FULL PAPER *Pathology*

Porcine Circovirus Induces B Lymphocyte Depletion in Pigs with Wasting Disease Syndrome

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ABSTRACT. To disclose the mechanism of cellular injury following porcine circovirus (PCV) infection, 12 pigs were examined by the terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling (TUNEL) method and immunohistochemistry. Histologically, the lymphoid tissues were characterized by marked apoptosis of lymphocytes, lymphocyte depletion, and macrophages and giant cells containing numerous inclusion bodies with or without apoptotic bodies. Immunohistochemically, there were many lysozyme-positive macrophages in the lymphoid follicles, while the number of CD79a-positive B lymphocytes was scanty. Apoptotic cells, which were proved to be TUNEL positive, revealed CD79a positivity. Although detectable mainly in the cytoplasm of macrophages, PCV antigens were found also in the nuclei of macrophages and apoptotic lymphocytes. Ultrastructurally, the presence of PCV virions was confirmed in apoptotic bodies phagocytosed by macrophages. These findings suggested that lymphocyte depletion with apoptotic death of B lymphocytes was caused by PCV, and that some of the inclusion bodies were phagolysosomes derived from the apoptosis. Thus, PCV may trigger the development of wasting disease syndrome by producing an immunocompromised state in pigs.

KEY WORDS: apoptosis, B lymphocyte, lymphocyte depletion, porcine, porcine circovirus.

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Postweaning multisystemic wasting syndrome (PMWS) has recently emerged as an important disease that affects pigs shortly after weaning and fattening [6, 13]. A circovirus-like virus was isolated from pigs affected with PMWS in Canada, U.S.A. and Europe in 1998 [3, 9]. This virus exhibited 68% nucleotide sequence homology with the porcine circovirus (PCV) contaminant of PK/15 cell cultures [12], and had limited antigenic cross-reactivity with PCV [3]. The new circovirus isolates were referred to as PCV2 and the original PCV as PCV1 on the basis of genomic and antigenic analyses [4]. This has led to the speculation that a new or modified pathogenic PCV may have emerged in the pig populations of several countries.

Microscopically, lymphocyte depletion, histiocytic infiltration and multinucleated giant cells were frequently observed in the lymphoid organs [6, 24]. A prominent finding was the presence of cytoplasmic inclusions in histiocytes [22, 23]. Rosell *et al.* [22] reported that the target cells for PCV replication were the monocyte/macrophage lineage and antigen-presenting cells. In addition, PCV antigen was detected in small round cells resembling small lymphocytes in lesions with severe lymphocyte depletion [22, 23]. Previous *in vitro* studies showed PCV replication in monocytes and macrophages, but there was no evidence of replication in lymphocytes in cultures [2, 20]. Although PMWS is considered to cause immunosuppression based on the histology (lymphocytic depletion) and concurrence of *Pneumocystis carinii* infection [6, 9, 22], its mechanism is still unclear. To clarify the pathogenesis of PCV infection, we performed *in situ* DNA strand break analysis and histological, immunohistological and ultrastructural examinations of the lymphoid systems from 12 piglets, and herein discuss the mechanism of systemic immunosuppression caused by PCV.

MATERIALS AND METHODS

The materials examined were extracted from our previous study of natural PCV infection in pigs [23]. Briefly, 12 pigs (50–80 days old) with loss of body condition from 3 farms were euthanized and necropsy was performed immediately (Table 1). Samples of the spleen, tonsil and lymph nodes (including superficial inguinal, mesenteric, mediastinal and submandibular lymph nodes) were collected and fixed by immersion in 10% neutral buffered formalin. Fixed samples were dehydrated, embedded in paraffin wax, sectioned at 3 μm, and stained with hematoxylin and eosin (HE).

