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Apoptosis of bursal lymphoid cells in chickens experimentally infected with IBDV(SH/92)

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Abstract. Chickens at 3-weeks of age were inoculated with a highly virulent strain (SH/92) of Infectious Bursal Disease Virus(IBDV) through ocular and cloacal routes. The infected chickens were killed at 6, 12,, 24, 48, 72, and 96 hrs post inoculation (PI) and Bursa of Fabricius(BF) were collected. The sizes of bursal follicules in infected chickens decreased at 48 to 96 hrs PI. Histologically, the cellular changes were first evident at 12 hrs PI and characterized by condensation of nuclear chromatin of bursal lymphocytes, indicating apoptosis. By 24 hrs PI, apoptotic lymphocytes dramatically increased. In addition, infiltration of heterophils were also seen in the follicles and in the interfollicular connective tissues. At 48 hrs PI, cystic cavities were observed in the follicles. As the infection advanced, the bursal follicles showed atrophy, accompanied by disappearance of heterophils and reduction in numbers of lymphocytes in the cystic cavities which was replaced by proteineous materials. The nuclei of most affected lymphocyte stained positively with the in situ end labeling for apoptosis. Electron microscopy showed viral particles with crystalline array in the lymphocytes of BF infected with IBDV. These results indicated that SH/92 IBDV infection in chickens caused increased apoptosis in the BF.

Key words: apoptosis, bursa of Fabricius, chicken, infectious bursal disease.

Introduction

Infectious Bursal Disease Virus (IBDV) infection of newly hatched chickens causes considerable worldwide economic losses in the poultry industry. IBDV belongs to the genus Avibirnavirus of the family Birnaviridae[1] which mainly effect the B-cells.[2,3] It is also known to cause depletion of lymphocytes in the bursa of Fabricius (BF), subsequently leading to immune deficiency[4] and influenced the immunosuppression against specific antigens.[5-8]

The apoptosis has been reported in the chickens infected with viruses such as chicken anemia virus,[9,10] influenza virus,[11] Newcastle disease virus,[12] Marek's disease virus[13] and IBDV. Vasconcelos and Lam[14] reported that IBDV can induce apoptosis of peripheral blood lymphocytes in chickens and they also showed apoptosis in ovo.[14,15] In addition, flow cytometric study showed low bursal cellular proliferation and increased the number of apoptotic cells in IBDV infection.[16]

Recently, a highly virulent strain, SH/92 of IBDV isolated by Mo et al.[17] was associated with severe pathological lesions in the cloacal bursa of chickens. The objective of this study was to

determine the morphological evidence of apoptosis in bursal lymphocytes infected with SH/92 IVDV strain

Materials and Methods

Viruses

The highly virulent IBDV (SH/92) was isolated from farm with history of IBD outbreak. The virus was provided by the National Veterinary Research Institute(NVRI), MAF, Anyang, Korea. The strain has been classified as highly virulent IBDV with 80% mortality when inoculated to 5-week-old chickens.[18]

Chickens

Specific Pathogen Free (SPF) white leghorn chickens, 3 week old, were provided by the NVRI. Chickens were maintained in isolated unit with filtered air. The inoculation was performed using both eyedrop and cloacal routes with 0.1㎖ of bursal homogenates containing the viruses ($10^{4.8}$ mean embryo infectious doses [EID_{50}]/0.1㎖) in a dividing doses via each route. Chickens were humanly killed at 6, 12, 24, 48, 72, and 96 hrs PI. The BFs were collected in order to assess the bursal weight index, and to carry out histological and electron microscopic studies.

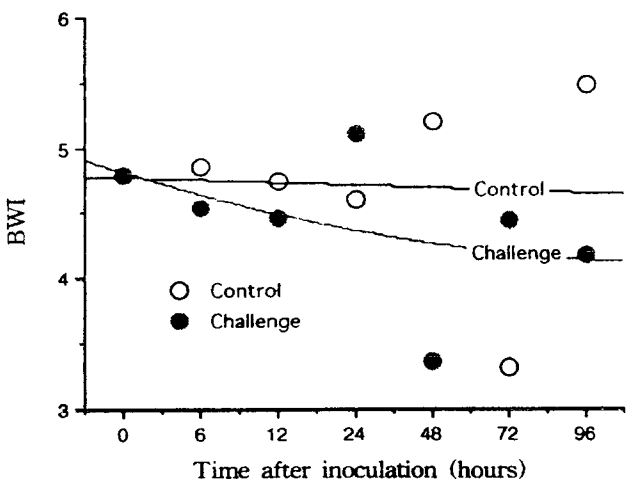

Fig. 1. The changes of the bursal weight index(BWI) after IBDV inoculation in the chicken.

