Porcine Reproductive and Respiratory Syndrome Virus Replicates in Testicular Germ Cells, Alters Spermatogenesis, and Induces Germ Cell Death by Apoptosis†

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Like other arteriviruses, porcine reproductive and respiratory syndrome virus (PRRSV) is shed in semen, a feature that is critical for the venereal transmission of this group of viruses. In spite of its epidemiological importance, little is known of the association of PRRSV or other arteriviruses with gonadal tissues. We experimentally infected a group of boars with PRRSV 12068-96, a virulent field strain. By combined use of in situ hybridization and immunohistochemistry, we detected infection by PRRSV in the testes of these boars. The PRRSV testicular replication in testis centers on two types of cells: (i) epithelial germ cells of the seminiferous tubules, primarily spermatids and spermatocytes, and (ii) macrophages, which are located in the interstitium of the testis. Histopathologically, hypospermatogenesis, formation of multinucleated giant cells (MGCs), and abundant germ cell depletion and death were observed. We obtained evidence that such germ cell death occurs by apoptosis, as determined by a characteristic histologic pattern and evidence of massive DNA fragmentation detected in situ (TUNEL [terminal deoxynucleotidyltransferase-mediated digoxigenin-UTP nick end labeling] assay). Simultaneously with these testicular alterations, we observed that there is a significant increase in the number of immature sperm cells (mainly MGCs, spermatids, and spermatocytes) in the ejaculates of the PRRSV-inoculated boars and that these cells are infected with PRRSV. Our results indicate that PRRSV may infect target cells other than macrophages, that these infected cells can be primarily responsible for the excretion of infectious PRRSV in semen, and that PRRSV induces apoptosis in these germ cells in vivo.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales* (7), together with equine arteritis virus, lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (24). The infection of domestic swine by PRRSV is characterized by respiratory dysfunction, late-term abortion, and a high incidence of stillborn, mummified, and debilitated newborn pigs (24). PRRSV is considered to cause the most economically significant infectious disease of swine that has emerged in the 1990s. As is characteristic of arteriviruses, PRRSV is a small enveloped RNA virus which replicates primarily in macrophages. The PRRSV particle is 50 to 65 nm in diameter, with a central isometric nucleocapsid approximately 30 to 35 nm in diameter (24). The genome of PRRSV is a single-stranded polyadenylated RNA 15 kb in length which contains eight open reading frames (ORFs). Of the structural genes (ORFs 2 through 7), ORF 7 is known to code for the nucleocapsid protein, and ORFs 2, 3, 4, 5, and 6 code for envelope proteins (25). The genome organization and the replication of arteriviruses, which takes place via a 3′-coterminal nested set of subgenomic mRNAs, is similar to that of coronaviruses (7, 33).

PRRSV primarily infects pulmonary alveolar macrophages during acute infection. Several laboratories have provided clear evidence that the alveolar macrophage and macrophages from several other tissues are the primary cell type that sustains the in vivo replication of the virus (24, 33, 36). PRRSV is known to infect individual animals for long periods of time, and during that time the virus may be transmitted by contact or through semen (8, 9, 27, 35, 41). The ability of PRRSV to be shed in semen has proven to be a principal factor in the venereal transmission of the infection (41) and constitutes a significant drawback for the use of artificial insemination in modern swine production. It has been reported that PRRSV infection causes significant diminishment of semen quality in boars (i.e., decreased spermatozoal motility and increased number of spermatozoa with distal cytoplasmic droplets) compared to semen from uninfected control animals (8, 35). Although it is known that the PRRSV clearly seems to be shed in the cellular fraction of swine semen for at least several weeks (9), nothing is known about the mechanisms of pathogenesis underlying this seminal association of PRRSV. Specifically, whether the above-mentioned effects are due to a direct effect of the viral replication in the cells of the testis or due to a secondary effect of the infection in other cells has not been determined.

In this study, we demonstrate that PRRSV infects cells of the spermatic epithelium in the testicular seminiferous tubules but not mature spermatozoa. By immunohistochemistry (IHC) and in situ hybridization (ISH), we detected PRRSV in abnormal cells of spermiogenic origin, including primordial spermiogenic cells and multinucleated giant cells (MGCs). We found these cells both in the ejaculate, where the presence of these cells is considered to be a sign of abnormalities in the divisional mechanism of primary spermatocytes, and in the seminiferous tubules of PRRSV-infected boars. We also document that a major consequence of the PRRSV infection of the testis is

epithelial germ cell death, which takes place following a characteristic path of apoptosis. In summary, we have demonstrated that an important pathogenic feature which is essential for the venereal transmission of arteriviruses is based on a newly described ability of this group to replicate in germinal cells. This ability to infect male gonadal cells is a strategy that seems to have been consistently conserved among certain viruses, including viruses of vertebrates (4, 5, 11, 16, 38) and invertebrates (12, 13, 34). In all likelihood, such specialized tropism represents a viral evolutionary advantage, as it results in an efficient mechanism for the sexual transmission of the infection and for the perpetuation of the virus in a given host population. Apart from constituting a well-documented exception to the premise that arteriviruses replicate primarily in macrophages, the viral property herein reported provides a novel model for virus-induced alterations of spermatogenesis and apoptotic death of germ cells.

MATERIALS AND METHODS

Virus strains and cell cultures. The PRRSV 12068-96 strain was used for these studies. This strain was isolated in September 1995 from serum of a neonate during an outbreak of a severe reproductive form of porcine reproductive and respiratory syndrome affecting a breeding farm located in Johnson County, Nebraska. The virus was propagated twice in MARC-145 cells (18) at 37°C, and the titer was determined by microtiter assay in the same cells. MARC-145 cells were maintained at 37°C in Eagle's minimum essential medium (MEM) with 10% fetal calf serum.

Animal infection. We used 33 5- to 6-month-old boars obtained from an unvaccinated PRRSV-free, specific-pathogen-free herd. Blood was obtained from all boars prior to the onset of the experiment, and serum was tested by using a commercial PRRSV enzyme-linked immunosorbent assay (ELISA; Idexx Laboratories, Portland, Maine) to assay for the presence of PRRSV antibodies. Twenty-six boars were inoculated intranasally with the PRRSV 12068-96 strain, using an inoculum of $10^{3.8}$ 50% tissue culture infective doses/2 ml, delivered in volumes of 1 ml/nostril. Seven uninfected boars, which were matched to the principals in age, breed, and farm of origin, were used as controls. Different isolation room areas (biosafety level 2) were used to separate control boars and those infected with PRRSV 12068-96. The progress of the experimental infection was monitored by periodic clinical examination and sampling of serum which was used for the assessment of viremia and antibody response. Groups of infected and control boars were killed and necropsied according to the following schedule. Two infected boars and one control boar were necropsied at 7, 13, 25, and 60 days postinfection (dpi), respectively, and in all cases reproductive tissues were removed and fixed in 10% neutral formalin. For studies on seminal ejaculates, another six infected and three uninfected boars (all of them trained for semen collection prior to the inoculation) were used for periodic collection of semen ≥3 times per week from 0 to 60 dpi. Of these, two infected boars and one control boar were necropsied at 9, 25, and 60 dpi, respectively. Also in these cases reproductive tissues were removed and fixed in formalin at necropsy. The remaining 12 infected boars, used to monitor the length of viremia, were not killed but instead used for a separate study on PRRSV in vivo persistence.

