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African Swine Fever Virus Infection in the Argasid Host, *Ornithodoros porcinus porcinus*

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The pathogenesis of African swine fever virus (ASFV) infection in *Ornithodoros porcinus porcinus* was examined in nymphal ticks infected with the ASFV isolate Chiredzi/83/1. At times postinfection (p.i.) ranging from 6 h to 290 days, ticks or dissected tick tissues were titrated for virus and examined ultrastructurally for evidence of virus replication. The ASFV infection rate in ticks was 100% in these experiments, and virus infection was not associated with a significant increase in tick mortality. Initial ASFV replication occurred in phagocytic digestive cells of the midgut epithelium. Subsequent infection and replication of ASFV in undifferentiated midgut cells was observed at 15 days p.i. Generalization of virus infection from midgut to other tick tissues required 2 to 3 weeks and most likely involved virus movement across the basal lamina of the midgut into the hemocoel. Secondary sites of virus replication included hemocytes (type I and II), connective tissue, coxal gland, salivary gland, and reproductive tissue. Virus replication was not observed in the nervous tissue of the synganglion, Malpighian tubules, and muscle. Persistent infection, characterized by active virus replication, was observed for all involved tick tissues. After 91 days p.i., viral titers in salivary gland and reproductive tissue were consistently the highest detected. Successful tick-to-pig transmission of ASFV at 48 days p.i. correlated with high viral titers in salivary and coxal gland tissue and their secretions. A similar pattern of virus infection and persistence in *O. porcinus porcinus* was observed for three additional ASFV tick isolates in their associated ticks.

African swine fever (ASF) is a highly lethal disease of domestic pigs for which animal slaughter and area quarantine are the only methods of disease control. African swine fever virus (ASFV), the causative agent of ASF, is a large double-stranded DNA virus which is the only member of an unnamed family of viruses. ASFV is the only known DNA arbovirus (4, 6, 12). The natural arthropod host for ASFV is *Ornithodoros porcinus porcinus* (Walton) ticks (40). Some confusion exists in earlier reports since ticks that should be classified as *O. porcinus porcinus* are often referred to as either *O. moubata porcinus* or simply *O. moubata* (59).

ASFV can infect hosts through either a sylvatic cycle or a domestic cycle. In the sylvatic cycle, ASFV infects warthogs (*Phacochoerus aethiopicus*) and bushpigs (*Potamochoerus* spp.) as well as ticks of the genus *Ornithodoros* (7–10, 36, 55). In sub-Saharan Africa, warthogs occupy burrows which are frequently infested with large numbers of *O. porcinus porcinus* ticks (38, 45, 57, 58), and a correlation, though not absolute, has been established between ASFV infection of warthogs and the presence of *O. porcinus porcinus* ticks in burrows (57). In ASFV-enzootic areas, adult warthogs are typically nonviremic, although most are seropositive (28, 41, 46, 53, 58), and virus can usually be isolated only from lymph nodes (28, 41). Young warthogs, which are confined to the burrow for the first months of life, are most likely to be infected through feeding of infected *O. porcinus porcinus* ticks. Infection in young warthogs is subclinical, with viremic titers ranging from 2 to 3 $\log_{10}$ 50% hemadsorption dose (HAD_{50})/ml (56, 57), a level sufficient to infect a low percentage of naive ticks (42, 58, 30). The sylvatic ASFV cycle is further maintained by transovarial (43) and venereal (44) transmission in ticks. In burrows containing ASFV-infected ticks, infection rates are typically low (<2%), with the highest rate occurring in adult females (40, 45, 57, 65). The mechanism of ASFV transmission from the sylvatic cycle in Africa to the domestic cycle is most likely through feeding of infected ticks on pigs (41, 58), since direct contact between infected warthogs and domestic pigs has failed to result in transmission (36, 10, 28, 58), except in a single case (8). The virus may be transmitted between domestic pigs by either direct or indirect contact (33).

Various characteristics of ASFV infection have been studied in a number of *Ornithodoros* spp. ticks. The first association of ASFV with a tick was made by Sanchez-Botija (50), who reported isolation of ASFV from *O. erraticus*, a tick native to the Iberian peninsula and later considered important to maintenance of ASFV in an enzootic cycle in that region (51). In the first experimental infection, striking differences were found in the percentage of *O. moubata porcinus* ticks infected by two different ASFV isolates, a low infectious dose for ticks (ranging from of 0.9 to 4 $\log_{10}$ HAD_{50}) was demonstrated, and transmission out to 469 days postinfection (p.i.) was successful with single ticks (42). Experimental ASFV infection and transmission to pigs has been demonstrated for *O. savignyi*, a tick found in Africa (34), *O. coriaceus* (23, 25) and *O. turicata* (25), ticks indigenous to the United States, and *O. puertoricensis* (25, 14), a tick indigenous to the Caribbean. A 40% mortality rate was found in infected *O. coriaceus* (25) and *O. puertoricensis* ticks (15). *O. marocanus*, which was formerly referred to as *O. erraticus*, transmitted ASFV out to 588 days p.i., although 73% mortality was reported for infected ticks (16, 17). A number of reports have not found significant virus-induced mortality in *O. moubata porcinus* ticks (22, 40–44). In contrast, mortality rates were 35% higher in infected *O. moubata porcinus* females in

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the only study to examine mortality during the gonotrophic cycle (26).

Specific aspects of ASFV infection in the natural host remain poorly understood. Greig (22) experimentally infected *O. moubata porcinus* ticks with pathogenic ASFV isolates and used virus titration and immunofluorescence of dissected tissues to determine that the midgut was the initial site of viral replication and the site of longest persistence. Several other tissues were also found to have detectable levels of virus, although the midgut was the only tissue which was consistently positive. The presence of ASFV has been demonstrated in hemocytes of infected *O. coriaceus* ticks by electron microscopy and immunofluorescence studies, but the presence or nature of virus replication was not addressed (13).

Here we describe the pathogenesis and persistence of ASFV infection in *O. porcinus porcinus* ticks. Our data indicate that initial ASFV replication occurs in phagocytic digestive cells of the midgut epithelium, with secondary replication occurring in undifferentiated midgut cells at later times p.i. Generalization of virus infection from the midgut to other tick tissues required 2 to 3 weeks. Secondary sites of virus replication include hemocytes (type I and II), coxal gland, salivary gland, connective tissue, and reproductive tissue. Successful tick-to-pig transmission correlated with relatively high viral titers in salivary and coxal glands. Persistent infection in the tick involves continuous viral replication in several tissues and is associated with minimal cytopathology.

MATERIALS AND METHODS

ASFV isolates. Chiredzi/83/1 (Ch1) was isolated from *Ornithodoros* spp. ticks collected near Chiredzi, Zimbabwe (26), and was obtained from the Plum Island Animal Disease Center reference collection. Pretoriuskop/96/4/1 (Pr4) and Crocodile/96/1 (Cr1) were isolated from *O. porcinus porcinus* ticks collected from warthog burrows in Kruger National Park, Republic of South Africa, in September 1996. Nooitverwacht/96/6 (No6) was isolated from *O. porcinus porcinus* ticks collected from a warthog burrow in the Northern Province, Republic of South Africa, in September 1996. For Pr4 and Cr1, virus isolations were made by homogenizing individual ticks or small pools of ticks in 0.5 ml of medium (RPMI 1640; Gibco BRL) supplemented with 20% fetal bovine serum (HyClone Laboratories, Inc.) and 1× antibiotic-antimycotic (Gibco BRL) in sterilized Ten Broeck grinders. The homogenates were centrifuged at 10,000 × *g* for 1 min. The supernatants were diluted 1:10 and 1:100, and 100 μl was placed in multiple wells of a 96-well plate (Primaria) containing porcine peripheral blood mononuclear cells prepared as previously described (21, 37). After 24 h, 20 μl of diluted (2% [vol/vol]) porcine erythrocytes was added to each well, and samples were monitored for hemadsorption and cytopathic effect for a minimum of 7 days. For isolate No6, repeated virus isolation attempts from approximately 200 ticks, in pools of 3 to 10 ticks, failed to yield a virus isolate. Subsequently, 200 additional ticks from the same collection were allowed to feed on a naive pig. The pig developed ASF, and virus was isolated from the spleen and blood. Restriction endonuclease analysis of genomic DNA from all isolates confirmed the presence of a double-stranded DNA genome approximately 180 kbp in length (30).

Ticks. All ticks used in these experiments were *O. porcinus porcinus* (59). Ticks used for experiments with Ch1 isolate were from a colony maintained for an indeterminate period of time at the Plum Island Animal Disease Center. Ticks used for experiments with ASFV isolates Cr1, Pr4, and No6 were field-collected nymphs (stage N2 or N3) from the same collections which yielded the respective virus isolate. Natural ASFV infection rates for each collection were less than 2%. All ticks were held en masse in polycarbonate jars with a sand substrate. Jars were maintained in sealed desiccator boxes over a saturated NaCl solution, which provided a relative humidity of approximately 78%, at 26°C. Desiccator boxes were held in a diurnal chamber with 12-h light/12-h dark photoperiod.