For identification of cell populations in the lymphoid organs (including the lymph nodes, tonsil and spleen), immunohistochemical staining was carried out by the avidin-biotin-peroxidase complex (ABC) method with an ABC kit (BioGenex Laboratories, San Ramon, CA, U.S.A.). The following antibodies were used: rabbit anti-human CD3 polyclonal antibody, mouse anti-human CD79a(HM57) monoclonal antibody, rabbit anti-human lysozyme polyclonal antibody (Dako, Glostrup, Denmark) and rabbit anti-bovine S100 protein polyclonal antibody (Nichirei, Tokyo, Japan) [11, 27, 28]. The specificity of these antisera was ascertained in normal porcine lymphoid tissues. PCV in the tissues was examined by the streptavidin-biotin-peroxidase complex immunoperoxidase technique (SAB) (Nichirei, Tokyo, Japan) using hyperimmune rabbit antiserum to PCV1 (CCL-33) as described previously [23]. Sections were lightly counterstained with Mayer's hematoxylin and assessed by light microscopy.

To investigate the presence of cells with DNA strand breaks, which are a characteristic finding for the apoptotic process, paraffin-embedded specimens of the lymphoid tis-

Table 1. Immunohistopathological features of the lymphoid tissues of pigs with wasting disease syndrome

Piglet no.	Age (days)	Number of positive cells[b)					
		CD79a-positive B lymphocytes	lysozyme-positive macrophages	CD3-positive T lymphocytes	S100-positive dendritic cells	PCV antigen-positive cells	TUNEL-positive cells
1	50	230 ± 52	296 ± 51	723 ± 97	12 ± 3	37 ± 7	88 ± 28
2	80	220 ± 45	303 ± 53	819 ± 90	9 ± 2	79 ± 19	196 ± 30
3	70	175 ± 43	331 ± 72	805 ± 127	10 ± 3	70 ± 17	202 ± 34
4	50	143 ± 24	306 ± 32	770 ± 97	9 ± 2	61 ± 22	59 ± 20
5	80	133 ± 29	341 ± 33	747 ± 89	8 ± 3	70 ± 20	73 ± 34
6	80	125 ± 33	280 ± 47	701 ± 84	7 ± 3	79 ± 18	147 ± 43
7	50	116 ± 33	262 ± 40	728 ± 151	12 ± 1	85 ± 15	118 ± 20
8	70	109 ± 26	348 ± 73	769 ± 108	8 ± 3	69 ± 17	119 ± 24
9	70	104 ± 23	340 ± 41	753 ± 86	11 ± 4	79 ± 11	200 ± 45
10	80	50 ± 18	398 ± 73	790 ± 108	8 ± 3	61 ± 10	172 ± 41
11	60	25 ± 9	433 ± 89	758 ± 98	9 ± 2	83 ± 14	269 ± 23
12	60	25 ± 9	518 ± 84	790 ± 111	11 ± 2	108 ± 21	99 ± 21
13[a)	70	450 ± 52	97 ± 20	775 ± 108	10 ± 3	0	10 ± 3
14[a)	70	364 ± 37	81 ± 24	738 ± 102	9 ± 3	0	11 ± 5
15[a)	70	402 ± 68	60 ± 7	759 ± 138	7 ± 2	0	9 ± 5

a) Control.
b) Per 10 fields of × 200 magnification.

sues, were examined by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end labeling (TUNEL) procedure [5]. The reagents used were obtained from an Apoptag kit (Oncor, Gaithersburg, MD, U.S.A.). In addition, representative specimens from 3 piglets aged 70 days without any history of PCV infection demonstrable by histopathological, immunohistological and isolation techniques served as controls.

The mean numbers of CD79a-positive B lymphocytes, CD3-positive T lymphocytes, lysozyme-positive macrophages, S100-positive dendritic cells, TUNEL-positive cells and PCV antigen-positive cells were directly counted under microscope in ten randomly selected fields of × 200 magnification in each section.

For electron microscopical examination, small blocks taken from the lymph nodes demonstrable to be positive for PCV1 were examined with a transmission electron microscope (TEM, JEM-1010, JEOL, Tokyo, Japan) as described previously [23].

