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Detection of African swine fever viral antigens in paraffin-embedded tissues by use of immunohistologic methods and polyclonal antibodies

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SUMMARY

Tissues obtained from pigs inoculated with African swine fever virus (ASFV), fixed by vascular perfusion using glutaraldehyde, and embedded in paraffin or araldite were used for an immunohistologic electron microscopic study. To detect ASFV antigens, 4 methods were used on paraffin sections with or without pretreatment of the tissues. Use of biotinylated anti-ASFV antiserum combined with avidin-biotin complex and peroxidase proved to be the most suitable method, and antigen was detected in tissues infected with 2 ASF viruses of different virulence.

Use of the glutaraldehyde fixation method should ensure optimal morphologic (structural and ultrastructural) data while allowing an immunohistologic study, and add to knowledge of the pathogenesis of ASF.

African swine fever (ASF), an important disease of domestic pigs, is caused by an icosahedral cytoplasmatic DNA virus classified as a member of the family Iridoviridae.[1]

The ASF virus (ASFV) isolates differ in virulence, hemadsorption, and antigenic properties.[2] Thus, the E70 ASFV isolate is classified as highly infective and highly virulent, whereas the E75 ASFV isolate is considered highly infective and moderately virulent.[3]

The ASFV replicates primarily in mononuclear phagocyte system (MPS) cells,[2-4] although other replication sites have been reported.[5-7] When generalized infection develops, high concentration of virus is found in lymph nodes, spleen, lungs, liver, and kidneys, which are the main sites of secondary replication.[1-3]

Use of immunologic techniques on fixed tissue greatly facilitates the study of viral pathogenesis by allowing the identification of specific cell types infected and the associated tissue changes. Tissue fixation by vascular perfusion with glutaraldehyde results in excellent ultrastructural detail with good preservation of membranes.[8] However, this fixation method is not usually used for immunohistologic studies because it has greater cross-linking potential than does formalin, which is used for light microscopy and is not suitable for detection of many antigens.[9]

The objective of the study reported here was to detect ASFV antigens in glutaraldehyde-fixed, paraffin-embedded tissues of pigs inoculated with 2 ASFV isolates by use of several immunohistochemical techniques and preparatory treatments of tissue sections.

Materials and Methods

Pigs—Eighteen Large White × Landrace male pigs were studied, each with live weight of approximately 20 kg at the beginning of the study and free of parasitic and infectious diseases. Pigs were allotted to 2 groups (1 and 2), each containing 9 animals, and were inoculated with different strains of ASFV. The 2 groups were further allotted to 3 subgroups, each consisting of 3 pigs. Two pigs from each subgroup were inoculated IM with 5×10^5 50% hemadsorbing doses of either the highly virulent E70 isolate or the attenuated E75 isolate of ASFV.[a] The third subgroup served as a control. Pigs were euthanatized on post-inoculation day (PID) 3, 5, or 7 (group 1) or on PID 9 or 11. Two pigs of group 2 were found dead on PID 12. All pigs euthanatized during the study were anesthetized with azaperone and thiopental prior to vascular perfusion with glutaraldehyde.[4]

Collection of specimens—Organs were fixed by perfusion with 2.5% glutaraldehyde in 0.1*M* phosphate buffer. Specimens of lungs, liver, spleen, and lymph nodes were embedded in paraffin wax or araldite.

For the structural study, sections of various thickness were taken, depending on the type of embedding. Staining techniques used were H&E and toluidine blue. For ultrastructural examination, 60-nm sections embedded in araldite were stained with uranyl acetate and lead citrate.

Antisera—Porcine-origin, anti-ASFV hyperimmune serum[b] was obtained. For conjugation with horseradish per-

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[a] Courtesy of INIA, Madrid, Spain.

[b] Courtesy of Laboratorio Regional Pecuario, Córdoba, Spain.

oxidase,[c] the IgG fraction of hyperimmune serum was isolated by ammonium sulfate precipitation, according to a reported method.[10] After desalting by dialysis and gel chromatography,[d] the purified IgG fraction was conjugated with horseradish peroxidase.[10] Conjugated and nonconjugated antisera were stored at −40 C until use. Biotinylated swine IgG directed against ASFV was obtained.[a]

Preparation of sections—Endogenous peroxidase activity was inhibited on deparaffinized tissue sections by preincubation with 2.5% H_2O_2 in methanol, followed by 2 rinses in 0.05*M* tris-buffered saline solution (TBSS). At that point, sections were treated either with enzymatic agents (trypsin,[e] a protease from *Streptomyces griseus*[f]) or with nonionic detergents,[g,h] to compare both procedures for detecting ASFV antigens in glutaraldehyde-fixed tissues.

