

# **Glomerular pathology in surviving pigs experimentally infected with african swine fever virus**

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**Summary.** Twelve miniature pigs were inoculated with an attenuated African swine fever virus to study glomerular involvement in surviving pigs.

In acute phase, kidneys were severely affected and displayed a glomerular capillary thrombosis with fibrin deposition in vascular lumen, detected by immunofluorescence. Fibrin-positive deposits were progressively cleared between one to three months after infection in surviving pigs. The histological picture in kidneys of surviving pigs, up to one post-infection year, showed a focal and segmental glomerulonephritis with hyalinosis, and IgM and C3 deposition was detected by immunofluorescence. Its pathogeny as an evolutive stage of acute glomerular injury is pointed out.

**Key words:** ASFV, Glomerular, Pathology

## **Introduction**

Kidneys are organs widely affected in African swine fever (ASF), playing a partially known role in pathobiology, evolution and potential lethality of the disease (Mebus et al., 1983; Anderson, 1986).

Pathological changes of ASF consist of multisystemic hemorrhagic events and microcirculatory thrombosis in a disseminated intravascular coagulation (Pan, 1987), displaying diverse qualitative and quantitative disturbances in platelets and blood coagulation test (Edwards et al., 1984, 1985; Anderson et al., 1987).

Pathogeny is still obscure, and effects could be explained by direct viral injury (Moulton and Coggins, 1968; Colgrove et al., 1969), by modifications induced in monocyte macrophage system as main target cell (Anderson, 1986; Pan, 1987), immune-complex deposition (Slauson and Sánchez-Vizcaíno, 1981), etc.,

or a combination of these mechanisms (Pan, 1987).

The renal pathology in ASF has been described with a variety of morphological pictures ranging from hemorrhagic parenchymal necrosis (Maurer et al., 1958; Nunes Petisca, 1965a,b) and exudative glomerulopathy (Maurer et al., 1958; Moulton and Coggins, 1968; Konno et al., 1971), to a subtle chronic glomerulopathy. Moreover, infected cells were detected both in glomeruli and interstitial tissues, using anti-ASF virus serum (Colgrove et al., 1969; Pan, 1987; Sierra et al., 1989), and immune-complex deposition was also observed in glomeruli and tubules of infected pigs (Slauson and Sánchez-Vizcaíno, 1981).

The purpose of the present study was to identify and define the morphological changes in kidney from experimental ASF infection with respect to their physiopathology.

## **Materials and methods**

### *Virus*

The ASFV strain Spain 75, partially attenuated by four passages in CV1 cell line (ATCC, CCL 70) was used.

### *Animals*

Eighteen six-month-old miniature pigs, SLA<sup>DD</sup>, were used in these experiments. Twelve were inoculated intramuscularly with 10<sup>4</sup> hemadsorption units (HAD 50) of ASF virus per pig. Six non-infected pigs were used as controls (pigs 1-6). Controls and test pigs were sampled at weekly intervals to determine changes of circulating antibodies and viremia. Five pigs died between post-infection days (PID) 8-13, and surviving pigs after transient clinical illness, were sacrificed on PID 8, 30, 90, 120, 150 and 365 (2) (pigs 7-13). Control pigs were sacrificed at the same time of inoculated.

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### Pathological studies

At necropsy, tissue and blood were collected for virus isolation and tissues were fixed in the following fixatives 10% buffered formalin; B-5; Dubosq-Brazil; Zenker and Bouin; and some samples were embedded in O.C.T. compound (Miles Lab., Inc., Elkhart, IN) and quick-frozen in methylbutane, cooled with liquid nitrogen and then stored at -70° C. Fixed tissues were then embedded in paraffin, sectioned and stained with H & E, Masson trichrome, Periodic-acid Schiff (PAS), methenamine silver, phosphotungstic acid-haematoxylin (PTAH), and Congo Red.

### Special stains

**Immunofluorescence:** Frozen kidney samples for studying immune-complex and fibrin deposition were cut at 4 µm, mounted and air-dried. The slides were then washed in two changes of 0.01 M phosphate-buffered saline (PBS) at pH 7.4 to remove unbound serum proteins.