RESULTS

Macroscopical and microscopical lesions: At necropsy, enlargement of inguinal, and mesenteric lymph nodes was the most obvious lesion in the 12 affected pigs. Microscopically, in the lymph nodes, severe lymphocytic depletion characterized the indistinct lymphoid follicles, and there were many apoptotic lymphocytes in these follicles (Fig. 1). Macrophages with sharply demarcated spherical, basophilic, cytoplasmic inclusions were present mainly in the germinal centers of follicles, although they were also detected in the T lymphocyte-dependent zone (Fig. 2). Most of them contained lymphocytic apoptotic bodies as well as inclusion bodies, and it was occasionally difficult to distinguish between these two types of bodies. The larger the number of bodies was, the more severely the lymphoid organs were affected. Several multinucleated giant cells were seen in the severely affected lymphoid follicles. The spleen and tonsils showed lesions similar to those in the lymph nodes.

Cell populations in the lymphoid organs: The lymphoid tissue had a small number of CD79a-positive B lymphocytes (Fig. 3) and many more lysozyme-positive macrophages (Fig. 4) in the lymphoid follicles compared with those of the control cases (Table 1). Apoptotic lymphocytes in the lymphoid follicles were CD79a positive. CD3-positive T lymphocytes and S100-positive dendritic cells subpopulations in the lymphoid tissues were similar to those in the control cases.

Immunohistochemical detection of PCV antigen: PCV inclusion bodies were strongly stained with the antiserum to PCV (Figs. 5 and 6). Many apoptotic lymphocytes (Fig. 6) and phagocyted apoptotic bodies (Fig. 7) were labeled by polyclonal antibodies to PCV. PCV antigen was occasionally detected in the nuclei of both lymphocytes and macrophages (Fig. 5).

Cells with DNA strand breaks: In contrast to the control cases, numerous TUNEL-positive lymphocytes and apoptotic bodies were present in B lymphocyte-dependent areas (Fig. 8). The former had karyopyknotic or karyorrhectic nuclei, which corresponded to the PCV-positive nuclei in immunostained sections (Fig. 9). The TUNEL-positive reaction was considered to be accelerated with the increase of intranuclear PCV.

Electron microscopical evidence: In the nuclei of apoptotic lymphocytes, condensed chromatin was arranged along the nuclear envelope as homogeneous masses, and often assumed a half-moon configuration. Viral particles of circovirus, granular and small (size range 17–20 nm), were found in such nuclei (Fig. 10) as well as in the nuclei and cytoplasm of macrophages. Although intracytoplasmic par-

