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The growth of a virulent strain of African swine fever virus in domestic pigs

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Many isolates of African swine fever virus (ASFV), especially those of relatively recent origin in reservoir species of wild Suidae, produce an extremely high mortality in domestic pigs (DeTray, 1963; Scott, 1965*a*). Others which have been maintained for prolonged periods in domestic swine populations or, more particularly, in cell culture systems, have a reduced virulence but animals which survive infection often remain virus carriers (Botija & Jover, 1964; Scott, 1965*a*, *b*). The process of infection has not been studied quantitatively for either type of virus, however, and there are no published data on which to base an assessment of differences in their *in vivo* behaviour.

Similarly, little is known about the route of infection in ASF, although it is commonly assumed to be by nuzzling or ingestion, with primary invasion of the upper respiratory or alimentary tracts (Scott, 1965*a*, *c*). In addition, the selection of tissues from sick or dead animals for diagnosis by virus isolation should depend on a knowledge of the distribution of the virus at various stages of the disease but such information appears to be lacking.

For these reasons we have carried out a sequential and quantitative study of the development of ASF infection in pigs infected by the intranasal route with a strain of virus causing virtually 100% mortality. The results should serve as a basis for future comparative investigations with attenuated strains.

MATERIALS AND METHODS

Virus strain

The virus used was the Tengani strain, which caused an epizootic of ASF in Malawi in 1962 characterized by a very high mortality in domestic pigs and which was presumably derived from warthogs (Cox & Hess, 1962; Matson, 1960). The virus was stored at about $-70°$ C. in the form of portions of a pig spleen (EV/59) which were thawed as required and used to prepare 10% (w/v) suspensions in phosphate-buffered saline, pH 7·2 (PBS of Dulbecco & Vogt, 1954); the suspensions were clarified by low-speed centrifugation and titrated in pig bone marrow (PBM) cultures. Occasionally such suspensions were stored at 4° C. for up to 14 days before use; this procedure probably did not lead to any loss of infectivity, as no fall of titre was demonstrable after at least 8 weeks under these conditions.

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On subcutaneous inoculation in British pigs the Tengani strain of virus produced a significant rise of temperature after 2–7 days, depending on the dose of virus administered, and death followed invariably within the following 10 days.

Experimental animals

Large White pigs of about 45–55 lb. live weight were bought from commercial sources. They were housed in isolation units and their rectal temperature was recorded every morning; in the great majority of them temperatures higher than 104° F. could be regarded as definitely abnormal.

Intranasal infection of pigs

The animals were held in dorsal recumbency whilst 1 ml. of infected spleen suspension was dropped slowly into both nostrils. Part of the inoculum was undoubtedly blown out during expiration, part of it may have been swallowed but the absence of coughing indicated that fluid probably did not gain access to the trachea. Titration in pigs showed that the inoculum contained $10^{7\cdot2}$ ID 50 of ASFV.

Harvest of tissues

Two to five pigs were killed by captive-bolt pistol at intervals after inoculation which varied from 16 hr. to 7 days (Tables 1–4). They were exsanguinated as fully as possible by severing the neck vessels or by cardiac puncture with a wide-bore trocar. Blood for titration was collected into one-third the final volume of 1·5% ethylene-diamine-tetra-acetic acid:disodium salt (EDTA) in 0·7% NaCl. Two pigs, nos. FI/71 and FW/8, died of the disease but their bodies were still warm and the blood was unclotted at the time of collection of samples.

The skin was cleansed with running tap water and a range of tissues, as shown in Tables 3 and 4, was collected into small Petri dishes; separate sterile instruments were used for each tissue. Lymph nodes were carefully dissected out from the surrounding connective or adipose tissue, and the nomenclature used for those of the head and neck was that advocated by Saar & Getty (1964). Alimentary and respiratory mucosae were gently washed with copious quantities of tap water before excision; less cohesive mucosae, as in the intestines, were scraped off with a scalpel.

The 'oral' mucosa consisted of portions dissected from the folded region lying between the base of the tongue and the epiglottis. 'Nasal' mucosa was stripped from the middle and lower thirds of the dorsal turbinate bones. The 'retropharyngeal' mucosa was dissected from the dorsal wall of the pharynx lying immediately beneath the base of the skull and the ventral straight muscles of the head; this region has a median fold and the mucosa contains numerous lymphoid follicles. The mucosa of the trachea was obtained from its lower third, immediately above the bifurcation, and bronchial mucosa as a pool of scrapings from both main-stem bronchi. Caecal and colonic mucosae were always taken from the same regions, near the apex of the caecum and near the apex of the spinal coil of the colon.

Slices of kidney included both cortical and medullary tissue, whilst 'brain' consisted of parts of the grey and white matter, near the cruciate fissure of the cerebral

hemispheres. 'Bone marrow' for titration was prepared as a 10 % (10^{-1}) suspension of packed cells from a femur, processed as described by Plowright (1964).

Detection and titration of infectivity in tissues

After further washing with PBS where desirable to free them from blood (e.g. for the nasal mucosa), tissues were weighed in quantities varying from about 0·3 to 1·0 g., chopped with scissors or crossed scalpels and homogenised in Ten Broeck grinders to yield 10 % (w/v) suspensions in complete PBM culture medium, containing per ml. 300 units of penicillin, 100 units of neomycin and 150 units of nystatin. Suspensions were always prepared within about 3–4 hr. of removal of tissues from the pig.

Dilutions in a 10-fold series were prepared from these crude suspensions and a minimum of two dilutions were each inoculated in a dose of 0·2 ml. into five tubes of PBM cells. For detection of virus 10^{-1} and 10^{-2} dilutions of solid tissues were normally used, whereas blood was inoculated undiluted and at 10^{-1} dilution. Undiluted blood was removed after 4–18 hr. by washing 2–3 times with PBS in order to remove excess erythrocytes and thus allow haemadsorption to be seen; the other inocula were not removed at all. Although severe cytotoxic effects occasionally resulted with 10 % tissue suspensions or undiluted blood, enough cells were usually left attached to permit detection of minimal quantities of virus by haemadsorption.

For virus titrations four tenfold dilutions usually spanned the end-point satisfactorily but when the titre was unexpectedly high or low, samples were retitrated from 10 % suspensions kept at 4° C. for periods up to 2–3 weeks.