Virus isolation. The MARC-145 cell line was used for virus isolation assays on serum samples. The growth medium consisted of MEM, 10% fetal calf serum, and gentamicin (50 μg/ml). Virus was isolated by inoculating 96-well plates containing a confluent monolayer of MARC-145 maintained at 37°C. If cytopathic effect was evident, the identity of the virus was confirmed by an indirect immunofluorescence assay using a monoclonal antibody (MAb) specific for the N protein of PRRSV (SDOW-17) (26) followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). In the absence of cytopathic effect, the cells were frozen and thawed, and 50- to 100-μl aliquots of medium and cells were transferred to four-chamber Lab-Tek slides (Nunc, Inc., Naperville, Ill.). These second-passage cultures were incubated for 5 to 6 days and examined for the presence of PRRSV antigen by immunofluorescence assay.

Semen collection. Semen was collected ≥3 times/week by the gloved-hand method, using trained boars and a dummy sow (19). Raw semen was filtered through a triple layer of gauze and diluted 1:500 to 1:1,000 in MEM. Diluted raw semen was applied to Superfrost/plus slides (Fisher Scientific, Pittsburgh, Pa.) by centrifugation at $200 \times g$ for 5 min in a cytocentrifuge and fixed in either acetone (for immunocytochemistry) or PLP fixative (4% paraformaldehyde, 100 mM L-lysine dihydrochloride, and 10 mM sodium *m*-periodate in phosphate-buffered saline [PBS]) (for ISH). For immunocytochemical evaluation, slides were fixed in acetone for 5 min and air dried. Slides were stored at −20°C until use. For ISH, slides were fixed in PLP fixative for 5 min. The slides were then washed twice in PBS (pH 7.4) for 5 min, dehydrated through an ethanol series, and stored at 4°C until use.

Preparation of tissues for histopathology, IHC, ISH, and TUNEL assay. For histopathology, IHC, ISH, and in situ terminal deoxynucleotidyltransferase-mediated digoxigenin-UTP nick end labeling (TUNEL) staining, tissue sections (5 μm) were adhered to Superfrost/plus slides. Deparaffinization was done by heating the sections for 20 min at 65°C, and the sections were rehydrated through graded alcohol and washed with PBS (pH 7.4). All reproductive tissues of the boars were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for routine histopathological examination. Tissue sections (5 μm) were used for IHC, ISH, and fragmented DNA in situ.

Detection of acrosomal antigen of boar germ cells, swine macrophage marker, and PRRSV antigen. Immunolabeling was performed via the standard three-step immuno-alkaline phosphatase technique using a streptavidin-biotin-based alkaline phosphatase system (Dako Corp., Carpinteria, Calif.). MAbs were the primary antibodies. These MAbs consisted of MAb against swine acrosomal antigen of boar spermatocytes and spermatozoa (clone ACR-2; Caltag Laboratories, Burlingame, Calif.) (31), anti-swine macrophages (ATCC hybridoma 142.1, which produces MAb against the SWC3a antigen of swine macrophages (22), and PRRSV nucleocapsid protein (SDOW-17, obtained from National Veterinary Services Laboratories, USDA Animal and Plant Health Inspection Service, Ames, Iowa) (26). MAbs were coated on the slides at dilutions of 1:100 (ACR-2), 1:50 (SWC3a), and 1:250 (SDOW-17). All sperm cell (cytospun) preparations and tissue sections were first treated with 3% H_2O_2 in PBS for 20 min at room temperature, followed by three PBS washes and digestion with 0.05% protease XIV (Sigma Chemical Co., St. Louis, Mo.) in Tris buffer (50 mM Tris buffer [pH 7.6]) for 5 min at 37°C. Following several PBS washes, semen preparations and sections were incubated in a block solution (5% normal goat serum in PBS) for 30 min at room temperature. All slides were incubated with diluted primary antibody in PBS for 3 h at 4°C. After three 10-min washes with PBS, the slides were incubated with biotinylated goat anti-mouse linking antibody for 1 h at room temperature. After three 10-min washes with PBS, slides were incubated with streptavidin-alkaline phosphate for 30 min at room temperature. Following the last PBS wash, slides were developed by incubation with the alkaline phosphatase substrate (Vector Laboratories, Inc., Burlingame, Calif.) until color development was observed (a process that typically requires 5 to 15 min), and the color reaction was stopped by washing the slides in deionized water. For detection of acrosome antigen and swine macrophages, slides were counterstained with 0.5% methyl green. For the detection of PRRSV antigen, the slides were counterstained with Mayer's hematoxylin.

ISH for PRRSV RNA. The hybridization probe (a 433-bp cDNA) was produced by reverse transcription-PCR as previously described by Mardassi et al. (23) and labeled via random priming reaction with digoxigenin-dUTP (Boehringer Mannheim Corp., Indianapolis, Ind.). The cDNA probe represents a region of the PRRSV genome in ORF 7 (nucleotides 51 to 483 [23, 40]) that is also present in all subgenomic mRNAs produced by active replication of PRRSV within a cell. This probe selection consequently provides a high degree of sensitivity. The ISH was carried out as previously described (40). Briefly, tissue sections were deparaffinized, rehydrated, and digested with proteinase K (Gibco BRL Life Technologies, Inc.) for 20 min in PBS at 37°C. The concentrations of proteinase K were 4 μg/ml for sperm preparations and 20 μg/ml for tissue sections. Specificity controls included previously verified PRRSV-positive tissue sections or cytospun preparations of PRRSV-infected cultured cells or PRRSV-infected sperm cells which were treated with RNase A (Boehringer Mannheim) at 100 μg/ml in 10 mM Tris-Cl (pH 7.4) for 30 min at 37°C to remove target RNA (signal extinction control). After digestion, all cell preparations and tissue sections were refixed in 4% paraformaldehyde in PBS for 5 min. After two 5-min washes with PBS, sections were prehybridized for 60 min at 65°C in a mixture of 50% deionized formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 1× Denhardt's solution, 2 mM EDTA, and 500 μg of salmon sperm testis DNA (Sigma) per ml. The labeled probes (0.5 ng/50 μl) were diluted in 300 μl of the prehybridization mixture and heated for 5 min in a 95°C heating block. The probes were quenched on ice before being applied to the semen and tissue sections. The slides were rinsed briefly in 2× SSC, and 30 μl of probe mixture was added to each slide and sealed with rubber cement. The hybridization was performed overnight at 56°C. After hybridization, the slides were washed twice in 4× SSC for 3 min at room temperature, once in 2× SSC for 10 min at 50°C, once in 0.2× SSC containing 60% formamide for 10 min at 50°C, twice in 2× SSC, and twice in 0.2× SSC for 5 min at room temperature. After the washing steps, immunological detection was carried out by using antidigoxigenin immunoglobulin conjugated with alkaline phosphatase (Boehringer Mannheim) (dilution, 1:500) and which was incubated for 2 h at room temperature. Finally, tissue sections and/or sperm cells preparations were incubated with color substrate solution. The color solution consisted of 45 μl of nitroblue tetrazolium salt and 35 μl of BCIP (5-bromo-4-chloro-3-indolylphosphate, toluidinium salt), and the reaction was incubated for 3 to 6 h in the dark. The color reaction was stopped with deionized water. Counterstaining was done with 0.5% methyl green.

