NOTE ***Pathology***

Acute Hepatitis in a Piglet Experimentally Inoculated with Tissue Homogenates from Pigs with Postweaning Multisystemic Wasting Syndrome

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(Received 23 January 2003/Accepted 10 June 2003)

ABSTRACT. Five 2 day-old colostrum-deprived piglets were inoculated with tissue homogenates from pigs with postweaning multisystemic wasting syndrome. One of the five piglets developed icterus and died 23 days post-inoculation. Histologic examination revealed acute hepatitis. Porcine circovirus type 2 (PCV-2) antigen and nucleic acid were detected in hepatocytes and phagocytic cells. Ultrastructurally, hepatocytes and phagocytic cells had large numbers of cytoplasmic inclusions, which were composed of electron-dense paracrystalline arrays of small non-enveloped viral particles approximately 17 nm in diameter. Apoptotic hepatocytes were confirmed by the TUNEL method and electron microscopic examination. These findings may indicate that hepatocellular necrosis is associated with replication of PCV-2. Apoptosis of hepatocytes also contributes to the pathogenesis of hepatic lesions in this case.
KEY WORDS: hepatitis, PCV-2, PMWS.

J. Vet. Med. Sci. 65(9): 1041–1045, 2003

Porcine circovirus type 2 (PCV-2) is an agent that is associated with naturally occurring and experimentally induced postweaning multisystemic wasting syndrome (PMWS) [1]. In most cases of PMWS, clinical signs appear at 5 to 12 weeks, and are characterized by progressive weight loss, dyspnea, palpable lymphadenopathy, and less frequently, pallor, diarrhea, and jaundice [1, 12]. Lesions associated with PCV-2 infection include lymphohistiocytic to granulomatous lymphadenitis, interstitial pneumonia, hepatitis, nephritis, myocarditis, enteritis, and pancreatitis [1, 12]. Among these lesions, hepatitis is frequently observed in cases of PMWS and hepatocytes seem to be a target cell for PCV-2 infection and replication [13]. Gnotobiotic pigs with experimentally-induced PMWS died of liver failure associated with severe granulomatous hepatitis [7, 8]. However, little is known about the pathogenesis of hepatic damage in PMWS. In this communication, the hepatic lesions of experimentally-induced PMWS in a piglet were investigated.

Eight colostrum-deprived conventional piglets were obtained from a sow that lacked detectable antibodies to PCV-1 and PCV-2 by immunoperoxidase monolayer assay (IPMA) [2] using porcine kidney cells infected with PCV2 isolate. The serum collected from the sow before farrowing was negative for PCV-1and PCV-2 by polymerase chain reaction (PCR) [4,9]. For challenge, a 10% (W/V) lymphoid tissue homogenate was prepared from pigs diagnosed as PMWS. The inoculum contained $10^{3.5}$ $TCID_{50}$/m*l* infectious PCV-2 viruses when titrated on PCV-free porcine kidney (PPK3F) cell monolayers. The inoculum was shown to be free from PCV-1, porcine parvovirus (PPV), porcine reproductive and respiratory syndrome virus (PRRSV) by PCR and from PPV by hemagglutination test. Five piglets were inoculated intranasally with the viral inoculum at 2 days of age and three piglets were untreated and kept as uninfected controls. Three inoculated piglets died and the others were euthanized under deep anesthesia by intramuscular injection of ketamine hydrochloride at 35 and 42 days post-inoculation (DPI). All animal procedures in this study complied with the Nippon Institute for Biological Science's Guidelines for Care and Use of Laboratory Animals (1999).

Mild to severe diarrhea was seen in all inoculated piglets during the first week of the experiment. Two piglets (Nos. 4 & 5) died at 7 DPI. One piglet (No. 6) had elevated body temperatures of 40.0 to 40.7°C between 18 and 22 DPI. This piglet became dull and anorectic at 22 DPI, then became dysstatic and died at 23 DPI. Uninoculated control piglets remained clinically normal throughout the experimental period. The macroscopic changes are summarized in Table 1.