Fractionation of blood

Blood (40 or 80 ml.) with a final concentration of 0·5 % EDTA was centrifuged for 30 min. at about 2300*g*. in a refrigerated machine and the supernatant was then removed and clarified by a second cycle of centrifugation under the same conditions; the upper part of the supernatant was regarded as 'plasma'. The leucocytes, mixed with red cells, were aspirated from the surface of the cellular layer of the first centrifugation and resuspended and deposited twice in 20–40 ml. Ca:Mg-free saline containing 0·02 % EDTA. The washed leucocytes were then deposited in tubes of 7 mm. internal diameter and the superficial, whitish or purple fractions removed for further washing in 40 ml. of 0·02 % EDTA. Finally, the 'leucocyte' concentrates were resuspended in one-tenth of the original volume of normal pig serum or complete culture medium to constitute the 'leucocyte fraction'.

'Erythrocyte' fractions were prepared by taking about 2 ml. of the lower part of the deposit from the primary centrifugation and washing 3–5 times in 20–40 ml. of EDTA saline. Finally, a 10 % (v/v) suspension was prepared in PBS or complete culture medium.

The packed cell volume in all original blood samples was determined by a standard method in haematocrit tubes.

Tissue cultures and infectivity end-points

Cultures were prepared from PBM cells washed out from fragments of red marrow derived from the long bones of Yorkshire-type pigs of about 25–35 lb. live weight. The cells were resuspended in medium to a concentration of 5·5–6·0 × 10^6/ml. and dispensed in 2 ml. quantities into tubes 150 × 16 mm. which were incubated for 2–4 days at 37° C. in stationary racks before inoculation. The medium used at first consisted of modified Eagle's basal medium (Macpherson & Stoker, 1962) with 10 % tryptose phosphate broth (Difco) and 25 % unheated normal pig serum; this mixture was later changed to a simpler one containing Earle's balanced salt solution with the same addition of serum. No change of medium was normally carried out during the life of the cultures but 0·2 ml. of a 1 % suspension of washed pig erythrocytes was added to each tube on the penultimate day of incubation after virus inoculation; this procedure was essential to obtain clearcut haemadsorption end-points.

Microscopic examinations for haemadsorption and cytopathic effects (Malmquist & Hay, 1960) were usually carried out on the 4th, 6th and 7th, 8th or 9th days after inoculation. End-points were recorded on the 7th to 9th days and titres per gramme of solid tissues or per ml. of blood were calculated by the method of Thompson (1947). The sensitivity of PBM cultures for the detection of the Tengani strain of ASFV was approximately the same as that of pigs.

RESULTS

Clinical reaction in pigs

In seventeen of eighteen pigs the incubation period, i.e. the time after intranasal inoculation when the rectal temperature first exceeded 104° F., was 3 or 4 days; in the remaining animal it was 5 days, the mean for the group being 3·8 days. With the Tengani strain of virus peak temperatures of 105·0 to 108·4° F. were usually attained 24–48 hr. later and death commonly occurred 2–6 days after the onset of pyrexia with a mean death time of 2·9 days in seventy-eight animals, infected by various routes with different doses of virus. At 24 hr. and later after infection it was always possible to recover virus from one or more tissues, i.e. infection never failed following intranasal instillation of virus.

The route of infection with ASFV

The upper respiratory tract

Tables 1 and 2 give details of all virus recoveries from thirteen pigs which were killed during the first 48 hr. after infection. In two cases, nos. FW/99 and FI/33 a trace of virus only was demonstrable in the nasal mucosa, whilst in a further ten animals no virus at all was detected in this tissue. This was remarkable in view of the very large inoculum (> $10^{7·0}$ ID 50) which had been employed and the high stability of ASFV at 37° C. (Plowright & Parker, 1967). In the remaining pig, no. FX/0, the titre of virus in the nasal mucosa was $10^{3·2}$ HAD 50/g, which almost certainly implied viral proliferation there before generalization had occurred, as indicated by the absence of virus in the blood, spleen and bone marrow

By 24–40 hr. virus was consistently present in the retropharyngeal mucosa, in increasing quantities which were undoubtedly associated with local proliferation. One of two pigs showed definite localization in this tissue by 16 hr., without detectable transfer to the local retropharyngeal lymph nodes. In three of four pigs killed at 24–40 hr., virus was already present in the retropharyngeal nodes but to a lower titre than in the mucosa; after 48–72 hr., however, the lymph node titre exceeded that in the mucosa (Tables 1–3, Fig. 1).

Table 1. *The distribution of ASFV in the tissues of pigs killed 16–24 hr. after intranasal infection*

	Time after infection					
	16 hr.		24 hr.			
Tissue	FW/98*	FW/99	FU/20	FU/87	FW/84	FW/85
Lymph nodes:						
Medial retropharyngeal	—	—	2·8	3·4	—	—
Lateral retropharyngeal	—	—	NT	—	3·4	—
Parotid	—	—	—	2·4	—	—
Right bronchial	—	—	—	1·6	—	—
Left bronchial	—	—	—	⩾ 4·2	—	—
Mucosae:						
Nasal	—	Tr†	—	—	—	—
Retropharyngeal	2·8	—	NT	NT	4·6	4·2
Tracheal	—	—	—	1·6	NT	—
Bronchial	—	—	—	5·8	—	—
Lung:						
Hilar	—	—	—	2·8	—	—
Diaphragmatic	—	—	—	4.2	—	—

* = pig. no.
† = Trace. Haemadsorption in one of five tubes inoculated with 10 % (w/v) suspension.
NT = not tested.
The figures in each column are $\log_{10}$ HAD 50/g.

The lower respiratory tract

In two of thirteen pigs which were killed during the first 48 hr. after infection (nos. FU/87 and FI/33) there was highly suggestive evidence for a primary 'complex' of infection in the bronchial mucosa and left bronchial lymph node but the retropharyngeal mucosa was not, unfortunately, examined in either of these instances. In both of these animals, the highest recorded titre of virus was in the bronchial mucosa, with as much or more infectivity demonstrable in the left bronchial as in the medial retropharyngeal lymph node (Tables 1 and 2). Virus recovered from the 'lung' tissue of these animals could obviously have been due to the inevitable inclusion of bronchial or bronchiolar mucosae. In addition, generalisation had definitely occurred by 48 hr. in pig FI/33 since the spleen titre was already $10^{3\cdot0}$ HAD 50/g. and virus was demonstrable in the blood; early generalization had also probably occurred in one other animal (no. FU/88) killed at 48 hr., as a trace of infectivity was found in the bone marrow but not the spleen (Table 2).