A membrane feeding apparatus for individual tick feedings was patterned after that of Mango and Galun (32). Individual ticks were fed by placing each one in a polystyrene chamber made from the top end of a culture tube. This chamber was sealed at one end with stretched Parafilm M. The sealed end was placed in a well of a 12-well tissue culture plate which held 0.5 ml of heparinized pig blood. The blood was maintained at 39°C by floating the plate in a water bath for the duration of the feeding. Ticks were allowed to feed to repletion before being removed from the membrane and placed into a sterile multiwell tissue culture plate for coxal fluid collection. Ticks were observed for 2 h; if coxal fluid had not been produced by this time, the ticks were moved to a holding container. If coxal fluid was produced, it was collected by flushing the well with 0.5 ml of medium. Following feeding, blood from beneath the membrane was collected; the well and membrane were washed with an additional 0.5 ml of medium, and this was added to the blood sample. Samples were held at −70°C until assayed for virus titer as described above.

Infection of ticks. Three groups of ticks were fed on separate pigs infected by intramuscular injection with 2 $\log_{10}$ HAD_{50} of the ASFV isolate Ch1. Viremic titers on the day of tick feedings were 7.7 ± 0.2, 8.0 ± 0.3, and 7.9 ± 0.2 $\log_{10}$ HAD_{50}/ml for the three feedings on Ch1-infected pigs, 8.3 ± 0.3 $\log_{10}$ HAD_{50}/ml for the feeding on the Pr4-infected pig, 8.3 ± 0.3 $\log_{10}$ HAD_{50}/ml for the feeding on the Cr1-infected pig, and 7.8 ± 0.2 $\log_{10}$ HAD_{50}/ml for the feeding on the No6-infected pig. Tick feedings were conducted on pigs anesthetized with xylazine and ketamine HCl (1 mg/lb of body weight, intravenously) 5 to 6 days p.i. During the feeding, ticks were contained in chambers, made from sections of polyvinyl chloride pipe with wire mesh glued to either side, which were held in contact with the medial surface of the hindlimb. Only fully fed ticks were used for subsequent experiments.

Virus titrations. Individual whole ticks were ground in 0.5 ml of medium in sterilized Ten Broeck grinders. The samples were stored at −70°C. Immediately prior to titration, samples were thawed at 37°C, sonicated for 1 min, and centrifuged for 1 min at 10,000 × *g*. Supernatants were serially diluted, and replicate samples were added to porcine peripheral blood mononuclear cells as described above. Titers were calculated by the method of Reed and Muench (49).

To determine the virus titers in isolated organs, ticks were dissected under a binocular microscope and tissue samples were taken for determination of virus titer as follows. Individual ticks were weighed on an analytical balance so that titers could be reported on a per-milligram-of-tick-body-weight basis. Ticks were placed in a petri dish containing black dissecting wax ventral side down in a depression melted into the wax surface and held in place by forcing the legs into the softened wax. Immobilized ticks were covered with 100 μl of Grace's insect cell culture medium. After an incision was made through the cuticle and around the lateral edge, the dorsal cuticle was removed. After removal of the cuticle, as much of the 100 μl of medium as could be collected was withdrawn and saved for assay of virus titer; this sample contained hemolymph, leaked gut and rectal sac contents, and any coxal fluid produced by the tick as it was being attached. The tick was then immersed in fresh medium, and any remaining leaked gut contents and rectal sac contents were washed away to facilitate dissection. The following organs were removed and saved for assay of titer: the midgut and all of the attached ceca, paired salivary glands, paired coxal glands (without the associated coxal accessory gland), the gonads (testis, ovary, or the immature organ from preadult ticks, excluding the accessory gland or Gene's organ), and the synganglion. Following removal, each tissue was washed three times in sterile Grace's insect cell culture medium and placed in an Eppendorf tube with 100 μl of medium. Following dissection, the tissues were ground with sterile plastic pestles (Pellet pestle; Kontes). The cuticle and any remaining tissues (including but not limited to the rectal sac, Malpighian tubules, tracheal system, muscles, and connective tissues) were saved and processed as described above. Viral titers were determined as described above.

To determine the viremic titer of infected pigs, heparinized blood drawn from the anterior vena cava was held at −70°C until used, thawed at 37°C, sonicated for 1 min on ice, serially diluted, and added to cells as described above.

Histological and ultrastructural procedures. At times p.i., ticks were immobilized in paraffin wax, surrounded by a pool of ice-cold fixative, and sliced in half along the sagittal plane to allow infiltration of the fixatives and embedding resins (19). To ensure complete infiltration of the embedding medium a small portion of the front and back cuticle was removed. Ticks were fixed with a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4°C and further processed with 2% osmium tetroxide for 2 h at 4°C followed by 2% aqueous uranyl acetate overnight at 4°C. Ticks were dehydrated in ethanol and/or ethanol with propylene oxide and embedded in either Spurr's resin or Embed 812 (Electron Microscopy Sciences, Port Washington, Pa.). For ultrastructural studies, 70- to 90-nm sections were collected on single slot grids coated with Formvar, stabilized with carbon (Electron Microscopy Sciences), and photographed with a Philips 410 electron microscope operated at 80 kV. Nymphal ticks were sampled most extensively in the front half, which contained two-thirds of the midgut and its associated diverticula, salivary glands, coxal glands, synganglion, and portions of the Malpighian tubules. At least four sections from each of two ticks were sampled for each time point. For adult ticks, reproductive organs were isolated by dissection then processed and examined as described above.

RESULTS

Infection of *O. porcinus porcinus* ticks with ASFV. Nymphal *O. porcinus porcinus* ticks (stage N2 or N3) were fed on domestic pigs infected with ASFV isolate Ch1. Titration of individual ticks immediately after feeding indicated they were infected with a mean dose of 5.7 $\log_{10}$ HAD_{50} of ASFV/mg of tick body weight. At times p.i. ranging from 6 h to 290 days, whole ticks or dissected tick tissues were titrated for infectious virus ($n = 3$) and examined ultrastructurally for evidence of