Tissue samples from liver, spleen, kidney, heart, lungs, tonsils, lymph nodes, and all grossly visible lesions were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.2), processed routinely for paraffin embedment, sectioned, and stained with haematoxylin and eosin (HE).

Immunohistochemical detection of PCV-2 antigen in formalin-fixed paraffin-embedded tissues was performed as described previously [5]. Briefly, liver and lymph node sections were reacted with 1:500 dilution of rabbit hyperimmune serum against PCV (Kindly provided by Dr. Imada, National Institute of Animal Health, Ibaraki, Japan) [11]. After reaction with the primary antibody at 4°C overnight, tissues were incubated with biotinylated goat anti-rabbit IgG before visualizing the reaction product by the streptavidin-biotin (SAB) immunoperoxidase method using a commercial reagent (Histofine SAB-PO Kit, Nichirei Corp., Tokyo, Japan). To investigate apoptosis in relation to the presence of PCV-2 antigen, paraffin embedded liver sections were examined by the terminal deoxy-nucleotidyl

Table 1. Summary of gross and microscopic lesions in piglets inoculated with tissue homogenates from pigs with PMWS

Piglets No.	Gross lesions/Microscopic lesions							
	Liver	Spleen	Kidney	Heart	Lung	Lymph nodes	Ileum	Colon
1* (23)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
2* (35)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
3* (49)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
4 (7)	-/-	-/-	-/-	-/-	-/-	-/-	1+[a)]/-	-/-
5 (7)	-/-	-/-	-/-	-/-	-/-	-/-	-/-	3+[b)]/3+[h)]
6 (23)	3+[c)]/3+[i)]	-/3+[j)]	-/-	2+[d)]/-	3+[e)]/3+[k)]	1+[f)]/3+[l)]	-/-	-/-
7 (35)	-/-	-/-	-/2+[m)]	2+[g)]/2+[n)]	-/-	1+[f)]/2+[l)]	-/-	-/-
8 (49)	-/-	-/-	-/1+[m)]	-/1+[n)]	-/-	-/-	-/-	-/-

* Uninfected controls. Days post-inoculation stated in parenthesis.
1+: mild, 2+: moderate, 3+: severe.
Gross lesions (a-g): a) congested, flaccid small intestine, b) mesocolonic edema, c) icterus, d) hydropericardium, e) pulmonary edema, f) lymphadenopathy, g) pale heart.
Microscopic lesions (h-n): h) mesocolonic edema with catarrhal colitis, i) acute hepatitis, j) granulomatous splenitis, k) edema of subpleural and interstitial tissue, l) granulomatous lymphadenitis, m) granulomatous nephritis, n) nonsuppurative myocarditis.

transferase-mediated dUTP-nick end labeling (TUNEL) procedure (Apoptosis *in situ* Detection Kit, Wako Pure Chemical Industries, Ltd., Osaka, Japan) [3].

For *in situ* hybridization (ISH), deparaffinized sections were treated with 0.2 N HCl at room temperature for 20 min and digested with proteinase K (Sigma-Aldrich Japan Inc., Tokyo, Japan) 0.75 mg/m*l* in PBS at 37°C for 20 min. After postfixation with 4% PFA in PBS for 5 min, the sections were immersed in 2 mg/m*l* glycine in PBS for 30 min and kept in 40% deionized formamide in 4 × standard saline citrate (SSC; pH 7.0) until use for hybridization. A digoxigenin (DIG)-labeled probe which was derived from ORF1 region of PCV-2 was PCR-amplified with primers 5' GGG TGT TCA CGC TGA ATA ATC CTT CCG 3' and 5' TCC TCC GAT AGA GAG CTT CTA CAG C 3' using DIG labeling mix. The probe was dissolved in a hybridization buffer which consisted of 2 × SSC containing 50% formamide, 5% dextran sulfate solution and 0.2% skim milk. Each probe/tissue preparation was covered with a clean coverslip, and placed in a 90°C oven for 10 min. Hybridization was carried out at 42°C for 12–16 hr. After repeated washes, sections were soaked once in 1% blocking reagent at room temperature for 1 hr and incubated with anti-DIG antibody conjugated with alkaline phosphatase diluted 1/200 in blocking reagent (Boehringer Mannheim, Co., Ltd., Tokyo, Japan). After three washes in buffer, substrate consisting of nitro-blue tetrazolium (NBT) and 5-bromocresyl-3-indolyphosphate (BCIP) was layered over the sections. Color was allowed to develop for 10 min in the dark, and development was then stopped by dipping the slides briefly in tri-ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Sections were counterstained with 0.5% methyl green.

