

Pathological and Immunohistochemical Studies of Subclinical Infection of Chicken Anemia Virus in 4-Week-Old Chickens

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ABSTRACT. Subclinical infection of chicken anemia virus (CAV) at 4 to 6 weeks of age, after maternal antibodies have waned, is implicated in several field problems in broiler flocks. In order to understand the pathogenesis of subclinical infection with CAV, an immunopathological study of CAV-inoculated 4-week-old SPF chickens was performed. Sixty 4-week-old SPF chickens were equally divided into CAV and control groups. The CAV group was inoculated intramuscularly with the MSB1-TK5803 strain of CAV. Neither mortality nor anemia was detected in the CAV and control groups. In the CAV group, no signs were observed, except that some chickens were grossly smaller compared with the control group. Sporadic thymus lobes appeared to be reddening and atrophied. Within the first two weeks p.i. of CAV, there was a mild to moderate depletion of lymphocytes in the thymus cortex and spleen in some chickens. Moreover, lymphoid depletion of the bursa of Fabricius, proventriculus and cecal tonsils was observed. Hyperplastic lymphoid foci were observed in the liver, lungs, kidneys and heart at the 4th week p.i. of CAV. Immunohistochemically, a moderate lymphoid depletion of CD4⁺ and CD8⁺ T cells in the thymus cortex and spleen was observed in some chickens within two weeks p.i. of CAV. CAV inclusions and antigens were detected infrequently in the thymus cortex and spleen. It could be concluded that the immunosuppression in subclinical infection with CAV occurs as a result of reduction of cellular immunity.

KEY WORDS: CAV, immunohistochemistry, immunosuppression, pathology, subclinical infection.

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Chicken anemia virus (CAV) causes transient aplastic anemia, generalized lymphoid atrophy, thrombocytopenia and increased mortality after infection of 1-day-old maternal antibodies-free chickens (clinical infection) [7]. Infection of older birds with CAV results in a subclinical disease and reduces resistance to other viral, bacterial, fungal and parasitic pathogens [19, 27, 30].

Clinical infection with CAV could be abolished through vaccination of breeders (maternal antibodies) in the first 3 weeks of age [18, 33] and by the age-related resistance (neutralizing antibodies) in chickens older than 3 weeks [10, 20, 32, 34]. The maternal antibodies persist until about 3 weeks of age [16, 18], and seroconversion to CAV starts from 5 to 9 weeks of age [16, 23] and continues to occur at 18 to 28 weeks in breeder flocks [16]. Despite vaccination to induce high levels of maternal antibodies, clinical disease associated with CAV infection has been observed in 2- to 4-week-old chickens from vaccinated and unvaccinated parent flocks, suggesting that the persistence of CAV in broiler breeders could lead to intermittent vertical transmission and a subsequent clinical outbreak of chicken anemia in progeny [2, 23].

Age-related resistance becomes complete by 3 weeks of age or even earlier in immunologically competent chickens [20, 34]. The degree of resistance may vary depending on the virulence of the virus, dose and route of infection [20]. The neutralizing antibodies as well as genomes of CAV detected in broilers between weeks 4 and 6 of age concurred with disappearance of maternal antibodies [23]. Also, seroconversion to CAV was detected in broilers at the time of slaughter [4, 17]. Moreover, a second peak of mortality has appeared in some vertically-derived CAV outbreaks around the age of 30 to 33 days in Sweden and Denmark [5]. The seroconversion as well as the second peak of mortality in CAV outbreaks is due to horizontally acquired infection after maternal antibodies have waned. These acquired horizontal infections are implicated in several field problems, which have negative adverse effects on broiler flock productivity. McNulty *et al.* [17] found significantly better economic performance in CAV seronegative flocks than in those that were seropositive. In addition, CAV infections in broilers were associated with increased slaughterhouse condemnation rates [3]. Also, CAV was more often detected in diseased flocks than in healthy flocks and accompanied by increased mortality and condemnation rates [8]. CAV is considered a significant risk factor for acquiring other diseases, like Marek's disease (MD), Newcastle disease (ND), coccidiosis, gangrenous dermatitis, some bacterial infections and respiratory disease [2, 8]. In order to understand these adverse effects of CAV on broilers, a study on the

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pathogenesis of CAV infection in 4-week-old SPF chickens should be conducted.