Detection and quantitation of fragmented DNA in situ. All experiments for establishing the presence of fragmented DNA by in situ reaction were performed by the TUNEL technique in sections from PRRSV-infected and mock-infected testes as previously reported (15, 37) and as described in the in situ cell death detection kit's insert (Boehringer Mannheim). After deparaffinization and rehy-

dration, tissue sections were digested with 20 μg of proteinase K (Gibco BRL) per ml in 10 mM Tris-HCl (pH 7.5) for 20 min at 37°C, followed by three 10-min washes in PBS. Tissue sections were covered with 50 μl of the TUNEL reaction mixture containing the terminal deoxynucleotidyltransferase and fluorescein-dUTP (Boehringer Mannheim) and incubated under a coverslip in a humidified chamber for 1 h at 37°C. The reaction was terminated by washing slides in PBS for 15 min at room temperature. The sections were then incubated with the anti-fluorescein-alkaline phosphatase conjugate (Boehringer Mannheim) diluted 1:3 in 100 mM Tris-HCl–150 mM NaCl (pH 7.5)–1% blocking reagent for 1 h at room temperature. After three 15-min washes in PBS, sections were stained by incubating the chromogenic substrate (X-phosphate–BCIP) for 5 to 15 min at room temperature and counterstaining with 0.5% methyl green. The number of apoptotic cells was quantified via light microscopy at a magnification of ×100 by square test grid (Klarmann Rulings, Inc., Manchester, N.H.). Quantitative evaluation was performed by using one complete cross section of the testes with a standardized area of 1.56 mm^2 per square, each square containing approximately 20 (±2) seminiferous tubules. Apoptotic cells were identified by TUNEL assay within seminiferous tubule cross sections, and the incidence of apoptosis was defined for each seminiferous tubule as three or more positive cells per cross section of tubule. A total of 400 to 600 seminiferous tubule cross sections were evaluated for each of the boars, and the data were expressed as percentages of the total.

RESULTS

Experimental infection with PRRSV. The pattern of PRRSV infection in the 26 inoculated animals was consistent with previous descriptions of the effect of PRRSV infection on adult male animals reported by other laboratories (8, 9, 35, 41) in that no clinical signs such as temperature elevation, loss of appetite, or other alterations of general condition were observed. The infection status of the animals was confirmed by (i) viral isolation from serum on MARC-145 cells at ≥7 dpi, (ii) detection of PRRSV-infected cells in ejaculates (discussed below), and (iii) development of specific antibodies evaluated by ELISA between 7 and 14 dpi in all of the inoculated animals but in none of the sham-inoculated controls. After seroconversion, the PRRSV serologic status of the animals remained positive for the rest of the experiment. No seroconversion of the uninoculated control animals took place throughout the experiment. The length of the viremia was monitored by viral isolation on MARC-145 cells from the sera of 12 of the animals. The presence of infectious virus in serum became undetectable, in most (11 of 12) cases at a period ranging between 30 and 58 dpi, according the following sequence for clearance of viremia: in four animals by 30 dpi, in one animal by 38 dpi, and in five animals by 58 dpi. At the same time, 1 of the 12 animals, which had tested positive for infectious PRRSV in serum at 7 and 14 dpi, had no detectable infectious PRRSV in serum by day 30 postinfection. Starting at 71 dpi, no animal was positive for infectious virus in serum, although some of the animals remained positive for longer periods of time when tested by PRRSV-specific reverse transcription-PCR (data not shown).

Effect of the PRRSV infection on the testis. Microscopic examination of the testes of the PRRSV 12068-96-infected animals had significant changes, especially in the samples collected between days 7 and 25 p.i. A consistent feature was the presence of MGCs, observed in numerous seminiferous tubules, especially at days 7 and 9 p.i. MGCs were round, contained either normal or abnormal nuclei, and were frequently observed in the degenerating tubules (Fig. 1). The MGCs were small (with 2 to 4 nuclei) or large (with 8 to 15 nuclei) (Fig. 1A), suggesting that these MGCs originated by meiotic division of the spermatocyte nuclei with incomplete cytoplasmic separation. MGCs were also sporadically observed in the lumen of epididymis and were surrounded by mature spermatoza, spermatids, and spermatocytes (data not shown). The MGCs were observed in correspondence with areas of abundant desquamation of the seminiferous tubules (Fig. 1B), in-

dicating severe hypospermatogenesis. The areas most affected by hypospermatogenesis were characterized by a complete lack of mature spermatids and a nearly complete absence of germ cells except for some spermatogonial cells. A prominent morphological feature in the testes of acutely PRRSV-infected boars was the presence of apoptotic cells. These apoptotic cells appeared to be derived mainly from spermatocytes, although occasional degenerate spermatogonia were observed. Degenerative changes in these cells included nuclear rupture, pyknosis and vacuolar degeneration, cytoplasmic condensation, and fragmentation of chromatin (Fig. 1C).

