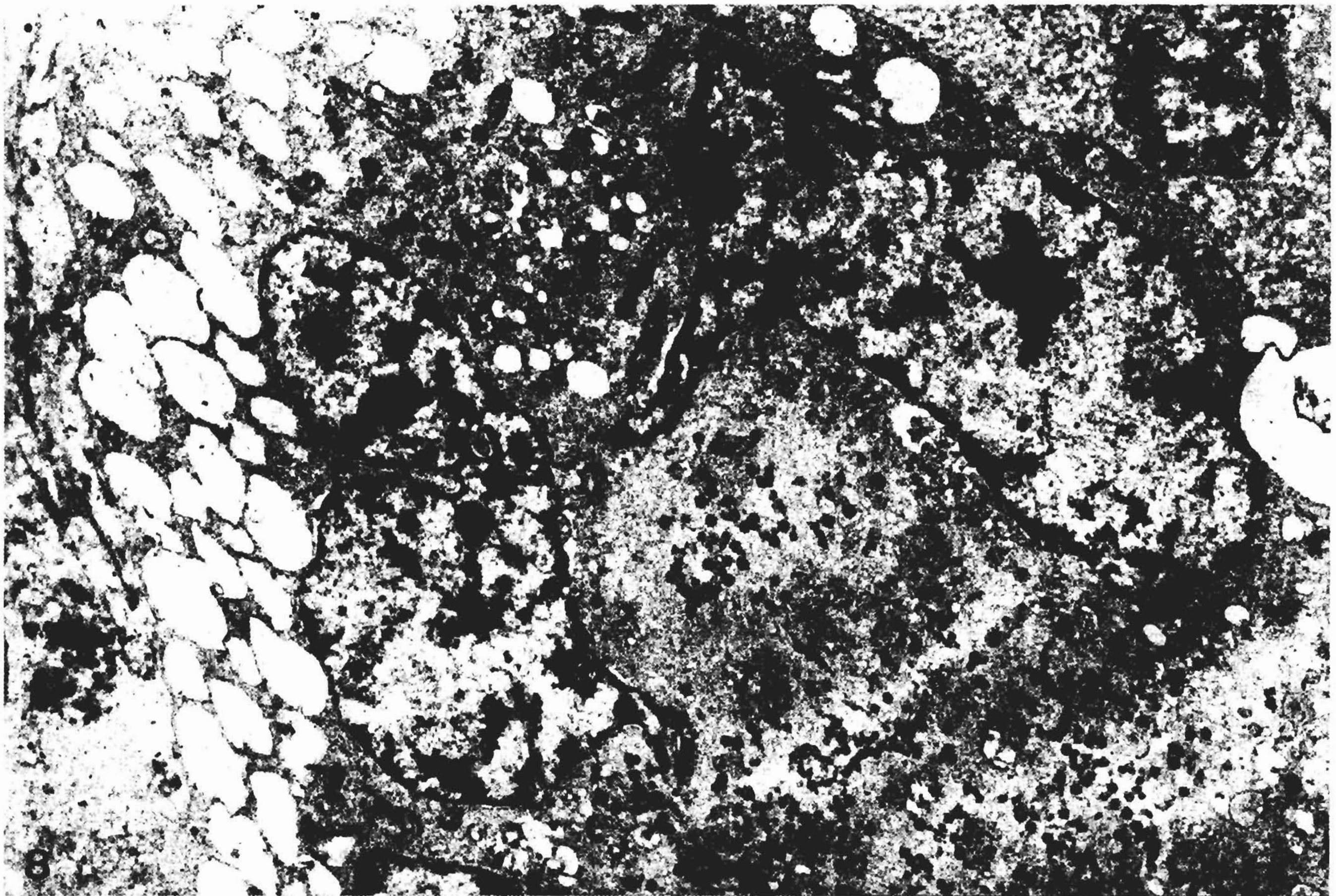



**Fig. 8.** 5 days postinjection. Virus replication in a megakaryocyte. Note the severe cytophatic effect. x 6,600

Counts on sections stained with antibody to antigen-related factor VIII showed a slight but significant drop in megakaryocytes per area. Nevertheless, the number of megakaryocytes undergoing apoptosis rose significantly between days 5 and 11, peaking at 7 days postinjection (Table 1).

Ultrastructurally, the nuclei of megakaryocytes in apoptosis appeared condensed, with abundant heterochromatin; cytoplasm was reduced to a fine perinuclear ring and contained few organelles. Chromatin arrangement was indicative of a high degree of maturity (Fig. 9).