Bursal weight index (BWI)

BF and body of each bird were weighed and the bursal weight index values were calculated as follows: Bursal weight/Body weight ×1,000.

Pathology

The BFs obtained from each bird were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H-E, and examined microscopically. For analysis of the bursal follicular size, the images of tissue sections were captured by a digital imaging camera. The images were analysed with the analySIS-Pro (SIS GmbH, Germany) software. Twenty follicles were measured and their means were calculated. Small pieces (1㎟) of fresh BF were pre-fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), then processed routinely, and observed under a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

In situ detection of fragmented DNA

BFs were stained using a commercial

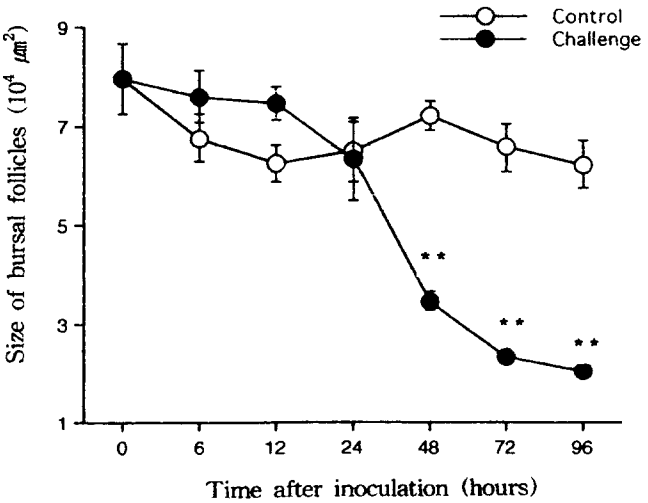

Fig. 2. The changes of bursal follicular size measured by Image Analysis System after inoculation of IBDV in chickens. **: Significant different compared to the control group ($p<0.01$).

apoptosis detection kit (TACS TM2 TdT, Trevigen, Gaithersberg, USA)[19]. Briefly, the procedure was as follows: After deparaffinization and rehydration, sections were digested by proteinase K (1.0 mg/mℓ proteinase K; Sigma) for 15 minutes and blocked endogenous peroxidase by 2% hydrogen peroxide for 5 minutes. The sections were incubated with labeling reaction solution containing dATP, dCTP, and dGTP, and biotin-11-dUTP for 1 hour at 37℃. The sections were also incubated with strept-HRP. Apoptotic nuclei were visualized by peroxidase-diaminobenzidine (DAB : 0.1 mg/mℓ for 10 min) reaction. The sections were then counter-stained with Harris hematoxylin.

Results

Bursal weight index

The BWI of challenged group decreased below those of the control group as the experiment progressed (Fig 1). The follicular sizes of bursa in infected chickens were reduced (Fig 2).

H-E and EM examination

Histological lesions were first detected in some bursal follicles at 12 hrs post-inoculation (PI). Condensation of nuclear chromatin of some lymphocytes was prominent in the medulla of the follicles. At 24 hrs PI, most lymphoid follicles were occupied by increased apoptotic cells accompanied by heterophil infiltration (Fig 3). Heterophils were seen in the follicles as well as in the edematous interfollicular connective tissues. Some lymphocytes were phagocytized by macrophages and heterophils (Fig 3). There were cystic cavities lined by epithelial cell and contained pyknotic lymphocytes in the follicles at 48 hrs PI. By 72 hrs PI, heterophils disappeared and the follicles showed atrophy. The numbers of apoptotic cells in the cystic cavities decreased with influx of proteineous materials (Fig 4). There was gradual loss of lymphoid follicle-associated epithelial cells which possessed endocytic capability and by 72 hrs PI the most follicles reduced to small cellular outlines. Severe lymphoid cellular depletion and plical atrophy were detected from 48 to 96 hrs PI.

Electron microscopically, bursal lymphocytes with condensed chromatin could be seen at 12 hrs PI. These apoptic cells were incresed in numbers at 24 hrs PI (Fig. 6). Virus particles were arranged as a typical crystalline array in the cytoplasm of affected lymphocytes (Fig. 7). These affected lymphocytes also showed disrupted plasmalemma. Infiltrated macrophages were filled with membrane bound masses of apoptotic bodies, myelin figures and viral particles (Figs 8,9). At 72 hrs PI, condensed chromatin, cellular and organelle swelling, vacuolization and interruptions in plasmalemmal continuity were observed in altered lymphocytes (Fig 10).