PRRSV replicates in testicular germ cells. By ISH, we were able to detect specific PRRSV-positive signals in two distinct areas of the testicle and epididymis: (i) in the interstitium of the testis, between seminiferous tubules (Fig. 2A) (as well as in the connective tissue of the epididymis, between the ducts) and (ii) in germ cells of the seminiferous epithelium (Fig. 2B and C). In the interstitium, the PRRSV-specific ISH signals were evident in a moderate number of single cells that were primarily located in the connective tissue septa adjacent to the blood vessels (Fig. 2A). This observation was particularly evident within the range of 7 to 30 dpi. An identical pattern of distribution of PRRSV-positive single cells in the interstitium was obtained when we used IHC with the MAb specific for PRRSV N antigen (data not shown). The morphology and distribution pattern of these single infected cells in the interstitium were suggestive of macrophages. Macrophages are known to be a conspicuous target of PRRSV replication in tissues. To confirm whether the PRRSV ISH and IHC signals observed in the interstitium and connective tissue of the epididymis were based in macrophages, we used IHC on replicate sections of the same piece of testis to look for the presence of cells positive for the swine macrophage phenotypic marker SWC3a. In this case the distribution pattern, morphology, and location of the SWC3a-positive cells were in close agreement with findings for the previously described PRRSV-positive cells (Fig. 2D). The SWC3a-positive cells were, also in this case, adjacent to small blood vessels in the interstitium (Fig. 2D). Likewise, analysis of a large number of testicular sections from uninfected control animals by IHC indicated that the interstitium and connective tissue of epididymis were the only areas of the testis where SWC3a-positive cells were apparent. The positive signal was absent in the seminiferous tubules of either the testis of PRRSV-infected or uninfected controls (data not shown). The PRRSV-positive signal obtained by ISH in the seminiferous tubules was detected primarily in spermatocytes and round spermatids (Fig. 2B and C). This was consistently observed up to 25 dpi but was particularly prominent between 7 and 9 dpi. The PRRSV-specific ISH signal was cytoplasmic, consistent with our previous observations in lung and lymphoid tissue (40). The PRRSV-positive signals were extensively observed in areas of the tubules characterized by the presence of homogeneous populations of round spermatids (Fig. 2B). The specificity of the ISH reaction in all cases was confirmed by (i) the absence of this signal in tissues from uninfected control animals and (ii) extinction of the specific signal after digestion of a consecutive testicular section with a solution of RNase A prior to ISH (Fig. 2E). The location of PRRSV infection in spermatid and spermatocyte-rich areas of the seminiferous tubules was also confirmed by performance of IHC with a MAb against the N protein of PRRSV, indicating a close correlation between the areas containing PRRSV RNA and PRRSV N antigen (Fig. 2F).