## Discussion

Analysis of cells and structures belonging to the two main bone marrow compartments, the hematopoietic cells and the hematopoietic microenvironment, showed that after inoculation with a moderately virulent strain, the most significant changes occurred in macrophages and megakaryocytes.

In the hematopoietic environment, macrophage activation was only observed from 3 days postinjection, while viral replication was observed at 5 days postinjection and occasionally at 7 days postinjection.

The absence of viral replication at 3 days postinjection and the large number of cells affected at 5 dpi agrees with earlier findings (Gómez-Villamandos et al., 1997) in bone marrow after inoculation with a highly virulent strain, thus confirming the role of bone marrow as a secondary viral replication site following intramuscular inoculation of highly and moderately virulent viruses. Nevertheless, other authors (Heuschele, 1967; Plowright et al., 1968; Hess, 1971) have detected high virus titers in this organ at 48 hours postinoculation (hpi), which may be due to the greater blood content of this organ rather than viral replication in stromal and hematopoietic cells at these intervals.

Macrophages harbouring viral replication showed a cytopathic effect characteristic of the virus (Haag and Larenaudie, 1965; Pan, 1987; Sierra et al., 1987, 1989). Moreover, replication sites close to virions contained polyribosomes which acquired a helix formation, designated "helicoid polyribosomes" (Ghadially, 1988). This has been observed in cells showing a physiologically or pathologically high degree of multiplication, in mitotically active cells, and in cells that had been infected by other viruses (Djaczenko et al., 1970); and it

