CHAPTER IV

RESULTS

Clinical Signs

All the pigs showed depression and decrease appetite within 3 dpi. The pigs in group A and B recovered to normal appearance in 10 dpi while the pigs in group C showed progressively depress and diarrhea on 5 dpi until the end of experiment. Some of them showed ataxia and seizure around 14-21 dpi. In group C, one pig gradually died on 10, 11, 12, 14, and 20 dpi except one pig survived until the end of experiment. Pigs in all groups had fever (rectal temperature >40^{o}C) which was different onset and duration. Pigs in groups A, B, and C showed average duration of the fever as 7.8, 8, and 10.3 days respectively.

White Blood Cell Count

Decreasing of white blood cell count (WBC) was found in all groups recognizing as leukopenia (WBC<9,000 cells/μl) as 2, 1, and 4 animals at 3 dpi in group A, B, and C, respectively. WBC of all pigs, except group C, were increased more 9,000 cells/μl since 7 dpi (Table 2). Leukopenia was continuously demonstrated in most pigs of group C until 10 dpi. While WBC in 2 of 5 pigs in group C returned to high level as leukocytosis and they finally died.

Virus Isolation

CSFV detected by IPMA was found in blood collected from pigs in group A and B at 3 dpi as 2/6 and 2/6 respectively then the virus disappeared from 7 dpi until end of experiment. The virus was detected in 3 of 6 pigs from group C at 3 dpi. At 7 dpi, the virus was found in all pigs (6/6) of group C and persisted until death or the end of observation (Table 3). It was noted that the concurrent of viremia and leucopenia

were recognized in early stage at 3 dpi but after 7 dpi, the viremia remained only in group C while WBC had been returned normal (Table A1 and A2, Appendix A).

Table 2 The number of pigs showed leukopenia (WBC<9,000 cells/μl) after challenge with CSFV from 0 to 21 dpi (n = 6 each group).

	Leukopenia on dpi					
Group	0	3	7	10	14	21
A	0/6	2/6	0/6	0/6	0/6	0/6
B	0/6	1/6	0/6	0/6	0/6	0/6
C	0/6	4/6	6/6	3/5	0/2	0/1

Table 3 Virus detection in pigs after the challenge with Bangkok 1950 strain of CSFV from 0 to 21 dpi (n = 6 each group).

	Virus detection on dpi					
Group	0	3	7	10	14	21
A	0/6	2/6	0/6	0/6	0/6	0/6
B	0/6	2/6	0/6	0/6	0/6	0/6
C	0/6	3/6	6/6	5/5	2/2	1/1

Serum Neutralizing Antibody

All of the pigs vaccinated with CSFV-vaccine (lapinized Chinese strain) at 3 wk of age, showed a significantly response different level ($P<0.05$) of SN titer against CSFV. The means of SN titer at 2 wk post vaccination in pigs group A and C

was gradually decreased. In contrast, pigs in group B which had a low level of maternal antibody produced a high antibody response within 2 wk post vaccination. After challenge one week, the antibody titers were markedly decreased in group A and C while group B was increased. At 14 dpi, pigs in group A showed a low seroconversion whereas the pigs in group B showed a high seroconversion. The SN titer of group A and B appeared to be constant at 21 dpi but it had been disappeared in group C since 14 dpi (Figure 4). The mean $\log_2$ SN titer of group B was higher than that of group A and C with a statistical significant ($P<0.05$) at 14 and 21 dpi (Table 4).