The alimentary tract

No virus was recovered from the 'oral' mucosa and tonsil of any of the first thirteen pigs or from the fundic mucosa, Peyer's patches and ileal mucosa of six of them. Hence, it can be concluded that the alimentary tract does not serve as a portal of entry for ASFV, at least following nasal instillation of virus.

Table 2. *The distribution of ASFV in the tissues of pigs killed 40–48 hr. following intranasal infection*

	Time after infection						
	40 hr.		48 hr.				
Tissue	FW/87*	FX/0	FH/55	FI/33	FU/21	FU/88	FW/86
Lymph nodes							
Medial retropharyngeal	3·2†	3·4	5·2	3·8	6·8	5·6	6·8
Lateral retropharyngeal	—	—	NT	—	—	4·4	—
Mandibular	—	—	3·0	1·8	—	2·8	—
Parotid	—	—	3·4	1·8	4·8	—	5·2
Right bronchial	—	—	—	—	—	—	—
Left bronchial	—	—	—	3·8	—	—	—
Mucosae							
Nasal	—	3·2	—	Tr‡	—	—	—
Retropharyngeal	4·6	5·0	NT	NT	NT	NT	5·0
Tracheal	—	—	—	2·0	—	—	—
Bronchial	—	—	—	4·2	—	—	—
Lung							
Hilar	—	—	—	—	—	—	—
Diaphragmatic	—	—	—	2·0	—	—	Tr
Spleen	—	—	—	3·0	—	—	—
Bone marrow	—	—	—	—	—	Tr	—
Blood	—	—	—	0·4	—	—	—

* = pig no.
† = $\log_{10}$ HAD 50/g. or per ml. (blood).
‡ = Trace. Haemadsorption in one of five tubes inoculated with 10 % (w/v) suspension.
NT = not tested.

The distribution of ASFV in the blood of infected pigs

The fractionation procedures which were adopted produced plasma which was free from all cellular elements but failed to produce 'erythrocyte' fractions free of leucocytes, although the numbers of the latter were materially reduced and in one instance virtually eliminated (pig no. FW/18, Table 5). Similarly, the leucocyte fractions in three of four cases had nucleated counts which were 1·6 to 1·9 times greater than those of the original blood whilst erythrocyte counts were greatly reduced (8 to 55 times) (Table 5).

The results of virus titrations are given in Table 6. Virus free in the plasma had a very high titre varying from $10^{7\cdot0}$ to $10^{7\cdot4}$ per ml. and constituted 4–18 % of the total infectivity. The titre of 'leucocyte' fractions was invariably lower than that of the original blood, by 0·8–1·4 log units, in spite of the greater concentration of

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leucocytes. The total recovery of virus in 'erythrocyte' fractions was calculated from the titre of 10% red cell suspensions and the packed cell volume; it varied from 20 to 100% with a mean of 45·6%, the highest figure being recorded for pig no. FW/18 in which the leucocyte count had been reduced about 170 times.

The generalization of ASFV in pigs

General

Tables 3 and 4 give figures for the virus content of thirty-one tissues harvested from eighteen pigs which were killed 3–7 days after infection. The behaviour of

Table 3. *The distribution of ASFV in the tissues of pigs killed 72–96 hr. following intranasal inoculation*

	Time after infection								
	72 hr.				96 hr.				
Tissue	FI/61*	FI/68	FU/92	FW/2	FI/59	FI/62	FI/69	FU/93	FV/90
Lymph nodes									
Medial retropharyngeal	8·0†	8·0	9·0	8·2	6·2	8·0	8·6	8·4	9·0
Lateral retropharyngeal	—	5·2	—	3·0	5·2	7·2	7·0	—	8·8
Mandibular	6·0	6·8	7·2	7·2	6·6	5·4	5·8	7·0	9·2
Parotid	6·0	6·6	6·4	4·6	5·4	7·2	6·0	5·8	8·4
Right bronchial	3·8	3·0	—	4·0	6·0	5·6	6·8	3·6	7·2
Left bronchial	2·8	2·6	—	3·0	4·8	6·0	6·8	2·4	7·0
Dorsal sup. cervical	1·6	—	—	3·0	5·8	4·0	5·8	—	7·6
Prefemoral	—	NT	—	2·6	4·6	4·8	5·6	—	7·8
Gastro-hepatic	3·0	NT	—	3·4	5·2	5·8	6·0	—	7·2
Mesenteric	1·6	2·4	—	3·4	5·0	4·8	5·6	—	6·8
Caecal	3·0	3·0	—	2·8	5·2	5·8	6·2	—	6·2
Pharyngeal tonsil	5·4	5·4	—	6·6	5·0	6·2	6·0	—	7·2
Peyer's patches	2·6	NT	—	—	NT	5·2	5·2	—	6·0
Spleen	6·4	6·6	4·0	7·2	7·0	8·8	7·4	5·2	9·0
Bone marrow	4·8	4·2	1·8	4·2	6·4	7·0	6·6	4·6	8·8
Blood	5·6	4·2	—	5·4	5·8	7·6	6·2	2·0	7·0
Nasal mucosa	2·6	4·2	4·6	3·6	4·6	5·2	5·0	3·0	7·2
Retropharyngeal musoca	NT	NT	NT	7·2	NT	NT	NT	—	6·2
Tracheal mucosa	3·4	2·4	—	Tr‡	4·0	4·6	5·2	—	5·6
Bronchial mucosa	3·2	2·4	—	4·2	5·2	5·4	4·6	Tr	7·4
Hilar lung	5·4	3·4	1·6	5·6	6·0	7·2	8·4	2·4	8·4
Diaphragmatic lung	5·6	3·8	2·0	5·6	5·4	7·2	8·0	2·2	8·8
Oral mucosa	3·2	2·4	—	—	4·4	5·0	5·2	—	5·6
Fundic mucosa	2·2	2·4	—	3·2	NT	NT	NT	—	6·2
Ileal mucosa	—	—	—	—	4·8	5·0	5·0	—	6·0
Caecal mucosa	2·6	2·4	—	3·4	5·0	5·2	5·0	—	5·8
Colonic mucosa	2·6	—	—	2·6	4·8	5·6	5·6	—	6·2
Liver	4·8	5·0	—	5·4	3·8	6·2	7·0	2·6	8·6
Kidney	4·2	3·2	—	4·0	4·2	6·2	5·2	—	7·0
Myocardium	3·0	2·8	—	3·6	4·4	5·2	5·4	—	6·6
Brain	—	—	—	2·8	NT	4·4	4·0	—	6·2

* = pig no.
† = $\log_{10}$ HAD 50/g. or ml.
NT = not tested.
‡ = Trace. Haemadsorption in one of five tubes inoculated with 10% (w/v) suspension.

