Korean Journal
of Veterinary Service

Kor J Vet Serv(2002) 25(4) : 347~355

Apoptosis in experimentally infected chicks with avian infectious bronchitis

Sun-Kyong Song[1], Jong-Hoon Lee, Yeon-Cheol Choi,
Yong-Uk Shin, Il-Guc Park

Puyeo Branch, Chungnam Livestock and Veterinary Research Institute, Puyeo, 323-808, Korea
(Received 2 April 2002, accepted in revised from 1 May 2002)

Abstract

This experiment was performed to investigate apoptosis during undergoing pathogenesis of *avian infectious bronchitis virus*(IBV)-infected chicks. Sixteen days old chicks were infected with IBV, Massachusetts-41 strain(M-41, 10^4-10^5 EID_{50}) experimentally, they were autopsied to remove trachea, lung, kidney and cloacal bursa at 6 hr, 12 hr, 1 day, 3 day and 7 day post infection(PI) respectively for H-E and TUNEL staining. Grossly, mild serous, catarrhal exudate was observed in the trachea, nasal passages and sinuses nasal from 4 day PI. The cloacal bursa was swollen from 3 day PI. Histopathologically, the trachea was seen mild cellular infiltration, edema of the mucosa and submucosa, vascular congestion and mild hyperplasia of the epithelium from 6 hr PI and the changes were seen a little more severely on 7 day PI. It was observed that the cloacal bursa was getting more and more hyperplasia through the experiment. The nuclei degeneration were shown in the kidney on 7 day PI. No specific changes were seen in the lung. In TUNEL analysis, apoptotic cells showed sharp increasing at 12 hr PI and reaching a maximum on 1 day PI in the trachea, lung, kidney and cloacal bursa. And then apoptotic cells decreased gradually returning to a level of the control by 7 day PI in all the removed organs.

Key words : IBV, Apoptosis, Histopathologic, TUNEL analysis

Introduction

Avian infectious bronchitis virus(avian IBV) is classified into coronaviridea and the genus of coronavirus. Avian IBV is an acute, highly contagious respiratory disease of chicks characterized by tracheal rales, coughing, and sneezing. In addition, young chicks may have nasal discharge and in laying flocks there is usually drop in production. The first report of avian IBV was by Schalk and Hawn(1931). During the

[1]Corresponding author
Phone : +82-41-833-8611, Fax : +82-41-830-2598
E-mail : skc@hanmail.net

1940s IBV was a serious respiratory disease of laying flocks and caused marked losses in egg production. The prevalence and economic importance of the disease resulted in efforts to control it by immunization with various kinds of live and killed IB vaccines[1]. However, it is well known that such vaccines are not necessarily protected from infection with virulent IBV[2~5]. Thus, for the control of this disease, it seems necessary to make a more detailed analysis of the properties of IBV.

IBV changes its antigenicity following *in vivo*[6,7] and *in vitro*[8] passages. Otsuki et al[7] reported that IBV changes its organ tropism as well. Prior to 1956 avian IBV was considered to be caused by a single antigenic type of virus as exemplifed by the pathogenic Massachusetts-41 strain and the Beaudette, nonpathogenic, embryo lethal-42 strain. However, since that time a number of isolants have been identified from outbreaks of IBV in chicks that are different antigenically from the organ Massachusetts type such as Cunningham 1970, Cowen et al 1971, Winterfield et al 1971, Johnson et al 1972, Fields 1973, Van der Heide et al 1973, Hopkins 1974 and so on[1].

Apoptosis[12] is genetically programmed cell death[10,11] and a complex process that removes aging or injured cells from body[12] with features that distinguish it form necrosis. Apoptosis cells usually shrink and condense, display surface alterations and cleave DNA into large and often small oligonucleosomal-sized(200-bp) fragments that form a ladder pattern on agarose gels while organelles and the plasma membrane retain their integrity requiring ATP and the activation of specific proteases[13]. Necrotic death, on the other hand, comprises cell and organnelle swelling and ultimately followed by cell dissolution. As a result, necrosis is strongly proinflammatory *in vivo,* whereas apoptosis cells are rapidly phagocytosed and thus generate minimal inflammation[13]. It is conceivable that apoptotic pathways converge to one or very few common final executive steps. These comprise the tumor suppressor p53 or the regulatory role of Bcl-2-family members[14].