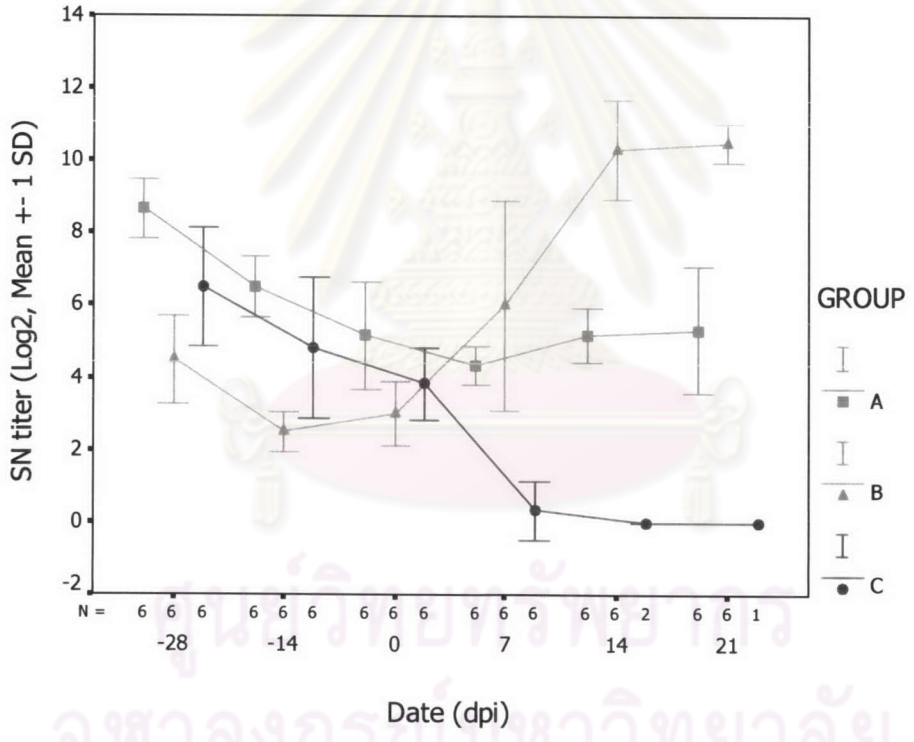

Figure 4 The $\log_2$ (means $\pm$ SD) of SN titer at various days of experiment. Pigs were vaccinated with CSFV vaccine (lapinized Chinese strain) at 3 weeks of age and challenged with Bangkok 1950 strain of CSFV 2 weeks post vaccination.

Table 4 The means SN titer of three week-old pigs vaccinated with lapinized Chinese strain of CSFV vaccine (at -14 dpi) followed by challenging with Bangkok 1950 strain of CSFV. Data represents the mean $\pm$ SD of SN titer (log_2) from 1 wk of age (-28 dpi) to 8 wk of age (21 dpi). The means were compared between groups in each dpi.

Group	SN titer (Log_2 mean$\pm$SD) on dpi					
	-28 (2 wk prior vaccinate)	-14 (vaccinate)	0 (challenge)	7	14	21
A	8.67 ± 8.2^{a}	6.50 ± 0.84^{a}	5.17 ± 1.47^{a}	4.33 ± 0.52^{a}	5.17 ± 0.75^{a}	5.33 ± 1.75^{a}
B	4.50 ± 1.22^{b}	2.50 ± 0.55^{b}	3.00 ± 0.89^{b}	6.00 ± 2.9^{b}	10.33 ± 1.37^{b}	10.5 ± 0.55^{b}
C	6.50 ± 1.64^{c}	4.83 ± 1.94^{c}	3.83 ± 0.98^{ab}	0.33 ± 0.82^{c}	0.0	0.0

a, b, c ($P<0.05$). different superscripts in the different rows means significantly difference

Macroscopic Findings

The gross pathological findings were mainly demonstrated in all pigs of group C that died during observation as thymic atrophy (6/6) and petechiation or ecchymosis at skin, subcutaneous tissues, kidneys, lymph nodes, intestinal mucosa included stomach, cecum, and colon (6/6). Mild to moderate hemorrhages of thymus, tonsil, and heart muscle were found in 3/6, 2/6, 2/6 of pigs in group B respectively, whereas petechial hemorrhage of heart muscle was found in 3/6 of pigs in group A. Multifocal splenic infarction and tonsillar necrosis were found in 4 of 6 pigs in group C (Figure 5).

Histopathological Findings

The pigs of group C that died after CSFV challenged demonstrated a moderate to severe depletion of lymphoid tissues in tonsil, thymus, spleen, lymph

nodes, and Peyer's patches. The depletion of lymphoid cells was found in germinal centers as well as peripheral lymphoid tissues (Figure 6). Thymus was the most affected tissue in which all of lymphoid cells in cortex and medulla were predominantly destroyed. Severe depletion of lymphoid cells was also seen in tonsil, spleen, and Peyer's patches but less prominent than that of thymus. All vaccinated pigs in groups A and B showed un-remarkable lesions and any depletion of lymphoid cells could not be recognized (Table 5).