For electron microscopy, small pieces of the PFA-fixed liver were postfixed in 1% osmium tetroxide, and embedded in epoxy resin (Epok 812, Okenshoji Co., Ltd., Tokyo, Japan). Semithin sections were stained with 1% toluidine blue. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined with a Hitachi H-600L transmission electron microscope.

Two piglets (Nos. 6 & 7) had lesions in the lymph nodes, spleen, tonsil and Peyer's patches. These lymphoid lesions were characterized by multifocal to diffuse infiltrates of epithelioid macrophages with basophilic cytoplasmic inclusion bodies, formation of syncytial cells, depletion of lymphocytes, and necrosis of follicular centers. Lesions in other piglets are summarized in Table 1. PCV antigen and nucleic acid were detected in the lesions in liver, spleen, kidney, heart, lungs and lymph nodes (Nos. 6–8). PCV-2 DNA was detected in the lymph nodes, tonsils, liver, spleen, heart, and lungs of three piglets (Nos. 6–8) by type specific PCR. PCV-1 was not demonstrated in any tissues of inoculated and control piglets by PCR.

Hepatic lesions were observed only in one piglet (No. 6)

Fig. 1. Liver; piglet. A hepatocyte has a swollen and vesicular clear nucleus and small cytoplasmic inclusion bodies (arrowheads). HE. Bar=10 μm.

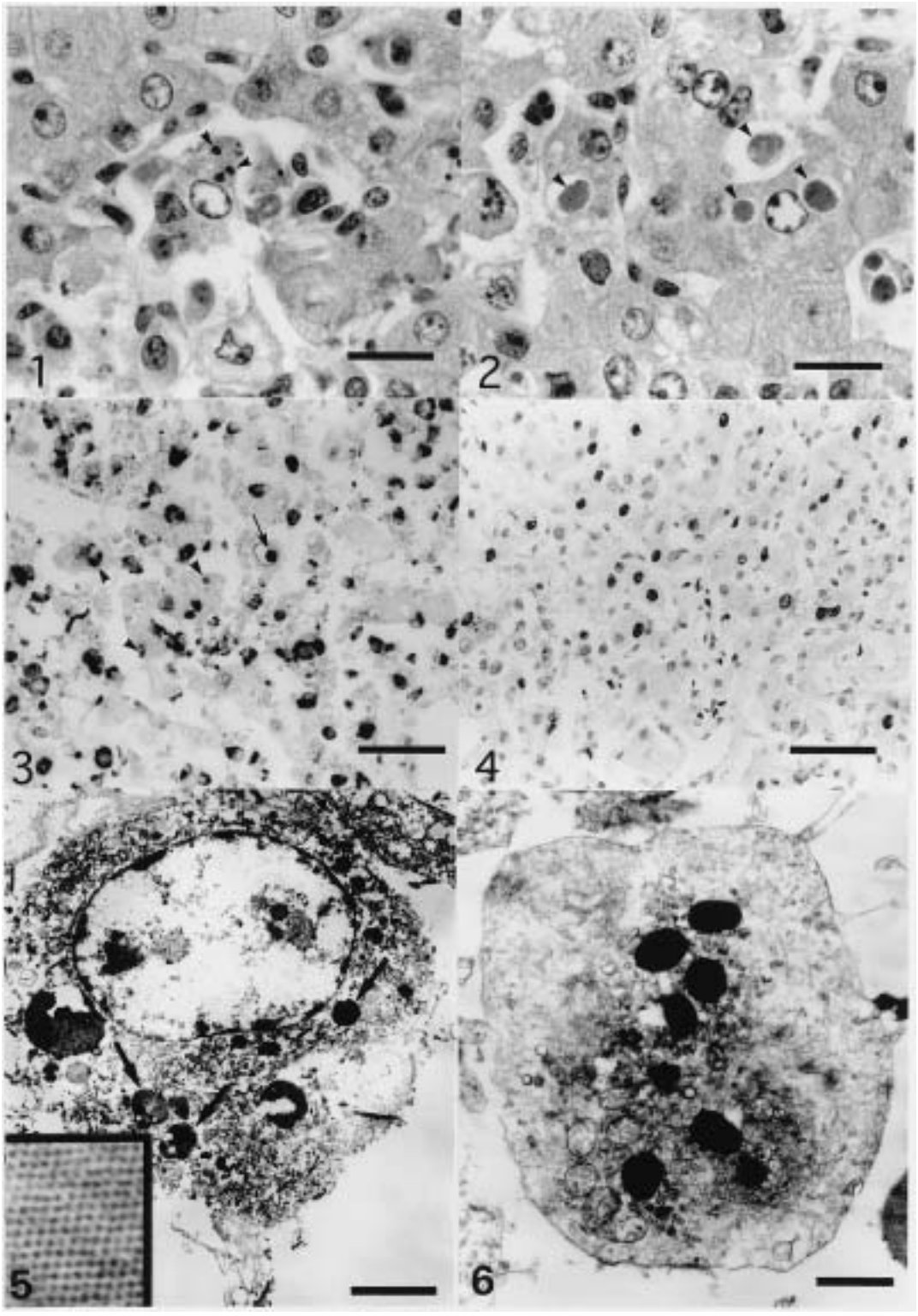
Fig. 2. Liver; piglet. Acidophilic bodies are frequently present in the hepatocytes (arrowheads) and sinusoid. HE. Bar=10 μm.