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individual animals in a group was reasonably regular with three exceptions which will be discussed later; these were nos. FU/92, FU/93 and FW/7 and figures for the two last were excluded in the calculation of means used to prepare Figs. 1–6 inclusive. Dissemination and growth of virus in the pig will now be described with reference to tissue and organ systems, omitting the three animals mentioned above.

Table 4. *The distribution of ASFV in the tissues of pigs killed 5–7 days following intranasal infection*

	Time after infection								
	5 days				6 days			7 days	
Tissue	FI/70*	FI/73	FV/21	FW/3	FI/71	FW/7	FW/16	FW/8	FW/17
Lymph nodes									
Medial retropharyngeal	7·6†	7·6	8·8	8·4	8·8	8·6	9·2	8·4	8·8
Lateral retropharyngeal	7·6	8·2	9·0	8·6	8·2	8·2	9·0	9·2	9·0
Mandibular	6·4	8·0	9·2	8·2	8·2	8·2	8·6	9·0	9·2
Parotid	7·0	7·8	8·6	9·2	8·2	8·4	9·4	9·6	8·8
Right bronchial	7·5	7·8	8·6	8·4	8·2	4·8	8·4	9·4	9·0
Left bronchial	6·4	7·4	8·2	8·8	7·6	4·0	8·4	9·2	8·8
Dorsal sup. cervical	7·0	7·2	8·6	8·6	8·2	7·8	9·0	9·0	8·8
Prefemoral	6·8	7·4	8·2	9·0	8·2	4·8	8·8	9·2	8·8
Gastro-hepatic	6·4	6·4	8·8	9·2	8·2	4·8	8·4	9·0	9·2
Mesenteric	6·0	5·6	7·4	8·0	7·2	5·6	9·2	9·2	9·0
Caecal	7·2	6·8	7·6	8·0	8·2	4·8	8·0	9·0	8·6
Pharyngeal tonsil	6·6	6·0	7·6	8·2	8·0	4·2	8·2	9·0	9·0
Peyer's patches	4·0	5·0	6·2	6·4	NT	4·2	6·2	NT	7·6
Spleen	7·4	NT	8·6	9·0	8·4	8·0	8·8	9·2	9·0
Bone marrow	6·2	5·4	8·2	8·8	7·8	7·2	8·8	8·2	8·8
Blood	8·0	8·2	7·4	8·2	7·4	7·8	8·6	8·8	8·4
Nasal mucosa	6·2	NT	7·2	8·0	7·2	4·6	8·0	8·4	8·0
Retropharyngeal mucosa	NT	NT	5·6	6·8	NT	6·8	8·4	7·2	8·6
Tracheal mucosa	3·8	NT	5·8	6·2	6·0	2·6	6·4	8·2	6·0
Bronchial mucosa	4·4	NT	6·2	6·8	5·6	5·8	6·6	NT	6·6
Hilar lung	5·2	NT	8·8	8·0	8·4	5·8	8·4	8·8	8·4
Diaphragmatic lung	5·9	NT	8·2	7·8	8·2	6·0	8·6	8·6	8·8
Oral mucosa	3·2	NT	5·8	6·0	5·0	3·6	5·8	7·4	6·3
Fundic mucosa	4·6	NT	5·8	6·4	5·6	4·4	5·8	7·2	7·0
Ileal mucosa	2·7	NT	6·4	6·2	6·6	4·4	6·0	NT	7·8
Caecal mucosa	3·7	NT	6·2	5·8	5·2	4·4	6·4	NT	7·8
Colonic mucosa	5·2	NT	6·2	5·4	6·4	4·4	6·6	NT	7·8
Liver	7·2	NT	8·2	8·0	8·0	4·4	8·4	8·8	8·4
Kidney	5·0	NT	6·8	7·6	7·2	5·0	6·6	7·4	8·0
Myocardium	4·6	NT	6·2	7·0	6·2	5·2	6·8	6·6	6·0
Brain	3·6	NT	6·2	6·8	5·6	4·6	6·0	6·8	6·6

* = pig no. † = $\log_{10}$ HAD 50/g. or ml. NT = not tested.

Lymphopoietic tissues

Following on the early localization of virus in the retropharyngeal nodes, especially the medials, it was found that maximal infectivity titres ($10^{8·0}$–$10^{9·0}$ HAD 50/g.) were present in these structures at 72 hr. (Table 3) and that the mean titre had reached a plateau by that time which was maintained to death, with

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possibly no further significant increase (Fig. 1). In two other cephalic nodes, the mandibular and parotid, the virus was also well established at 72 hr., but mean titres continued to rise a further 2 $\log_{10}$ units by the end of the experiment; during the period 48–72 hr. after infection there was much more virus in these nodes than in the blood (Fig. 2). The lateral retropharyngeal nodes were not consistently infected until the 4th day (Table 3) but the majority of the bronchials, alimentary

Table 5. *Fractionation of the blood of infected pigs*

		Original blood		Erythrocyte fraction		Leucocyte fraction	
Pig no.	Day of disease	RBC count*	WBC count†	RBC count	WBC count	RBC count	WBC count
FW/16	3	ND	33·3	ND	2·0	ND	52·0
FW/18	2	5·0	18·0	ND	0·03	0·09	33·9
P 91	4	4·2	24·6	0·8	1·1	0·5	47·5
P 100	3	2·6	19·0	1·4	1·0	0·1	21·8

* = $\times 10^6$/mm.^{3} † = $\times 10^6$/ml. ND = not done.