There have been few studies on the effects of CAV infection in chickens older than three weeks [14, 34], and most of them did not include the pathomorphological and immunohistochemical changes. The pathological lesions due to CAV infection in older chickens, after the beginning of age-related resistance, have been insufficiently studied. The aim of the present work was to study the pathogenicity of CAV inoculated into 4-week-old SPF chickens by investigating the hematocrit value, pathomorphology and immunohistochemistry to detect CAV antigen, CD4⁺ and CD8⁺ T-lymphocytes.

MATERIALS AND METHODS

Chickens: All used chicks were derived from a specific-pathogen-free (SPF) White Leghorn line P2 flock free from antibodies to adenovirus, avian infectious bronchitis virus, CAV, infectious bursal disease virus (IBDV), Marek's disease virus (MDV), Newcastle disease virus, reovirus and subgroup J avian leukosis virus and hatched at our laboratory at Iwate University. The chicks were housed in small isolated boxes with food and water *ad libitum* in a sterilized, isolated room.

Virus: *Chicken anemia virus.* The MSB1-TK5803 strain was passaged 10 times in MDCC-MSB1 cells after isolation from infected chickens [6].

Experimental design: Sixty 1-day-old chicks were divided into two groups (control and CAV). Twenty chickens were inoculated with CAV (MSB1-TK5803 strain) intramuscularly at 4 weeks of age. The challenge dose was $10^{7.5}$ mean tissue culture infective dose (TCID₅₀)/0.1 ml per bird for the MSB1-TK5803 strain of CAV. The control group received sham inoculation with physiological saline at the same time. All chicks were euthanized humanely by exsanguination under ether anesthesia. Chickens were sampled weekly from both groups for 4 successive weeks.

Histopathology: Samples of visceral organs, thymus, bursa of Fabricius and spleen were collected and immersed into 10% neutral buffered formalin for fixation. Femurs were split longitudinally to expose the bone marrow. After fixation, femurs were decalcified with 5% formic acid for 2 weeks. Samples were dehydrated and embedded in paraffin wax in the usual manner, sectioned (4 µm thick) and stained with hematoxylin and eosin.

Immunohistochemistry: Samples from spleen and thymus from the CAV and control groups were placed in a drop of optimal cutting temperature embedding medium and cooled in nitrogen vapor before immersion in liquid nitrogen and storage at -80°C. Samples were cut into 8 µm thick sections using a cryocut cryostat. Sections were fixed in 4°C acetone for 10 min and allowed to air dry for 90 min.

Antibodies. Antiserum to CAV was raised as previously described [9] for detection of CAV antigen. The CT4 and CT8 monoclonal antibodies (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.) were used to recognize chicken homologues of CD4 and CD8α, respectively.

Immunohistology. Acetone-fixed sections were treated with a 0.03% solution of H₂O₂ in PBS (phosphate buffer solution, pH 7.6) at room temperature for 10 min to block the endogenous peroxidase activity. Slides were washed 3 times in PBS 5 min each. Monoclonal antibodies were diluted to predetermined optimal dilutions in Dako REAL™ antibody diluent (Tris buffer, pH 7.2, containing 15 mmol/l NaN₃, protein; Code S2022, Dako, Glostrup, Denmark). The avidin-biotin peroxidase complex (ABC) method for immunoperoxidase staining was carried out to detect CAV antigen [9]. Slides were covered with antiserum to CAV diluted 1:100 in a humid chamber at 4°C overnight and then incubated with biotinylated goat anti-chicken IgG (Vector Labs, Peterborough, England) diluted 1:100 in PBS for 30 min. The slides were covered with a preformed avidin and biotinylated horseradish peroxidase macromolecular complex (VECTASTAIN ABC Standard kit, Vector Labs) for 45 min. The peroxidase activity was developed with a substrate of 0.5 mg 3, 3 diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, U.S.A.) per ml Tris-HCl buffer, pH 7.6. Slides were counterstained with hematoxylin for 10 sec, dehydrated and finally mounted with DPX mountant. Negative and positive antigen controls were included in ABC staining. Due to the scarcity of CAV antigen in the spleen and the nonspecific reaction in the ABC method, an indirect immunofluorescence assay (IFA) was performed using anti-CAV chicken hyperimmune serum and rabbit FITC-conjugated anti-chicken IgG antibodies. The labeled streptavidin-biotin peroxidase complex technique using a LSAB+ System-HRP kit (code k0679, Dako, Carpinteria, CA, U.S.A.) were used to stain CD4⁺ and CD8⁺ T cells. The staining procedure was done according to the steps mentioned in the kit's manual.