Apoptosis is inhibited or increased depending on kinds of diseases. The infectious diseases associated with inhibition of apoptosis are *papillomavirus* in woman uterine[15] and the infectious diseases associated with increased apoptosis are AIDS[16], *Salmonella typhimurimu*[17], *S typhi* in human keratinocytes[18], *Escherichia coli*[19], *Shigella flexneri*[20], *Helicobactor pylori* in human gastric epithelial cells[21], *Cytomegalovirus* in human retina[22], *Corinebacterim diphtherae, Bordetella pertussis* and *Listeria monocytogenes*[23].

Two coronaviurses have been shown to induce apoptosis. Infection of four different cell lines with the porcine coronavirus transmissible gastroenteritis virus induced casepase-dependent apoptosis[24,25]. Infection of cultured macrophages and other cell cell lines with the murine coronavirus mouse hepatitis virus was also shown to induce apoptosis[26,27]. But there are few studies of apoptosis with avian IBV in chicks. In this study, we investigated the effects of avian IBV-infection on apoptosis in various organ at early phase during undergoing pathogenesis in chicks.

Materials and Methods

Animals and Virus

This study used 16 days old chicks and avian IBV, Massachusetts-41 strain (M-41)

provided by National Veterinary Research and Quarantine Service. Chicks was inoculated intratracheally with IBV strain M-41(10^4-10^5 EID_{50}). These chicks were housed on wire and were kept in separate isolated rooms throughout the investigation period. All chicks were observed for clinical signs each time. And then 6 hr, 12 hr, 1 day, 3 day and 7 day after infection, infected and controls chicks were autopsied respectively and trachea, lung, kidney and cloacal bursa were removed.

Histopathological analysis

Serial organs is fixed with 10% formailin, embede in praffin, and sectioned to 5㎛ thickness. The sections were stained with H-E for the light microscopic examination.

TUNEL staining

The sections were held with the ApopTag plus Peroxidse Kit(Intergen, NY, USA) in a coplin jar. Paraffin sections were deparaffinized and pretreated with proteinase K (Sigma Chemicals, NY, USA 20㎍/㎖). After washing in phosphate buffered saline (PBS), sections were quenched endogenous peroxidase in 3.0% hydrogen peroxide and applied working strength TdT enzyme(incubation at 37℃ for 1 hr). The reaction was stopped by putting PBS. The sections were then incubated in a humidified chamber with Anti-Digoxigenin conjugate for 30 min at room temperature. After 4 times washing in PBS, the sections were applied with enough peroxidase substrate to develop color. And counterstained with hematoxylin, according to standard procedure.

Statistical analysis

TUNEL-positive cells counted at three fields of the sections made from every specimen each time were average out and expressed as number of TUNEL-positive cells per 1,000 cells. As no significant difference was among controls groups during experiment, the number of TUNEL-positive cells in controls were combined. To compare the significance of experimental and control chicks, the results were analyzed with one-way analysis of variance and presented the level of significance as $p<0.01$.

Results

Clinical signs and gross lesions

All infected chicks showed mild respiratory signs from 2 day PI. Grossly mild serous, catarrhal exudate was observed in the trachea, nasal passages and sinuses nasal from 4 day PI. The swollen cloacal bursa was seen swollen from 3 day PI and no severe lesions were observed the removed organs during the experiment.

Histopathological observation

Microscopic changes were mild cellular infiltration and edema of the mucosa and submucosa, vascular congestion, mild hyperplasia of the epithelium in the trachea from 6 hr PI. The changes were seen more severely on 7 day PI showing epithelial cells lost their cilia and increasing cellular infiltration and edema. It was observed that the cloacal bursa was getting more and more hyperplasia through the experiment. No specific changes showed in the lung and kidney(Fig 1).