Table 6. *The distribution of ASFV in the blood of infected pigs*

		Infectivity in:*			
Pig no.	Day of disease	Whole blood	Plasma	Erythrocyte fraction	Leucocyte† fraction
FW/16	3	8·6	7·4 (4 %)	7·6 (37 %)	7·4
FW/18	2	7·8	ND	7·4 (100 %)	6·4
P 91	4	8·0	7·4 (18 %)	6·8 (20 %)	7·0
P 100	3	7·8	7·0 (12 %)	6·8 (26 %)	7·0
Mean recovery (%)			11·3	45·6	

* = $\log_{10}$ HAD 50/ml. Figures % in brackets refer to total estimated recovery in fraction.
† = total virus recovery not calculated owing to poor recovery of leucocytes.
ND = not determined.

and superficial body nodes already showed small quantities of virus at 72 hr. The mean titres of the prefemoral and dorsal superficial cervical (prescapular) nodes are shown in Fig. 2; they continued to rise until the 7th day when they eventually exceeded that of the blood.

Virus growth was always well established in the spleen at 72 hr., the mean titre reaching a plateau by the 4th day, with only a slow rise subsequently; throughout the experiment there was more virus in the spleen than in the blood (Fig. 3). With the exception of pig FU/92 virus had begun to proliferate in the pharyngeal tonsil at 72 hr. but titres were generally lower at first than those in the cephalic nodes (Table 3). Peyer's patches did not contain a considerable quantity of virus until the 4th day and titres were lower than those in the blood, following very closely those in the intervening ileal mucosa (Fig. 5).

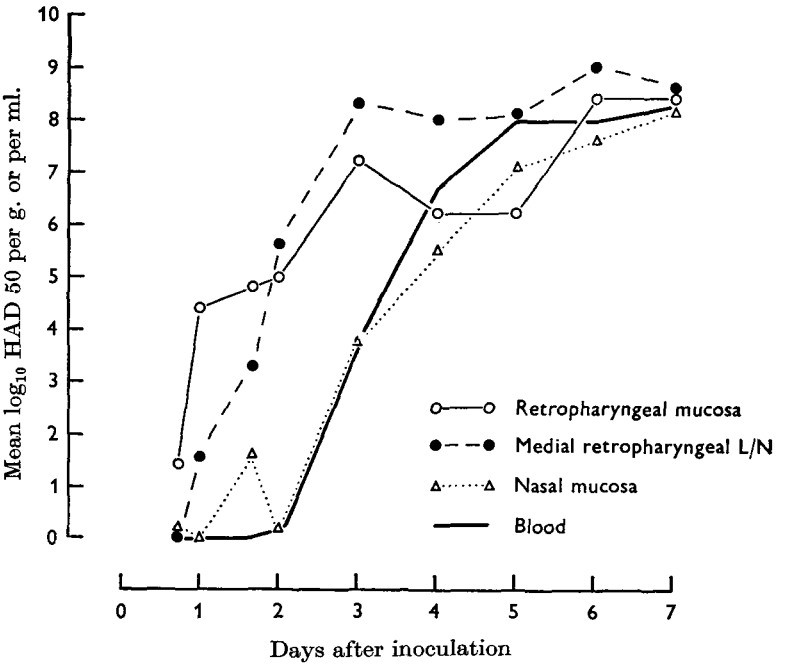

Fig. 1. Upper respiratory tract.

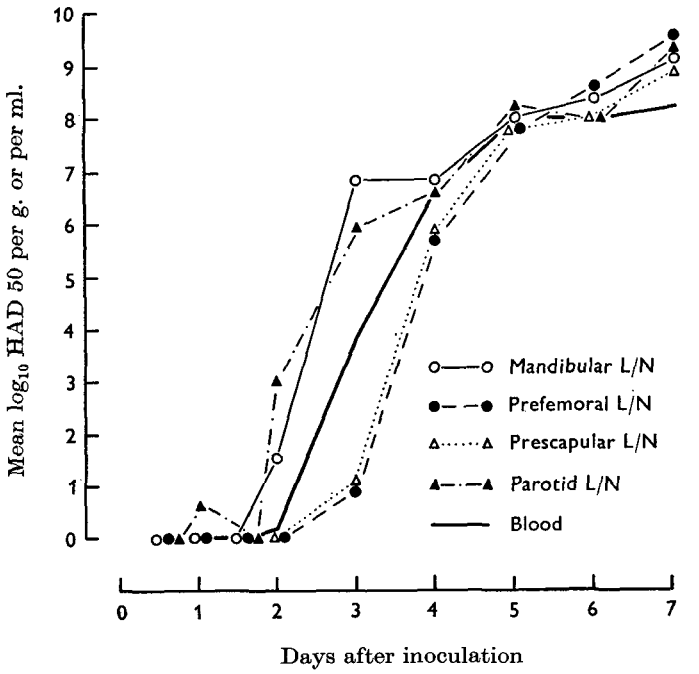

Fig. 2. Lymphoid tissues.

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Blood and haemopoietic tissues

Large quantities of circulating virus were present at 72 hr. and the mean titre continued to rise to a plateau commencing on the 5th day: from this point onwards mean titres varied between $10^{8\cdot0}$ and $10^{8\cdot2}$ HAD 50/ml. It was evident from these figures that the virus content of many tissues after generalization could be largely influenced by their blood content and hence the mean blood titre was used as a 'reference' line in Figs. 1–6.

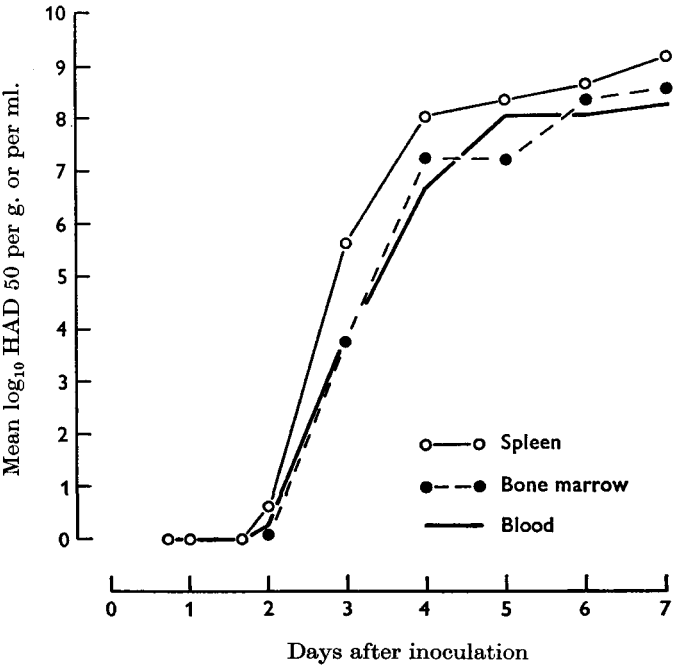

Fig. 3. Haemopoietic tissues.