Statistical analysis: The statistical analyses were undertaken using one-way ANOVA. The findings were expressed as means ± standard deviation (SD). They were performed to compare the packed cell volumes (PCVs) of the CAV and control groups using Statistical Package for the Social Sciences for Windows (SPSS, version 15.0, Chicago, IL, U.S.A.).

RESULTS

PCV: The PCV of the control group ranged from 27 to 35%. The PCV of the CAV group ranged from 29 to 38%. No significant difference between the mean PCVs of the CAV and control groups was observed. No anemic birds were detected in the CAV or control group, considering a chicken with a PCV of 25% to be anemic [20].

Clinical signs: No clinical signs of disease were observed during the experiment, except that the chickens were grossly smaller in the CAV group compared with the control group. No mortalities occurred after CAV inoculation.

Gross changes: Sporadic thymus lobes (ranging from one to 3 lobes) distributed in one or both sides of the neck appeared to be reddening, glistening and atrophied at 1 and 2 weeks p.i. of CAV. In contrast, the control group had whitish and firm thymic lobes.

Table 1. Histopathological lesions in SPF chickens (4-week-old) intramuscularly inoculated with the MSBI-TK5803 strain of CAV

Histopathological lesion	Age of birds (Post inoculation of CAV at 4 weeks of age)			
	5 weeks	6 weeks	7 weeks	8 weeks
	Thymus			
Focal depletion in the thymus cortex	4/5	5/5	0/3	0/5
Starry-sky appearance	4/5	5/5	0/3	0/5
Apoptotic bodies	3/5	1/5	0/3	0/5
Intranuclear inclusions	1/5	3/5	1/3	0/5
Repopulation of the thymus cortex	0/5	0/5	3/3	5/5
Spleen				
Lymphoid depletion	1/5	4/5	1/3	0/5
Bursa of Fabricius				
Follicular lymphoid depletion	1/5	1/5	0/3	3/5
Cyst formation (1 or 2 cysts)	1/5	1/5	1/3	1/5
Inflammatory cell infiltration	1/5	1/5	1/3	3/5
Liver				
Swelling of hepatocytes	2/5	1/5	0/3	0/5
Vacuolar degeneration	0/5	2/5	0/3	0/5
Lymphoid foci	0/5	0/5	2/3	5/5
Proventriculus				
Lymphoid depletion	0/5	3/5	2/3	1/5
Granulocytic cell infiltration	0/5	3/5	2/3	3/5
Cecal tonsils				
Granulomatous reaction	0/5	1/5	1/3	1/5
Heart				
Lymphoid foci	0/5	0/5	0/3	2/5

Histopathological changes: The histopathological lesions of the CAV group are summarized in Table 1.