Sections of the experimental and normal pig kidney specimens were incubated in a moist chamber at room temperature for 30 to 45 minutes with the following monoclonal antibodies (MAb) and polyclonal antibodies anti-pig IgG and IgM; rabbit anti-pig IgA; fluorescein-conjugated rabbit anti-pig gammaglobulins, fibrinogen and C3. Thereafter, sections were washed again in three changes of 0.01M PBS and incubated for 30 to 45 minutes with fluorescein-conjugated anti-mouse or anti-rabbit according to the cases. After washing, coverslips were applied with buffered glycerin and the specimens examined with a fluorescence microscope.

**Indirect immunoperoxidase:** Slides were incubated with the MAb ascitic fluid diluted 1:40 in Tris-buffered saline (TBS) pH 7.6 for 1 hour. After rinsing with TBS, slides were incubated with peroxidase-conjugated rabbit anti-mouse Ig (Dako, Copenhagen, Denmark) at 1:20 dilution in TBS pH 7.6 with 30% normal swine serum. After extensive washing, peroxidase was developed and slides were counterstained with Carazzi's haematoxylin, dehydrated and mounted with DePex (Serva). Negative controls were performed with an irrelevant MAb.

### Antiserum production

Fluorescein-conjugate anti-pig gammaglobulins and anti-pig fibrinogen antisera were produced in rabbits by multiple injections of porcine serum proteins and commercial porcine fibrinogen (Sigma Chem. Co, St. Louis, MO) in complete and incomplete Freund's adjuvants.

MAb reactive with porcine peripheral blood lymphocytes (PBL) were obtained from Dr. J.K. Lunney: 74-12-4 reactive with T-helper lymphocytes; 76-2-11 reactive with cytotoxic/suppressor lymphocytes; 74-22-15 reactive with macrophages and granulocytes (Pescovitz et al., 1984) and 5C9 anti porcine IgM (Paul et al., 1985).

Monoclonal antibody C3 reactive with porcine IgG,

and MAb 1G1 reactive with peripheral blood monocytes/macrophages and granulocytes, were produced in our own laboratory.

### Results

The twelve pigs inoculated developed viremia, clinical evidence of disease and the surviving pigs were viremic up to PID 41. Specific anti-ASFV antibodies were detected by indirect immunofluorescence at titres ranging from 1/40 on PID 26, to 1/5120 on PID 82 and decreased progressively thereafter.

Pathological features observed in the pig sacrificed on PID 8 (acute phase), included multivisceral hemorrhages and splenomegaly. Nevertheless, the only findings in the organs from surviving pigs were kidney pallor with tiny brown spots dispersed in cortical areas, fibrinous pericarditis in pig 11, reddish skin papules in pig 12, and myocardial fibrosis in pig 13.

Glomeruli in the pigs sacrificed on PID 8 (pig 7) exhibited focal to diffuse PAS-positive material occluding the capillary lumina (Fig. 1), fibrin intracapillary deposits strongly stained with phosphotungstic acid-haematoxylin (PTAH), and there was formation of early crescents.

There was evidence of thrombosis in arterioles and interlobular arteries and renal cortical necrosis with broad areas of haemorrhagic ischemic devitalized tissue. Tubular casts with intense PAS positivity, and swelling of proximal tubular epithelial cells with different grades of detachment, were prominent outside ischemic areas.

The disease developed a high degree of uniformity in kidneys from surviving pigs; light microscopic examination revealed focal and segmental hyalinosis from PID 30 to 365. Glomeruli in juxtamedullary cortex became preferentially affected, and pathological changes were obvious in no more than 10-30% of glomeruli per section examined. Glomerular volume increased and hyaline material (PAS-positive, Congo Red-negative) expanded the mesangium. With Masson trichrome, there was no underlying fibrosis. The increase in mesangial matrix was accompanied by increased in mesangial cell number (Fig. 2).