After pretreatment, sections were rinsed once for 10 minutes in TBSS, then were incubated for 30 minutes at 20 to 22 C (room temperature) in a blocking solution consisting of 0.015% normal goat serum in TBSS.

Immunohistologic Techniques

Four procedures were performed on paraffin-embedded tissues from pigs infected with the E75 ASFV isolate: direct immunoperoxidase, indirect immunoperoxidase (IIP), avidin-biotin complex with peroxidase (ABC-I) and modified (2-step) methods (ABC-II). This last technique, ABC-II, was the only one applied on tissues from pigs inoculated with the E70 ASFV isolate.

Direct immunoperoxidase—Tissue sections were incubated for 1 hour at 37 C with peroxidase-labeled swine IgG anti-ASFV diluted 1:10 in phosphate-buffered saline solution (PBSS).

Indirect immunoperoxidase—Nonconjugated swine IgG anti-ASFV and peroxidase-labeled rabbit anti-swine IgG[i] were used after determination of the optimal dilutions by titration (1:120 and 1:90, respectively). Incubation time of 1 hour at 37 C and 16 hours at 4 C yielded optimal results.

Three-step ABC-peroxidase technique—This technique was performed as follows. Nonconjugated swine IgG anti-ASFV (1:120, 1 hour at 37 C), biotinylated goat IgG anti-swine IgG[j] (1:100, 30 minutes at 20 to 22 C), and ABC[k] (1 hour at 20 to 22 C) were used.

Two-step ABC-peroxidase technique—The ABC-II was performed as follows. Sections were incubated either 1 hour at 37 C or overnight at 4 C with biotinylated swine IgG anti-ASFV diluted 1:10 in PBSS. The ABC was then applied for 1 hour at room temperature. This technique was also used for 10% formalin-fixed samples obtained on PID 12 to compare formalin and glutaraldehyde fixation.

To detect viral antigens that could have been masked by immunoglobulins, the elution technique described by Porter et al,[11] using a buffer acid 3 hours after deparaffination and endogenous peroxidase inhibition, was applied to glutaraldehyde and formalin-fixed tissue sections. The substrate for peroxidase, 3,3-diaminobenzidine tetrahydrochloride[l] (DAB) was used as described.[10] After rinsing, slides were counterstained with hematoxylin, then washed, dehydrated, and mounted in routine manner.

Tissues from control pigs were used as negative controls for all 4 immunoperoxidase methods. In addition, specific anti-ASFV serum was substituted in infected tissues with the following reagents: normal goat serum, normal rabbit serum, and nonimmune swine serum.

Results

Histopathologic Study

Isolate E75 ASFV experiment—At PID 9, lymphoid follicle hyperplasia and scattered cell necrosis in the subcapsular area were seen. Necrosis was more frequent at PID 11, and was associated with hemorrhages. Severe necrosis, similar to that described for the highly virulent virus, was seen at PID 12.

Similar lesion development was observed in the spleen. Necrosis was first seen in the red pulp and, at PID 12, the white pulp was also affected with abundant erythrocytes and fibrin.

In the liver, stellate macrophage hyperplasia with hemadsorption was observed at PID 9. At PID 11, abundant intrasinusoidal necrosis of neutrophils and lymphocytic cells was observed. Multifocal necrosis of hepatocytes and severe interstitial tissue edema were the most relevant hepatic lesions.

At PID 9, the interalveolar septae contained abundant mononuclear cells. At PID 11, diffuse intravascular cell necrosis of circulating cells and alveolar macrophages associated with cell debris and fibrin were evident. These lesions were more numerous at PID 12.

Isolate E70 ASFV experiment—The lymph nodes and spleen had progressive cellular necrosis, beginning in the subcortical lymph node areas and the red pulp at PID 3. The whole parenchyma was involved by PID 7.

Only scattered pyknotic cells were observed in hepatic sinusoids at PID 3. However, progressive multifocal parenchymal necrosis was evident at PID 5 and 7.