Thymus. The lesions in the thymus cortex were mainly constituted focal or diffuse mild to moderate lymphoid depletion. In the first week p.i., focal depletion of lymphocytes in the thymus cortex (Fig. 1a) with abundant macrophages and swollen lymphoblasts was seen. Lymphoblasts had markedly enlarged nuclei with pale chromatin. Small circular acidophilic inclusion bodies were observed in some macrophages and lymphoblasts (Fig. 1b and 1c). Others exhibited apoptotic changes such as pyknosis, fragmentation (karyorrhexis) and apoptotic body formation. The latter are sometimes phagocytized by macrophages (Fig. 1d). In the second week p.i., the degree of lymphoid depletion in the thymus cortex became more severe. Focal and extensive diffuse lymphoid depletions were observed. Swollen reticulo-epithelial cells and large lymphoblasts with karyomegaly were distinctly observed, giving the thymus cortex a starry-sky appearance. The inclusion bodies were frequently detected in the lymphoblasts and reticulo-epithelial cells. However, apoptotic changes were rarely detected. In the 3rd and 4th weeks p.i., the thymus cortex in all birds was repopulated with large thymocytes, except in one chicken in the 3rd week p.i. that exhibited slight focal cortical lymphoid depletion. Inclusion bodies were seen in some large cortical lymphoblasts in this chicken.

Spleen. Some periarterial lymphoid tissue (PALT) in the white pulp showed mild to moderate lymphoid depletion. Exudation of fibrinoid substances around the sheathed arteries was observed in some chickens. Lymphoid depletion of the spleen was prominent in the 2nd week p.i. of CAV. No remarkable histopathological changes were observed in the 3rd and 4th weeks p.i. in the CAV group.

Bursa of Fabricius. In the 1st and 2nd weeks p.i., the bursal lesions exhibited mild to moderate lymphoid depletion of the lymphoid follicles with mild granulocytic infiltration in subcapsular and interfollicular tissue of some chickens (Fig. 1e). A few birds had a bursa with a few large cysts. In the 3rd and 4th weeks p. i., the bursae were either completely repopulated with lymphocytes or moderately depleted with inflammation. The inflammatory lesion of the bursa was characterized by moderate depletion of the lymphoid follicles with heterophil, macrophage and plasma cell infiltration (Fig. 1f). This lesion was observed in 3 out of 8 chickens and was associated with secondary granulomas in their cecal tonsils.

Cecal tonsils. The cecal tonsils showed mild diffuse lymphocytic depletion of the follicles within the first two weeks p.i. of CAV. A few chickens had chronic granulomas in the lamina propria. The lymphoid follicles close to the granulomatous lesions were severely depleted. Granulomatous lesions were observed in 3 out of 18 chickens.

Proventriculus. Mild to moderate depletion of the lymphoid follicles in the proventricular mucosal and glandular tissues of some chickens was observed in the 2nd, 3rd and 4th weeks p.i. Granulocyte infiltration was seen in the 2nd week p.i. However, heterophils, macrophages and plasma cells were seen in the 3rd and 4th weeks p.i.

Liver. Hepatic lesions were mild and sporadic in weeks 1 and 2 p.i. The lesions included hepatic cell swelling, vacuolar degeneration and mild lymphocytic cellular reaction around blood vessels in the portal triad. However, hyperplastic lymphoid foci, especially around blood vessels, were observed in the hepatic tissues in the 3rd and 4th weeks p.i.

Heart, kidneys and lungs. Hyperplastic lymphoid foci were observed in the heart, kidneys and lungs. Most of these