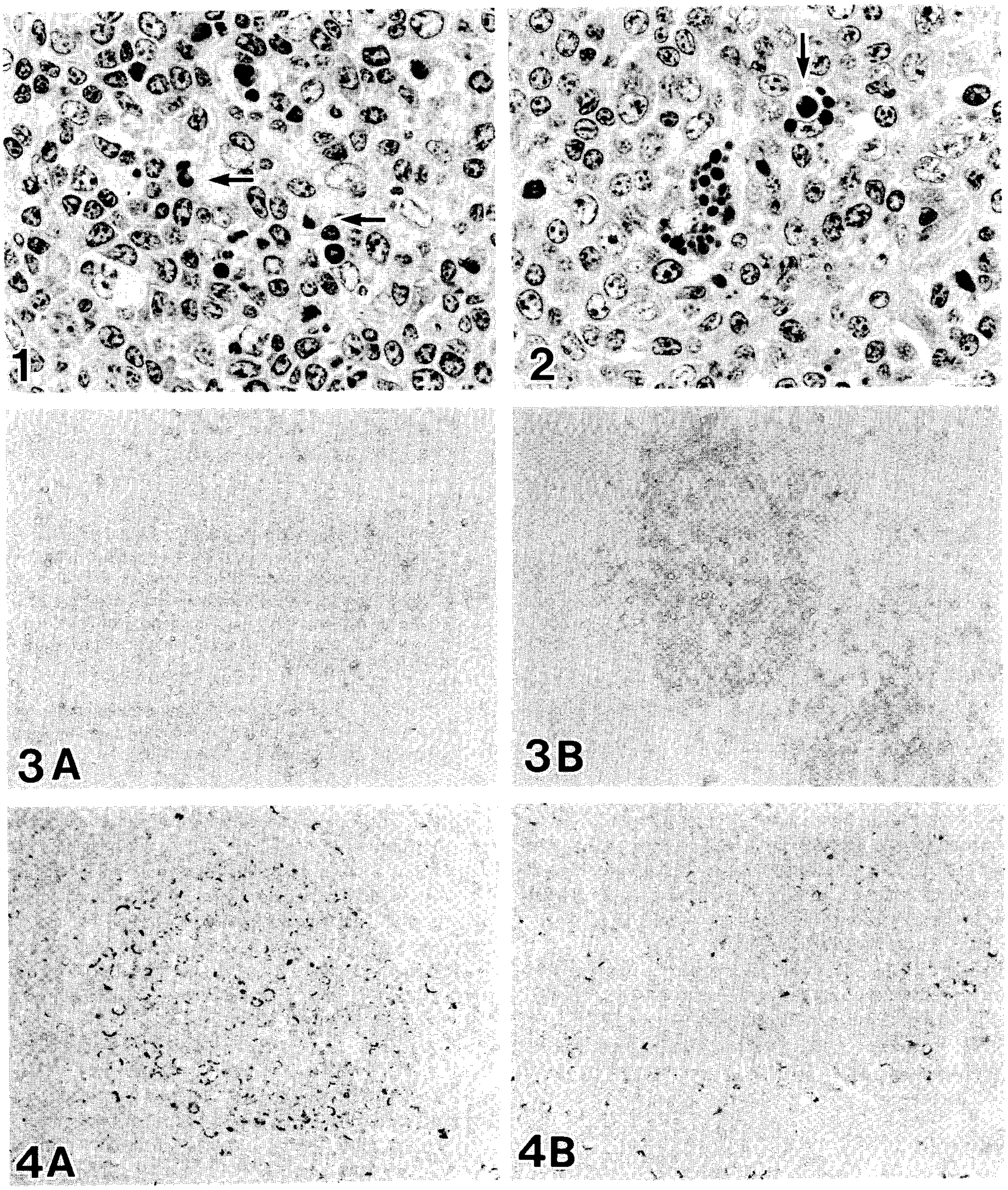
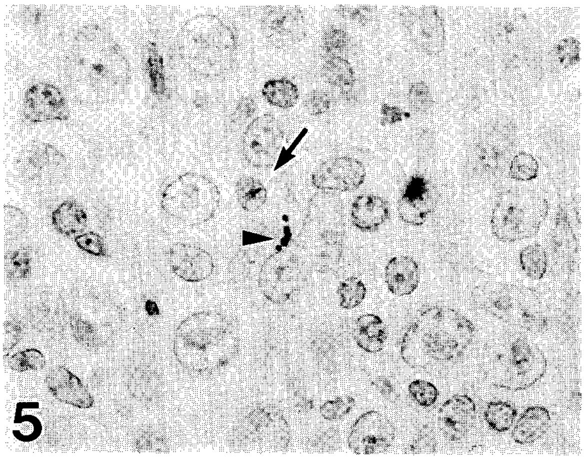


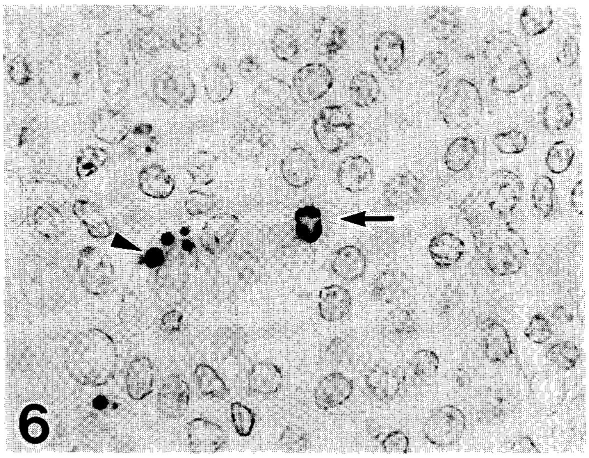
Fig. 1. Bronchial lymph node. Karyopyknosis is observed in apoptotic lymphocytes (arrows) in the lymphoid follicular zone with infiltration of macrophages. HE. × 630.

Fig. 2. Mesenteric lymph node. Macrophages with numerous cytoplasmic inclusion bodies are phagocytosing an apoptotic body (arrow) in the lymphoid follicle. HE. × 630.