In the lungs, the main changes were confined to the interstitial tissues. Thus, increased numbers of septal cells were observed at PID 3, and abundant necrotic cells were seen by PID 5 and 7.

Immunohistologic Results

Glutaraldehyde-fixed, paraffin-embedded tissues from pigs infected with ASFV-E75 and euthanatized at PID 9 were used to perform the pretreatments and the various immunoperoxidase techniques (Table 1). Two variables were considered—specific staining and background staining.

[c] Peroxidase V type VII, Sigma Chemical Co, Munich, Germany.
[d] Sephadex G-25, Pharmacia Fine Chemicals, Uppsala, Sweden.
[e] Trypsin, Fluka Co, Buchs, Switzerland.
[f] Pronase, Sigma Chemical Co, Munich, Germany.
[g] Tween 20, Panreac, Barcelona, Spain.
[h] Triton X-100, Panreac, Barcelona, Spain.
[i] RASw/IgG (H+L)/PO, Nordic Immunological Laboratories, Tilburg, The Netherlands.
[j] Biotinylated anti-swine IgG (H+L) immunoglobulin produced in goats, Vector Laboratories, Burlingame, Calif.
[k] ABC Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif.
[l] 3,3'-Diaminobenzidine, Sigma Chemical Co, Munich, Germany.

Table 1—Staining reactions obtained on paraffin-embedded tissues, using various pretreatments and staining techniques

Variable	Pretreatment				
	NT	Pronase	Trypsin	0.1% Tween	0.1% Triton
Exposure time (min)		10	30 to 60	10 to 15	10 to 15
Temperature		RT	37 C	RT	RT
pH		ND	7.6	7.6±0.2	7.6±0.2
Method/reaction					
DIP					
SS	−	+	−/+	+	+
DBS	++	++	++	++	++
IIP					
SS	−/+	++	+/++	++	++
DBS	++	++	++	+/++	++
ABC-I					
SS	−/+	++	++	+++	++/+++
DBS	++	+/++	+/++	+	+/++
ABC-II					
SS	−/+	+/++	+/++	++/+++	++
DBS	−	+	+	−	−

Immunostaining: − = absent; −/+ = uncertain; + = scanty; +/++ = moderate; ++ = abundant; ++/+++ = abundant to highly abundant; +++ = highly abundant. NT = no pretreatment; RT = room temperature (20 to 22 C); DIP = direct immunoperoxidase; IIP = indirect immunoperoxidase; ND = not determined; SS = specific staining; DBS = diffuse background staining; ABC-I = avidin-biotin complex with peroxidase; ABC-II = modified (2-step) methods.

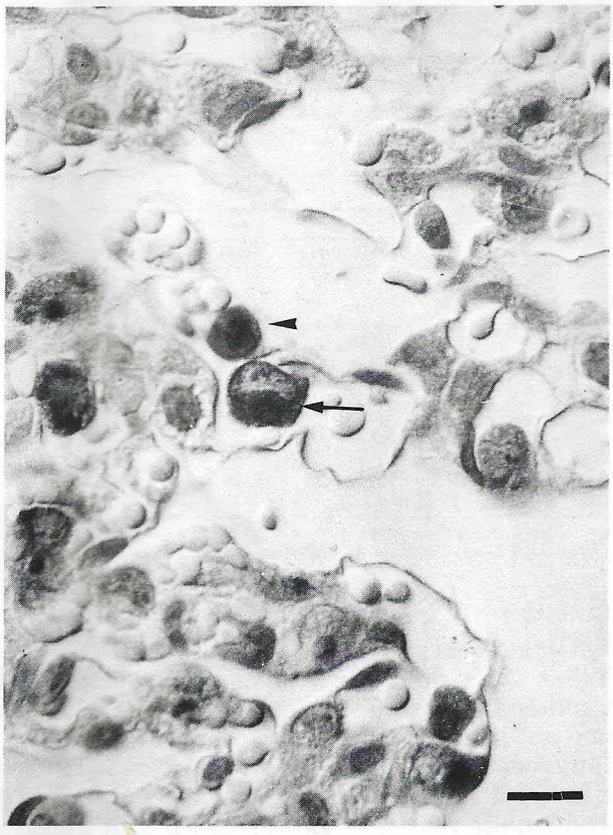

Figure 1—Photomicrograph of intravascular pulmonary macrophage (arrow) and monocyte (arrowhead) with positive cytoplasmic immunoreaction. Lung specimen was obtained 9 days after inoculation of the pig with African swine fever virus (ASFV) strain E75. Phase-contrast microscopy; avidin-biotin complex (ABC) I method; bar = 25 μm.