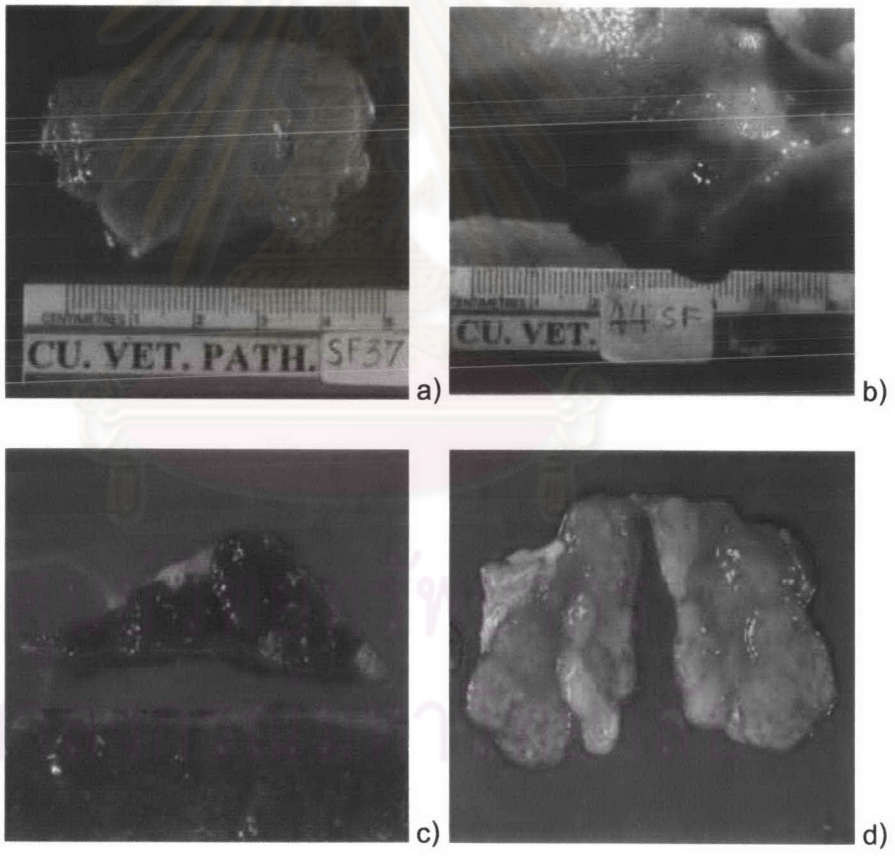

Figure 5 The gross lesions of lymphoid tissues from pigs of group C challenged with CSFV. (a) hemorrhage of thymus, (b) tonsil, (c) splenic infarction, and (d) superficial inguinal lymph node.