Immunohistochemistry

Lymphocytic nuclei showing signs of pyknosis or karyorhexis in the bursal follicles were stained by the modified TUNEL method. A significant increase in the number of positive cells was observed at 24 hrs PI, followed by a gradual decrease (Fig 5).

Discussion

In the present investigation, histopathological evaluation of the bursal lymphoid cells demonstrated that apoptosis leads to lymphoid depletion after an experimental infection with a highly virulent strain (SH/92) of IBDV in chickens. BWI values and follicular size by image analysis strongly indicated a drastic reduction in the number of bursal lymphocytes due to IBDV infection. The formation of cystic cavities was associated with the follicles containing pyknotic lymphocytes. Infiltration of inflammatory macro-

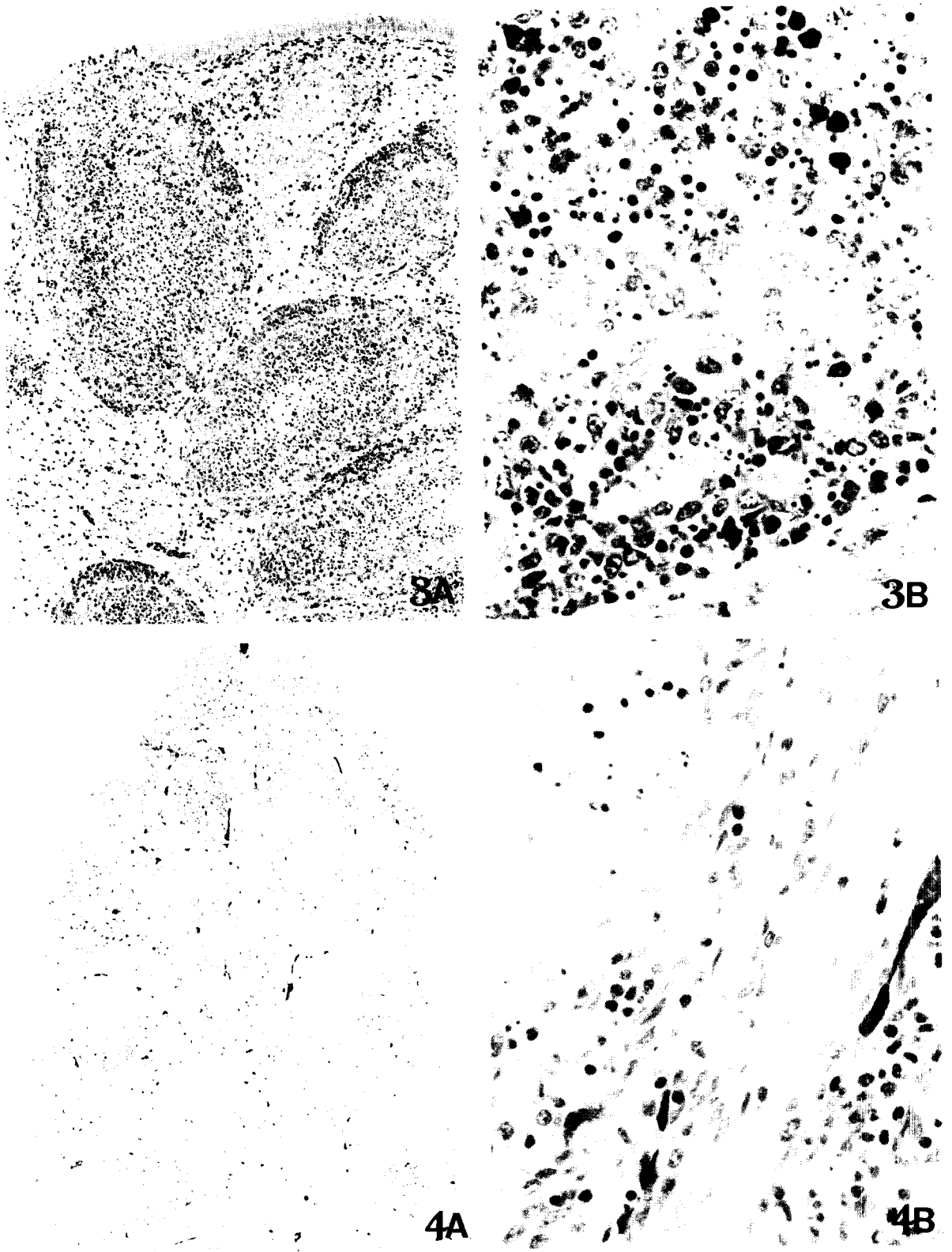

Fig. 3. At 24 hrs PI, apoptotic cells are observed throughout the follicles accompaning with a number of heterophil and macrophage infiltration. H-E stain(A; ×100, B; ×400).