TUNEL analysis

The results of TUNEL analysis were summarized in Table 1. Six hour after the

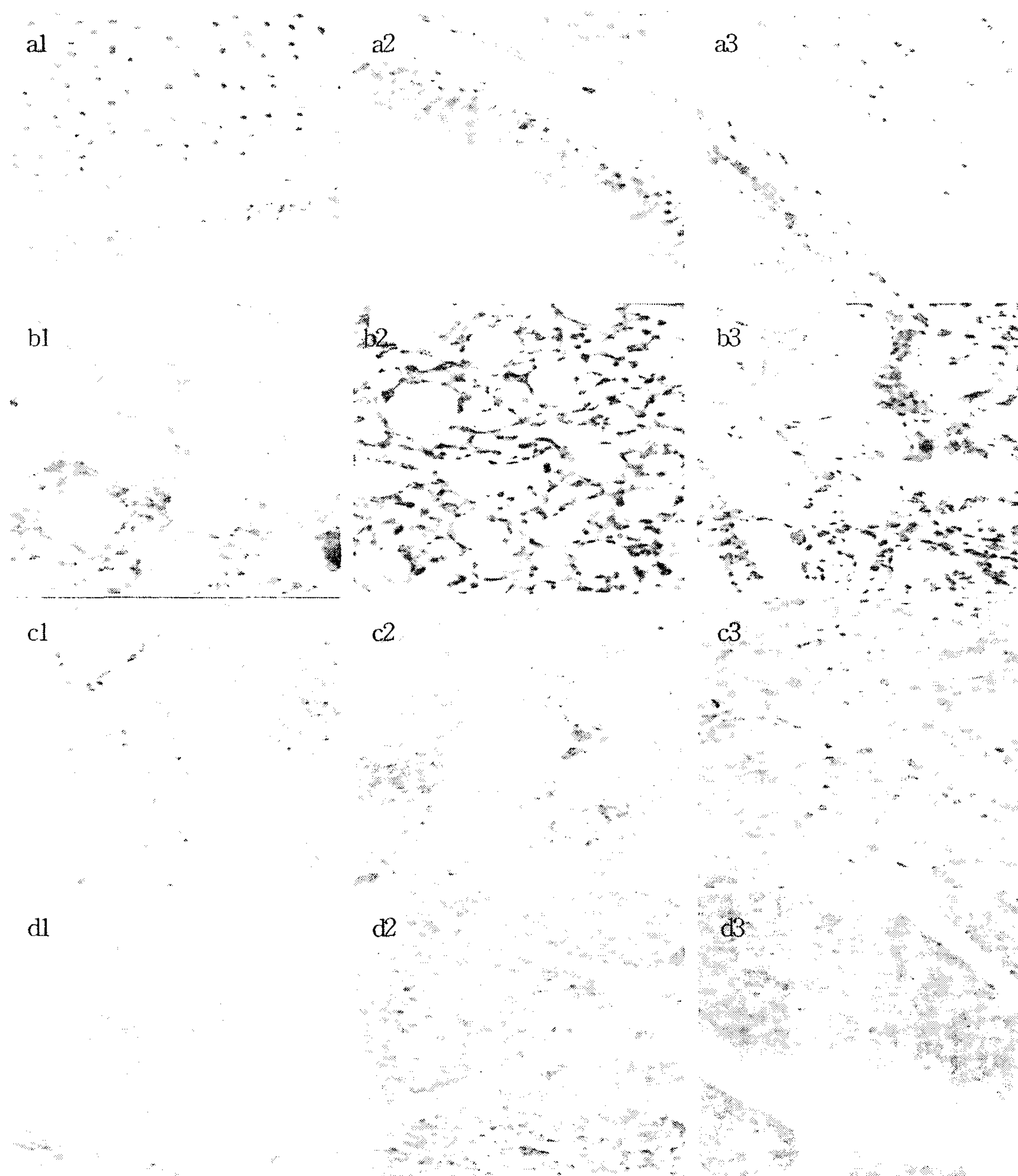

Fig 1. A: Trachea ; a1, 6 hr PI., showing no specific changes. a2, 1 day PI., showing cellular infiltration and edema of the mucosa and submucosa. a3, 7 day PI., showing epithelial cilia loosing and increasing cellular infiltration, edema and hemorrhage. H-E, ×400.
B: Lung ; b1, 6 hr PI., b2, 1day PI., b3, 7 day PI., showing no specific changes. H-E, ×400.
C: Kidney ; c1, 6 hr PI., c2, 1day PI., showing no specific changes. c3, 7 day PI., showing nuclear degeneration. H-E, × 400.
D.: Cloacal bursa ; d1, 6 hr PI., showing no specific changes. d2, 1 day PI., showing lymphoid follicles hyperplasia. d3, 7 day PI., showing more severe lymphoid follicles hyperplasia. H-E, stain. × 400.