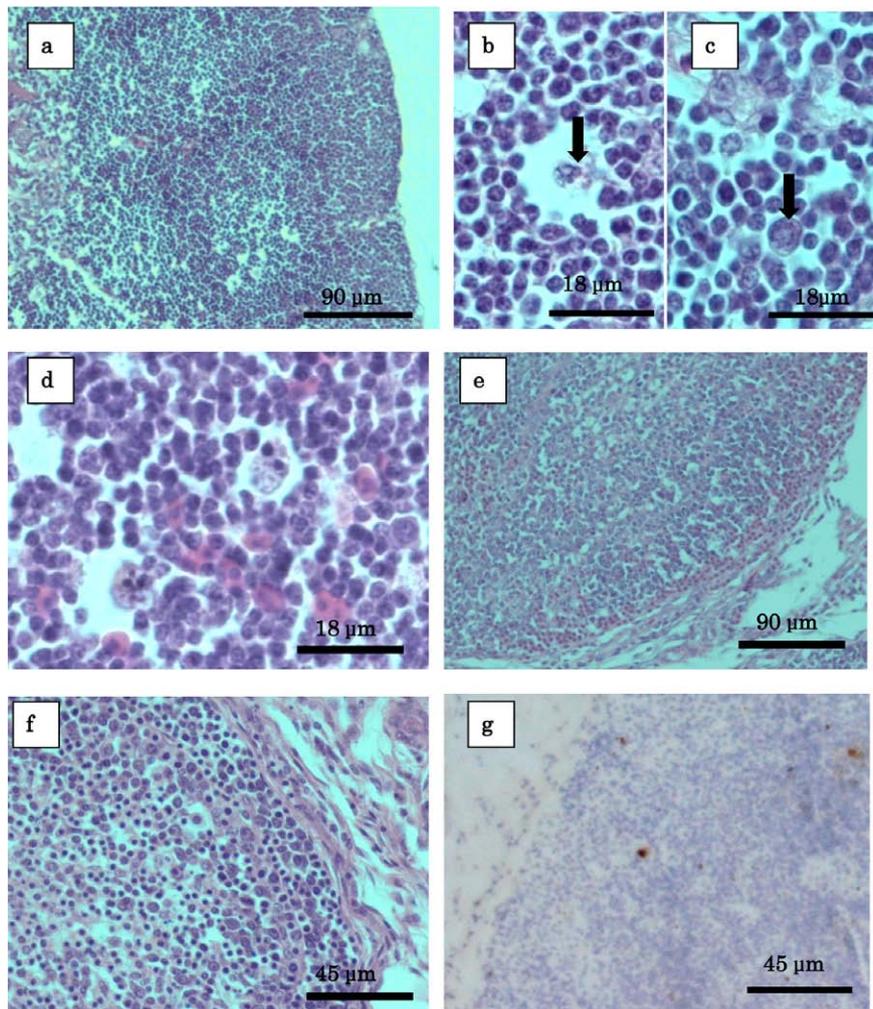


Fig. 1. Histopathological findings of CAV infection in tissues from 4-week-old SPF chickens stained with HE (a–f). (a) Thymus cortex showing focal and diffuse lymphoid depletion in a chicken at 2 weeks p.i. of CAV. (b) Thymus cortex with macrophages containing intranuclear CAV inclusions in a chicken at 2 weeks p.i. of CAV. (c) Thymus cortex with large lymphoblasts containing intranuclear CAV inclusions in a chicken at 2 weeks p.i. of CAV. (d) Thymus cortex with macrophages containing apoptotic bodies in a chicken at 1 week p.i. of CAV. (e) Bursa of Fabricius, large lymphoid follicle with eosinophilic granulocyte infiltration in subcapsular and interfollicular tissue of a chicken at 2 weeks p.i. of CAV. (f) Bursa of Fabricius showing moderate depletion of lymphoid follicle with inflammatory cell infiltration of a chicken at 3 weeks p.i. of CAV. (g) The thymus cortex revealed lymphoid tissue containing CAV antigen in a chicken at 1 week p.i. of CAV. Frozen section. ABC immunoperoxidase technique, counterstained with Mayer's hematoxylin.

foci were seen in the 4th week p.i.

Bone marrow. There were no prominent or remarkable lesions in bone marrow. Bone marrow appeared fatty, and no inclusions were seen in the hematopoietic cells.

Immunohistochemistry: Thymus. Although CAV antigens were scarce, they were detected in thymus cortex with or without prominent cortical lymphoid depletion (Fig. 1g). The CAV antigen staining was intranuclear and usually stained fine granules, but there was occasional intense staining of large intranuclear inclusions. This was detected mainly

within the first two weeks p.i. of CAV.