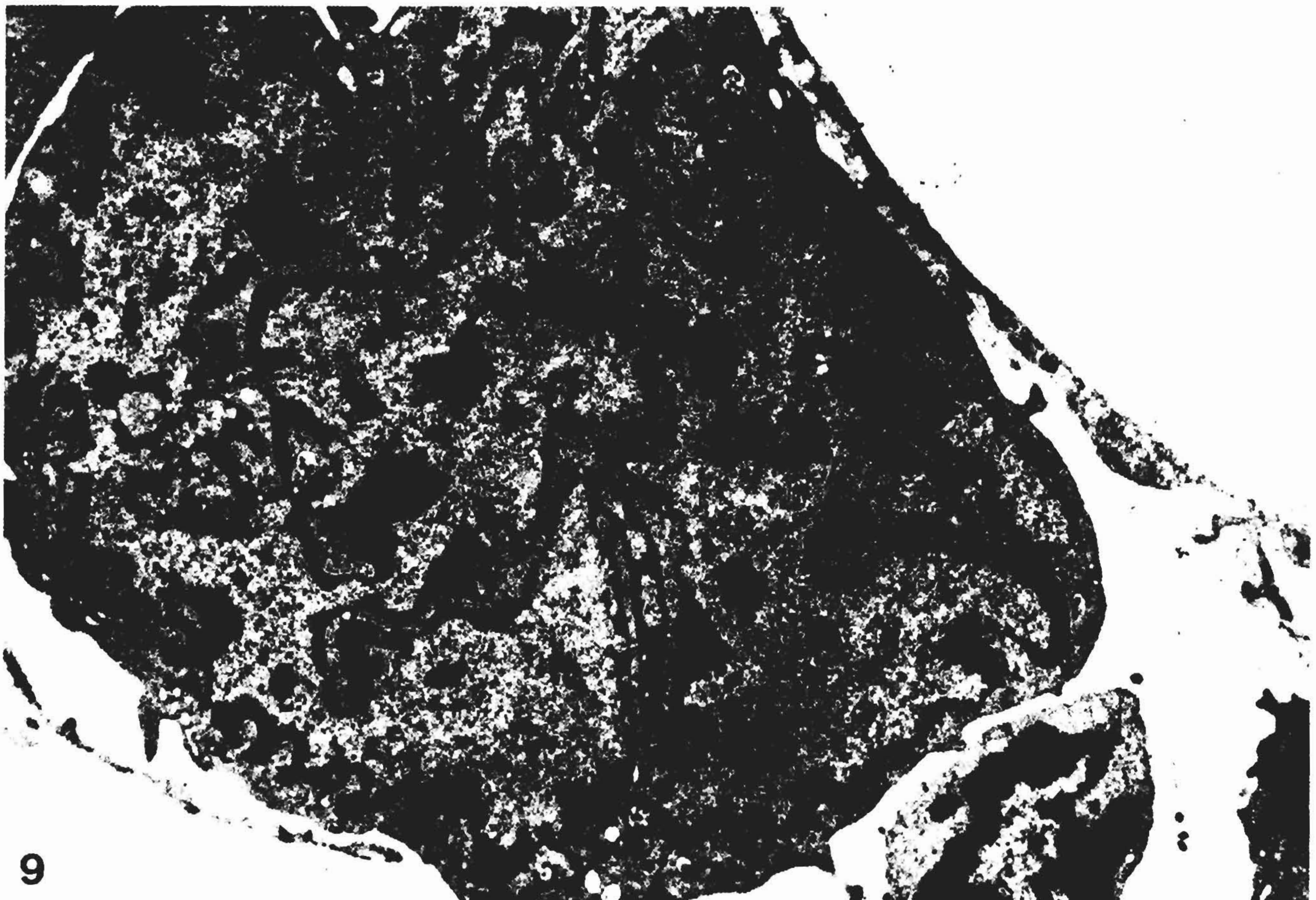



**Fig. 9.** 7 days postinjection. Apoptotic megakaryocyte. The nucleus appears condensed and the cytoplasm is reduced to a fine perinuclear ring. x

seems to be associated more with a change in the nature of the cellís messenger RNA than any polyribosome alteration (Ghadially, 1988). In ASF virus-infected cells, the production of abnormal messenger RNA is induced by the virus, which itself possesses a polymerase DNA-dependent RNA capable of synthesising complementary RNA from viral DNA (Kuznar et al., 1980; Salas et al., 1986) and the enzymes necessary for early RNA messenger synthesis (Viñuela, 1985).

The absence of viral particles from 9 dpi onwards may be related to the triggering of a mainly humoral immune response (Sánchez-Vizcaíno et al., 1981), which could create adverse conditions for virus replication. The virus may therefore "hide" in the monocyte/macrophage, replicating again when conditions are favourable. This hypothesis, requiring confirmation through future studies, is borne out by several findings. Viremia, as confirmed by hemabsorption testing, remains constant throughout the experiment, despite the absence of virions. Moreover, viral DNA detection tests using PCR showed the persistence of the virus over long periods of time (Carrillo et al., 1994).

In other microenvironment cells, neither cell activation nor viral replication were detected. Reticular and endothelial cells retained normal morphology, although Gómez-Villamandos et al. (1997) report different findings following inoculation with a highly virulent virus. Integrity of these cells, taken in conjunction with abundant mitosis observed in hematopoietic centres from 5 dpi onwards, suggests bone marrow compensation, which might account for the altered leukocyte formula in pigs inoculated with moderately virulent strains (Genovesi et al., 1988).

The second main cell group undergoing structural and ultrastructural modification, either through viral replication or indirectly-related mechanisms derived from viral infection, is the hematopoietic cells. Earlier results indicated a marked parallel between the time of appearance of apoptotic megakaryocytes and the transitory thrombocytopenia developed in these animals (Gómez-Villamandos et al., 1998). The considerable increase in the number of megakaryocytes undergoing apoptosis may be of vital importance in the development of thrombocytopenia. The term "apoptosis" refers to the programmed destruction of the cell mediated by the active selection of certain endogenous nucleases (Wyllie and Morris, 1982; Arends et al., 1990; Hatfill et al., 1992). This cell-death program may play an important role in the functional kinetics of hematopoiesis and megakaryocytopoiesis (Hatfill et al., 1992). Only a small number of megakaryocytes undergoing apoptosis are found in healthy bone marrow (Zucker-Franklin et al., 1989). Increased apoptosis associated with ASF seems to be due to the accelerated maturation of these cells owing to the release of cytokines by MPS cells (Gómez-Villamandos et al., 1998).

Finally, virus replication in immature neutrophils from 5 dpi agrees with *in vitro* findings reported by Casal et al. (1984), who detected replication in 4% of neutrophils following inoculation of both moderately- and highly-virulent isolates, and with the *in vivo* findings of Carrasco et al. (1996), after inoculation with a highly-virulent isolate, to an extent, sidelines the hypothesis advanced by Genovesi et al. (1988), according to which viral antigen in neutrophils is due to passive transport of the virus, as is the case with erythrocytes (Dardiri and Hess, 1970; Wardley and Wilkinson, 1977; Quintero et al., 1986). Viral replication in mature and immature neutrophils may play a major role in the spread of the virus through the organism given that it occurs in the early stages of the disease, characterised by neutrophilia and neutrophil accumulation due to the liberation of chemotactic factors by activated macrophages (Carrasco et al., 1996).

Virions were observed in the cytoplasm of a small number of lymphoid cells, though there was no evidence of replication. Similar findings were reported from immunocytochemical and *in situ* hybridization studies by Mínguez et al. (1988) and Galo and Nunes Petisca (1990), and from in vitro studies by Casal et al. (1984); lymphoid cells act as agents of passive diffusion of the virus throughout the organism.

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