PRRSV induces germ cell death by apoptosis. To confirm our histopathological observation indicating the presence of apoptosis in cells of the seminiferous tubules (Fig. 1C), we
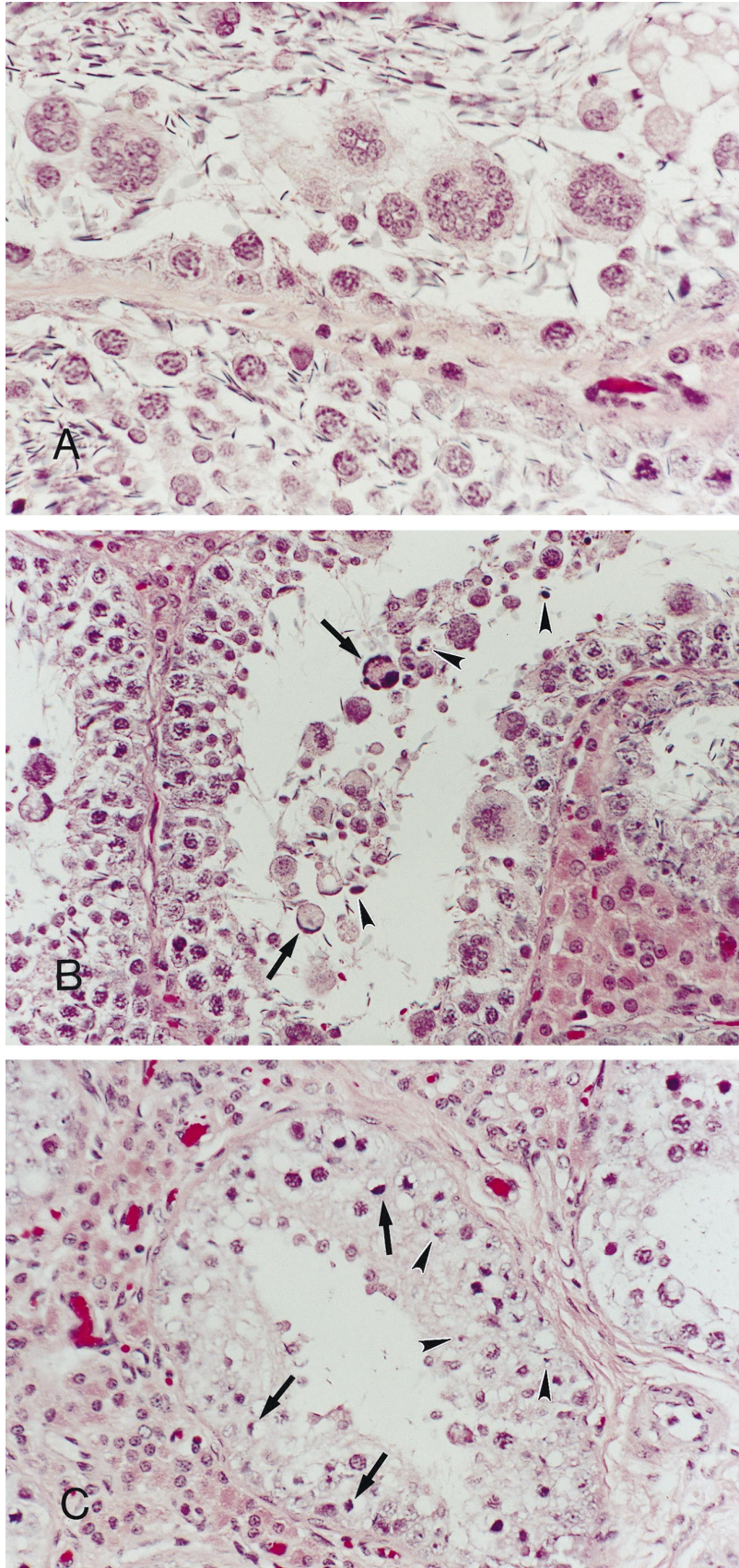
FIG. 1. Hematoxylin-eosin-stained sections of testes taken from PRRSV-infected boars at 7, 9, and 25 dpi. (A) Section of testicular seminiferous tubule (25 dpi). Marked tubular atrophy characterized by presence of MGCs is seen. Magnification, ×1,152. (B) Section of testicular seminiferous tubules (9 dpi). Degeneration of spermatic epithelial cells, marked hypospermatogenesis, and reduced number of epithelial layers are evident. The numerous pyknotic germ cells (arrowheads) and degenerating MGCs (arrows) are seen. Magnification, ×720. (C) Section of testicular seminiferous tubules (7 dpi). Spermatocytes and spermatids are absent. Individual germinal epithelial cells have condensed chromatin (arrows). Several apoptotic bodies in epithelial layer (arrowheads) are seen. Magnification, ×720.
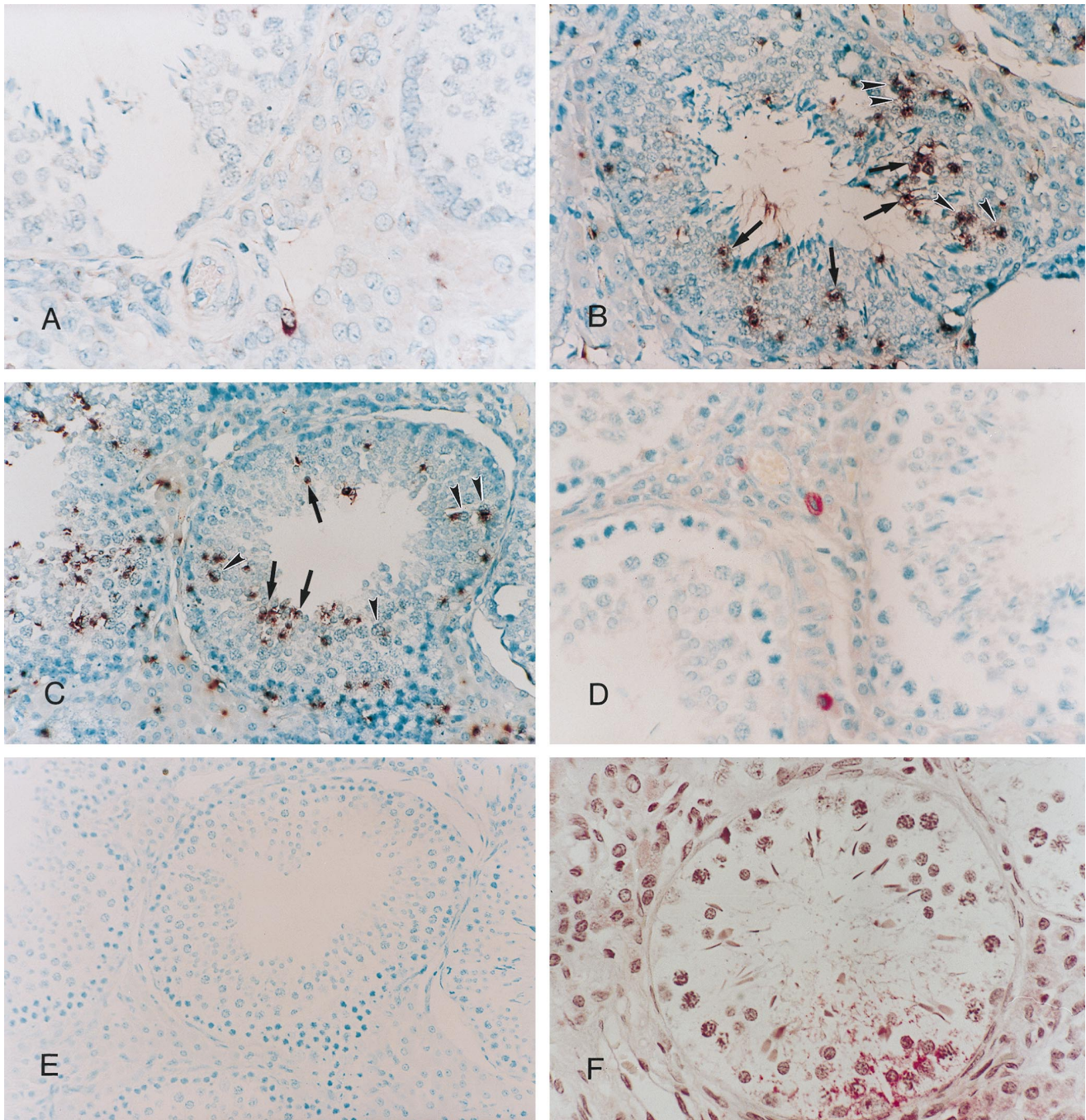
FIG. 2. (A through E) Tissues counterstained with methyl green; (D and F) IHC staining for swine macrophages (D) and PRRSV antigen (F) in testes taken from infected boars at 7 and 25 dpi; (A, B, C, and E) ISH performed on sections of testicle taken from PRRSV-infected boars at 7, 9, and 25 dpi. Tissue sections were hybridized with a digoxigenin-labeled, PRRSV-specific probe (433-bp cDNA). Methyl green counterstained. (A) A positive PRRSV ISH signal in a macrophage in the interstitium of the testis of a PRRSV-infected boar at 7 dpi. Magnification, ×883. (B) Positive ISH signals were seen in the cytoplasm of the spermatocytes (arrowheads) and spermatids (arrows) in the seminiferous tubules from a PRRSV-infected boar at 7 dpi. Magnification, ×552. (C) PRRSV-positive signals were seen in spermatids (arrows) and few spermatocytes (arrowheads) of a PRRSV-infected boar at 9 dpi. Magnification, ×431. (D) Detection of porcine macrophage surface antigen in macrophages located in the testicular interstitium (replicate section of the testis corresponding to the same area as in panel A) at 7 dpi. Magnification, ×883. (E) Serial section of RNase A-treated section of the testes shown in panel C. Positive ISH signals were not detected. Magnification, ×431. (F) Localization of PRRSV-infected germ cells in seminiferous tubules (25 dpi). PRRSV-infected antigens appear as a red precipitate in the cytoplasm of cells with location consistent with spermatids and spermatocytes. Alkaline phosphatase with hematoxylin counterstain. Magnification, ×883.