Fig. 3. Bronchial lymph node. Small number of CD79a-positive B lymphocytes in the lymphoid follicle in an affected piglet compared with those of a control piglet. A) PCV-infected piglet. B) control piglet. Anti-CD79a. ABC. × 200.

Fig. 4. Mesenteric lymph node. A large number of lysozyme-positive macrophages in the lymphoid follicle in an affected piglet compared with a control piglet. A) PCV-infected piglet. B) control piglet. Anti-lysozyme. ABC. × 200.





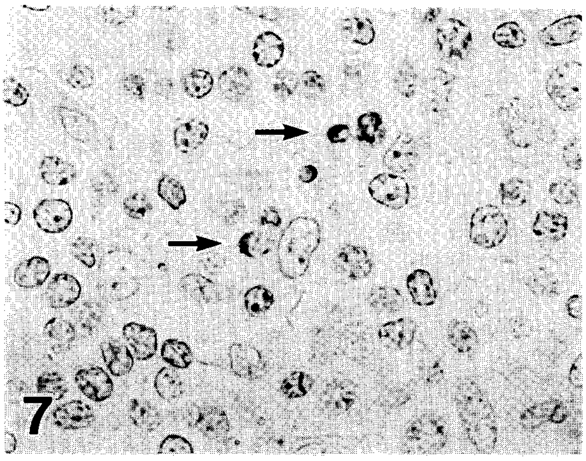


Fig. 5. Bronchial lymph node. PCV antigen is detected in the nucleus of a lymphocyte (arrow) and in the cytoplasm of a macrophage (arrowhead) of the lymphoid follicle. Anti-PCV. SAB. × 1,000.

Fig. 6. Mesenteric lymph node. Apoptotic lymphocyte has PCV antigen just beneath the intact nuclear envelope (arrow), and inclusion bodies in a macrophage (arrowhead) are also labeled by polyclonal antiserum to PCV in the lymphoid follicle. Anti-PCV. SAB. × 1,000.

Fig. 7. Bronchial lymph node. Phagocyted apoptotic bodies (arrows) in macrophages of the lymphoid follicle are labeled by polyclonal antiserum to PCV. Anti-PCV. SAB. × 1,000.

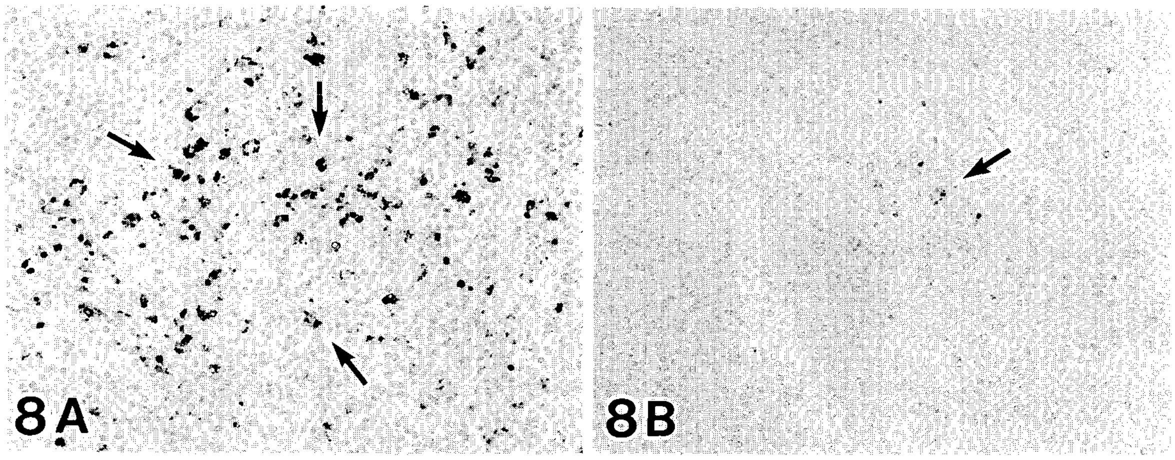
ticles were plentiful, intranuclear ones were usually few in number and loosely aggregated. Particles showing the same features were observed in apoptotic bodies, which were enclosed by double membranes (Fig. 11), but some particles were present without surrounding membranes in the cytoplasm of macrophages (Fig. 12).