The bone marrow was consistently infected from 72 hr. onwards but in only two of eighteen animals was the titre significantly greater than that in the blood; these were pigs FV/20 and FU/93 (Table 3); in two other cases (FI/70 and FI/73) the bone marrow titre was 1·8 and 2·8 $\log_{10}$ units lower than that of the blood. The mean lines for virus in the blood and bone marrow were approximately the same throughout the infection (Fig. 3).

Respiratory tract

The titre of virus in the retropharyngeal mucosa was exceeded by that in the associated retropharyngeal nodes from 48 hr. onwards though virus presumably continued to multiply in the former (Fig. 1). The bronchial and tracheal mucosa, after also probably being involved in early viral penetration in a minority of animals, never subsequently assumed an important role in supporting virus growth (Fig. 4). The titre of virus in the nasal and retropharyngeal mucosae was usually high from the 5th day onwards (Tables 3 and 4) and this, together with any contri-

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butions from the lower respiratory tract, could presumably result in a high rate of nasal excretion.

The lungs contained virus consistently on the 3rd day and titres considerably in excess of those in the blood were seen in 2/5 pigs on the 4th day (nos. FI/69 and FU/20; Table 3) but not thereafter; the mean line followed very closely that of the blood, being about 2 log units higher than those of the tracheal and bronchial mucosae and continuing to increase until death or destruction on the 7th day.

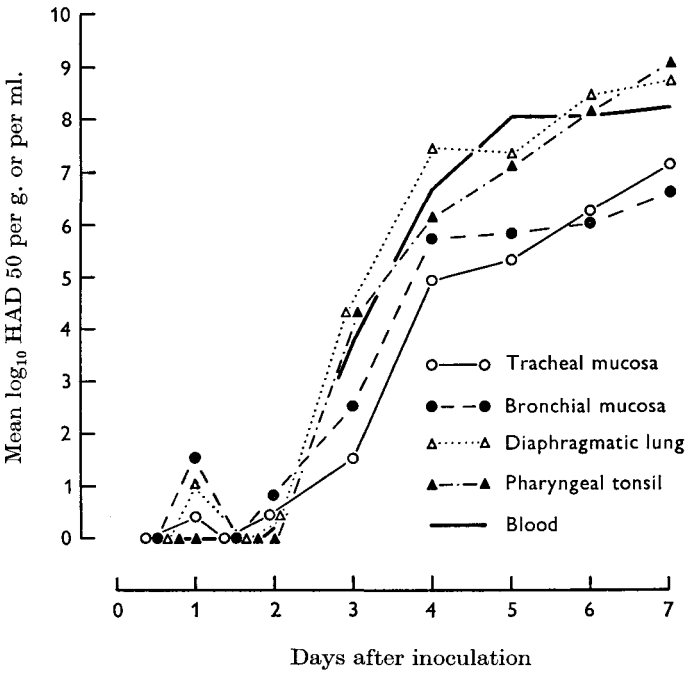

Fig. 4. Respiratory tract.

It was, incidentally, of interest to observe the close agreement between titrations of 'diaphragmatic' and 'hilar' lung; differences were never greater than 0·7 log units, the mean difference for eighteen pairs of observations being 0·20 log units (Tables 3 and 4). Assuming that there was no real difference between the viral content of tissue from these two locations, the reproducibility gives an indication of the accuracy within cell batches of the titration technique.

Alimentary tract

Virus had generally localized in the oral, fundic, caecal and colonic mucosae by the 3rd day but it was not present in the ileum until the 4th day. Virus titres in all these tissues were of approximately the same magnitude in individual pigs, with the possible exception of no. FI/70 (Table 4). The mean titres ran almost parallel, increasing rapidly to the 4th day and thereafter more slowly to death. There was always much more virus in the blood than in the alimentary tract, the difference often being of the order of 1 to 2 or more $\log_{10}$ units in individual pigs, as well as in the mean (Tables 3 and 4; Fig. 5).

Parenchymatous organs and central nervous system

On the 3rd day the liver usually contained much virus, which subsequently increased in an almost identical manner to that in the blood; this was true both of individual pigs and of the mean line (Tables 3 and 4; Fig. 6). Exceptionally, however, pigs such as no. FV/20 (Table 3) did show more virus in the liver than in the blood; it can probably be concluded that the liver, like the bone marrow, is a site of virus production particularly when its behaviour is compared with that of other highly vascular tissues such as kidney, myocardium and brain.

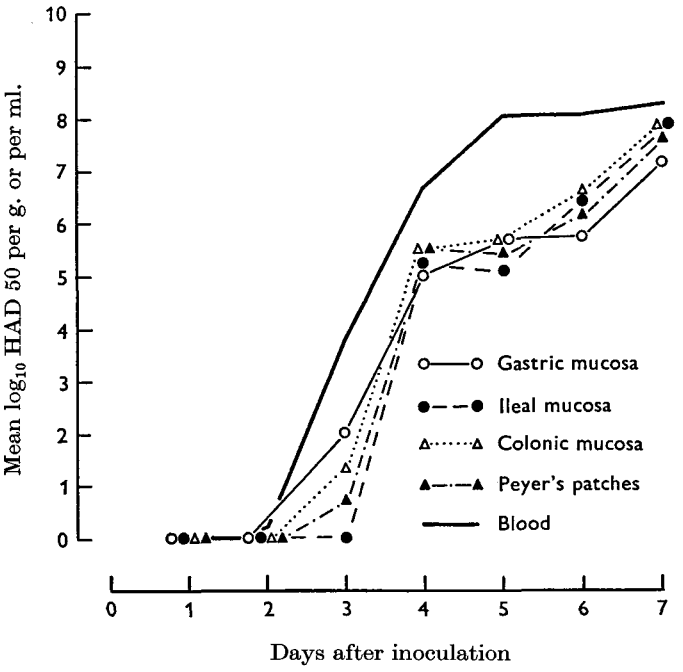

Fig. 5. Alimentary tract.

Vitrus titres in the kidney were almost invariably lower than those encountered in the liver but often exceeded those in the myocardium, which in turn usually contained more virus per gramme than did the brain (Fig. 6). There was no evidence of viral proliferation in the kidney, myocardium or brain, if titres in these tissues were compared with the degree of viraemia.