The specific reaction was always observed in the cytoplasm of infected cells. The brown color conferred by the DAB product appeared homogeneously distributed throughout the cytoplasm as globular dark-brown inclusions (Fig 1–4). The background varied from absent to intense, the latter being diffuse and homogeneous brown staining of the whole section.

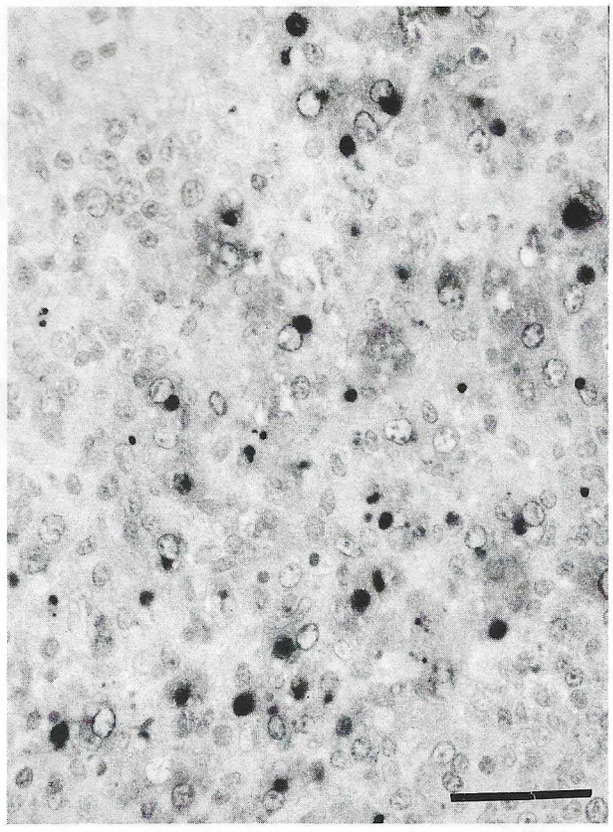

Figure 2—Photomicrograph of a section of a lymph node containing infected macrophages 9 days after inoculation of the pig with E75 ASFV; ABC-II method; Bar = 100 μm.

The quantity of cells containing such specific immunoreaction was substantially higher when pretreatments and IIP methods, including ABC-I and -II, were applied. Pretreatments also resulted in lower nonspecific, background staining associated with various techniques. On this basis, use of nonionic detergent pretreatment combined with the ABC-II method was best to detect the highest number of infected cells with lowest background staining (Tables 2 and 3).

When IIP and ABC-I were used (Table 2), nonspecific staining reaction was always observed in tissues regardless of the primary reagent used (swine anti-ASFV serum or normal swine, goat, or rabbit sera), as a result of specific binding of the peroxidase-conjugated rabbit anti-swine IgG to plasma cells, macrophages, and plasma. In noninfected tissue sections, plasma cells and plasma were labeled, but macrophages (including stellate macrophages) appeared unstained. All these nonspecific staining reactions were lacking when the ABC-II method was used, and nonspecific staining of mast cells was observed when ABC methods were used, irrespective of the specificity or nonspecificity of the primary reagent.

Using only the ABC-II technique after elution of bound antibody, partial increase of ASFV antigen was seen, especially in macrophages of the lungs and liver at PID 11 and 12 (Table 3).

The ABC-II technique was applied to tissues from pigs infected with the E70 isolate of ASFV. Immunoreactive

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Method/reaction					
DIP					
SS	−	+	−/+	+	+
DBS	++	++	++	++	++
IIP					
SS	−/+	++	+/++	++	++
DBS	++	++	++	+/++	++
ABC-I					
SS	−/+	++	++	+++	++/+++
DBS	++	+/++	+/++	+	+/++
ABC-II					
SS	−/+	+/++	+/++	++/+++	++
DBS	−	+	+	−	−