None of the pathogenic organisms such as porcine parvovirus, porcine reproductive and respiratory syndrome virus, cytomegalovirus and *Mycoplasma* without PCV were identified or isolated in the lymphoid samples with routine histopathological, virological and bacteriological isolation techniques.

DISCUSSION

This study describes the histopathology of the lymphoid organs in 12 natural cases of PCV infection, together with the cell population, distribution of PCV, and *in situ* DNA strand break analysis. Lymphocytic depletion, an increase in the number of macrophages and unique cytoplasmic inclusions were characteristic of PCV infection in the lymphoid organs, as observed by others [15, 17, 19, 25]. This tissue destruction was characterized by apoptosis [16, 29] of CD79a and TUNEL-positive B lymphocytes and a decrease in the number of CD79a-positive B lymphocytes. Furthermore, PCV antigen and virions were confined in the apoptotic bodies phagocytosed by macrophages, and the other organisms were not found. These findings suggested that PCV induced apoptosis in B lymphocytes and led to B-lymphocyte depletion and systemic immunosuppression in pigs. Those results agree with most of those of previous reports, but the morphological findings, intranuclear virions and *in situ* DNA strand break analysis of B lymphocytes in follicles were not reported in those studies [7, 10, 14, 24].

PCV belongs to the same virus family as chicken anemia virus (CAV) which causes immunosuppression [1, 21]. Apoptin, a protein encoded by CAV, induces apoptosis in various cultured human tumorigenic and/or transformed cell lines, e.g. in leukemia, lymphoma or Epstein-Barr virus transformed B lymphocytes, but not in normal cells [8]. Our histopathological investigations, along with previous reports [1, 8, 21, 22] suggest the following mechanism for systemic immunosuppression in piglets infected with PCV. PCV directly infect dividing cells, including B lymphocytes and macrophages, and induces apoptosis directly in individual B lymphocytes through cellular infection, but it can not induce apoptosis or necrosis of macrophages. The lymphocytic apoptotic bodies with PCV are trapped within macrophages and giant cells in germinal centers and serve as a source of infection for cells that reside in or migrate through the lymph



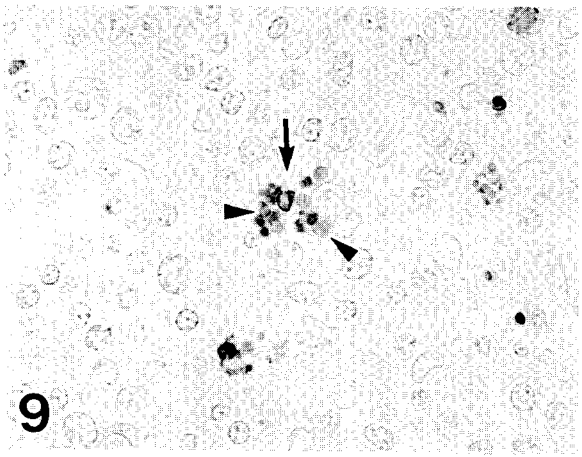


Fig. 8. Mesenteric lymph node. A lymphoid follicle in an affected piglet has a large number of TUNEL-positive lymphocytes and TUNEL-positive apoptotic bodies (arrows) in the cytoplasm of macrophages compared with those of a control piglet. A) PCV-infected piglet. B) control piglet. TUNEL. × 200.

Fig. 9. Mesenteric lymph node. TUNEL-positive apoptotic body (arrow) and inclusion bodies (arrowheads) in the cytoplasm of macrophages in the lymphoid follicle. TUNEL. × 630.

nodes throughout the course of infection even during the early and often prolonged asymptomatic period. Macrophages avidly phagocytose apoptotic cells, and the viral particles in apoptotic debris might spontaneously transfect macrophages and lead to the production of new virions as previously described [26]. The induction of apoptosis in a large number of B lymphocytes in the lymphoid organs appears to be one of the mechanism of PCV pathogenesis and might be an explanation for the dramatic reduction in the number of B lymphocytes in PCV-infected pigs. Therefore additional infectious agents such as porcine parvovirus [18] and porcine reproductive and respiratory syndrome virus [23] would produce severe clinical disease in PCV-infected pigs. However, it is not clear whether PCV have apoptin-like material or not. We suspect, based on our results and previous studies [1, 8, 21] that apoptin-like material encoded by PCV induces predominantly apoptosis in B lymphocytes. Future studies should focus on the identification of apoptin-like material encoded by PCV and the mechanism of induction of apoptosis by this material and apoptotic genes.