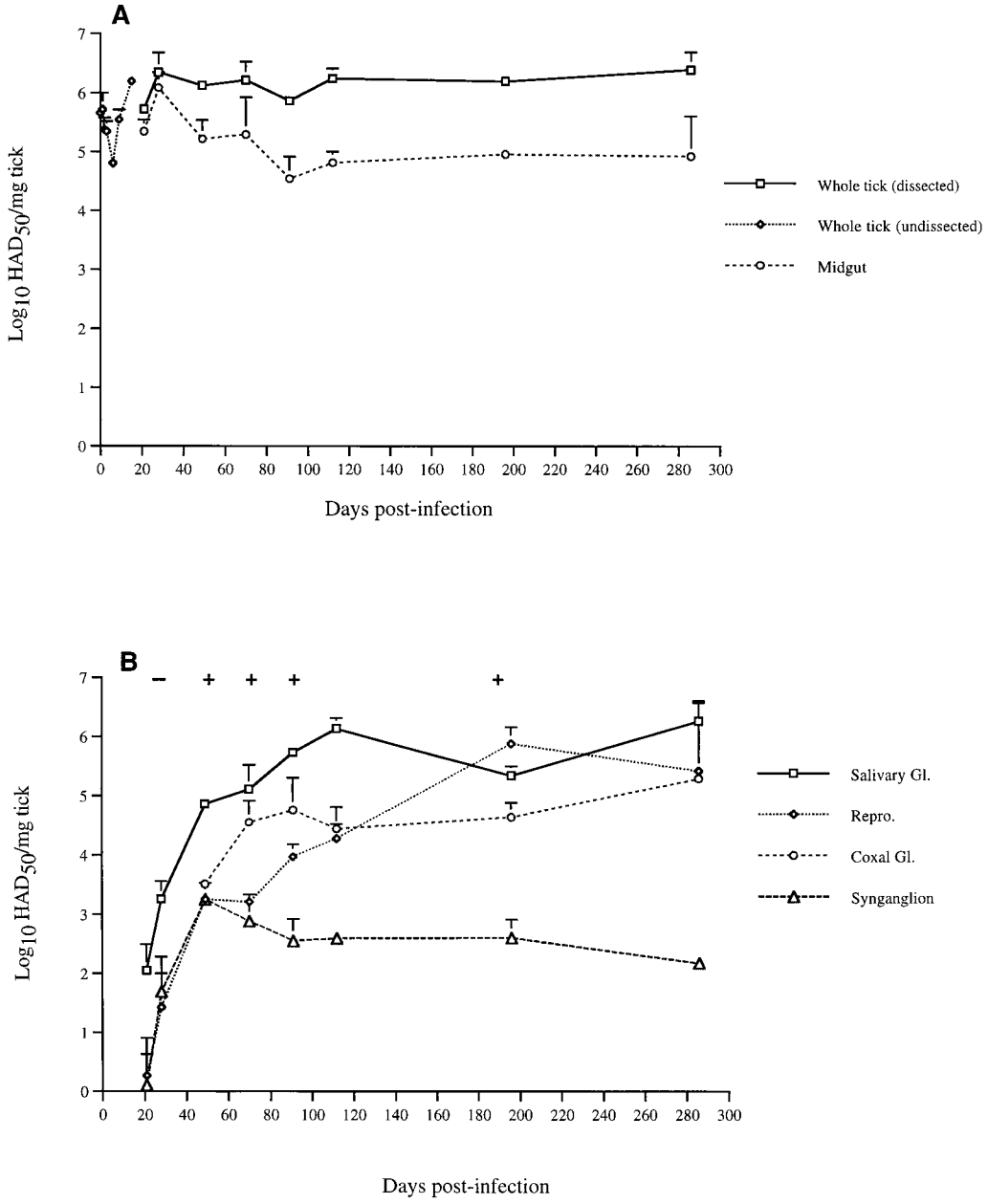


FIG. 1. ASFV titers in Ch1-infected ticks. Whole ticks and dissected tick midguts (A) plus additional tissues from the same ticks (B) were titered at times p.i. Values are expressed as mean titers ± standard errors of the means. Unsuccessful (−) and successful (+) tick-to-pig transmission attempts are indicated.

ASFV replication ($n = 2$). Virus titration data are shown in Fig. 1, and ultrastructural findings are summarized in Table 1. All ticks examined over the course of this study ($n = 78$) were found to be ASFV infected.

ASFV infection of *O. porcinus porcinus* was not associated with a significant increase in tick mortality. A group of ASFV infected ticks ($n = 60$) held separately for the duration of the study (290 days) exhibited a cumulative mortality rate of 6.6%, compared to 2.9% for an age- and feeding status-matched control group ($n = 35$). Additionally, no mortality was observed for ticks used in the pathogenesis experiments.

Initial ASFV replication occurs in phagocytic digestive cells of the midgut. Titration of individual whole ticks at 1, 2, 3, 6, 9, and 15 days p.i. demonstrated that virus levels decreased slightly, to 4.8 $\log_{10}$ HAD_{50}/mg at 6 days p.i., before increasing to 6.2 $\log_{10}$ HAD_{50}/mg at 15 days p.i. (Fig. 1A). Within 24 h postfeeding, ultrastructural analysis demonstrated phagocytic digestive midgut cells containing erythrocytes within phagolysosomes. Virions were observed adsorbed to erythrocytes, both within the phagolysosomes and in the midgut lumen (Fig. 2A, B, and D). Beginning at 4 days p.i., nascent virus factories were observed in the cytoplasm of phagocytic digestive cells (Fig. 2C). Virus factory structures were characterized by a small number of curvilinear (partially formed) viral capsids in electron-lucent regions of the cytoplasm (Fig. 2E). After 9 days p.i., virus factories were consistently observed in the phagocytic digestive cells of the midgut (Table 1). At 9 to 15 days p.i., infected cells containing large numbers of viral particles were

TABLE 1. Ultrastructural evidence of ASFV replication in *O. porcinus porcinus* ticks

Days postfeeding[a]	Factories observed[b]					
	Midgut	Hemocyte	Coxal gland	Connective tissue	Salivary gland	Reproductive tissue
0.25	−	−	−	−	−	NE
1	−	−	−	−	−	NE
2	−	−	−	−	−	NE
3	+	−	−	−	−	NE
4	+	−	−	−	−	NE
7	+	−	−	−	−	NE
9	+	−	−	−	−	NE
15	++	−	−	++	−	NE
21	++	+	−	++	−	NE
28	++	+	−	++	−	NE
42	++	++	+	++	++	NE
49	++	++	+	++	++	NE
70	++	++	+	++	++	NE
91	++	++	+	++	++	NE
168	++	++	+	++	++	NE
252[c]						
Male	++	++	+	++	++	++
Female	++	++	+	++	+	++

[a] Two ticks examined for each time point.
[b] —, no factories observed; +, 0 to 1 factories/section; ++, 2 to 15 factories/section; NE, not examined. No factories were observed in synganglia, Malpighian tubules, or skeletal muscle.
[c] Isolated organs from a single adult male and female.

projecting into the lumen (Fig. 3). Sloughed infected epithelial cells were also observed in the midgut lumen (data not shown). At 21 days p.i., infection of less differentiated midgut epithelial cells was noted (Fig. 4A). Peak numbers of infected midgut cells were observed at 21 days p.i. These data indicate that phagocytic midgut epithelial cells are the initial site of virus replication, replication in less differentiated cell types occurs at later times, and the midgut contains the majority of ASFV through 28 days p.i.

Generalization of ASFV infection requires 15 to 21 days. Before 15 to 21 days p.i., only midgut tissues exhibited ultrastructural evidence of virus infection and replication. At 21 days p.i., undifferentiated cells of the midgut contained numerous virus factories. A large number of mature virions were observed both adjacent to and within the basal lamina (BL) of the midgut, with an occasional virion on the hemocoel side of the BL, suggesting a possible route of movement of virus from the primary site of replication, the midgut, across the BL into the hemocoel (Fig. 4B). At 15 to 21 days p.i., ultrastructural analysis demonstrated the first appearance of viral replication in both type I and type II hemocytes and connective tissue (Fig. 5). At this time point, and all subsequent time points, infected hemocytes were observed with virions budding from the plasma membrane (Fig. 5B). Virus titration data from individual ticks tissues at 21 days p.i. support the ultrastructural data described above. At 21 days p.i., high viral titers were detected only in the midgut (Fig. 1A). However, small amounts of virus (2 $\log_{10}$ HAD_{50}/mg of tick) were consistently found in the salivary gland at this time, indicating that virus infection of this tissue had already occurred (Fig. 1B). These data suggest that critical early events in midgut are necessary for successful generalization of ASFV infection in *O. porcinus porcinus* ticks.

Generalization and persistence of ASFV infection in *O. porcinus porcinus* ticks. Whole tick titers, which after 21 days p.i. were calculated by summation of the titers of all dissected and undissected tissues, reached their peak (6.4 $\log_{10}$ HAD_{50}/mg of tick) at 28 days p.i. and were maintained at this approximate level throughout the 290-day sampling period (Fig. 1A). Virus titers in the midgut increased to 6.1 $\log_{10}$ HAD_{50}/mg of tick at 28 days p.i., the highest titer detected for this tissue, and then persisted at titers of approximately 5.0 $\log_{10}$ HAD_{50}/mg of tick for the duration of the study.