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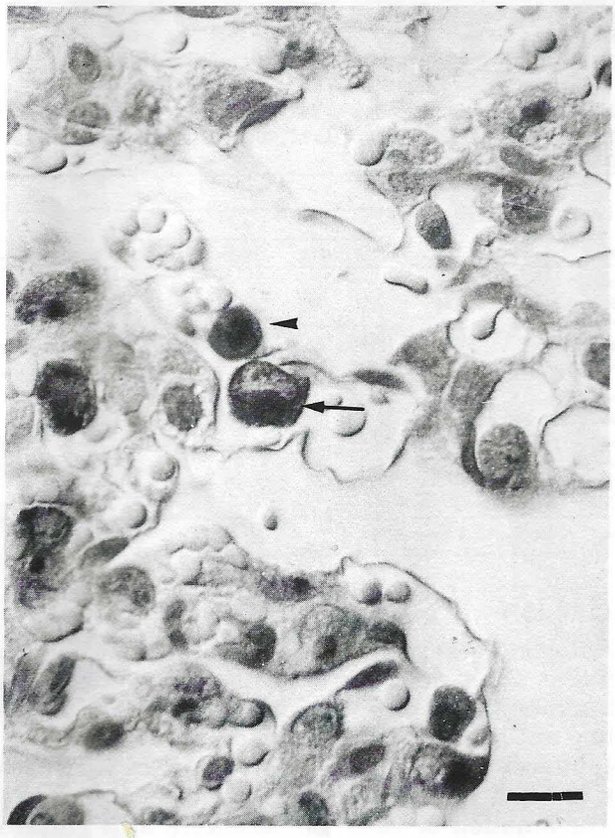

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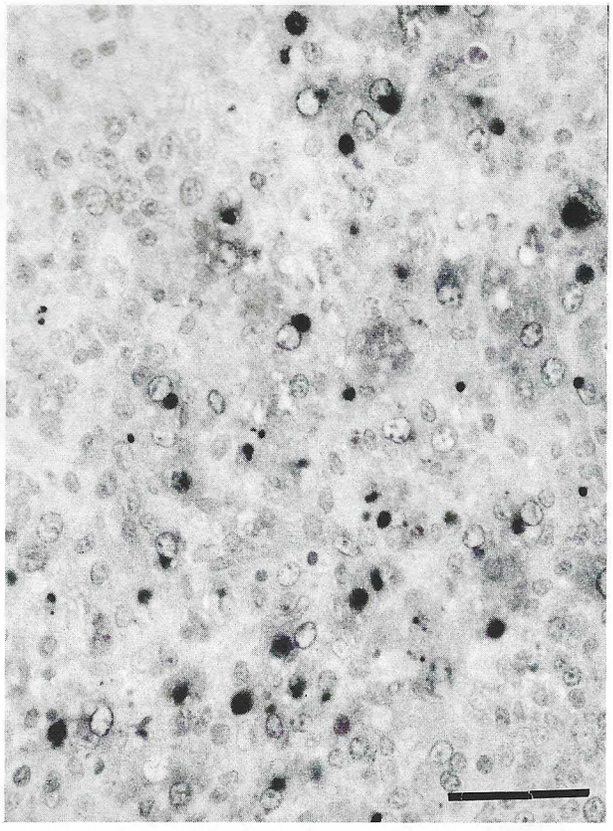

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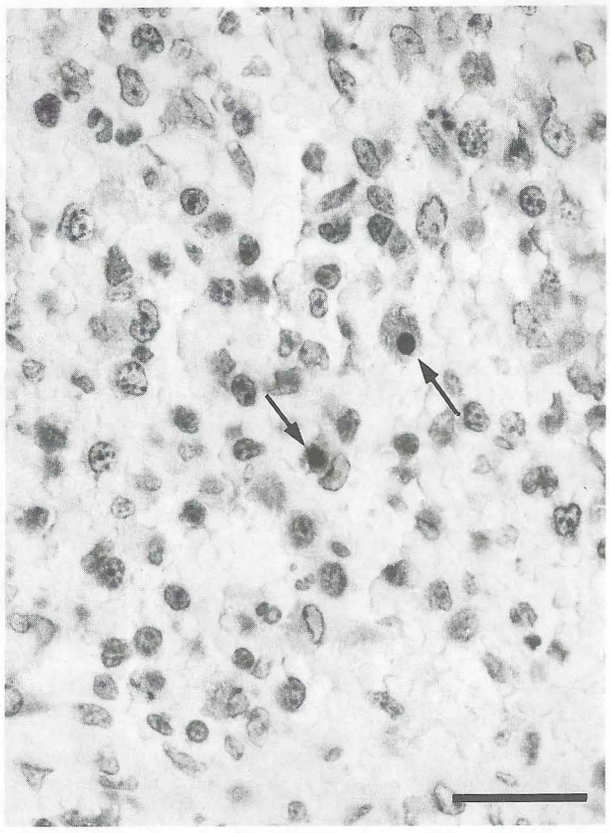

Figure 3—Photomicrograph of a section of the spleen with 2 immunoreactive macrophages (arrows). Specimen was obtained 11 days after inoculation of the pig with E75 ASFV. ABC-II method; bar = 100 μm.