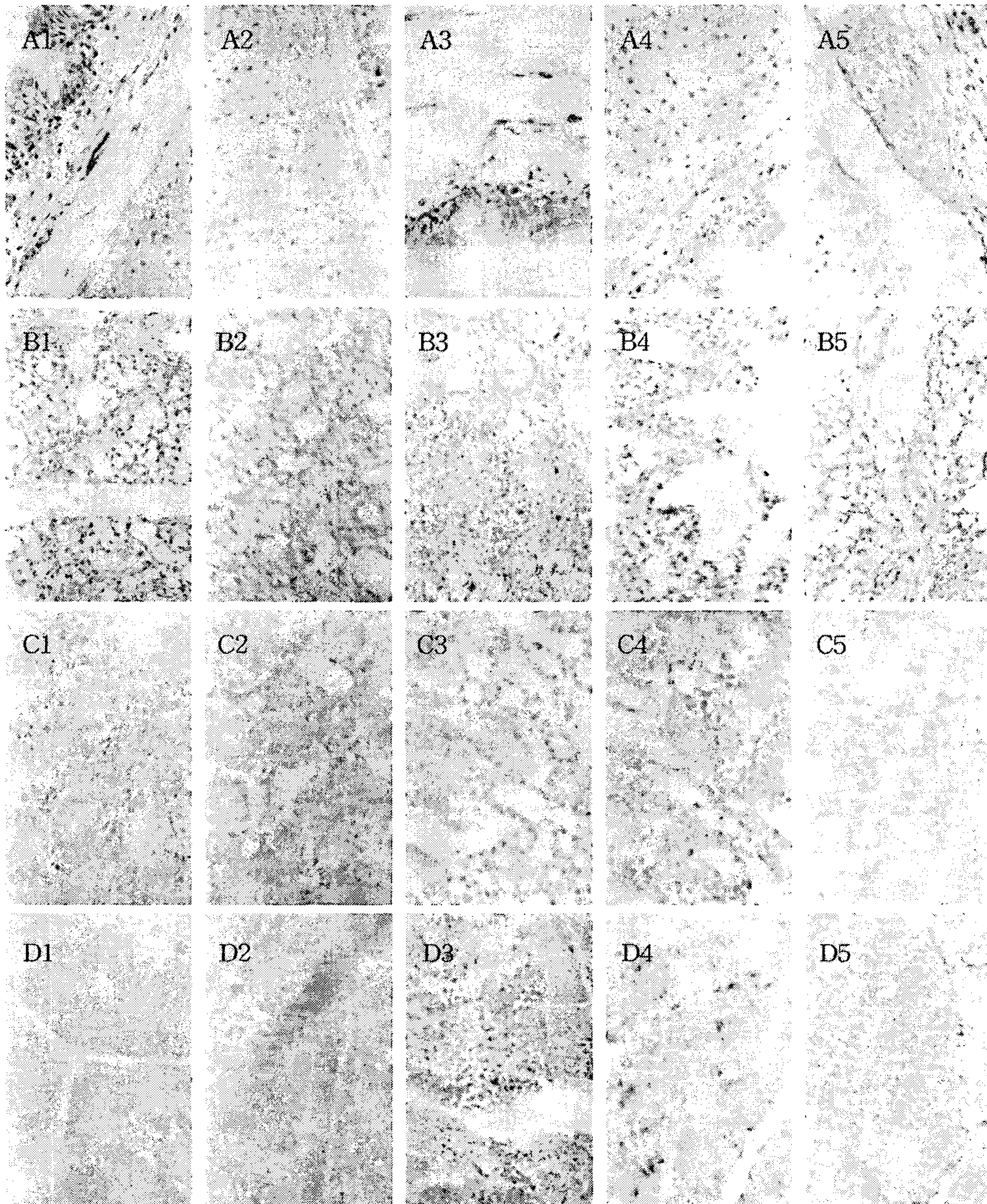

Fig 2. A : Trachea, showing increase of apoptotic cells by 1 day PI and decrease with time-course (A1: control, A2: 6 hr, A3: 12 hr, A4: 1 day, A5: 7 day). TUNEL stain, × 400