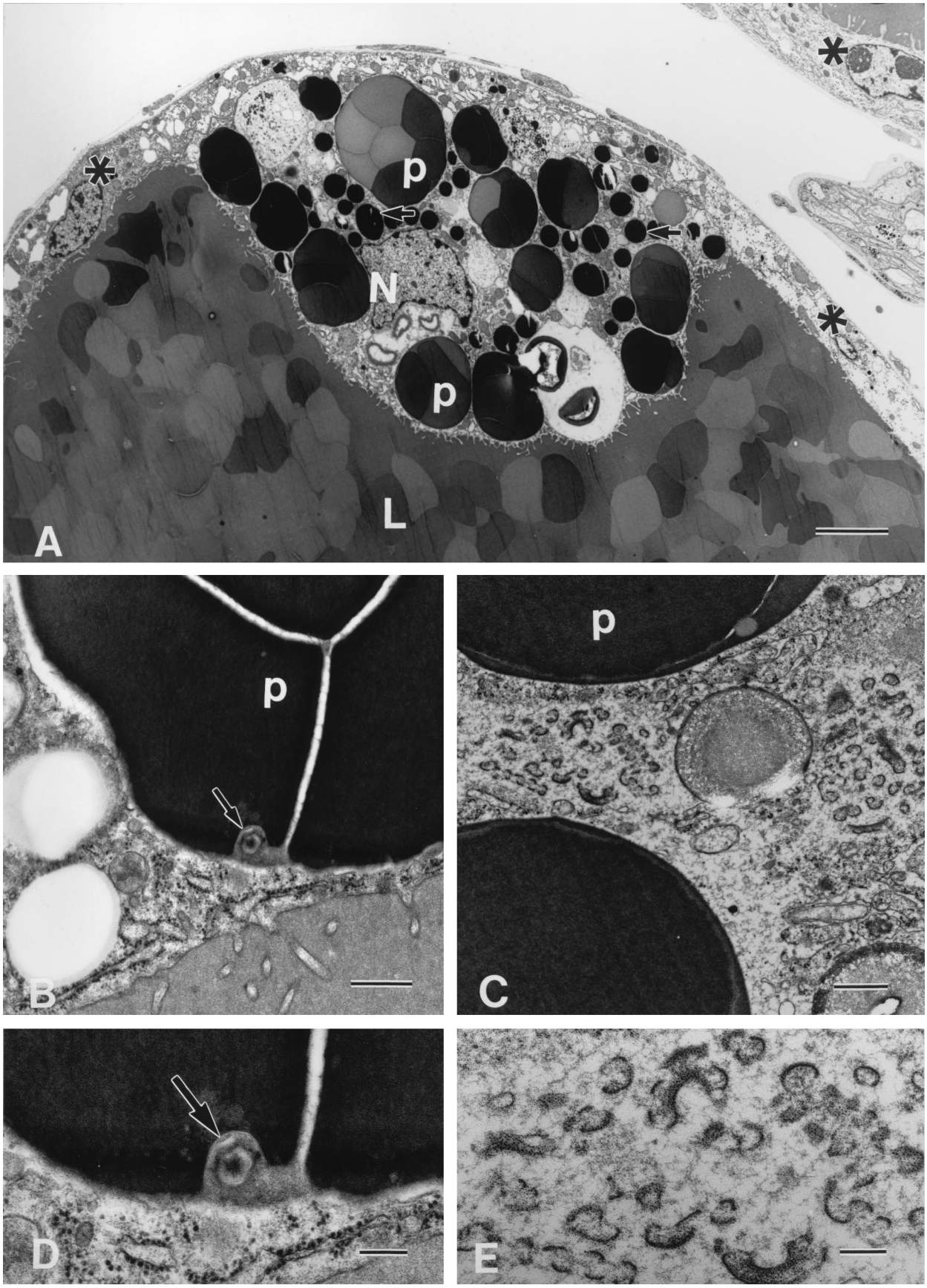
Coxal gland titers of ASFV rose to peak levels of approximately 4.5 $\log_{10}$ HAD_{50}/mg of tick by 70 days p.i. and persisted at this level throughout the 290-day experimental period (Fig. 1B). Virus replication was observed in both cells of the filtration membrane and cells of the collecting tubule of the coxal gland, and numerous virions were observed budding into the lumen of the filtration membrane (Fig. 6). Viral titers in the reproductive tissue continued to rise through 28 weeks p.i. In males, virus was observed budding from the accessory gland and the connective tissue lining the sperm ducts. In females, active virus replication was observed in nurse cells and in developing oocytes (data not shown).

ASFV titers in salivary gland increased 10,000-fold between 21 and 112 days p.i., reaching a peak titer of 6.1 $\log_{10}$ HAD_{50}/mg of tick. Similar high ASFV titers were detected at later sampling points (Fig. 1B). Virus replication was first observed in the connective tissue of the salivary gland at 21 days p.i. (Fig. 7B). At 42 days p.i., virus factories and mature virions were observed in the granular cells of the salivary gland, with virions accumulating in secretory granules (Fig. 7C). At later times, these observations were more pronounced.

In addition to cells of specific organs, connective tissue cells surrounding most organs and tissues were observed to contain replicating virus, which may have contributed to the viral titers of dissected tissues. Most notably, the connective tissue surrounding the synganglion contained replicating virus, although virus was never observed in the nervous tissue proper. In addition, evidence of virus replication was not detected in either the Malpighian tubules or skeletal muscle.

Successful tick-to-pig transmission of ASFV correlates with high viral titers in salivary and coxal glands. Virus transmission was first attempted at 21 days p.i. by feeding 19 ticks on an anesthetized, naive pig (Table 2). The pig failed to demonstrate any signs of ASFV infection over a 21-day observation period. Subsequent attempts at virus transmission via tick feeding at 48, 71, 92, and 187 days p.i. were successful, with characteristic ASF disease in infected pigs. The time to onset of febrile response ranged from 2 to 4 days, with death occurring from 6 to 8 days postfeeding (Table 2). These transmission results correlate well with the dramatic rise of viral titers in salivary and coxal glands that occurred between 21 days p.i., a time when transmission was unsuccessful, and the time of successful transmission at 48 days p.i. (Fig. 1B). Additionally, ASFV virions were first observed in secretory granules of the

FIG. 2. ASFV uptake and early stages of replication in midgut digestive cells. (A) At 24 h postfeeding, intact erythrocytes have been taken into phagolysosomes (p) from the blood meal in the lumen (L). Hematin crystals (arrows) are scattered throughout the cytoplasm. Undifferentiated epithelial cells (asterisks) occur on either side of the digestive cell. N, nucleus of digestive cell. Bar, 5 μm. (B) Phagolysosome (p) of a digestive cell 72 h postfeeding. Ingested erythrocyte has an enveloped ASFV particle associated with it (arrow). Bar, 0.5 μm. (C) Nascent virus factory in digestive cell at 96 h postfeeding. Curvilinear forms of assembling virus particles occur in a more electron-lucent region of the cytoplasm. p, phagolysosomes; bar, 0.5 μm. (D) High-magnification view of ASFV particle (arrow) in panel B showing possible deterioration of virus structure. Bar, 0.2 μm. (E) High-magnification view of nascent virus factory in panel C. Bar, 0.2 μm.



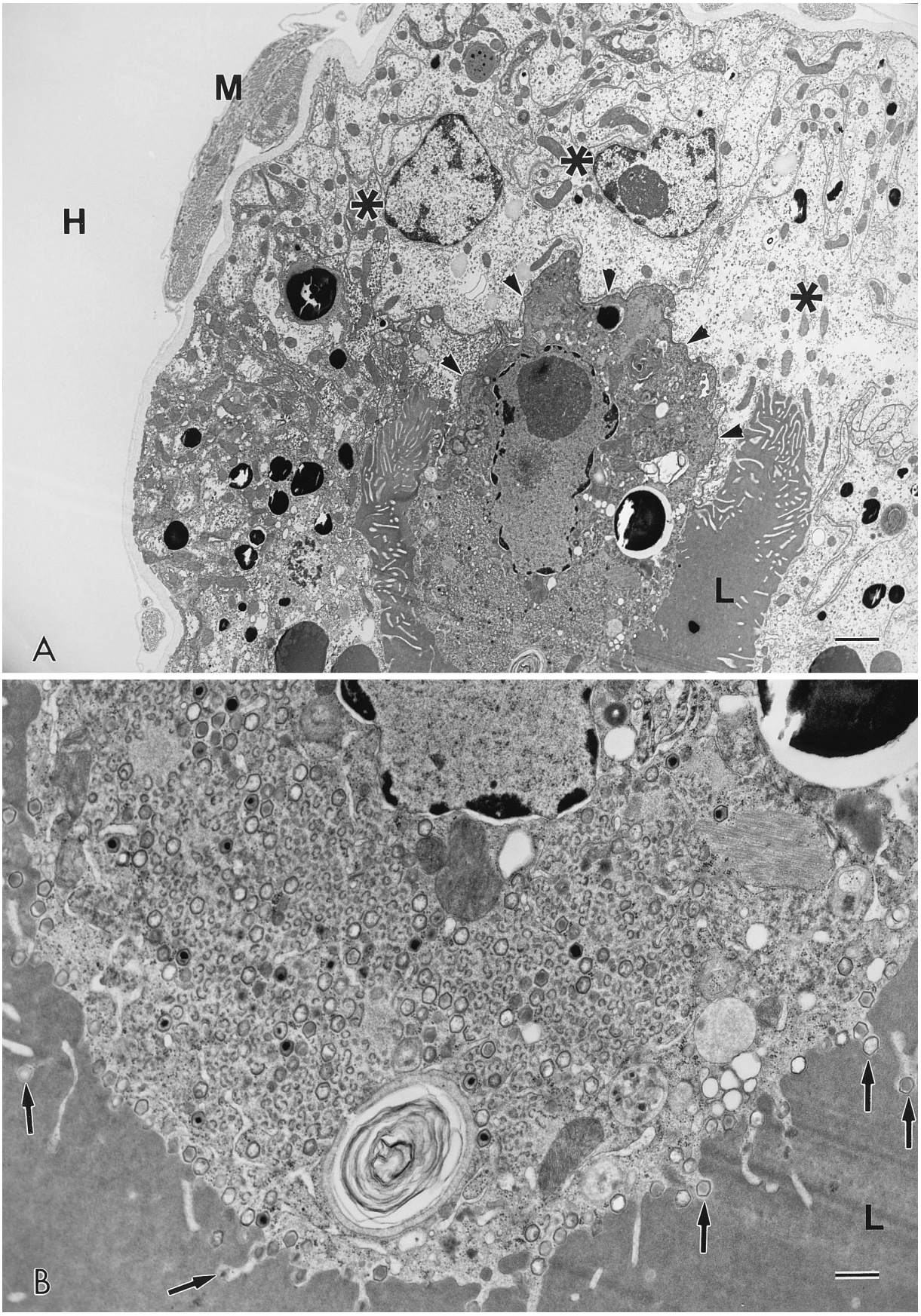


FIG. 3. Replication of ASFV in midgut digestive cells at 15 days postfeeding. (A) A digestive cell with an extensive virus factory projects into the midgut lumen (L) and is separated from undifferentiated epithelial cells (asterisks) via septate junctions (arrowheads). H, hemocoel; M, muscle; bar, 5 μm. (B) High-magnification view of the mature virus factory in panel A showing virus in various stages of assembly. Virions (arrows) are budding into the lumen (L). Bar, 0.5 μm.