Frequently, there was leakage of proteinaceous fluid in the urinary space with vacuoles and foamy macrophages. This was associated with prominence of parietal epithelial cells, which contained protein resorption droplets with occasional formation of early crescents and adhesions to Bowman's capsule (Fig. 2).

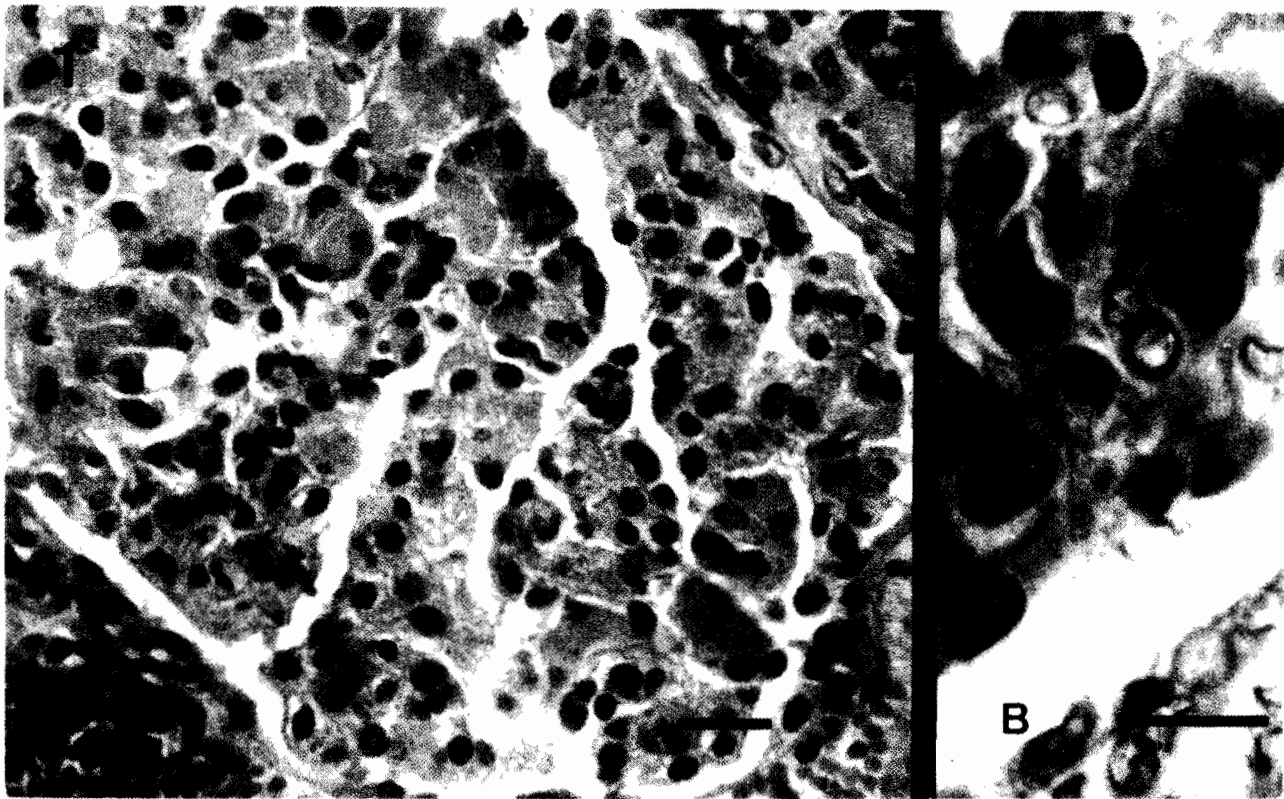
In the surviving pigs sacrificed early post-infection (pigs 8, 9, 10, 11), there were additional findings that appeared less prominent in pigs sacrificed a year after acute injury: visceral epithelial cells were often hypertrophic and detached from the basement membrane which appeared denuded in its outer aspect with a double-contour or garland pattern (Fig. 3A).