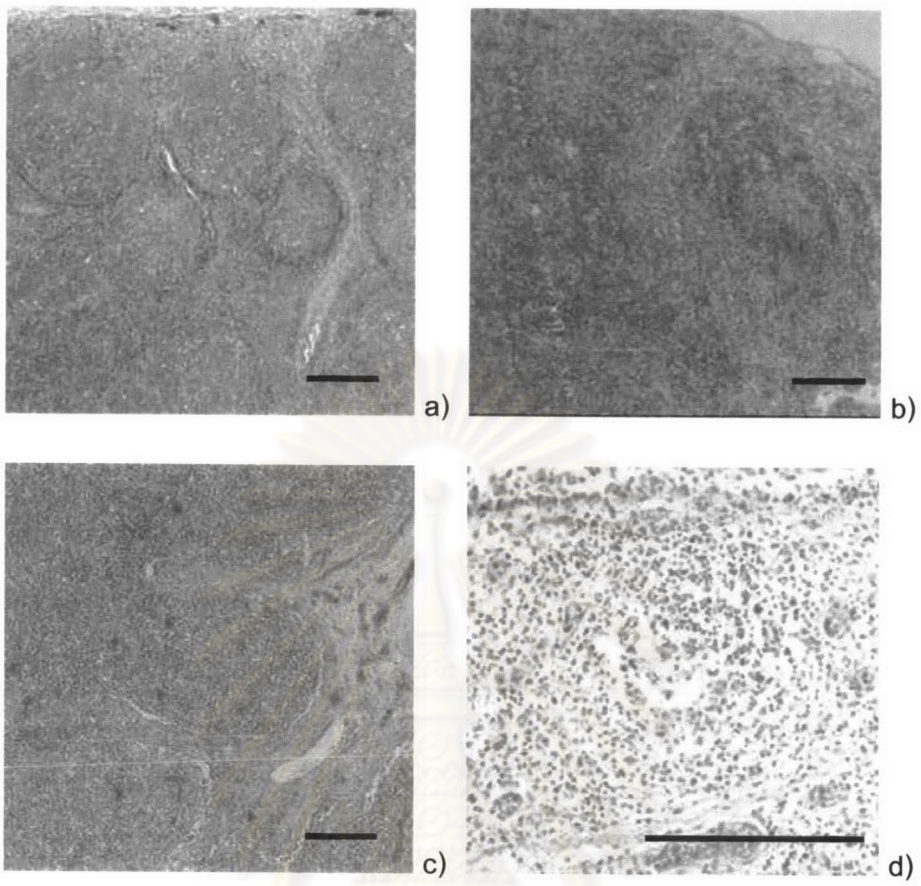

Figure 6 The histopathological findings of lymphoid depletion in lymph nodes according to the criteria. (a) no depletion (-) in pigs group A and B, (b) mild depletion (+), (c) moderate depletion (++), and (d) severe depletion (+++) in pigs group C. (HE, bar = 80μm)

CSFV-Ag Positive Cells in Lymphoid Tissues

All lymphoid tissues of the pigs in group C showed a positive staining with IHC using anti-CSFV gp55 MAb whereas, no positive cell was detected from pigs of groups A and B. The mainly positive stained cells found in follicle and paracortical area were lymphoid cells and mononuclear-phagocytes and the positive staining localized in the cytoplasm of the cells (Figure 7). Other cells were also stained by the MAb such as reticulo-endothelial cells, the cells lining at basement membrane and connective tissue cells i.e. fibroblasts. The result showed that tonsil was the most intensively positive tissue with mean $\pm$ SD of positive cells at 35.3 $\pm$ 6.5/0.1 mm^2 which was the highest

among those of the others ($P<0.05$). The tissue with the lowest positive cell number was thymus (11.2 ± 4.2 cells/0.1 mm^2) ($P<0.05$). The positive cells were detected in spleen, lymph node, and Peyer's patches as 27.2 ± 6.8, 23.4 ± 2.7, and 17.5 ± 5.7 cells, respectively. Nevertheless, the means of positive cells among dead pigs were not different (Table 6).