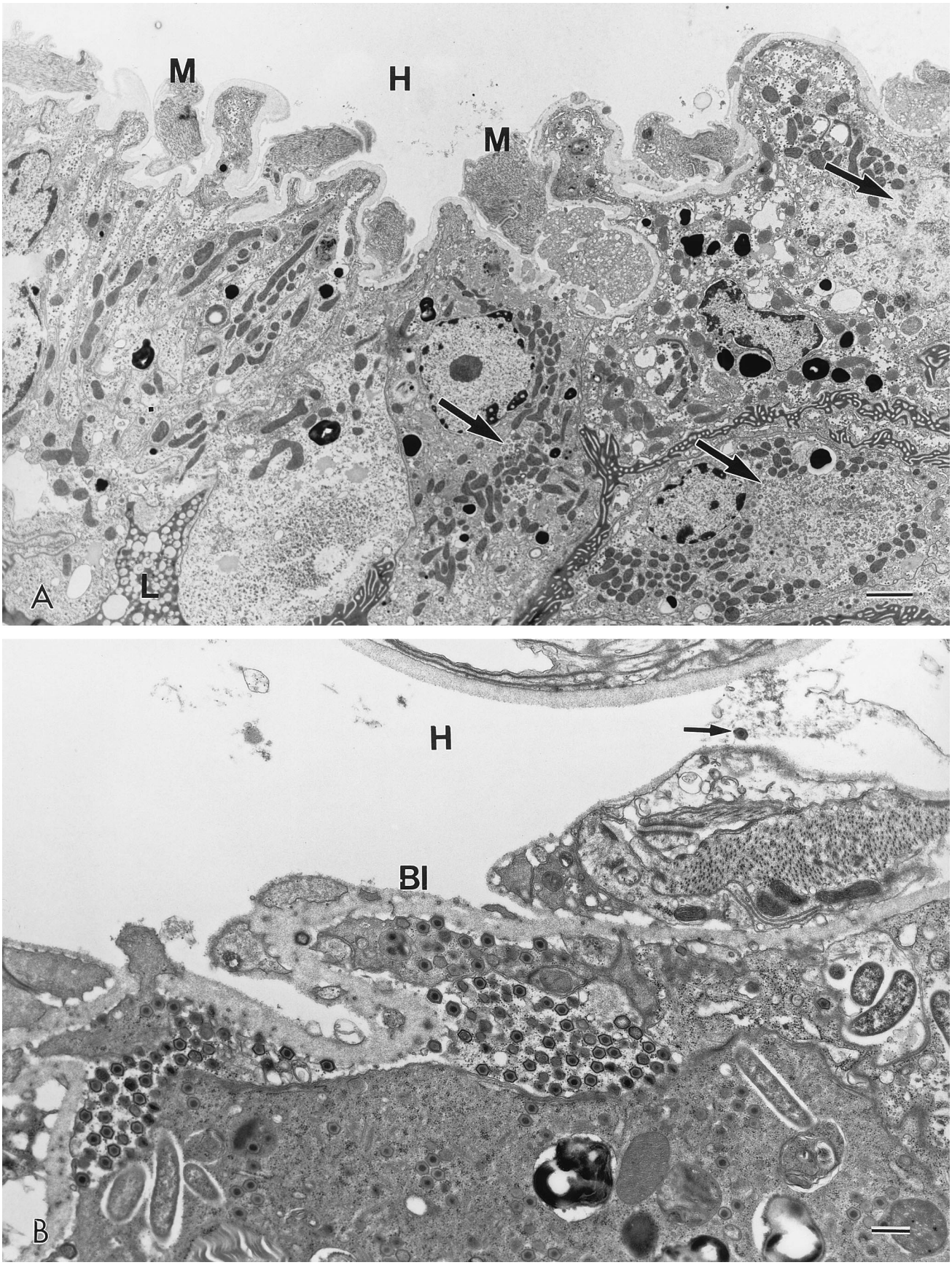


FIG. 4. Replication and generalization of ASFV in undifferentiated midgut cells at 21 days postfeeding. (A) Three undifferentiated epithelial cells with virus factories (large arrows). M, muscle; H, hemocoel; L, lumen; bar, 5 μm. (B) Accumulation of ASFV under the basal lamina (Bl). Arrow, virus free in the hemocoel (H); bar, 1 μm.

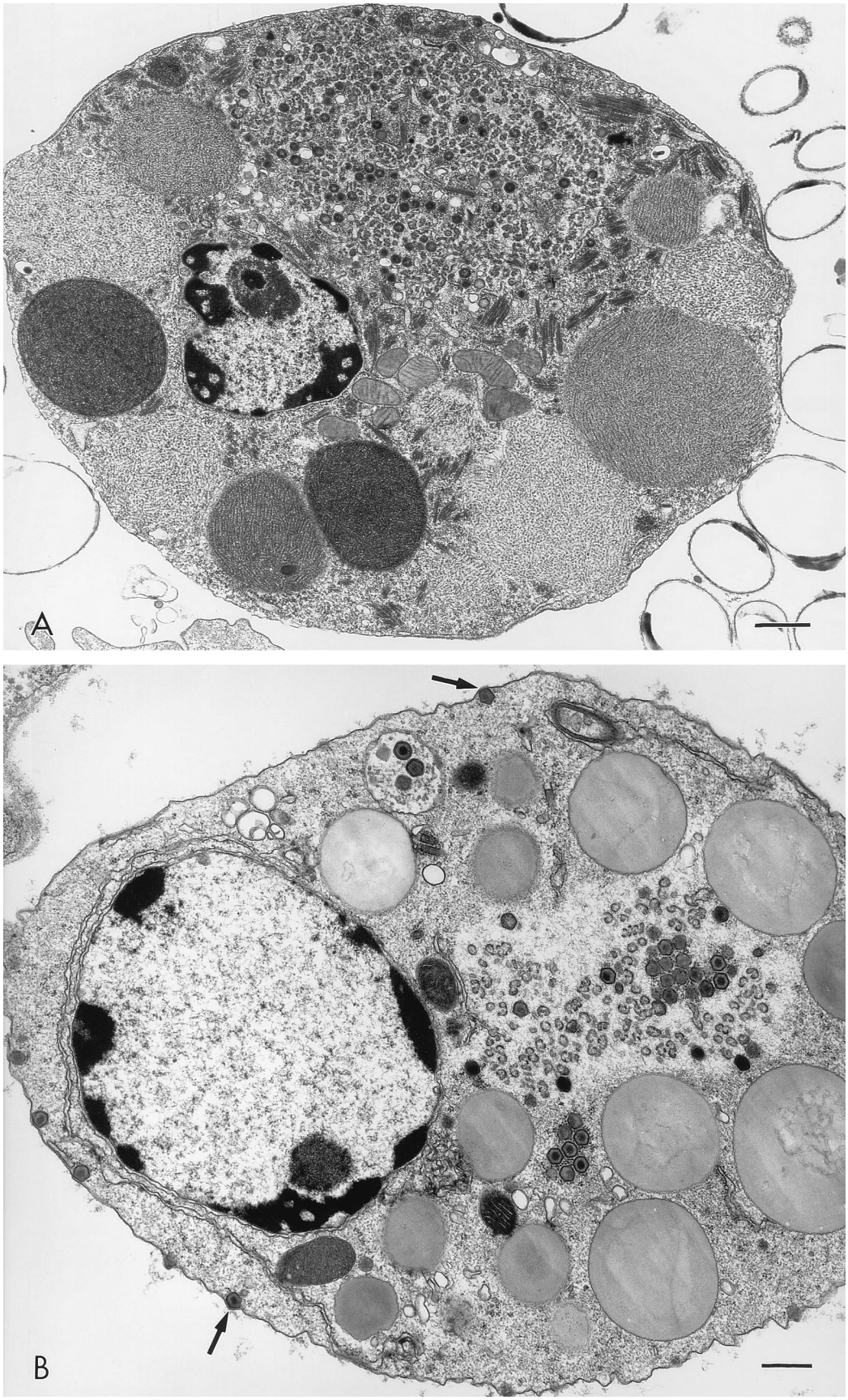


FIG. 5. ASFV in hemocytes. (A) Type I hemocyte containing an extensive virus factory with many crystalline arrays at the periphery. Bar, 1 μm. (B) Type II hemocyte with virus factory and budding virus particles (arrows). Bar, 0.5 μm.