Thickening of capillary walls with possible subendothelial deposits was observed occasionally up to PID 30 (Fig. 3B). Some capillary lumina were occluded by formation of hyalinosis lesions, distributed in

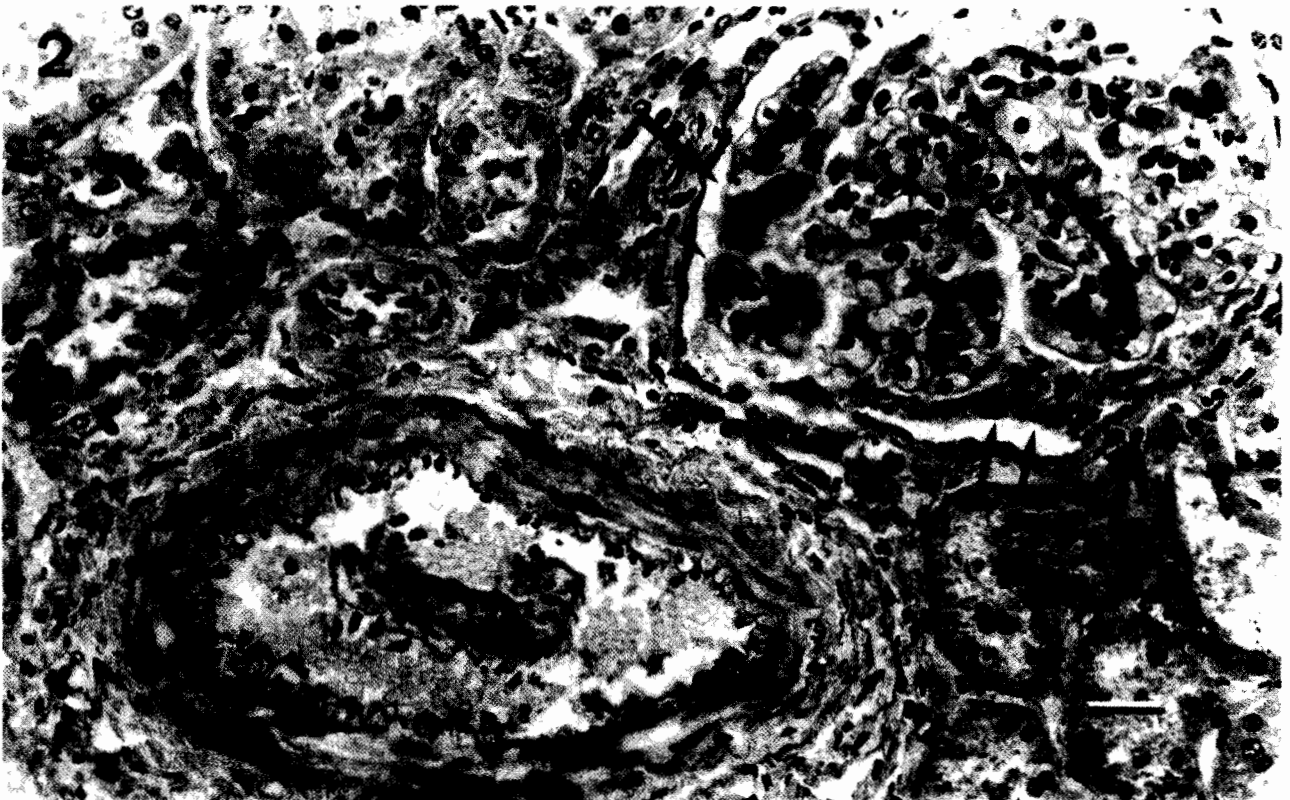
**Table 1.** Immunofluorescence staining scores in glomeruli of survivor pigs at different postinfection times

PIGS	1 - 6	7	8	9	10	11	12 - 13
SACRIFICE DATA	UNINFECTED CONTROL	8 PID	30 PID	90 PID	120 PID	150 PID	365 PID
Ig M	-	Not Done	4 + granular difuse	4 +	-	3 +	3 +
Ig G	1 +	Not Done	Not Done	1 +	1 +	-	1 +
Ig A	-	Not Done	-	-	-	-	-
TOTAL GAMMAGLOBULINS	-	Not Done	1 +	1 +	1 +	1 +	1 +
C <sub>3</sub>	-	2 +	2 + focal	3 +	2 +	1 +	1 +
FIBRIN	-	4 +	4 +	-	-	-	-