performed the TUNEL reaction on sections of testes of PRRSV-infected and control uninfected animals. By this method, intense specific staining was observed in nuclei and nuclear fragments with the morphological characteristics of apoptosis in the PRRSV-infected animals. Apoptotic bodies of various sizes exhibited distinct staining and the cytoplasm of apoptotic cells were also often stained, suggesting leakage of DNA fragments out of the nucleus (Fig. 3A and B). In the
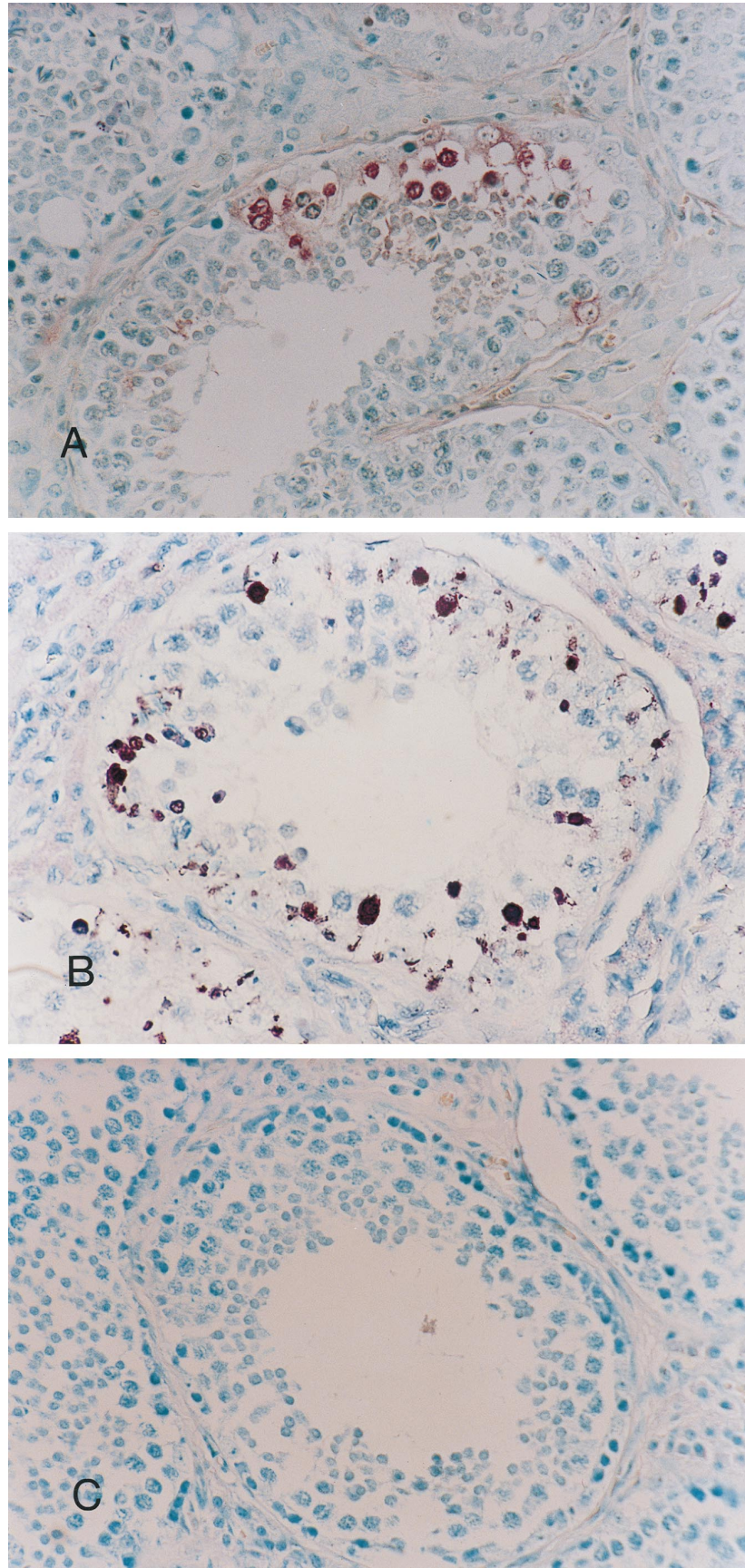
FIG. 3. TUNEL assays performed on testes taken from PRRSV-infected boars at 7 and 25 dpi and from a control animal. Methyl green counterstain. (A) TUNEL-positive cells were observed in a seminiferous tubule from a PRRSV-infected boar at 7 dpi. Magnification, ×563. (B) Abundant TUNEL-positive cells were observed in a seminiferous tubule from PRRSV-infected at 25 dpi. Magnification, ×720. (C) Typical section of seminiferous tubule from a PRRSV-negative control boar. TUNEL-positive cells, whose overall incidence in uninfected animals is significantly low compared to PRRSV-infected animals (Fig. 4), are not evident. Magnification, ×720.
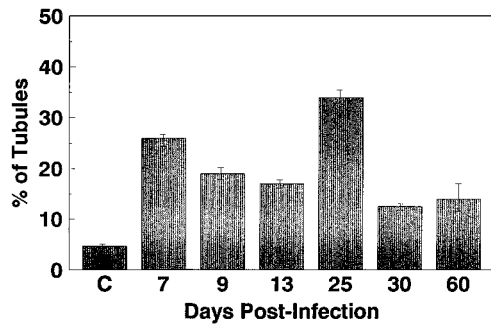
FIG. 4. Quantitation of apoptosis TUNEL assay at various times after PRRSV infection. The incidence of TUNEL-positive cells in control boars corresponded to $4.0 \pm 1.0\%$ ($n = 3$). Data are presented as percentages of total tubules. A significant increase in the frequency of apoptosis over the control animals was observed in the infected animals after 7 dpi.

sections of testis of uninfected control boars only sporadically were individual TUNEL-positive germ cells detected, and most commonly the tubules were completely devoid of apoptotic signals (Fig. 3C). In those samples where MGCs were commonly observed, MGCs often stained TUNEL positive (data not shown). As apoptotic events were readily recognized in the epithelium of seminiferous tubules, it was feasible for us to quantitate the rate of apoptotic cells labeled by TUNEL. Figure 4 shows the incidence of apoptosis in PRRSV-infected and control animals, expressed as the percentage of seminiferous tubules that showed more than three positive events per cross section. Germ cell apoptosis in seminiferous tubules had significantly increased after PRRSV infection between 7 and 25 dpi ($P > 0.05$), with two distinct peaks at 7 and 25 dpi. At days 30 and 60 p.i., the number of apoptotic events decreased significantly, although the number of positive cells was always greater than in the control animals (Fig. 4). It is interesting that the peak of PRRSV ISH signals in the testicular germ cells and the apoptotic activity in those cells seemed to peak simultaneously (at 7 to 9 dpi). However, by analysis of serial tissue sections processed for PRRSV ISH and TUNEL assays, we concluded that the two activities do not necessarily correlate on a cell-to-cell basis (data not shown). In all cases, the extent of apoptosis (i.e., number of cells involved) was more evident than that of PRRSV infection.

PRRSV is detected in the nonspermatozoa cell fraction of ejaculate. Simultaneously with the observation on the testicular sites reported above, we studied, by ISH, the localization of PRRSV in cells of the ejaculates of a group of PRRSV-infected and control animals. An evident cytological alteration observed in the ejaculates of the animals following PRRSV infection was a significant increase in the number of nonspermatozoa cells. In the ejaculates of all six PRRSV-infected boars from which semen was sequentially obtained, we observed PRRSV-infected (ISH-positive) nonsperm cells (Fig. 5A to C) starting as early as 3 dpi. The alkaline phosphatase precipitate typical of the ISH signal was observed in the cytoplasm of cells that, by size and morphology, we considered to be of the primary spermatocyte (Fig. 5A) and spermatid (Fig. 5B) types, and also in cells presenting the unequivocal morphology of MGCs (Fig. 5C). The positive ISH signal in nonsperm cells of the ejaculates was most frequently seen between 7 and 14 dpi, when five to eight positive cells per cytocentrifuge spot were observed. The number of positive signals then decreased toward 30 dpi and became undetectable after 46 dpi. Throughout the observation period, the spermatozoa were consistently negative for PRRSV ISH signal (data not shown).