Fig. 4. At 72 hrs PI, there are marked depletion of lymphoid cells in cortex and medulla with increased interfollicular spaces(A; ×100, B; ×400).

phages and heterophils, which phagocytized apoptotic lymphocytes, was a significant feature of this *in vivo* infection. This observation is consistent with previous studies by Kwon et al.,[18] which investigated the pathogenicity of IBDV (SH/92) isolated in Korea. These histopathological changes indicate that IBDV infection induced apoptotic death of bursal lymphocyte, resulting in lymphoid depletion

Immunohistochemical staining also demonstrated pyknotic/karyorhectic nuclei of the lymphocytes in the BF of challanged chickens. This immunohistological observation was also supported by Guanmin et al.,[20] who reported that the nuclei of the shrunk lymphocytes, characterized histologically by pyknosis or karyorhexis, were strongly stained by the modified TUNEL method.

The apoptotic process is under genetic regulation[21,22] and can also be launched by extracellular stimuli such as hormones, cytokines, killer cells, and a variety of chemical, physical, and viral agents.[23] Electron microscopically, we could not detect viral particles in apoptotic cells. Our results and the previous finding of Sharma et al.[24] who reported that apoptosis was not associated with active infection and replication of the IBDV in thymic cells, strongly suggest that apoptosis may represent required an additional stimulus. The hypothesis of concomitance is supported by the fact that apoptosis can also be caused by several nonviral stimuli, such as lymphotoxin, tumor necrosis factor (TNF), and glucocorticoids, and also by a lack of growth factors. These extracellular stimuli can converge on a common intracellular pathway, resulting in the activation of endogenous endonuclease.[25] TNF, a protein toxin secreted by activated macrophages and also by monocytes, has been considered as one of the factor which thought to induced both apoptotic and necrotic forms of cell lysis.[26,27] Clusters of virus particles were observed in the cytoplasm of macrophages as previously described by Cheville.[28] In our studies, an increase in macrophages, which contained clusters of virus particles, was observed in bursal follicles by 24 hrs PI. Therefore, it is possible that viral infection of the bursal lymphocytes and macrophages, the stimulated macrophage secreted TNF which in turn triggered apoptotic necrosis.

By 72 hrs PI, altered lymphocytes were characterized by nuclei containing condensed chromatin, cellular and organelle swelling, cellular vacuolization, and interruptions in plasmalemmal continuity (Fig. 10). These ultrastructural alteration in affected bursal lymphocytes are consistent with apoptotic necrosis.[23,29]

In conclusion, histopathological changes in the infected bursae observed in this study indicate that the SH/92 strain of IBDV induces apoptosis of the lymphocytic population. The severe lymphoid depletion in the BF following IBDV infection may result from active viral replication and extensive apoptosis which could contribute to the immunosuppression.

Acknowledgement

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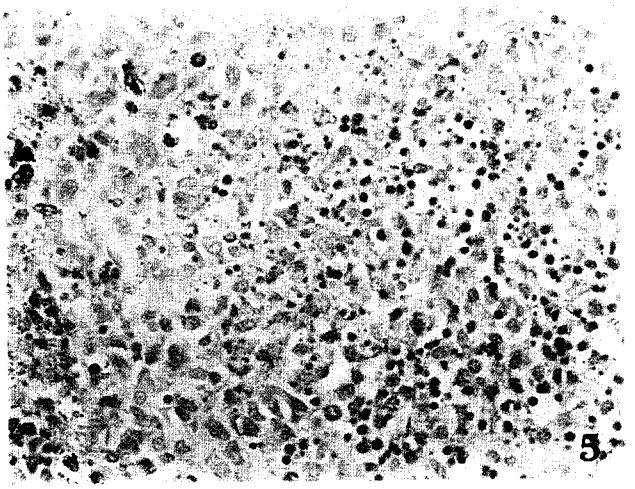