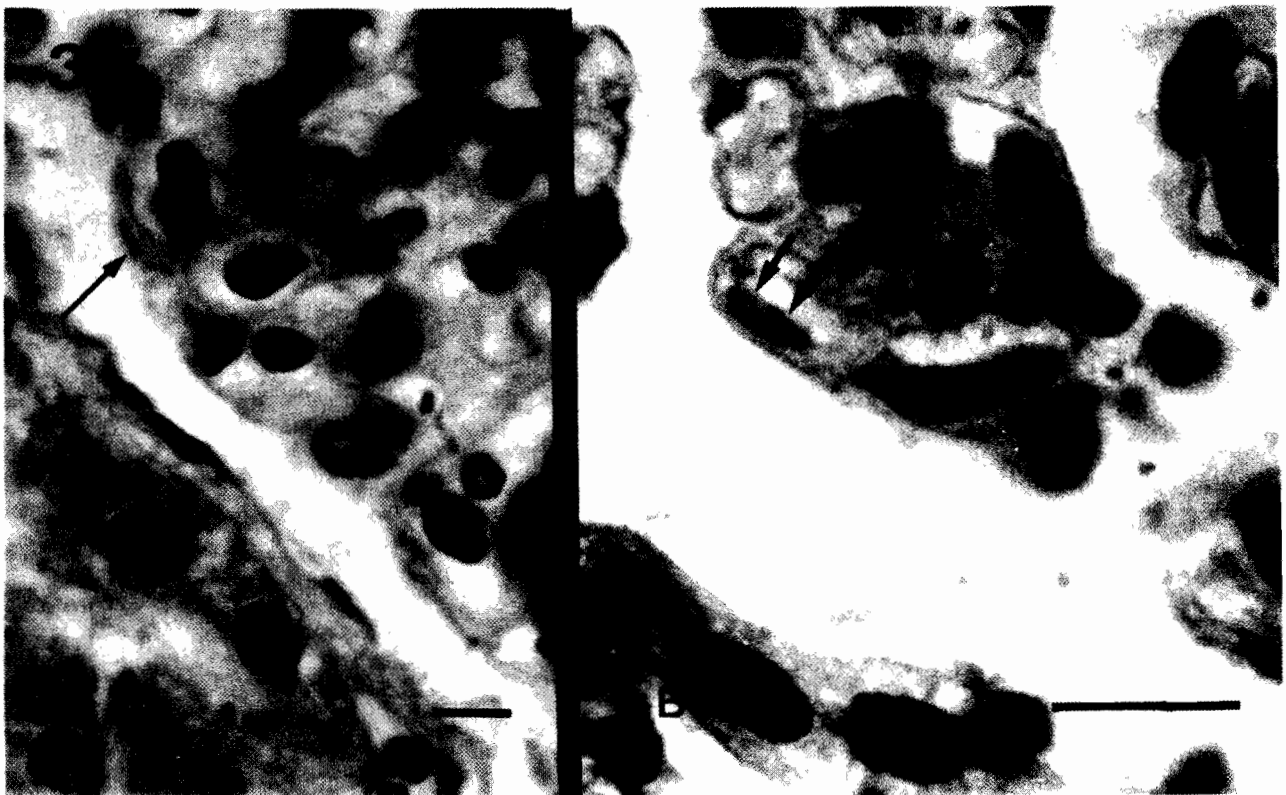
(- none; 4 + maximum)