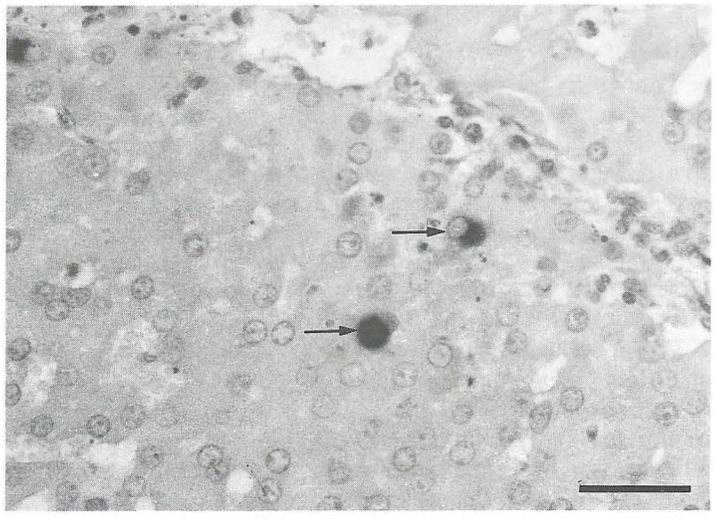

Figure 4—Photomicrograph of a positive immunoreaction in hepatocytes (arrows) at 7 days after inoculation of the pig with E70 ASFV ABC-II method; bar = 100 μm.

cells were mainly MPS cells, as was observed in tissues infected with ASFV-E75 (Table 4). However, other cells not belonging to the MPS were also found containing ASFV antigen. Ultrastructurally, all cells that were ASFV-positive by use of the immunoperoxidase techniques, were observed to contain virus particles (Fig 5 and 6), with the exception of nonspecifically stained cells (plasma cells and mast cells).

Table 2—Immunoreactive cells in infected tissues, using the DIP, IIP, ABC-I, and ABC-II methods (ASFV strain E75, at PID 9 and 11)

	DIP		IIP		ABC-I		ABC-II	
Tissue	9	11	9	11	9	11	9	11
Lymph nodes								
Free macrophages	+	−	++	+	+++	+	++/+++	ND
Fixed macrophages	+	−	+/++	+	++	+	++	ND
Spleen								
Free macrophages RP	+	−	++	+	+++	+	++/+++	ND
Fixed macrophages RP	+	−	++	+	+++	+	++/+++	ND
Macrophages WP	−	−	+	+	+	−/+	+	ND
Lungs								
Septa macrophages								
PIM + CM	+	−	+	+	+++	+	++/+++	+
IM	−	−	+	+	++	+	++	+
AM	−	−	−	−/+	−	+	−	++
Liver								
Stellate macrophages	+	−	++	+	+++	+	+++	++
CM	+	−	++	+	++	+	++	++
LIM	−	−	+	−	++	+	−	+
Hepatocytes	−	−	−	−/+	−/+	+	−/+	+

ASFV = African swine fever virus; PID = postinoculation day; RP = red pulp; WP = white pulp; PIM = pulmonary intravascular macrophages; CM = circulating monocytes; IM = (lung) interstitial macrophages; AM = alveolar macrophages; LIM = liver interstitial macrophages; ND = not done.
See Table 1 for key.

Table 3—Immunoreactive cells in infected tissues treated by the ABC-II method (ASFV strain E75) at PID 9, 11, and 12 with and without antibody elution[10]

	PID				
	9	11		12	
Tissue	Without	With	Without	With	Without
Lungs					
Septal macrophages					
PIM + CM	++/+++	+	+	ND	ND
IM	++	+	+	ND	ND
Alveolar macrophages	−	+	++	ND	ND
Liver					
Stellate macrophages	+++	+	++	−	+
CM	++	+	++	−	+
LIM	−	+	+	−	−
Hepatocytes	−/+	+	+	−	−
Endothelial cells	−	−	−	−	−

See Tables 1 and 2 for key.