B : Lung, showing increase of apoptotic cells by 1 day PI and decrease with time-course (B1: control, B2: 6 hr, B3: 12 hr, B4: 1 day, B5: 7 day). TUNEL stain, × 400

C : Kidney, showing increase of apoptotic cells by 1 day PI and decrease with time-course (C1: control, C2: 6 hr, C3: 12 hr, C4: 1 day, C5: 7 day). TUNEL stain. × 400.

D : Fabricius, showing increase of apoptotic cells by 1 day PI and decrease with time-course (D1: control, D2: 6 hr, D3: 12 hr, D4: 1 day, D5: 7 day). TUNEL stain. × 400.

inoculation, slow increasing of apoptotic cells were detected in the trachea(2.3 ‰), lung(1.6 ‰) and cloacal bursa(5.7 ‰). Apoptotic cells showed sharp increasing at 12 hr PI and reaching a maximum on 1 day PI in the trachea, lung, kidney and cloacal bursa. And then apoptotic cells decreased gradually returning to a level of the control by 7 day PI in all the removed organs(Fig 2).

Discussion

Virus-induced cell death is a complex and important aspect of the pathogenesis of virus infection. During the past 10 years, the ability of numerous viruses to elicit or inhibit apoptosis either directly or indirectly during their replication cycles has been demonstrated[28~30]. In this report, we show that infection of 16 days old chicks with the coronavirus IBV induces apoptosis experimentally. Characteristically morphologic and biochemical feature of apoptosis, such as blebbing of the plasma membrane, chromatin condensation, fragmented nuclei, and nicked DNA, were detected during IBV infection.

In a previous report, it was demonstrated that IBV K-34 strain also caused persistent infection in young chicks at or below 6 weeks of age[31,32]. Considerable mortality was observed in the 2-week-old chicks infected with the K-34 strain[33]. Futhermore, the clinical signs, particularly diarrhea, observed in the 2-week-old chicks was more severe and of longer duration than in the 4-and-6week-old ones[33]. So we performed this investigation with 16 days old chicks, however certain clinical signs was not observed by 7 day but mild respiratory sign. In addition, gross and histopathologic lesions got a little more and more clear by 7 day PI. But the lesions were not severe through the experiment. Although infected birds were housed on wire and were kept in separate room through out the duration of experiment, the reason the possibility of reinfection was not entirely eliminated, we could not perform for longer period than 7 days.

TUNEL stain results in all removed organs was shown increasing apoptotic cells from 6 hr PI and peaking at 24 hr PI. And then apoptotic cells decreased gradually returning to a level of the control by 7 day PI. When apoptosis was observed a maximum gross and histopathologic changes were not clear. In addition, the time apoptosis decreased to a level of control gross and histopathologic lesions were a little clearer.

Table 1. The number of apoptotic cells in the organs

Time \ Organs	Trachea	Lung	Kidneys	Fabricius
Control	0.8 ± 0.28	0.2 ± 0.12	0.1 ± 0.03	5 ± 1.2
6 hour	2.3 ± 0.27	1.6 ± 0.49	0.1 ± 0.014	5.7 ± 1.32
12 hour	23.4 ± 3.47**	9.3 ± 1.10**	11.8 ± 1.81**	14.6 ± 2.13**
1 day	27.8 ± 2.92**	22.9 ± 3.96**	13.6 ± 2.90**	16.23 ± 3.44**
3 day	1.3 ± 0.65	4.88 ± 1.12**	3.56 ± 1.09**	8.78 ± 2.22
7 day	1.0 ± 0.07	0.3 ± 0.02	3.64 ± 0.43	4.3 ± 1.93

Values (‰) of each organ were represented as a Mean ± SD.
**Significant difference from control values at $p<0.01$

This results suggested that apoptosis was induced early phase of pathogenesis in IBV infected chicks comparing with gross and histopathologic changes.