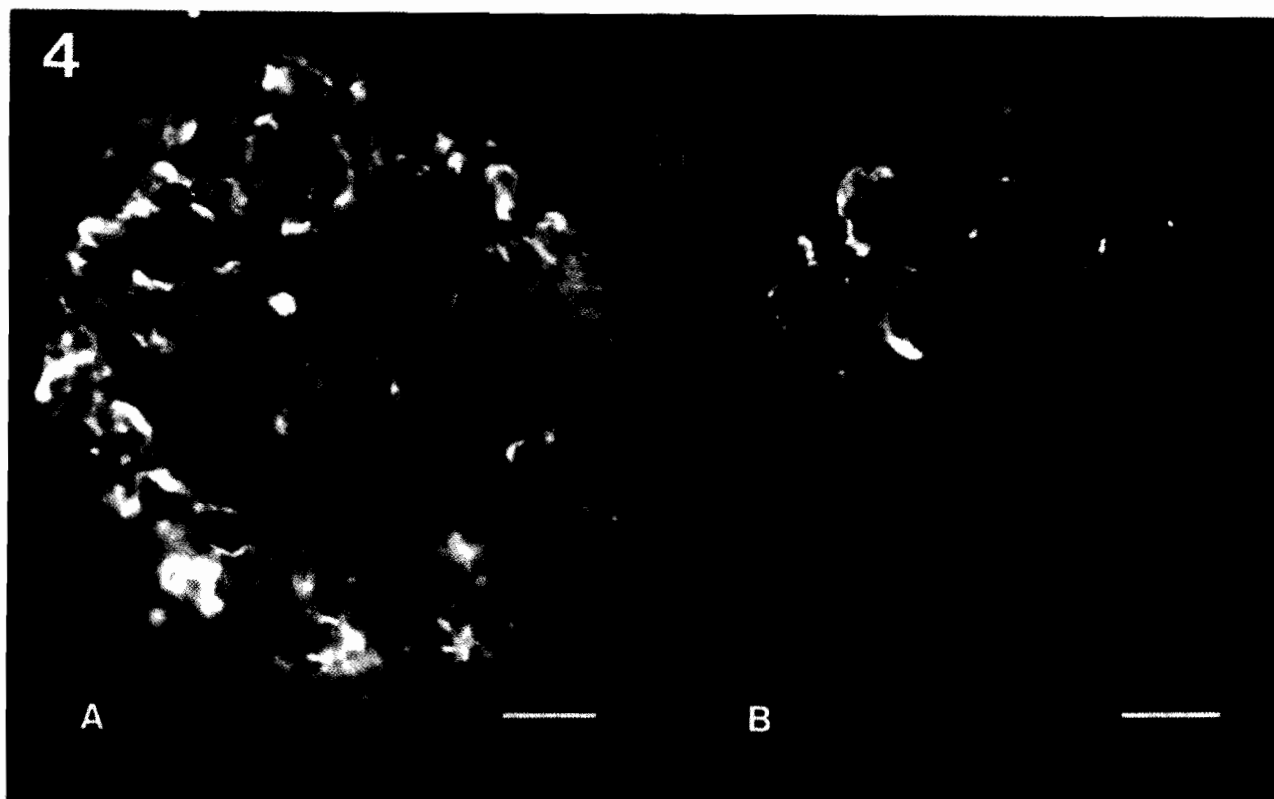
**Fig. 1.** Disseminated capillary thrombosis in glomerular tuft in acute african swine fever. B: Detail of fibrin thrombi. Pig 7, PID 8, H&E. Bar 10  $\mu$ m.



**Fig. 2.** Surviving pig glomerulus with broadening of mesangial matrix (arrow), double contour capillary walls (arrowhead) and proteinaceous fluid in the urinary space (double arrow). Small size artery with parietal changes. Pig 13, PID 365, H&E. Bar 20  $\mu$ m.



**Fig. 3.** Double contour of capillary walls (arrow). B: Occasional subendothelial deposits (double arrow). Pig 8, PID 30, H&E. Bar 5  $\mu$ m.



**Fig. 4.** Immunofluorescence. A: IgM-staining of mesangium and capillary wall, in a diffuse, finely granular pattern. B: C3 segmental capillary wall staining in a coarsely granular pattern. Pig 9, PID 90. Bar 20  $\mu$ m.

a segmental way, that were often localized at the glomerulo-tubular junction (tip opposite to hilum). Platelet or fibrin thrombi were occasionally seen, as well as congested and dilated capillaries with formation of microaneurysms. Glomeruli from control pigs exhibited no change.