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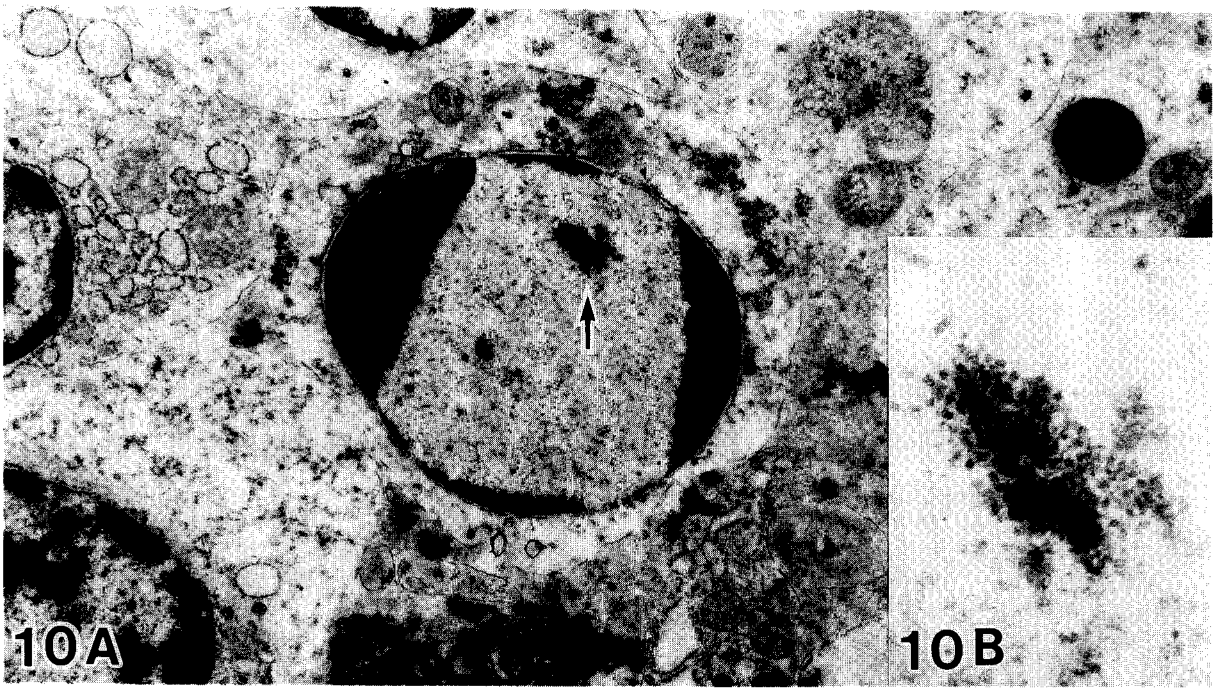