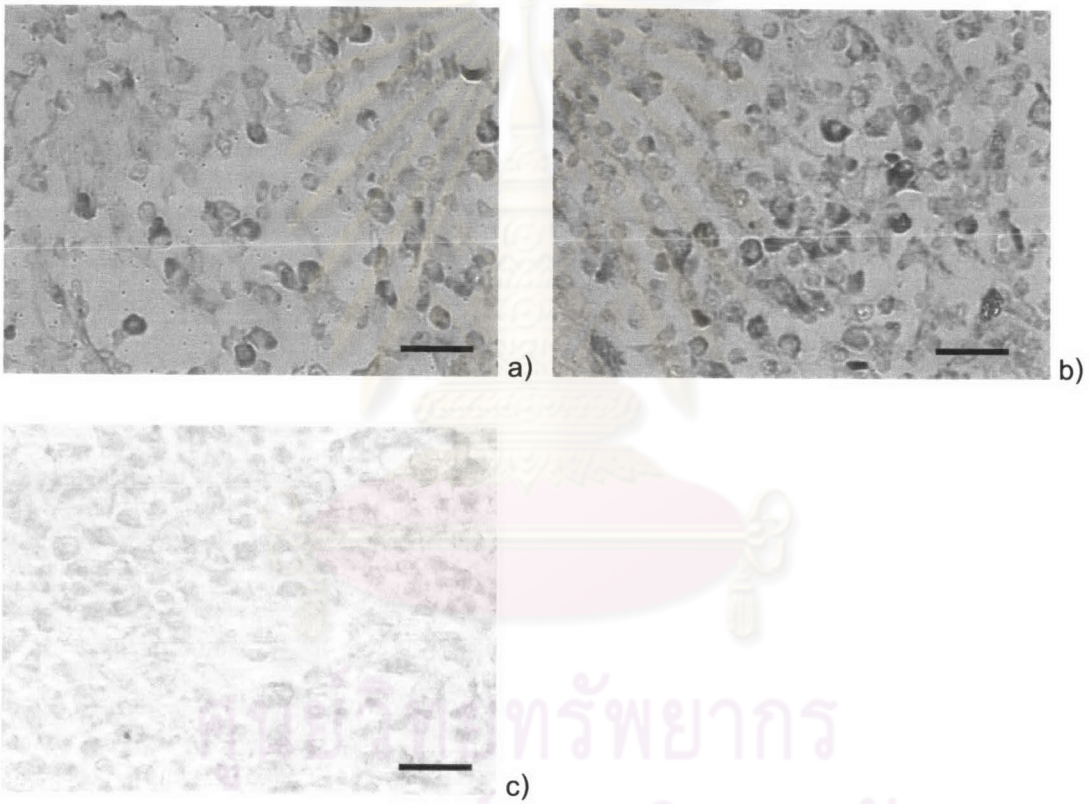

Figure 7 The immunohistochemistry (IHC) for detection of CSFV-Ag positive cells in lymphoid tissues using anti-CSFV MAb revealed positive result for lymph nodes of pigs from group C (a) Germinal center and (b) paracortical area showed positive cells stained by anti-CSFV gp55 MAb at 1: 50 (dark brown). The negative result was found in spleen of pigs in group A and B (c) (IHC-DAB, methyl green counterstain, bar = 20μm)

Table 5 The histopathologic grading of lymphoid depletion in lymphoid tissues following CSFV challenge in group C (n=6)

Group/ Pig no.	Tonsil	Thymus	Spleen	Mesenteric lymph nodes	Peyer's patch
A*	-	-	-	-	-
B*	-	-	-	-	-
C					
1	+++	+++	+++	+++	+++
2	+++	+++	+++	++	+++
3	+++	+++	+++	+++	+++
4	+++	+++	+++	++	+++
5	+++	+++	+++	++	+
6	+++	+++	+++	+++	+++

*The degree of lymphoid depletion were graded according to no lesion (-), mild (+), moderate (++), and severe (+++).

Table 6 Means ± SD of CSFV-Ag positive cells/0.1 mm^2 in lymphoid tissues collected from dead or euthanized pigs at 21 dpi. The means were compared among tissue types in group C. (n=6)

Group/ Pig no.	Tonsil	Thymus	Spleen	Lymph nodes	Peyer's patch	average
A	0	0	0	0	0	0
B	0	0	0	0	0	0
C	35.2 ± 5.0^{a}	11.3 ± 3.8^{b}	27.7 ± 7.7^{ac}	23.2 ± 4.2^{c}	17.5 ± 4.9^{bc}	22.9 ± 9.1^{c}

a, b, c ($P<0.05$), different superscripts in the different rows means significantly difference

Apoptosis and Quantitation of Apoptotic Cells in Lymphoid Tissues

The normal appearance of apoptosis in lymphoid tissues was found in all lymphoid tissue sections with a high variable in tissue types and groups. TUNEL method using in this study gave a positive result (dark brown color) in nucleus of cell that undergoing to die and in apoptotic bodies, the detritus of apoptosis that were a cluster of a lot of small matter of dead cells. Many macrophages phagocytizing the nuclear remnants or apoptotic bodies were found in the areas that apoptosis of lymphoid cells could be occurred. Apoptotic figure of lymphoid cells in lymph nodes was prominent in single nucleus while diffuse in a cluster of dead cells in Peyer's patch. The positive staining was different between vaccinated group (A and B) and group C. The apoptosis in group A and B was more prominent of apoptotic bodies than that of group C (Figure 8) The means $\pm$ SD of apoptotic cells counted in all lymphoid tissues from pigs in group A, B, and C were 14.8 $\pm$ 6.8, 23.4 $\pm$ 7.8, and 3.0 $\pm$ 1.7 cells/0.1 mm^2 respectively. The results showed that apoptosis occurred in all lymphoid tissues of group C was markedly lower than that those of groups A and B ($P<0.05$). In addition, apoptosis in thymus, spleen, and Peyer's patches of pigs in group B was significantly higher than those of group A ($P<0.05$) (Table 7 and Figure 9).