At interstitial level focal tubular necrosis in pigs 8, 9, 10, 12 was found. Vascular alterations were often observed and included hyaline or platelet thrombi identical to those seen in the glomeruli, parietal hypertrophy, subendothelial edema and periarteriolar fibrosis (Fig. 2). They were mostly restricted to arterioles and interlobular arteries.

Pig 8 showed a small focus of mononuclear peritubular infiltrate constituted mostly of macrophages (MAb 74-22-15 and 1G1) and suppressor T-lymphocytes (MAb T8) in the periphery.

Cellular proliferation typing in glomeruli and quantitation of cell number were done, both in infected and control pigs. Besides mesangial cell proliferation, there were a slightly higher number of macrophages in glomeruli from infected pigs when compared to controls. No significant differences were found with T4 and T8 antibodies.

Results obtained with immunofluorescence corresponding to sacrificed pigs are summarized in Table 1 and quantified on a scale of minus to 4+ (-, none; 4+ maximum) and all of them were positive for IgM and C3.

Glomeruli of control pigs were negative for IgM and C3, and baseline level of 1+ mesangial staining for IgG

was present. Surviving pigs showed capillary wall and mesangial staining for IgM in a diffuse and finely granular pattern (3 to 4+). There was also discontinuous capillary wall staining for C3, coarsely granular with some negative segments. Moderate C3 staining of tubular basement membrane was observed in three cases (pigs 10, 12, 13). In addition, slight staining for total gammaglobulins was present and IgG remained at baseline levels. IgA was negative.

In pig 7 (acute ASF), the thrombotic material existing in many glomerular capillary lumina exhibited strong staining for fibrin. Surviving pig 8, sacrificed early after infection (PID 30), showed some capillary lumina staining for fibrin in pigs 8 and 10 (PID 120) positivity of intertubular connective tissue was also seen. Fibrin was negative in surviving pigs sacrificed later. In pig 7, tubular cylinders were surrounded by C3 staining.

## Discussion

Experimental ASFV-infected pigs developed glomerulopathy, that in the animal sacrificed in extremis on PID 8, displayed the ASF acute-phase morphology: a glomerular capillary thrombosis. At glomerular level edema and vacuolization of endothelial cells and fibrin subendothelial deposits were observed, resulting in narrowing of the vascular lumen and thrombosis. Fibrin deposits altered filtration barrier and allowed an increase in permeability with leakage of fibrin and other proteins to the urinary space which was accompanied in our cases

by hyperplasia of the Bowman's capsule epithelium. These lesions typically occurred in the glomerular pole opposite to the hilum and often projected into the proximal tubule as an eosinophil cast. In the acute phase, various authors found evidence of disseminated intravascular coagulation (DIC) (Pan et al., 1975; Anderson, 1986). Microcirculatory thrombosis is partly due to altered coagulation parameters seen in ASF, such as increase in serum fibrin degradation products (Edwards et al., 1984), and disfibrinogenemy with low quality fibrin thrombi (Edwards and Dodds, 1985).

The quantitative effects in disseminated intravascular coagulation were demonstrated to depend on the degree of saturation of the mononuclear-phagocytic system and its ability to remove fibrin aggregates from circulation. The production of experimental DIC in rats was more effective if blockers of the monocyte-macrophage system are previously given (Galera-Davison et al., 1989). This system is severely compromised in ASF (Mebus et al., 1983; Mínguez et al., 1988).

Our finding of intraglomerular macrophages could be of interest because they could be a main local source of procoagulant mediators responsible for fibrin deposition, as demonstrated in experimental models of glomerulonephritis with macrophage infiltration (Tipping et al., 1987).