In the control group, CD4⁺ T cells were intensely stained in the thymus cortex; however, they were also found in the medulla (Fig. 2a). CD8⁺ T cells were stained mainly the thymus cortex, and no or faint staining was observed in the medulla (Fig. 2c). In the thymus cortex, the distributional density of CD8⁺ T cell immunostaining was equally or slightly stronger than that of CD4⁺ T cells. Just below the capsule, areas of no staining with both CT4 and CT8 antibodies were observed. In the CAV group, mild to moderate

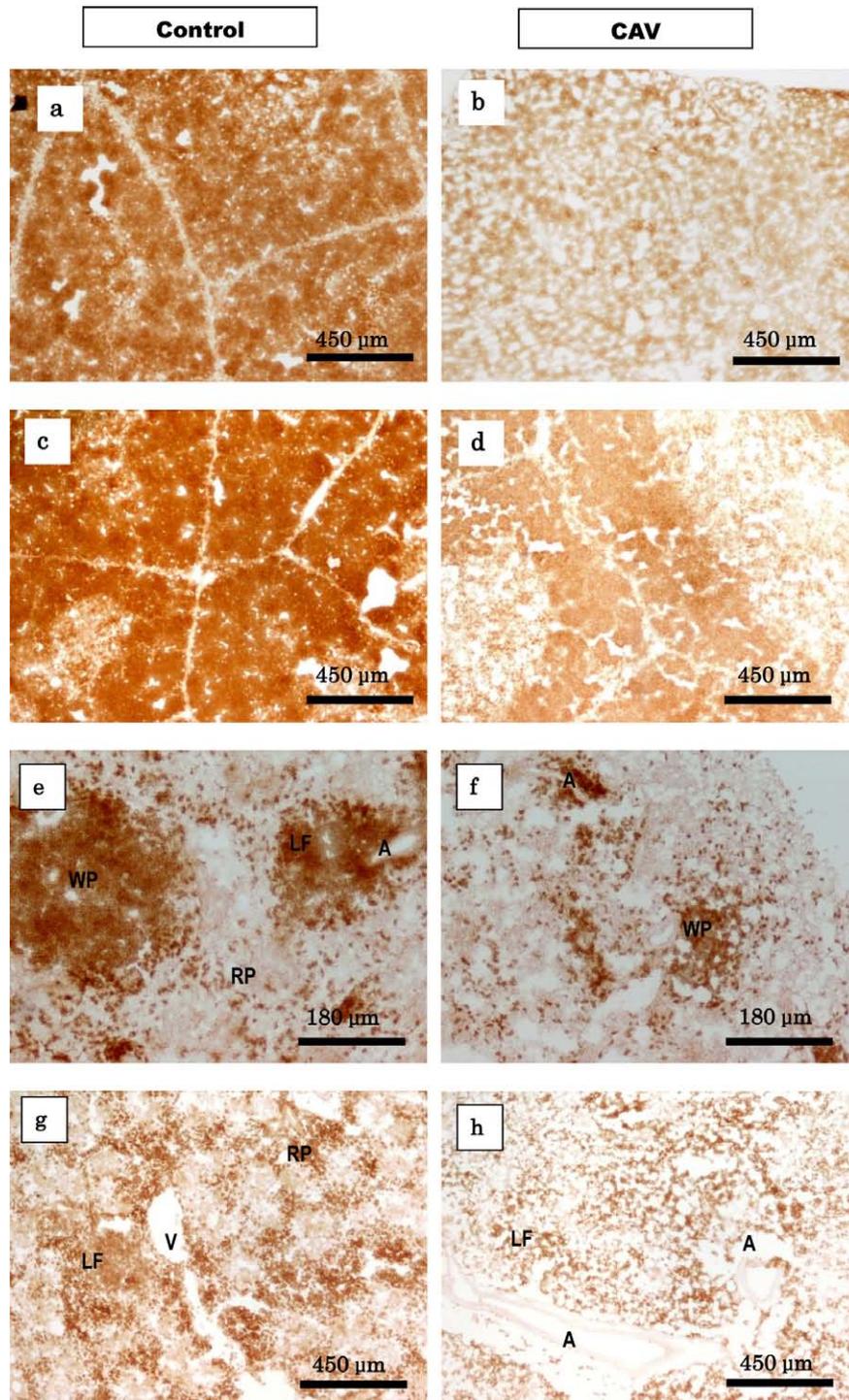


Fig. 2. Immunohistochemical staining of CD4⁺ and CD8⁺ T cells of the thymus and spleen in control and CAV groups using frozen sections and LSABC immunoperoxidase technique (a–h). (a, b) The distributional density of CD4⁺ T lymphocyte immunostaining in the thymus lobule appeared paler at one week p.i. in the CAV group (b) than in the control group (a). Note that the thymic lobulation is obscured in the CAV group. (c, d) The distributional density of CD8⁺ T lymphocyte immunostaining in the thymus lobule appeared paler at one week p.i. in the CAV group (d) than in the control group (c). Note the thinning of the cortex and widening of the medulla of the thymus in the CAV group. (e, f) Spleen showing depletion of CD4⁺ T cells from the PALT and PVLt in the CAV group at 2 weeks p.i. (f) in comparison with the control (e). (g, h) Spleen showing depletion of CD8⁺ T cells from the red pulp, PALT and PVLt at one week p.i. in the CAV group (h) in comparison with the control (g). WP: white pulp RP: red pulp A: arteriole V: venule LF: perivascular lymphoid follicles.

diffuse lymphoid depletion of CD4⁺ (Fig. 2b) and CD8⁺ T (Fig. 2d) cells was observed in the thymus cortex. Focal lymphoid depletion of CD8⁺ cells was observed in the thymus cortex of some birds. The lymphoid depletion was observed within the first two weeks p.i. of CAV. The thymus had almost intense staining with CT4 and CT8 antibodies at 3 and 4 weeks p.i. of CAV.