The previous worker found that infection of Vero cells with the coronavirus IBV induced caspase-dependent apoptosis with treating the general caspase inhibitor z-VAD-FMK[34]. In the presence of the caspase inhibitor, however, the infected cells continued to lose volume and eventually died of necrosis, indicating that cell death induced by IBV may recruit two biochemically distinct death processes, apoptosis and necrosis[35,36]. It is apparent that apoptosis may represent a by-product of the action of virus replication. On the one hand, apoptosis may facilitate the release of virus progeny and help virus to evade the immune surveillance by attenuating inflammation[37]. On the other hand, premature apoptosis, most likely evoked by host defence mechanisms, aborts virus infection and therefore limits virus productivity and infectivity. The intricate balance between life and death of infected cells must be regulated by viral products or by interaction between virus and host to ensure a successful infection cycle.

In conclusion, our results suggest that IBV-infection induced apoptosis at early phase of pathogenesis before gross and histopathologic changes occurred. This is understandable, as one of the main advantages of apoptotic cell death for virus infectivity is to facilitate the spread of virus progeny to the neighboring cells and to minimize the inflammatory reation evoked by virus-infected cells on the host.

References

1. Barnes HJ, Calnek BW, Reid WM, et al. 1984. *Diseases of Poultry*. Iowa State University Press. Eighth edition :
2. Davelaar FG, Kouwenhoven B. 1977. Influence of maternal antibodies on vaccination of chickens of different ages against infectious bronchitis. *Avian Pathol* 6 : 41~50.
3. Holmes HC. 1973. Neutralizing antibody in nasal secretion of chickens following administration of avian infectious bronchitis *virus. Arch. Gesamte Virusforsch* 43 : 235~241.
4. Raggi LG, Lee GG. 1958. Influence of age and congenital immunity upon immunization with an avian infectious bronchitis vaccine. *J Immunol* 81 : 155~160.
5. Raggi LG, Lee GG. 1965. Lack of correlation between infectivity, serological response and challenge results in immunization with an avian infectious bronchitis vaccine. *J Immunol* 94 : 538~543.
6. Kusters JG, Niesters HGM, Bleumink-Pluym NMC, et al. 1987. Molecular biology of infectious bronchitis virus in the Netherlands. *J Gen Virol* 68 : 343~352.
7. Otsuki K, Matsuo K, Maeda N, et al. 1990. Seletion of variants of avian infectious bronchitis virus showing tropism for different organs. 379-384 In : *Coronaviruses and Their Diseaes*. Plenum Press, New York :
8. Otsuki K, Tagawa Y, Tsubokura M. 1982. Antigenic variation of avian infectious bronchitis during replication in BHK-cell. *Arch Virol* 73 : 75~78.
9. Kerr JFR, Wyllie AH, Currie AR. 1972. Apoptosis: a basic biologic phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26 : 239~257.