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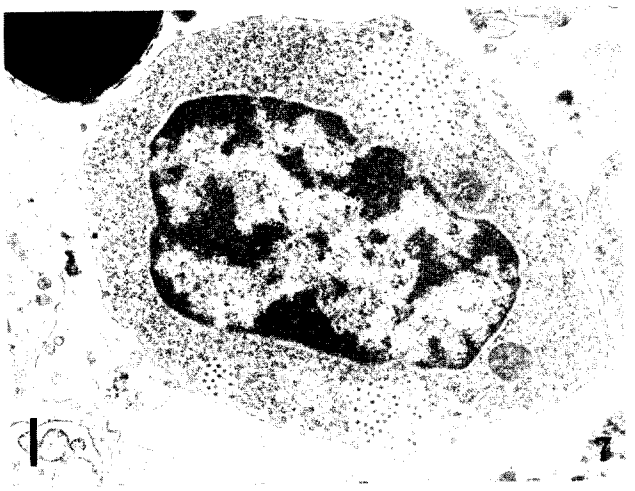

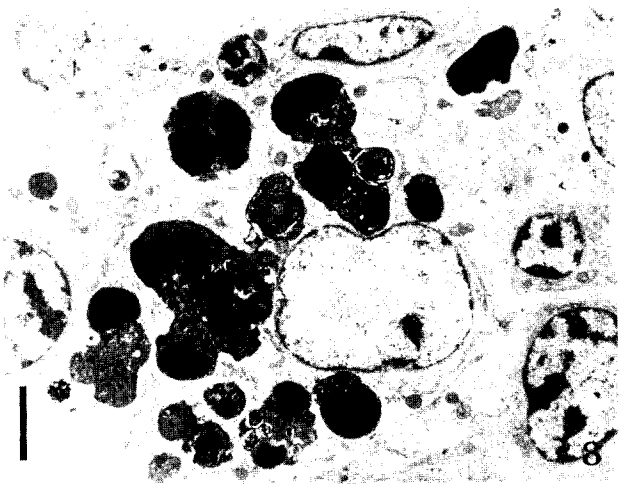

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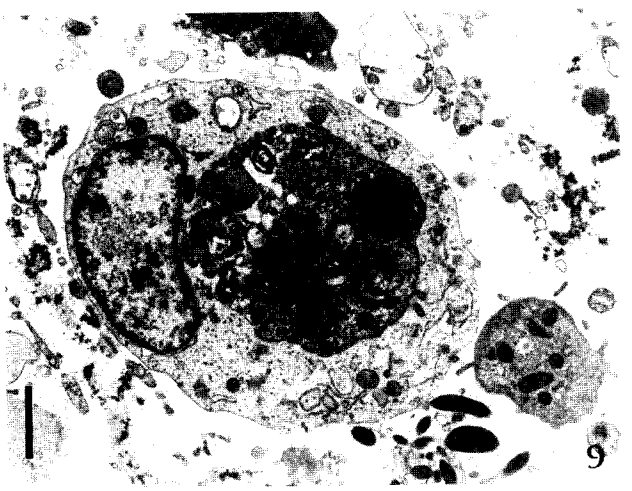

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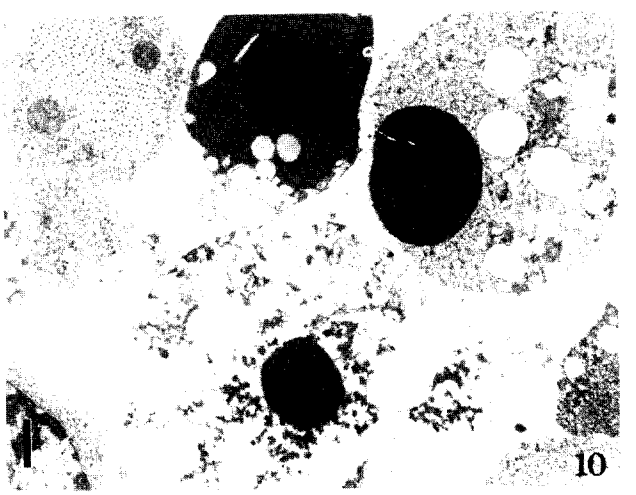

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Fig. 5. Bursal follicular cortex and medulla of IBDV-infected chickens at 24hrs PI). TUNEL-positive cells are greatly increased. ×400.

Fig. 6. Higher magnification of apoptotic cell with chromatin margination at 24 hrs PI. Bar=500㎚.

Fig. 7. Crystalline arrayment of IBDV is prominent in a bursal lymphocyte at 24 hrs PI. Bar=500㎚.

Fig. 8. A macrophage is filled with membrane bound masses of apoptotic bodies at 24 hrs PI. Bar=2㎛.

Fig. 9. A macrophage contains apoptotic body, heterophil debris and viral particles together at 72 hrs PI. Bar=2㎛.

Fig. 10. The altered lymphocytes are characterized by cellular swelling, organelle swelling, vacuolization and interruptions in plasmalemmal continuity at 72 hrs PI. Bar=1㎛.

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