Discussion

Our intent was to devise a single tissue-handling protocol that would provide a high-quality structural image with minimal loss of ultrastructural detail while maintaining antigenicity of the tissues.

Numerous immunohistologic studies of various antigens in fixed tissues have been reported.[11-13] However, the combination of vascular perfusion with glutaraldehyde as the fixation method[8] is not currently used in viral immunomorphopathologic studies.

Glutaraldehyde, a dialdehyde, has a greater cross-linking potential than does formaldehyde.[9] This, and the scarce amplification ability of the DIP method, might be the reasons why results using this technique had only relative diagnostic value in our study, although the DIP method has been successfully used for formalin-fixed, infected tissues.[10,13,14]

Use of 3 indirect techniques (IIP, ABC-I, and ABC-II)

Table 4—Detection of immunoreactive cells by use of the ABC-II method (ASFV strain E70) at PID 3, 5, and 7

Tissue	PID 3	PID 5	PID 7
Lymph nodes			
Free macrophages	+	++	++
Fixed macrophages	+	++	++
Spleen			
Free macrophages RP	++	++	+++
Fixed macrophages RP	++	++	++
Macrophages WP	+	−	−
Lung			
Septa macrophages			
PIM + CM	+	++	++
IM	−	+	+
Alveolar macrophages	−	−	−
Liver			
Stellate macrophages	+	+	++
CM	+	+	++
LIM	−	+	++
Hepatocytes	−	+	++
Endothelial cells	−	−	+

See Tables 1 and 2 for key.

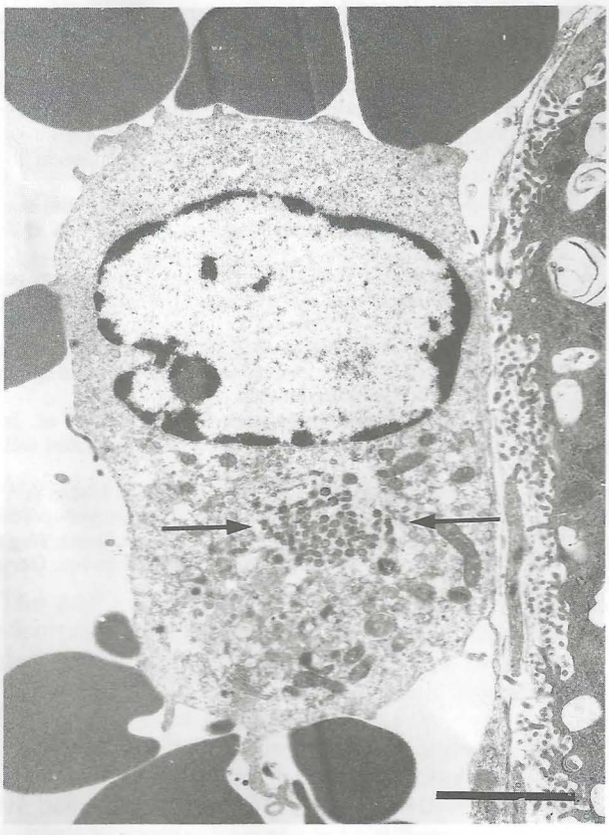

Figure 5—Transmission electron micrograph of an infected stellate macrophage containing a virus replication site (arrows) in the cytoplasm and hemadsorption. Specimen was obtained 9 days after inoculation of the pig with E75 ASFV; bar = 6 μm.

revealed high concentration of antigens in the tissue specimens, but only when pretreatment of tissue sections was applied. In this study, as has been previously observed,[14] detergent treatment had a series of advantages, compared with enzymatic treatment, namely: shorter treatment time, incubation at room temperature, no pH control needed, and lower background staining. Nonetheless, differences between the respective specific staining were not considerable.