To confirm the identity of the PRRSV ISH-positive nonsperm cells, we used a specific marker that enables the recognition of germ cells by IHC. The MAb clone ACR-2 (specific for acrosome antigen) labeling pattern at various stages of spermatogenesis allows the identification of germ cells (31). Extensive analysis of ejaculate samples indicated a consistent detection of acrosome antigen-positive cells, such as mid- and late pachytene primary spermatocytes and spermatids in the PRRSV-infected animals. One or two labeled bodies (nuclear pole) were observed in these nonsperm cells (Fig. 5D and E). Also, more differentiated spermatids exhibited a crescent-shaped, strongly labeled cap over the anterior part of the nucleus (data not shown). At the same time, extensive analysis of the same cell preparations with IHC for the SWC3a marker were consistently negative, indicating that the possible presence of macrophages among these nonsperm cells of ejaculates was negligible.

DISCUSSION

Our study provides evidence that PRRSV, a swine arterivirus, can infect spermatogenic epithelium. Such infection results in the release of PRRSV-infected germinal cells in semen, which therefore supports the venereal transmission of the infection and explains the reported infectivity of swine semen. Concomitantly with the venereal dissemination of the infectious virus, this type of gonadal infections entails a severe alteration of the function of the seminal tissues and results in a temporary (4, 16) or permanent (2, 13) virus-induced male sterility. Examples of such virus-induced orchitis and alterations in spermatogenesis are mumps testicular infection in humans (5), encephalomyocarditis virus in rodents (38), and canine distemper virus in racoons and (probably) dogs (16). Recently interest in this phenomenon has been invigorated due to the documented ability of human immunodeficiency virus type 1 (HIV-1) to infect germ cells (28), which results in venereal transmission of HIV-1 in semen and in the development of severe orchitis in patients with AIDS (11).

It is conceivable that the ability of PRRSV to infect germs cells, to alter spermatogenesis, and to release abnormal (infected) spermiogenic cells in semen of the host is shared by other arteriviruses. Equine arteritis virus is known to be shed in semen, and such capacity is known to be an important factor in the transmission of the disease (17, 30). Also, the occurrence of venereal transmission of LDV by infected semen has been postulated (1). A significant aspect of our findings is that we provide an unequivocal demonstration that the PRRSV seminal infection targets a type of cell that, being different from macrophages, does not fall within the commonly accepted hypothesis that explains the pathogenesis of arteriviruses on the basis of their ability to replicate in cells of macrophagic lineage (33, 36). In this scenario (conceived primarily on the basis of the LDV model), continually renewed nonessential macrophages in lungs, lymphoid tissues, and testis are permissive to arterivirus replication during acute and persistent infection (32). Some previous reports had already suggested that the arteriviruses could extend their tropism to cells other than macrophages (10, 36, 40).

In our experiments, the temporal relationship observed between the appearance and duration of PRRSV viremia and evidences for PRRSV testicular replication (detection of PRRSV RNA and N protein; histopathological and in situ detection of apoptotic death of target cells) seems to indicate that the testicular involvement would be a consequence of the establishment of viremia. The dissemination of PRRSV-infected macrophages via blood has explained the widespread
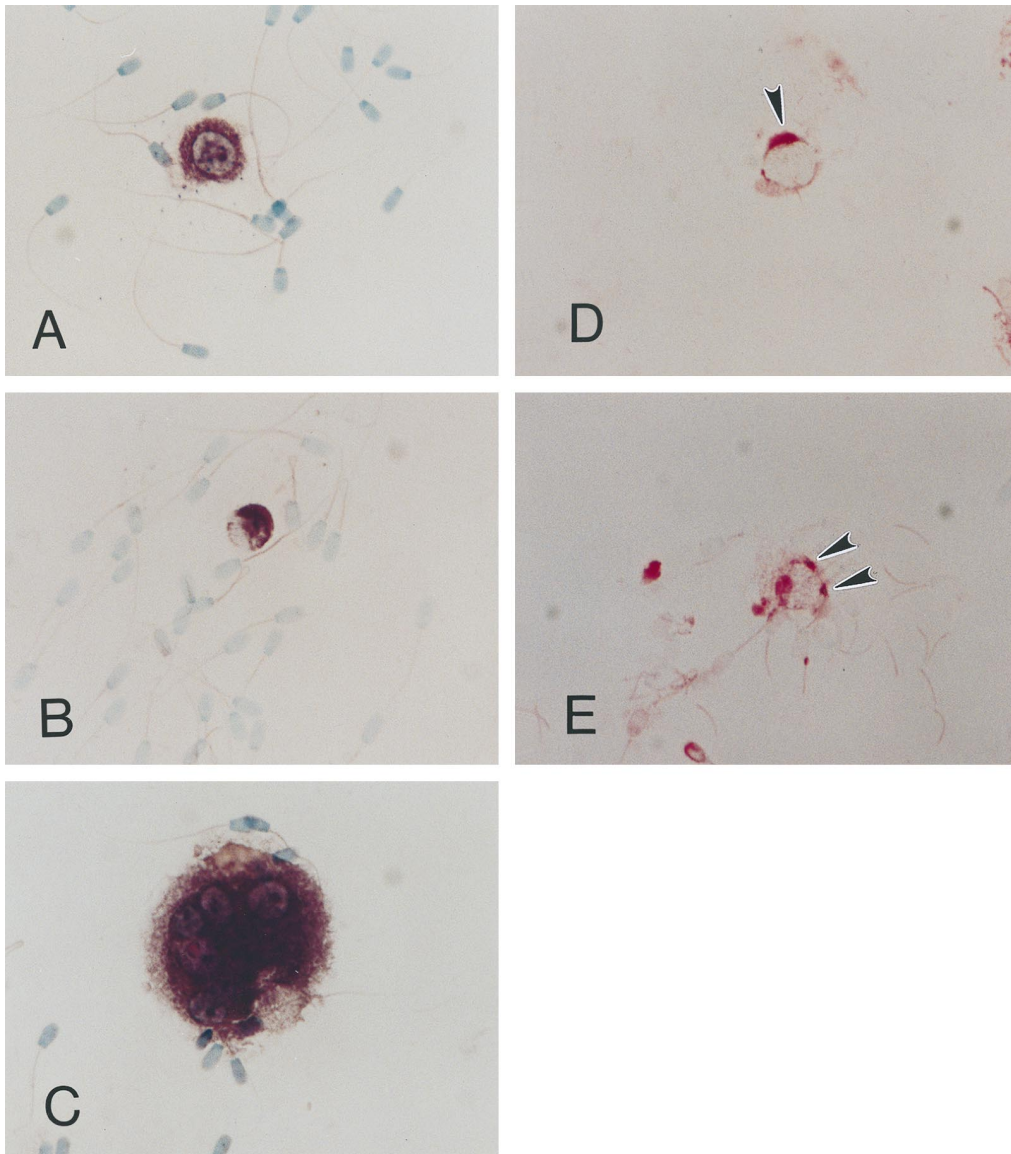
FIG. 5. (A to C) ISH on ejaculated semen collected at 7, 9, and 22 dpi (methyl green counterstain); (D and E) immunocytochemical staining for acrosome antigen in PRRSV-infected ejaculated semen collected at 7 and 25 dpi (alkaline phosphatase with methyl green counterstain). (A) Positive ISH signal in PRRSV-infected spermatocyte, 7 dpi; (B) positive ISH signal in PRRSV-infected spermatid, 9 dpi; (C) positive ISH signal in PRRSV-infected MGC, 22 dpi; (D) prominent acrosomal granule (arrowhead) observed in a spermatocyte, 7 dpi; (E) two acrosomal granules (arrowheads) observed in a spermatocyte, 25 dpi. Magnification for all panels, ×1,280.