Fig. 3. Liver; piglet. Both nuclear (arrow) and cytoplasmic (arrowheads) staining for PCV nucleic acid is apparent in hepatocytes, as well as cytoplasm of Kupffer cells and macrophages. ISH technique with methyl green counterstain. Bar=25 μm.

Fig. 4. Liver; piglet. TUNEL method stains many hepatocyte nuclei, as well as sinusoidal cells. TUNEL staining. Bar=25 μm.

Fig. 5. Electron micrograph. Liver; piglet. Hepatocyte with electron-dense, round to ovoid cytoplasmic inclusion bodies (arrows). Viral particles in paracrystalline array are approximately 17 nm in diameter (inset). Uranyl acetate, lead citrate. Bar=1 μm.

Fig. 6. Electron micrograph. Liver; piglet. Apoptotic bodies in a hepatocyte with condensed cytoplasm and lack of microvilli. Uranyl acetate, lead citrate. Bar=200 nm.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and were characterized by centrilobular necrosis of hepatocytes, dissociation of hepatic plates, Kupffer cell hyperplasia, and increased number of macrophages in hepatic sinusoids. Hepatocytes were often swollen and occasionally contained eosinophilic intranuclear inclusion bodies in their vesicular nuclei. In the cytoplasm of hepatocytes and Kupffer cells, there were often small, granular and basophilic to amphophilic inclusion bodies (Fig. 1). Scattered acidophilic bodies (Fig. 2), which sometimes contained several pyknotic fragments, were observed. It was sometimes difficult to distinguish apoptotic bodies from inclusions in anuclear hepatocytes by HE staining. A large amount of PCV antigen and nucleic acid were detected in the hepatocytes, Kupffer cells, and infiltrating macrophages (Fig. 3). PCV antigen and nucleic acid in hepatocytes were found frequently in the nucleus and sporadically in the perinuclear cytoplasm. In contrast, PCV antigen and nucleic acid in Kupffer cells and infiltrating macrophages were mainly detected in the cytoplasm and, to a lesser extent, in the nuclei. The distribution of PCV antigen and nucleic acid closely mimicked that of the inclusions in hepatocytes and Kupffer cells. The number of ISH positive cells was higher than IHC positive cells.