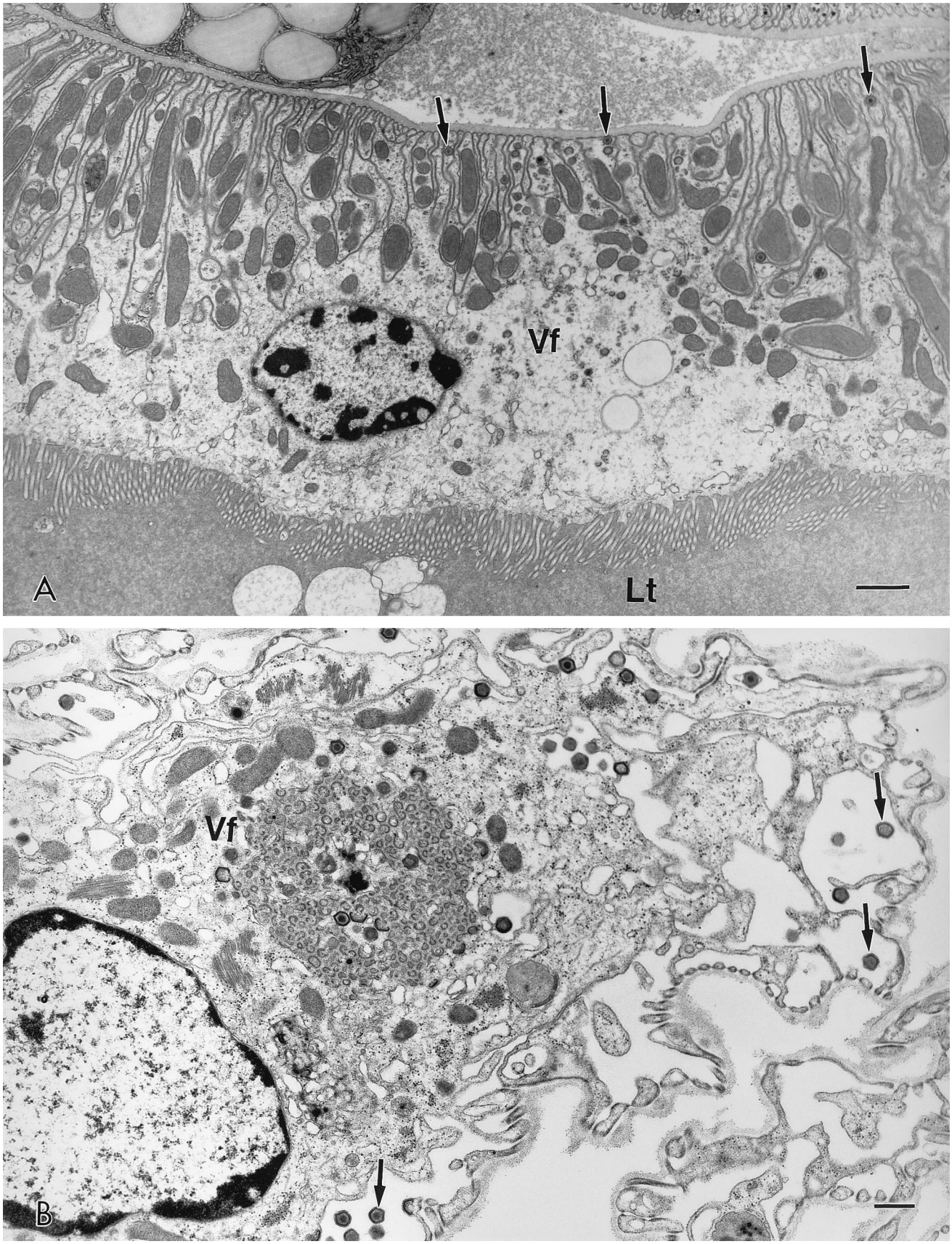


FIG. 6. ASFV in coxal gland. (A) A small virus factory (Vf), mature particles (arrows), and a condensed-chromatin-containing nucleus in the proximal tubule of the coxal gland. Lt, lumen of tubule; bar, 1 μm. (B) Cell of the filtration membrane portion of coxal gland with a virus factory (Vf) and released virions (arrows). Bar, 0.5 μm.

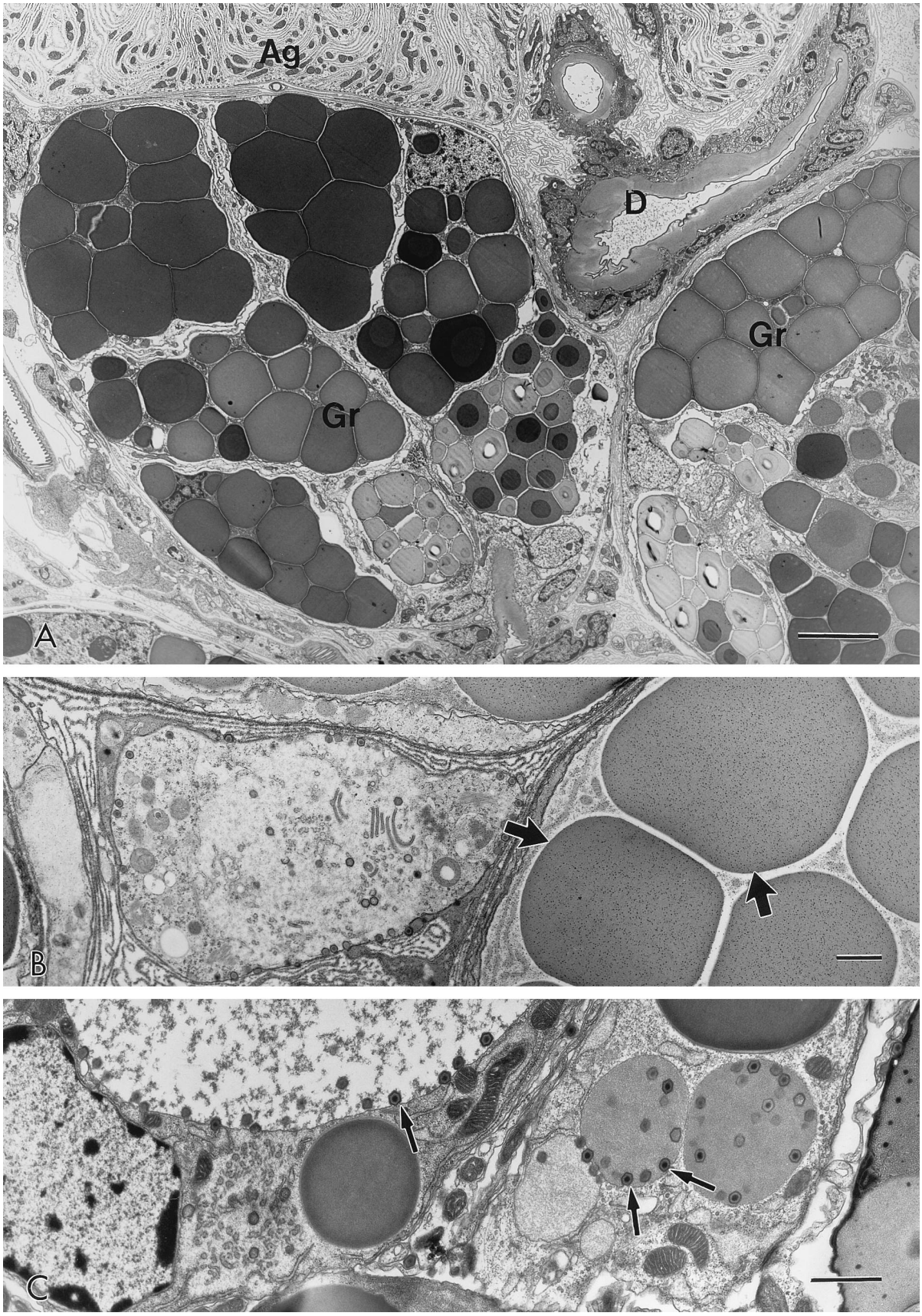


FIG. 7. ASFV in salivary gland. (A) Mock-infected salivary gland at 30 days postfeeding to show tissue structure. The salivary gland is composed of agranular (Ag) and granular (Gr) portions. Connective tissue cells occur at the edge and in the center of the gland. D, cuticle-lined ducts. (B) At 30 days p.i., replicating ASFV is found only in the connective tissue cells in the center of the gland. Secretory granules (arrows) do not contain virus. (C) At 42 days p.i., virus is observed budding into secretory granules (arrows). Bars: panel A, 5 μm; panels B and C, 1 μm.