Agarose Gel Electrophoresis for the Detection of DNA Ladder Formation of Apoptosis in Lymphoid Tissues

Detection of DNA ladder formation using agarose gel electrophoresis could not demonstrated a clear ladder in all lymphoid tissues. The results showed thick smear bands of DNA which were not different when compared among groups (Figure 1B, Appendix B). According to DNA ladder could not qualitatively differentiate the degree of apoptosis, it was excluded from data analysis.

Correlation between CSFV Infected Cells and Apoptotic Cells

Correlation between CSFV infected cells and apoptotic cells detected in lymphoid tissues of pigs in group C was assessed by correlation coefficient. The number of CSFV-positive cell was set as independent value and the number of apoptotic cell was set as dependent value. Upon statistical analysis using SPSS, linear regression was proved to have significance. The correlation coefficient (β_1) was -0.458 and coefficient of determination (r^2) was 0.21 which mean that the number of CSFV positive cells showed a little negative correlation to the number of apoptotic cells (Figure 10). When comparing in the same lymphoid tissues, a large number of CSFV positive cells were found while the apoptotic cells were low (Figure 11).

Table 7 Means $\pm$ SD of apoptotic cells/0.1 mm^2 in lymphoid tissues collected from dead pigs during the observation or euthanized pigs at 21 dpi. The means were compared between groups in each tissue type were showed in the right column. The mean $\pm$ SD of apoptotic cells was compared among groups as determined by the TUNEL method.

Group/ Pig no.	Tonsil	Thymus	Spleen	Lymph nodes	Peyer's patch	average
A (n=6)	20.2±3.1[a]	13.8±1[a]	8.7±4.3[a]	8.6±1.7[a]	15.6±5.2[a]	14.8±6.8[a]
B (n=6)	22.1±6.1[a]	31.5±3.4[b]	24.1±4.3[b]	11.9±3.3[a]	27.4±3.9[b]	23.4±7.8[b]
C (n=6)	2.74±7.8[b]	4.76±3.1[c]	2.28±0.56[c]	2.56±1.13[b]	2.9±1.29[c]	3.0±1.7[c]

[a, b, c] ($P<0.05$) different superscripts in the different rows means significantly difference