On the other hand, during the first postinfection year, glomeruli showed a picture of focal segmental glomerulonephritis with hyalinosis. Focal segmental glomerulonephritis with hyalinosis and other non-acute changes of the disease in surviving pigs, were probably produced through indirect changes in immune system, as was demonstrated for pneumonia (Pan et al., 1975). Immune-complex deposits (Slauson and Sánchez-Vizcaíno, 1987), lymphocyte function depression (Sánchez-Vizcaíno et al., 1981) and interaction of virus with monocyte-macrophage system (Pan et al., 1987; Mínguez et al., 1988), could be implicated in glomerular damage.

Acute and chronic serum sickness or experimental nephrotoxic nephritis are models of cell-mediated mechanisms of glomerular injury in which macrophages play a central role (Hunsicker et al., 1979); they have also been recently incriminated in experimental focal and segmental glomerulosclerosis (Matsumoto and Atkins, 1989). Mesangial cells, platelets and lymphocytes intervene in other cell-mediated mechanisms. Glomerular damage could also be based on antibody action or direct complement injury, either interacting with neutrophils or independent of inflammatory cells as in passive experimental Heymann nephritis (Glasscock et al., 1968). The type of glomerulonephritis developed depends on the localization of immune-complex deposition, and also on the glomerular response: cellular proliferation, basement membrane discontinuity, mesangial activation, etc. and the histological picture varies by means of distribution and severity of those parameters (Kincaid-Smith and Whitworth, 1987).

However, glomerular lesions may also be mediated

by non-immunological mechanisms, such as compensatory renal hypertrophy, coagulation, endothelial injury, release of products like prostaglandins, and cellular proliferation as occurs in experimental hypertension and after unilateral renal ablation (Olson and Heptinstall, 1988). Kidney pathology in the latter two events appeared histologically identical in surviving ASF-pigs.

In our cases, immune-complex deposition in glomeruli of infected pigs (IgM and C3) was found, which, besides optical findings, makes adequate the diagnosis of focal and segmental glomerulonephritis (Kincaid-Smith and Whitworth, 1987). The optical and ultrastructural findings reported from other authors as podocyte pedicellar fusion and basement membrane thickening found by electron microscopy, are also in agreement with this model of glomerulonephritis (Quezada and Whitworth, 1989). Depressed serum-complement values and glomerular immune-complex deposition composed of IgG and C3 were also detected in kidney of ASF-infected pigs, without demonstrable morphological lesion at PID 15. The authors considered the possibility of ulterior development of optical lesion (Tipping et al., 1987).

Also, forms of focal and segmental glomerulonephritis have been described in human viral diseases, in acquired immune deficiency disease (AIDS), and its pathogenesis is still unclear. The lesion is characterized by localized hyperplasia and vacuolization of the visceral epithelial cells, which coincides with our results. Immunofluorescence findings are frequently of IgM and C3 in the same pattern, though other immunoglobulin classes may also be localized (Rao et al., 1987).

Human pre-eclamptic glomerulonephritis (PEG) in toxemia of pregnancy, also displays a histological appearance of diffuse intracapillary thrombosis and in subsequent biopsies, fibrin thrombi of acute phase may be replaced by segmentary hyalinosis and hyaline thrombi or translucent areas (Kincaid-Smith and Whitworth, 1987), similar to those observed in our and other reports (Maurer et al., 1958; Moulton and Coggins, 1968; Tipping et al., 1987). In fact, heavy fibrin deposition on PID 8, was progressively cleared in PID 30 and 120.

In PEG as in ASF, epithelial Bowman's cells were prominent with an enlarged and vacuolated cytoplasm; this may be the consequence of protein reabsorption in the urinary space, as a result of an overload on the capability of the glomerulus to clear unfiltered macromolecules. In the resolving phase of PEG, during remodelling of basement membrane, it showed a double contour or garland image, similar to that of mesangiocapillary glomerulonephritis, sometimes persisting for months (Kincaid-Smith and Whitworth, 1987).

Other authors considered that this membrane pattern could be just due to the continuation of the mesangial matrix around capillary loops.

Although this matter should be the subject of further



studies, the same findings in surviving pigs of experimental ASF, as in the evolutive stage of PGE could mean evolution of an acute phase not severe enough to cause death in the animals.

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