TABLE 2. ASFV transmission to pigs by *O. porcinus porcinus* ticks

Virus	Days post-inoculation (ticks)	No. of ticks fed	Disease onset and progression in pigs			
			Days to onset of fever	Days to peak viremia	Peak viremic titer (log_{10} HAD_{50}/ml blood ± SEM)	Days to death
Ch1	21	19	Neg[a]	Neg	Neg	
	48	20	4	4	9.0 ± 0.3	6
	71	48	3	6	9.3 ± 0.3	8
	92	18	3	5	8.8 ± 0.3	6
	187	2	2	5	8.8 ± 0.4	6
	187	5	2	7	9.5 ± 0.0	8
No6	54	20	3	5	8.5 ± 0.0	13
Cr1	54	20	3	5	9.0 ± 0.3	6
Pr4	54	20	3	4	8.8 ± 0.3	5

[a] Neg, negative (transmission attempt was unsuccessful).

salivary gland and in the coxal gland of infected ticks at 42 days p.i. (Fig. 6 and 7C).

To determine the potential route(s) of virus transmission and to quantitate the level(s) of virus excreted, ticks were individually fed on artificial membrane feeders. Titratable virus was detected in both the salivary secretion and the coxal fluid excreted following feeding (Table 3). At 56 days p.i., salivary secretions from 4 of 13 (31%) ticks contained titratable virus, with titers ranging from 0.8 to 2.0 log_{10} HAD_{50}. All coxal fluid samples collected ($n = 6$) at this time contained virus, with titers ranging from 1.0 to 4.8 log_{10} HAD_{50}. At 84 days p.i., salivary secretions from 10 of 18 (56%) ticks contained virus, with titers ranging from 1.0 to 4.0 log_{10} HAD_{50}. All coxal fluid samples collected ($n = 8$) contained virus, with titers ranging from 1.8 to 4.3 log_{10} HAD_{50}.

Infection of *O. porcinus porcinus* with ASFV field isolates. Experiments similar to those described for the ASFV isolate Ch1 in colonized *O. porcinus porcinus* ticks were performed with ASFV isolates and *O. porcinus porcinus* ticks that were obtained from the same warthog burrow. Groups of ticks were fed on anesthetized pigs previously infected with 2 log_{10} HAD_{50} of the Pr4, Cr1, and No6 virus isolates. As with Ch1, tick infection rates with the Pr4, Cr1, and No6 isolates were 100%. At 227 days p.i., the cumulative mortality rates for Pr4-, Cr1-, and No6-infected ticks were 6.8% ($n = 44$), 1.9% ($n = 52$), and 11.0% ($n = 63$), respectively. Individual ticks ($n = 3$) were dissected at 3, 7, and 13 weeks p.i. Titration of dissected tissues revealed a pattern of virus infection and persistence similar to that found with Ch1-infected ticks (Fig. 8). At 21 days p.i., the midgut contained 5.4 to 6.5 log_{10} HAD_{50}/mg of tick body weight, while the synganglion, salivary gland, coxal gland, and reproductive tissue contained from 1 to 3 log_{10} HAD_{50}/mg of tick body weight. By 7 weeks p.i., salivary gland titers had increased over 100-fold, coxal gland titers had increased 10- to 50-fold, reproductive tissue titers had increased 100-fold, and synganglion titers had increased 50-fold. On average, midgut titers at 7 weeks p.i. were the highest of any tissue examined, with values ranging between 5.2 and 6.3 log_{10} HAD_{50}/mg of tick body weight. At 13 weeks p.i., salivary gland titers had increased further to 4.9 to 5.2 log_{10} HAD_{50}/mg of tick body weight. Ticks infected with Pr4, Cr1, and No6 isolates ($n = 20$/isolate) successfully transmitted virus to naive pigs at 56 days p.i. A typical ASF disease course was observed for infected animals.

DISCUSSION

In this report, we have described characteristics of ASFV infection, replication, dissemination, and transmission in *O. porcinus porcinus* ticks. Most work on the pathogenesis and dissemination of arboviruses within their vectors has been done with mosquitoes (64). However, ticks differ fundamentally from other hematophagous insects in their life history, feeding behavior, and digestive physiology and therefore pose different questions regarding virus-vector interactions. Few studies have addressed characteristics of arboviruses pathogenesis in ticks (1, 2, 5, 11, 22, 35).

After infection, the initial site of ASFV replication is in phagocytic digestive cells of the midgut epithelium. Similarly, Dugbe virus targets differentiated digestive gut cells of *Amblyomma variegatum* ticks soon after feeding, although hemocytes are considered to be the predominant cell supporting virus replication later in infection (2). In Qalyub virus infection of *O. erraticus* ticks, replication was detected only in midgut cells, and dissemination to other tissues was rarely observed (35). Powassan virus was detected exclusively in the midgut of *Dermacentor andersoni* ticks until 17 days p.i. (5). In contrast, midgut cells were infected in 20% or less of Thogoto virus infected *Rhipicephalus appendiculatus* ticks, and the synganglion appeared to be the main organ involved with virus replication and persistence (1). Midgut titers of these viruses were at least 1,000-fold lower than those observed for ASFV in this study.

Mechanisms of ASFV entry into midgut epithelial cells have not been previously described. Observations made here of ASF virions adsorbed to intact erythrocytes within phagolysosomes of midgut epithelial cells suggest that initial virus entry may be

TABLE 3. Quantitation of ASFV in salivary and coxal gland secretions from individual *O. porcinus porcinus* ticks

Tick no.	Log_{10} HAD_{50}/ml ± SEM[a]			
	56 days p.i.		84 days p.i.	
	Salivary secretion	Coxal fluid	Salivary secretion	Coxal fluid
2	DNF		2.0 ± 0.4	4.3 ± 0.3
3	DNF		1.0 ± 0.0	3.0 ± 0.3
5	DNF		3.3 ± 0.4	SNC
7	DNF		0	SNC
9	0	SNC	DNF	
11	DNF		0	SNC
12	DNF		1.3 ± 0.3	SNC
14	0	SNC	DNF	
16	0	1.0 ± 0.3	DNF	
18	DNF		0	4.0 ± 0.3
19	0	SNC	DNF	
20	DNF		0	1.8 ± 0.3
21	0.8 ± 0.0	SNC	1.5 ± 0.3	3.8 ± 0.3
23	2.0 ± 0.3	4.8 ± 0.3	DNF	
26	1.0 ± 0.3	1.8 ± 0.3	0	SNC
27	0.9 ± 0.0	SNC	3.5 ± 0.4	SNC
28	DNF		1.3 ± 0.3	SNC
29	DNF		2.0 ± 0.4	4.0 ± 0.3
31	0	2.8 ± 0.3	DNF	
32	0	3.0 ± 0.3	4.0 ± 0.3	4.0 ± 0.3
33	DNF		3.0 ± 0.4	SNC
34	0	SNC	0	SNC
37	0	3.5 ± 0.3	DNF	
38	DNF		0	SNC
40	0	SNC	0	4.0 ± 0.3

[a] DNF, did not feed; SNC, sample not collected.