Many nuclei of hepatocytes and some apoptotic bodies were positive by the TUNEL method. TUNEL-positive hepatocytes were present in and around the necrotic areas (Fig. 4). Electron microscopic examination revealed that cytoplasmic inclusion bodies, which were 0.3–3 μm in diameter, were scattered in the degenerative or anuclear necrotic hepatocytes as well as in phagocytic cells (Fig. 5). Inclusion bodies were electron dense and heterogeneous and contained non-enveloped viral particles, approximately 17 nm in diameter, which usually formed paracrystalline arrays (Fig. 5). There were apoptotic hepatocytes characterized by condensed chromatin surrounded by a nuclear membrane. Pyknotic fragments sometimes observed in the acidophilic bodies were composed of homogeneously electron-dense matrix without viral particles and were considered to be apoptotic bodies (Fig. 6). No intranuclear viral particles were observed in any hepatocytes, Kupffer cells, or infiltrating macrophages.

Clinical disease, gross and histological lesions observed in No.6 piglet were consistent with those in field and experimental cases of PMWS [1, 7, 12, 14]. PCV-2 infection was confirmed by immunohistochemistry, electron microscopy, ISH technique, and PCR test. These findings demonstrated that PMWS could be experimentally reproduced in a colostrum-deprived piglet by inoculation of tissue homogenates from pigs with PMWS. In previous studies, coinfections with other porcine viruses or immunostimulation have been shown to be important in the development of clinical PMWS [1, 7, 8]. In contrast, a recent report demonstrated that PCV-2 could induce clinical PMWS in piglets in the absence of other swine pathogens [10]. In the case presented here, the inoculum of tissue homogenates was shown to be free from PCV-1, PPV and PRRSV. No evidence of either environmental or laboratory contamination was observed in the No. 6 piglet.

There were some difference between the hepatic lesions of natural cases and the present No.6 piglet. Severe hepatic lesions observed in the natural cases of PMWS were characterized by extensive loss of hepatocytes, diffuse inflammatory infiltration, and periportal fibrosis [1, 12, 13]. These changes occurred in a later stage of the disease [13]. In the No. 6 piglet, lymphoplasmacytic infiltration was extremely mild and fibrosis was absent. These findings were probably related to a more acute disease accompanying the severe damage of lymphoid tissues in the present case. The severity and nature of the hepatic lesions may also be influenced by the age of piglet exposed to PCV-2 and the size of the infective dose.

A pathognomonic microscopic finding of PMWS is the formation of cytoplasmic inclusions, which were characterized by round, homogeneous, and magenta to basophilic, and botryoid clusters of variable sizes (5–25 μm in diameter) [1, 6, 12]. Cytoplasmic inclusions were formed in the cells of monocyte/macrophage lineage [1, 6, 12]. In the present study, cytoplasmic inclusions were detected in the liver as well as lymphoid tissues. Unlike lymphoid tissues, the inclusions in the hepatocytes were smaller in size (0.3–3 μm in diameter) of different stainability, and did not form botryoid clusters. To our knowledge, there are no reports on the formation of inclusion bodies in the hepatocytes. PCV antigen and nucleic acid were similarly detected in the hepatocytes with inclusion bodies. Intranuclear inclusions found in hepatocytes also contained PCV-2 antigen and nucleic acid but no distinct viral particles were detected in them by electron microscopy. It is not clear whether these inclusions are nuclear structures including immature viral components. Our findings may indicate that hepatocellular necrosis is due partly to replication of PCV-2. Furthermore, apoptotic hepatocytes were confirmed by TUNEL method and electron microscopy. Therefore, apoptosis may be also involved in hepatic lesions in this case. While the immune-mediated hepatocyte death was not excluded completely, lymphocytic infiltrates were rarely observed. This was reflected by a crippled immune status, evidenced by lymphocytic depletion in the lymphoid tissues in the piglet. The reason why only one of five piglets developed severe hepatitis remained unexplained. Conceivable explanations are differences in health status or immune response among piglets at the time of PCV-2 infection. Further studies are required to clarify the pathogenesis of hepatic damage in PMWS.

ACKNOWLEDGEMENTS. We thank Ms Hitomi Tomioka and Ms Nobuko Saito for their technical assistance.

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