presence of PRRSV in various tissues and organs (36). Our observation that infected macrophages can be detected in the interstitium of the testis supports the contention that the PRRSV infection of this tissue is seeded hematogenously by macrophages. It is conceivable that PRRSV replication in these migrating macrophages, which are relocated to the interstitium, in close apposition to the basement membrane of the seminiferous tubules, is responsible for transferring the virus to the underlying germ cells, either by cell-to-cell contact or by release of PRRSV into the extracellular milieu. This could explain the further progression of the viral replication to spermatogonias, spermatids, and spermatocytes. The disappearance of detectable viremia seems to coincide with the restoration of normal seminiferous tubular structure and function, again suggesting that hematogenous transportation of PRRSV-infected macrophages is an inherent requirement for

sustaining the germ cell infection. The restoration of normal spermatogenesis and disappearance of signs of PRRSV replication agree with the reported temporary period of sterility described for PRRSV-infected boars (35).

Another significant finding in our study is the observed association between PRRSV replication and the induction of apoptosis in the seminiferous epithelia. Suarez et al. (39) had previously demonstrated that the glycoprotein p25, encoded by PRRSV ORF 5, induces apoptosis when expressed in COS-1 cells. The same authors proved that apoptosis also occurs in PRRSV-infected cells (MA-104 and swine alveolar macrophages) (39). Now our observation of the occurrence of apoptosis at the level of tissues infected in vivo emphasizes the biological relevance of this property of PRRSV. It is clear from our studies that PRRSV replication localizes in the same areas of the testicle that exhibit histologic and in situ biochemical

evidence of apoptosis. In addition, the alterations of the seminiferous epithelia observed in PRRSV testicular infection, including cellular depopulation and desquamation of seminiferous tubules, formation of MGCs, and diffuse germ cell death, adhere to a common pattern of testicular germ cell death by apoptosis. A similar observation is noted in toxicant-induced sterility models (6) or deprivation of *Bax* function in a transgenic line of Bax$^{-/-}$ mice (20).

One important question is whether PRRSV replication and apoptosis colocalize to the same cells. Our analysis of serial sections of testicle by ISH for PRRSV and by TUNEL assay indicated that TUNEL-positive signals were more abundant in cells other than those containing just PRRSV. Therefore, while many cells were labeled for either PRRSV RNA or apoptosis, the majority were not labeled for both, and cell-to-cell correlation between both viral properties was not observed in our study. There are several possible explanations for this observation. (i) PRRSV RNA and/or antigen-positive germ cells, while undergoing extensive cell changes due to the ongoing infection, may not reflect the terminal morphologic changes of DNA fragmentation typical of advanced terminal apoptosis. TUNEL-positive cells, on the other hand, may have already altered or degraded cytoplasmic RNA of PRRSV beyond the capacity of detection by our techniques. A similar contention has been proposed for the observed lack of cellular colocalization observed between infection and apoptosis induced by murine reovirus (29). (ii) Different viruses seem to induce apoptosis at different times relative to virus replication. For example, Sindbis virus seems to induce apoptosis in close (cell-to-cell) parallel to virus replication (21), whereas certain strains of HIV may induce apoptosis before the virus can be detected in the infected cells (14). (iii) It is possible that PRRSV will induce apoptosis on bystander, uninfected cells of the seminiferous tubules similarly as described for HIV and simian immunodeficiency virus in lymphoid tissues (14). An alternative mechanism for such an effect of PRRSV infection could then be manifested by the local release of apoptogenic cytokines (i.e., tumor necrosis factor) (3) in the environment that surrounds the testicular seminiferous tubules. A putative source of these cytokines could be PRRSV-infected macrophages, which we know from the results of these experiments are consistently present in the interstitium of the testis. In addition, the enhancement of tumor necrosis factor secretion from virus-infected macrophages has been well documented (42).

An interesting finding in our experiments is the link observed between PRRSV replication in the germ cells of the seminiferous tubules of testis and the presence of PRRSV-infected cells in semen ejaculate. The ejaculated semen was observed to contain, besides spermatozoa, numerous nonspermatozoa cells which were determined by morphological analysis to be spermatocytes, spermatids, MGCs, and cytoplasmic droplets. These nonsperm cells were the basis of PRRSV ISH signals observed in the ejaculates of the PRRSV-infected animals. It might be argued that, based exclusively on cell morphology considerations, these ISH-positive cells could not be distinguished as to their exact phenotype and that therefore these cells could simply be, instead of spermiogenic cells, PRRSV-infected macrophages resident in the reproductive tract tissue and excreted in the semen. However, the use of immunocytochemistry with a monoclonal antibody specific against acrosomal proteins of boar germ cells and spermatozoa allowed the unequivocal identification of the infected cells. The presence of PRRSV RNA in spermatocytes and MGCs is commonly observed in ejaculated semen during early phase of this infection, especially from 7 to 25 dpi, by ISH. We did not detect any PRRSV-positive spermatozoa in ejaculated semen. Thus infection via semen, specifically through PRRSV-infected nonsperm cells, seems to be a major route of transmission of PRRSV.

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