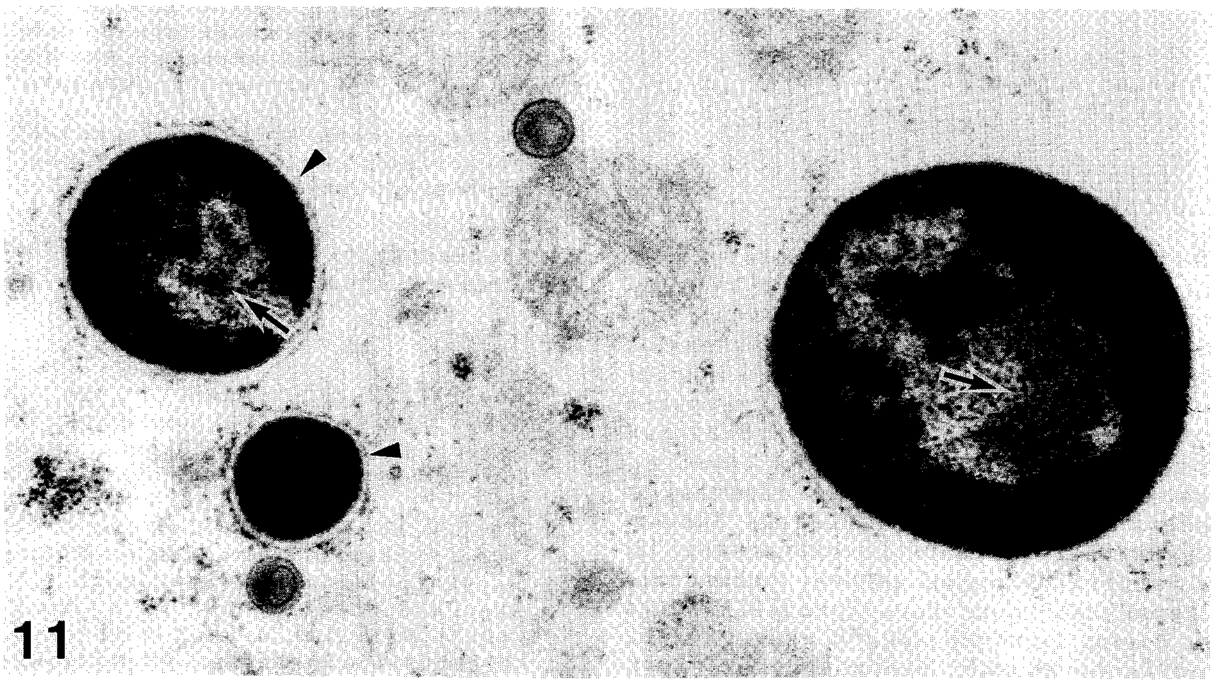

Fig. 10. Mesenteric lymph node. A) A lymphocyte shows degenerative changes with chromatin concentration just beneath the intact nuclear envelope, and has viral particles (arrow) in the nucleus. B) Higher magnification of an intranuclear aggregate of circovirus particles, approximately 17–20 nm in diameter, from. 10A. A) TEM. × 12,000. B) TEM. × 50,000.

Fig. 11. Bronchial lymph node. Apoptotic body with PCV (arrows) in sequestered cytoplasm, is phagocytosed by a macrophage in the germinal centre of the follicle. Phagocytosed apoptotic bodies (arrowheads) with chromatin content are enclosed by double membranes. TEM. × 34,800.

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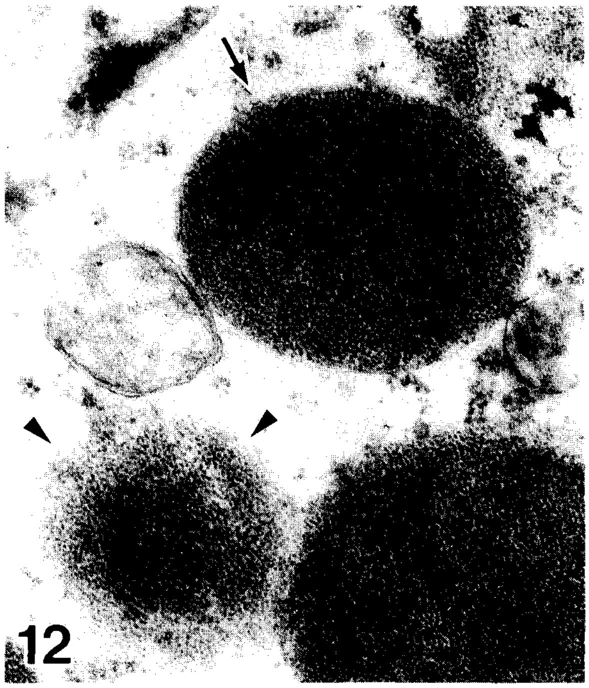

Fig. 12. Bronchial lymph node. Electron-dense, round-to-ovoid phagolysosomal bodies with sharp margins (arrow) and small inclusions composed of loosely aggregated indistinct electron-dense viral particles without membrane (arrowheads) are present in the cytoplasm of a macrophage. TEM. × 25,000.

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