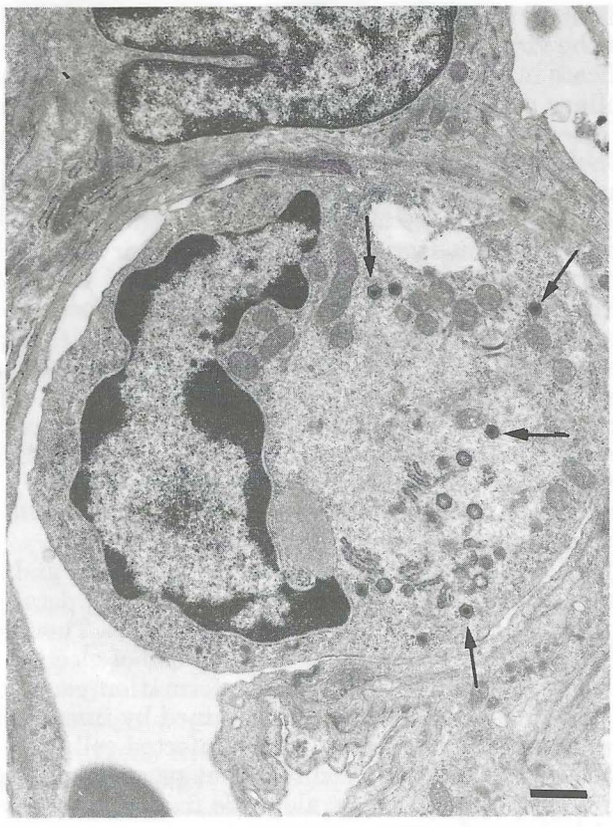

Figure 6—Transmission electron micrograph of an infected pulmonary intravascular macrophage containing a virus replication site and several virus particles (arrows). Specimen was obtained 9 days after inoculation of the pig with E75 ASFV; bar = 1 μm.

As others working with other viruses have reported,[12,13] the highest number of infected cells was observed when the ABC-I method was used. This was attributed to the amplification ability of the complex.[15]

The previously mentioned first antibody was raised in pigs. Use of biotinylated anti-ASFV antigen combined with ABC (ABC-II method) as a 2-step technique, proved to be most suitable. Specific immunoreactivity and amount of nonspecific background staining was the best.

Nonspecific staining of mast cells[16] was encountered in test sections, as well as in control sections, using the ABC-I, but not the ABC-II method. It appears that besides pH or ABC itself,[16] other reasons could be involved in this nonspecific phenomenon.

The finding of immunoreactive material in the cytoplasm of ASFV antigen-containing macrophages when specific antiserum was substituted for nonspecific reagents in tissue sections from experimentally infected, but not from control pigs, points to the existence of antigen-antibody complexes engulfed by those cells, a fact observed in cases when moderately virulent isolates are involved. These viruses induced specific IgM and IgG antibodies at PID 4 and 6 respectively.[2] The high titer of antibodies that are nonneutralizing but fix complement,[3] may be responsible for masking of antigens observed at PID 11 and 12, and could be related to false-negative results observed by use of immunofluorescence in pigs with subacute and chronic ASF.[2]

In the studied organs, ASFV antigen distribution was similar to that reported in immunohistologic studies car-

ried out on frozen samples.[3,17] In this study, we have seen that the virus affected principally the MPS cells, but could replicate in other cell types, such as hepatocytes and some capillary endothelial cells, especially at the end stage of the disease when highly virulent viruses are involved.

The ultrastructural study of infected tissues[2,3,7] was clearly improved when the proposed fixation method was used. Thus, some details were only recognized by perfusion, as the hemadsorption phenomenon[18] or infected pulmonary intravascular macrophages defined by electron microscopy.[4] The globular, granular, or diffuse specific staining encountered in infected cells agreed with results obtained by others, in either in vitro- or in vivo-infected cells,[2,3,17,19] studied by light and electron microscopy.[16,19] We believe that the globular structures observed as immunoreactive material at the structural level correspond to the replication sites observed at the ultrastructural level. The granular reaction product would correspond to phagosomes containing viral material.

The parallel ultrastructural study of infected and noninfected tissues indicated excellent morphologic detail and optimal correlation to the structural results. Though virus particles are clearly identified by use of electron microscopy,[4,6,19] further additional information concerning the biology of the virus can be obtained by immunoelectron microscopic studies of in vivo-infected cells.

Results of this study confirm that paraffin-embedded tissues perfused with glutaraldehyde fixative, can be used for detection of ASFV antigens when immunohistologic techniques, especially ABC-II, and various antigen-unmasking treatments have been applied. Thus, optimal morphologic, histologic, and immunohistologic data that should add knowledge about the pathogenesis of ASF can be obtained.

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