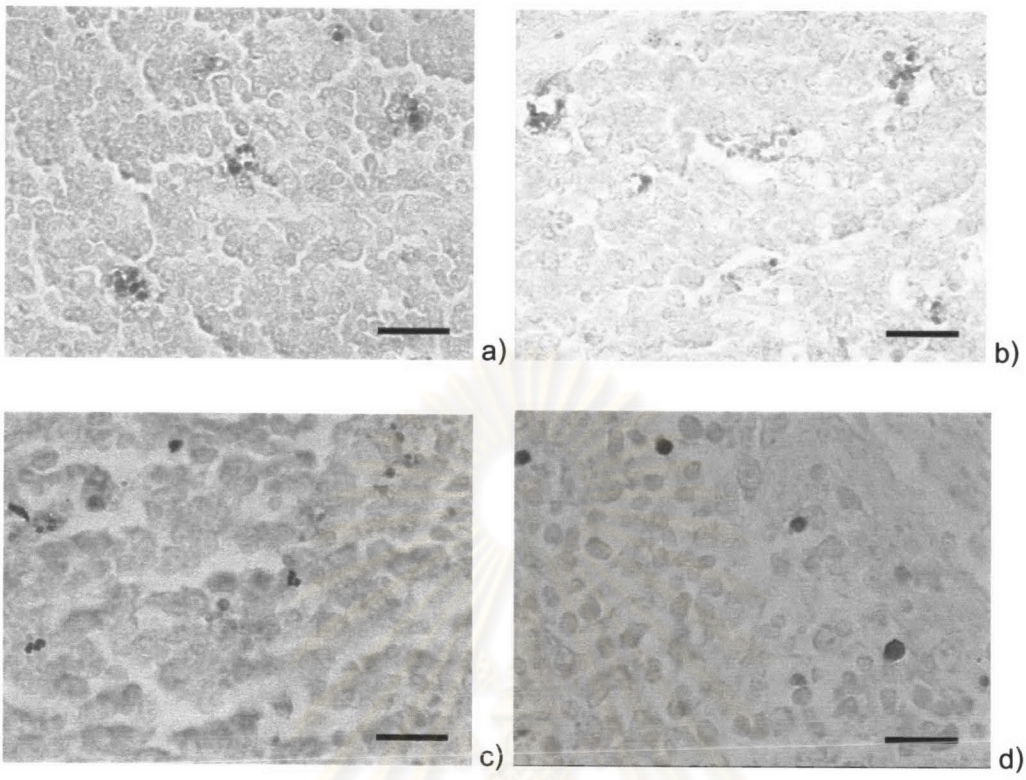

Figure 8 Microscopic findings of the apoptotic cells in the lymphoid tissues of pigs in group A and B. The positive staining was apoptotic bodies in phagocytic cells in thymus (light to dark brown) (a), Peyer's Patches (b), and spleen (c) of vaccinated pigs while the positive staining in spleen of pig in group C was presented in the nucleus of lymphoid cells (d) (TUNEL, DAB, methyl green counter stain, bar = 20μm)