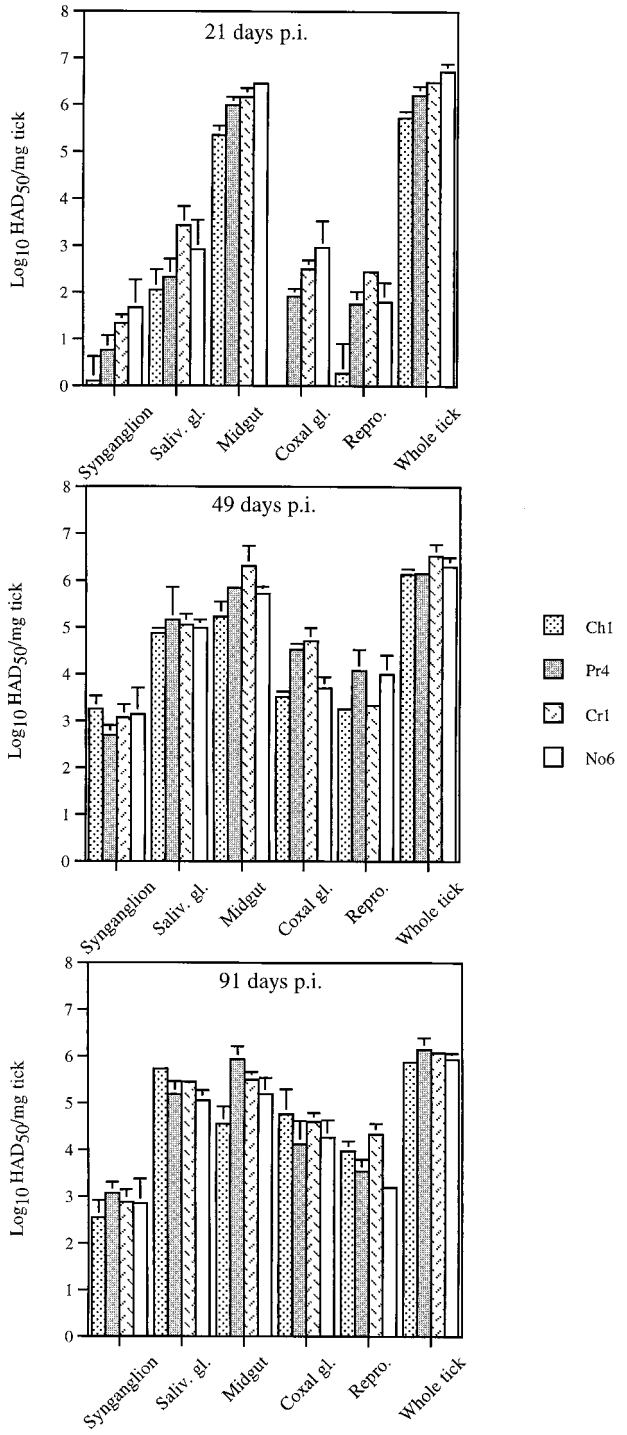


FIG. 8. Tissue titrations of ASFV-infected *O. porcinus porcinus* ticks. *O. porcinus porcinus* ticks (stages N2 to N4) collected from natural habitat were infected with ASFV isolates (Pr4, Cr1, and No6). At times p.i., ticks were dissected and tissue titers were determined. Values are expressed as mean titers ± standard errors of the means. Saliv., salivary; gl., gland.

via erythrocyte phagocytosis. Notably, almost all ASFV field isolates are hemadsorbing (31), and a significant proportion of the viremia in infected pigs is associated with the erythrocyte fraction (3, 20, 27, 39, 60). However, a second mechanism of virus entry is also likely to exist since undifferentiated epithelial cells become infected after hemolysis of the blood meal is complete. Receptor-mediated endocytosis of free virus in the midgut lumen at later times after infection may be an additional route of virus entry. A possible source of virus for infection of undifferentiated midgut cells is virions released from the initially infected midgut cells, which subsequently slough into the midgut lumen, releasing a large number of infectious particles. Isolation of nonhemadsorbing ASFV from ticks collected in warthog burrows (57) suggests the possibility of an entry mechanism that does not involve erythrocyte phagocytosis. Also, *O. porcinus porcinus* ticks have been experimentally infected with ASFV by feeding on cell-free virus preparations on an artificial membrane (52).

Interestingly, and in contrast to the report of Greig (22), a 15- to 21-day delay in generalization of ASFV infection in the tick was observed, suggesting a midgut barrier to generalization of infection. Such a barrier has been described for some arboviruses of mosquitoes (64). As with other arboviruses, the gut barrier to ASFV generalization may involve translocation of virus across the BL of the midgut into the hemocoel. The extensive replication and accumulation of ASF virions under the BL, similar to that seen in several arbovirus infections of mosquitoes (29, 62, 63), and the observation of virions within and immediately adjacent to the BL on the hemocoel side suggest that ASFV dissemination involves movement of virus across the BL. The delay observed may be due to the inefficiency of movement across the BL and/or to a requirement for high viral titers in proximity to the BL, a condition likely requiring extensive virus replication and thus time to achieve. The observation of ASFV replication in connective tissue and hemocytes early in generalized infection indicates their significance as secondary sites of virus amplification.

After dissemination and infection of a number of different tissues, a viral titer of over 6 $\log_{10}$ HAD_{50}/mg of body weight was maintained through out the 290-day sampling period. The total tick titers reported here correlate well with ASFV titers found in some naturally infected ticks (30, 45, 56, 57, 65). The titers of dissected salivary glands and reproductive tissue, which were the highest of any tissues studied after 91 days p.i., were found to rise to 5 to 6 $\log_{10}$ HAD_{50}/mg and persisted at this level through the sampling period. Maintenance of high ASFV titers in the salivary gland throughout infection is in contrast to observations made for ixodid ticks infected with other arboviruses, in which salivary gland viral titers increase after commencement of feeding (1, 2, 5, 11). This difference is likely necessitated by the different biology and life cycles of these two families of ticks. Unlike ixodid ticks, which feed on a vertebrate host for a number of days, thus allowing virus replication to be regulated in direct response to a transmission opportunity, *O. porcinus porcinus* ticks feed rapidly (in 1 h or less), requiring that virus already be present in tissues in quantities sufficient for transmission.

Successful transmission of ASFV to pigs by infected *O. porcinus porcinus* ticks coincided with high viral titers in salivary and coxal glands and the presence of virus in their secretions. Both salivary secretions and coxal fluid have previously been suggested as sources of virus for tick-pig transmission of ASFV (42). The observation here that coxal secretions were more often positive for virus than salivary secretions and that they contained relatively high titers of virus suggests that this may be a more important source of virus for transmission than previously recognized. Infection via coxal fluid may occur through contamination of abrasions and/or tick-feeding lesions at the skin surface. Ultrastructural visualization of ASF virions in secretory granules of the salivary gland indicates that this is a likely route of transmission during tick feeding. Although

high titers of ASFV were present in tick midgut at all times p.i., the presence of a proventricular valve at the esophagal terminus in argasid species precludes regurgitation as an operative transmission mechanism for ASFV (54).

Overall, our data indicate that ASFV is highly adapted to *O. porcinus porcinus* ticks. High levels of viral replication are present in diverse tissues and cell types, with little evidence of cytopathology and no significant increase in tick mortality. These data are consistent with observations made for ASFV-infected *O. porcinus porcinus* ticks under field conditions (30, 45, 56, 57, 65). The relatively constant high titers of virus maintained in the tick over long periods of time suggest that there is a mechanism for regulating viral replication at levels that are compatible with host survival. In the swine host, differentiated tissue macrophages and reticular cells are the major viral targets in vivo and, it has been suggested that the stage of monocyte differentiation may influence cell susceptibility to ASFV infection (24, 31, 61). Observations here of extensive replication in differentiated midgut epithelial cells and hemocytes suggest that viral replication might be regulated in part by the availability of cells at the appropriate state of differentiation. Alternatively, an immune-like host response to viral infection might be involved. Although no antiviral host responses have yet been described in arthropods (18, 48), it is reasonable to assume that they exist. ASFV infection of nonadapted European and North American *Ornithodoros* spp. results in significant tick mortality (14–17, 23, 25). Pathogenesis experiments with these ticks have not been done; thus, a direct comparison with our data on ASFV infection of *O. porcinus porcinus* cannot be made. However, it is possible that the inability of a nonadapted tick host to control virus replication and/or virus-induced pathology is responsible for the observed mortality.

In agreement with a previous study (22), the work presented here demonstrates that the midgut is the initial site of virus replication. However, a number of results described here for ASFV infection of *O. porcinus porcinus* ticks differ significantly from those reported by Greig (22). In these earlier experiments, which used an infectious dose ($\sim$5.0 $\log_{10}$ HAD_{50}) and virus isolation procedures similar to those used in our studies, tick infection rates varied from 54 to 97%, depending on the ASFV isolate and the collection of *O. porcinus porcinus* ticks used. Viral titers of infected tick tissues declined dramatically over the sampling period of 140 to 350 days, and salivary gland and reproductive tissues titers were never consistently higher than 2 $\log_{10}$ HAD_{50}. It is unlikely that these differences are due to experimental design and/or random variation; rather, they likely reflect the degree of virus-host adaptation. The four ASFV isolates examined in the present study were all of tick origin, and infection studies with three of them (Pr4, Cr1, and No6) were conducted with ticks from the same collection from which the virus was isolated. The significance of virus-host adaptation to infection outcome is also suggested by a previous study where markedly different infection rates (5% versus 95%) were observed when ticks from the same collection were infected with two different ASFV isolates (42).

In summary, our results demonstrate that *O. porcinus porcinus* ticks fed on viremic pigs develop a long-term, persistent infection with continuous and high levels of virus replication, experience minimal mortality, and efficiently transmit virus to pigs. Given these characteristics, it is likely that ASFV infection in its natural *O. porcinus porcinus* host represents a well-adapted and possibly coevolved biological system.

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