Irregular behaviour of a minority of pigs

Of eighteen pigs for which details are given in Tables 3 and 4, three apparently behaved in an irregular manner in that generalization was delayed, as in nos. FU/92 and FU/93 killed on the 3rd and 4th days, or that virus multiplication in many tissues was suppressed, as in pig FW/7.

The first two animals were infected at the same time, with the same inoculum, and neither had shown a temperature reaction by the time of sampling. Both had a severe portal cirrhosis, probably induced by Ascaris infection, but they were not

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clinically abnormal. It was possible that the delay in generalization could have been attributed to a smaller effective inoculum but this explanation was contra-indicated by the very high titres of virus ($10^{7\cdot0}$–$10^{9\cdot0}$ HAD 50/g.) found in the retropharyngeal and mandibular nodes; these titres were comparable to those found in other pigs killed at 72 or 96 hr. and considerably in excess of those determined in animals killed at 48 hr. The cultures used for the titration of all tissues from 'aberrant' pigs were of very high sensitivity, as shown by their simultaneous use for materials from other pigs, giving titres in excess of $10^{8\cdot0}$ HAD 50/g.

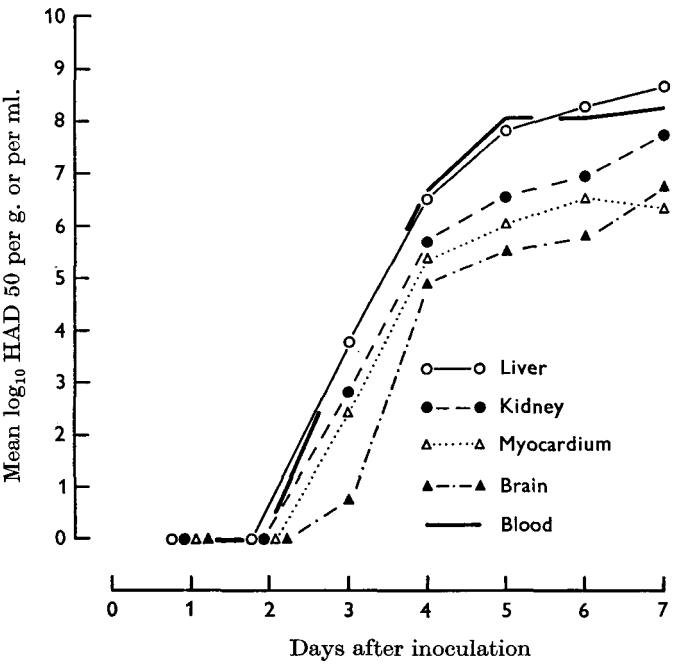

Fig. 6. Parenchymatous organs and brain.

A marked feature, in both pigs FU/92 and FU/93, was the absence of virus from many lymph nodes, even those of the head and neck; in addition no infectivity was detected in the tonsils or any part of the alimentary tract, including the oral mucosa, tonsil, Peyer's patches and mucosae of the stomach or small and large intestines. Low titres of virus were found in the lungs but no significant quantity in the mucosae of the respiratory tract, including in one case (no. FU/93) the retropharyngeal mucosa. Virus in the spleen was reduced at least 100-fold and whilst pig FU/92 was non-viraemic, a low-level viraemia ($10^{2\cdot0}$ HAD 50/ml.) was found in no. FU/93 (Table 3).

The third pig which behaved irregularly, no. FW/7, had shown a fever ($\geqslant$ 105° F.) for 3 days at the time when it was killed; it was infected simultaneously with no. FW/8 and showed some focal portal cirrhosis at autopsy. Its tissues were titrated in the same batch of cultures as those used for the animal FW/8, i.e. they were of maximal sensitivity. Again, virus titres were high in the cephalic nodes but about 3–4 $\log_{10}$ units lower than usual in the bronchial, alimentary and body nodes.

Spleen, bone marrow and blood virus had reached the expected titres but the respiratory mucosae, alimentary mucosae and parenchymatous organs showed titres which were often depressed by as much as 2 $\log_{10}$ units or more.

While no explanation can be offered for the unusual features of the infection in pigs FU/92, FU/93 and FW/7, with the exception of some possible association with the portal cirrhosis, it was decided to omit the figures for the last two animals in calculating the mean values shown in Figs. 1–6 inclusive. In the case of FU/92 the figures were included as it was considered possible that a 24 hr. delay in generalization could not definitely be regarded as abnormal.

DISCUSSION

Data obtained in this study (Tables 1 and 2) provided clear evidence that when ASFV was instilled into the nasal cavity of pigs it adsorbed to and began to proliferate in the retropharyngeal mucosa within 16–24 hr.; it did not persist, at least in infectious form, adsorbed to the turbinate mucosae and evidence for primary proliferation there was obtained in only one of thirteen pigs (no. FX/0). Virus was rapidly transported from the retropharyngeal mucosa to the retropharyngeal nodes, nearly invariably the medials, where it increased exponentially to reach virtually peak titres within 72 hr. (Fig. 1).

Efferents from the medial retropharyngeal nodes give rise to the tracheal duct, which discharges directly into the brachiocephalic vein (Saar & Getty, 1964); this must have facilitated the rapid dissemination of virus, presumably via the blood stream, to lymphopoietic tissues throughout the body, especially the spleen and lymph nodes. It would account, also, for the early involvement of the bone marrow, alimentary and respiratory mucosae as well as the parenchymatous organs (Table 3). The sequence of events in the great majority of pigs probably resembled that described in rabbits infected intranasally with vaccinia (Yoffey & Sullivan, 1939) or rabbit-pox viruses (Bedson& Duckworth, 1963); in both these instances primary proliferation occurred in the nasal mucosa, followed by spread to the superior, deep cervical lymph nodes, which in turn discharged virus after 12 hr. into the cervical lymph ducts (Yoffey & Sullivan, 1939).

The primary establishment of virus in the bronchial mucosa and associated lymph nodes of two pigs, with or without the involvement of the retropharyngeal route, suggests that infection by aerosols of small particle size can probably occur via the *lower* respiratory tract. This could be an alternative route of natural infection, as shown already for rabbit-pox (Bedson & Duckworth, 1963) and rinderpest in cattle (Taylor *et al.* 1965). It is necessary to observe, however, that transmission of ASFV by expired air could not be demonstrated by Montgomery (1921), even over a distance as short as 6 in.; he found, in addition, that muzzling was sufficient to prevent contact transmission and that feeding virus did not produce infection if the material was enclosed in a 'bait'.