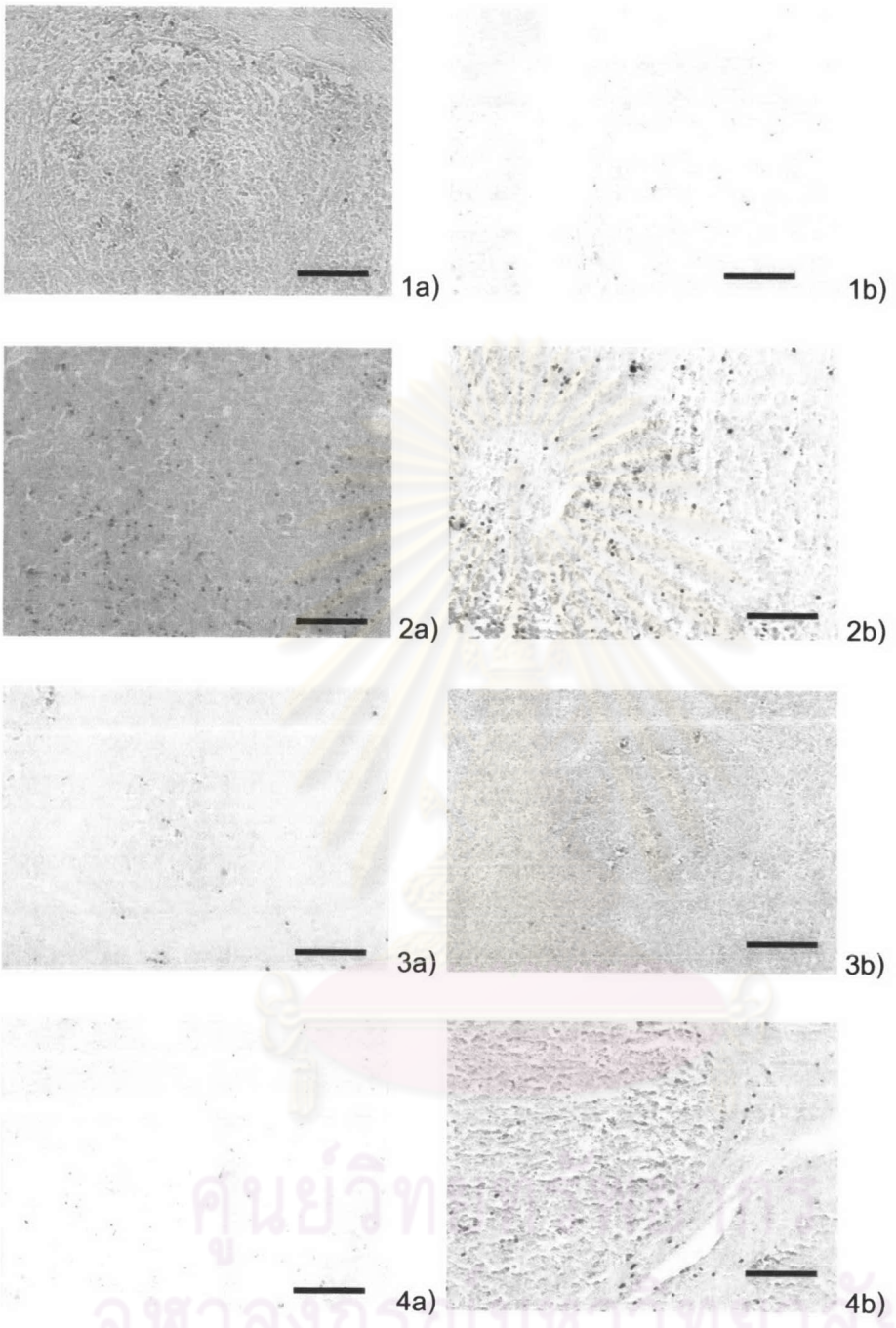

Figure 9 Microscopic findings of the apoptotic cells in the lymphoid tissues of pig in group A (column A) and B (column B). The positive cells appeared light to dark brown color in (1a, 1b) tonsil, (2a, 2b) thymus, (3a, 3b) lymph nodes, (4a, 4b) Peyer's Patches. (TUNEL, DAB, methyl green counterstain, bar = 80μm)

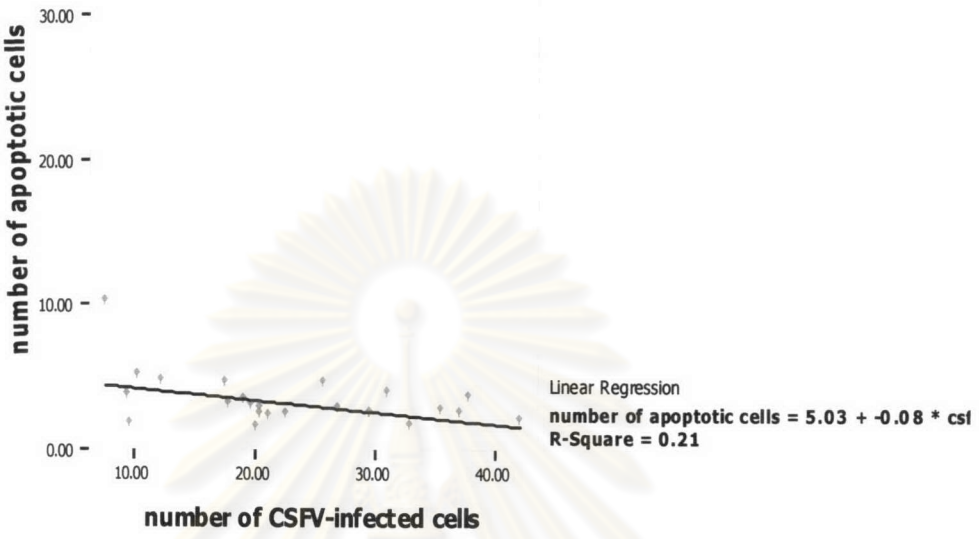

Figure 10 The associated scatter diagram indicating a linear relationship between CSFV-infected cells and apoptotic cells in lymphoid tissues.

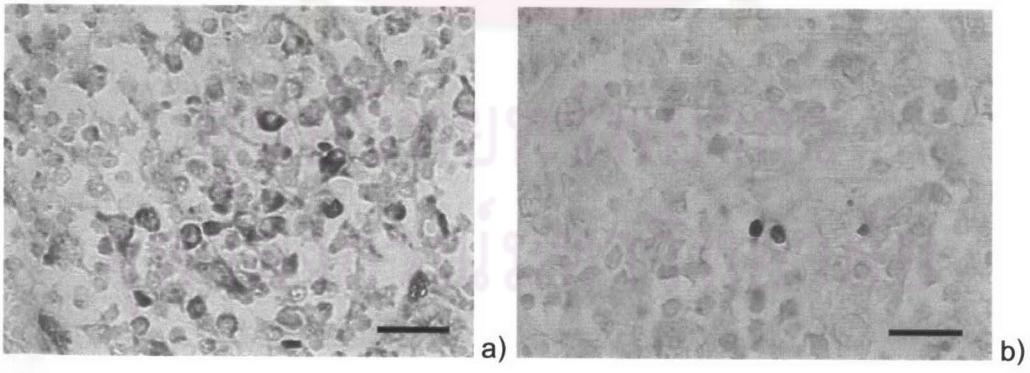

Figure 11 The comparative figures between CSFV-infected cells and apoptotic cells in group C. A large number of CSFV positive staining (dark brown) appeared in many lymphocytes and mononuclear phagocytes (a) while apoptosis appeared in a few lymphocytes (b) in lymph nodes of pigs in group C (a: IHC-DAB, methyl green stained counter ; b: TUNEL, DAB, methyl green counterstain, bar = 20μm)