10. Hockenbery D. 1994. Defining apoptosis. *Am J Pathol* 146 : 16~19.
11. Williams GT, Smith CA. 1993. Molecular regulation of apoptosis: genetic controls on cell death. *Cell* 74 : 777~779.
12. Geske FJ, Gerschenson LE. 2001. The biology of apoptosis. *Hum Pathol* 32 : 1029~1038.
13. Majno G, Joris I. 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 146 : 3~15.
14. Udo KM, Ankarcronab M, Nicoterab P, et al. 1994. p53 expression oxide-induced apoptosis. *FEMS Letters* 355 : 23~26.
15. Rogovskaya SI, Sukhikh GT, Zhdanov AV, et al. 2001. Apoptosis in woman uterine cervix in pathologies associated with human papillomavirus. *Bull Exp Biol Med* 131 : 576~582.
16. Th'ng JP. 2001. Histone modications and apoptosis : cause or consequence? *Biochem Cell Biol* 79 : 303~311.
17. Jesenberger BV, Katarzyna JP, Junying Y, et al. 2000. *Salmonella*-induced Caspase-2 activation on macrophages : A novel mechanism in pathogen-mediated apoptosis. *J Exp Med* 192 : 1035~1045.
18. Nuzzo I, Sanges MR, Folgore A, et al. 2000. Apoptosis of human keratinocytes after bacterial invasion. *Immunol Med Microbiol* 27 : 235~240.
19. Klumpp DJ, Weiser AC, Sengupta S, et al. 2001. Uropathogenic *Escherichia coli* potentiates type 1 pilus induced apoptosis by suppressing NF-kappaB. *Infect Immun* 69 : 6689~6695.
20. Zychlinsky A, Prevost MC, Sansonetti PJ. 1992. *Shigella flexneri* induces apoptosis in infected macrophage. *Nature* 358 : 167~169.
21. Kim JM, Kim JS, Jung HC, et al. 2000. Virulence factors of *Helicobacter pylori* in Korea isolate do not influence proinflammatory cytokine gene express and apoptosis in human gastric epithelial cell, nor do these factors influence the clinical outcome. *J Gastrornterol* 35 : 947 ~948.
22. Chiou SH, Liu JH, Hsu WM, et al. 2001. Up-regulation of Fas ligand expression by human *cytomegalovirus* immediate-early gene product 2 : a novel mechanism in *cytomegalovirus*-induced apoptosis in human retina. *J Immunol* 167 : 4098~4103.
23. Denise M, Bärbel R, Alexander EH, et al. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci USA* 93 : 9833~9838.
24. Eleouet JE, Chilmonczyk S, Besnardeau L, et al. 1998. Transmissible gastroenteritis coronavirus induces programmed cell death in infected cells through a caspase-dependent pathway. *J Virol* 72 : 4918~4924.
25. Sirinarumitr T, Kluge JP, Paul PS. 1998. Transmissible gastroenterititis virus induced apoptosis in swine testes cell cultures. *Arch Virol* 143 : 2471~2485.
26. An S, Chen CJ, Yu X, et al. 1999 Induction of apoptosis in murine coronavirus-infected cultured cells and demonstration of E protein as an apoptosis inducer. *J Virol* 73 : 7853~7859.
27. Belyavskyi Belyavskaya ME, Levy GA, Leibowitz JL. 1998. Coronavirus MHV-3-induced apoptosis in macrophages. *Virology* 250 : 41-49.
28. O'Brien V. 1998. Viruses and apoptosis. *J Gen Virol* 79 : 1833~1845.
29. Razvi ES, Welsh RM. 1995. Apoptosis in viral infections. *Adv Virus Res* 45 : 1~60.

30. Roulston A, Marcellus RC, Branton PE. 1998. Virus and apoptosis. *Ann Rev Microbiol* 53 : 577~628.
31. Alexander DJ, Gough RE. 1997. Isolation of avian infectious bronchitis virus from experimentally infected chickens. *Res Vet Sci* 23 : 344~347.
32. Alexander DJ, Gough RE. 1978. A long term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. *Res Vet Sci* 24 : 228~233.
33. Samuel BA, Koichi O, Mitsunobu H, et al. 1994. Experimental infection with avian infectious bronchitis virus (Kagoshima-34 strain) in chicks at different ages. *J Vet Med Sci* 56 : 443~447.
34. Liu C, Xu Hy, Liu XD. 2001. Induction of caspase-dependent apoptosis in cultured cell by the avian coronavirus infectious bronchitis virus. *J Virol* 75 : 6402~6409.
35. Columbano A. 1995. Cell death : current difficulties in discriminating apoptosis from necrosis in the context of pathological processes *in vivo*. *J Cell Biol* 58 : 181~190.
36. Majno G, Joris L. 1995. Apoptosis, oncosis and necrosis : an overview of cell death. *Am J Pathol* 146 : 3~15.
37. Savill J, Fadok V, Henson P, et al. 1993. Phagocyte recognition of cells nudergoing apoptosis. *Immunol. Today* 14 : 131~136.