Although the results of blood fractionation experiments were not entirely satisfactory they did show that about 90 % of the circulating virus was associated with the cellular fraction and that only 11 % was, on average, free in the plasma. The

finding that mean virus titres in the cephalic lymph nodes and spleen exceeded those in the blood throughout the infection and particularly during the earlier phases, strongly suggested that circulating virus was derived in large measure from these tissues. There was no evidence, however, that an important part of the viraemia was associated with intact leucocytes and the question therefore arises as to whether the virus was released in cell-free form from the producer 'target' tissues and subsequently adsorbed to erythrocytes in the circulation or whether 'carrier' erythrocytes were produced in the bone marrow.

Some evidence for the first of these alternatives was obtained in an experiment in which washed pig erythrocytes (25%, v/v) were mixed *in vitro* with culture-propagated virus at 37° C., and sampled at intervals by washing twice with chilled PBS and titrating in PBM cultures; the infectivity of the exposed erythrocytes rose from $10^{4\cdot4}$/ml. at 30 min. to $10^{5\cdot4}$/ml. at 3 hr., the titre of the original virus being $10^{5\cdot8}$/ml. The second alternative, i.e. the production of infected red cells in the bone marrow, was not supported by the relatively low titres of virus in packed cells derived from this tissue, which seldom exceeded those in the blood at the same time. Virus bound to erythrocytes would probably, as pointed out by Mims (1964), be cleared less rapidly from the circulation than plasma virus, which would be more readily taken up by macrophages; this would help to account for the attainment and maintenance of the very high levels of viraemia which were observed throughout the later course of the infection.

The predominant association of virus with the erythrocytes of the blood in ASF recalls a similar state of affairs in the pathologically-similar hog cholera. In this disease, caused by a completely unrelated virus, infectivity is also firmly adsorbed to the erythrocytes and could be demonstrated in large quantities in washed stroma; virus was also adsorbed by normal erythrocytes *in vitro* (Powick, 1937).

The distribution of virulent ASFV in the tissues of infected pigs shows striking correlations with the predominant lesions; these include a severe, necrotic and haemorrhagic lymphadenitis with karyorrhectic destruction of lymphocytes and also a fibrinoid angiitis, affecting particularly capillaries and arterioles (De Kock, Robinson & Keppel, 1940; Maurer, Griesemer & Jones, 1958). The high virus content of lymphopoietic tissues certainly does not conflict with the hypothesis that the virus grows in lymphocytes *in vivo*. Maurer *et al.* (1958) suggested that the vascular lesions, including those of the endothelium, were also a direct effect of the virus and, if this were confirmed, it could help to account for the high level of viraemia; similar considerations apply to hog cholera (Mims, 1964).

Heuschele, Coggins & Stone (1966) used the fluorescent antibody technique to examine smears from the tissues of pigs acutely infected with ASFV. They had difficulty with non-specific fluorescence of leucocytes but found specific, fluorescent globules in impression smears of spleen, gastric lymph nodes or, especially, liver and suggested that the cells containing them were macrophages. No precise information on the types of cell supporting the growth of ASFV can be obtained until difficulties in the application of the fluorescent-antibody technique to pig tissues have been overcome.

Our data may provide a partial explanation for the observation of Montgomery

(1921), repeated on several occasions by ourselves, that infected pigs during the first 12–24 hr. of fever do not transmit ASFV to other animals in pen contact with them. Thus, in addition to high titres in the retropharyngeal mucosa, virus was consistently present in appreciable quantities in the nasal mucosa at 72 hr after intranasal infection, i.e. at the beginning of or 24 hr. before pyrexia; it was also present at this time, albeit in smaller quantities, in the intestinal mucosae. But, apart from the primary site of entry, where proliferation could have been either in surface epithelium or in the associated lymphoid follicles, it is probable that much of the infectivity in the other locations mentioned was due to their blood content (see Tables 3 and 4); excretion would not begin, therefore, until the surface epithelium had allowed passage of virus from the circulation or produced virus itself. These considerations would not, of course, apply to excretion from the plasma by the urinary route, assuming that the virus could pass the glomerular barrier.

SUMMARY

Pigs were infected by the intranasal instillation of a large dose (*ca.* $10^{7.0}$ ID 50) of a highly virulent strain of African swine fever virus (ASFV) and the progress of the infection was studied by the 'routine titration approach' (Mims, 1964) using pig bone marrow cultures.

Virus growth was established within 16–24 hr. in the retropharyngeal but not in the alimentary or nasal mucosae or the tonsils. By 24–40 hr. the virus was consistently present in the retropharyngeal lymph nodes, almost invariably the medials; titres in these nodes exceeded those in the associated mucosa by 48–72 hr. Generalization, presumed to have occurred via the tracheal lymph ducts and the blood stream, was generally demonstrable after 72 hr., i.e. by the time of the onset of pyrexia or 24 hr. prior to this.

On average 11 % of the total infectivity in the blood was present in the plasma, with the rest assumed to be cell-associated. A mean of about 45 % of the total infectivity was recovered in erythrocyte fractions in which the concentration of leucocytes had been materially reduced; fractions with increased leucocyte counts contained relatively little virus and it was concluded that the great majority of circulating virus was closely associated with the erythrocytes. Adsorption of ASFV to normal pig erythrocytes was demonstrated *in vitro*.

The greatest concentrations of virus were recorded in the lymph nodes, especially those of the cephalic region, and in the spleen, where titres commonly attained $10^{8.0}$ to $10^{9.0}$ HAD 50/g. and exceeded those in the blood. They were, therefore, thought to be the source of much circulating virus, although there was some evidence that the liver, lungs and bone marrow may also have contributed, at least in some animals. There was no evidence that the mucosae of the alimentary and respiratory tracts or the kidney, myocardium and brain were a source of significant amounts of virus. The virus demonstrable in Peyer's patches did not exceed that in the intervening ileal mucosa.

Although contact transmission of ASF does not normally occur during the first 12–24 hr. of fever, considerable amounts of virus were usually present in the nasal

and intestinal mucosae at 72 hr. It was probable that this infectivity was due to the blood content and that excretion did not occur until the epithelium was breached.

Three pigs, all of which had lesions of a portal cirrhosis, showed a delayed or restricted generalization of virus, in comparison with the other twenty-eight animals which behaved according to a regular pattern.

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