Spleen. In the control group, the PALT and perivascular lymphoid tissue (PVLV) were stained mainly with CT4 antibody (Fig. 2e); however, the red pulp was stained pale with CT4 antibody. In contrast, CD8⁺ T cells were observed in clusters and intensely stained in the red pulp. The distribution intensity of the CD8⁺ T cells was diffuse and thick compared with CD4⁺ T cells. The latter were localized mostly in the PALT and PVLV. The CD8⁺ T cells were intensely stained in the PALT and some perivascular lymphoid follicles (Fig. 2g). In the CAV group, moderate depletion of CD4⁺ T cells of the PALT and PVLV was prominently observed (Fig. 2f). However, mild to moderate diffuse depletion of CD8⁺ T cells from the red pulp and localized lymphoid tissue (i.e., PVLV, PVLV and perivascular follicles) was more severe (Fig. 2h). Depletion of both CD8⁺ and CD4⁺ T cells in the spleen was observed in the CAV group within the first two weeks p. i.; however, it was more severe after 2 weeks p.i. than after one week p.i. The CAV antigen was rarely detected in spleen tissue.

DISCUSSION

In vaccinated breeder flocks, the majority of progeny chicks have maternally derived antibodies to CAV. The presence of these antibodies is protective in experimental infections with CAV [18, 33]. However, uneven or insufficient immunity of the parent flock resulted in vertical transmission of CAV to progeny with delivery of uneven maternal antibodies to hatched chicks, which might be very susceptible to the vertically transmitted virus as well as to environmental infection [2, 23]. Maternal antibodies to CAV usually disappear by about 3 weeks of age. A high prevalence of CAV antibodies was detected in older birds of egg-laying and meat-type breeds. In addition, many broiler flocks have actively acquired antibodies when examined at slaughter [4, 17]. Moreover, most of the breeder flock seroconverted from 8 to 12 weeks of age [16]. The detected antibodies were a response to horizontal infection with CAV between the 4th and 6th weeks of age [23] that appeared to be subclinical. In the present work, we inoculated CAV at 4 weeks of age (beginning of age-related resistance and when the maternal antibodies had waned) to study the pathological effect of CAV on 4-week-old, maternal antibody-free, immune-intact chickens. Our experiment results revealed that chickens inoculated with CAV exhibited a subclinical disease. Some chickens had mild to moderate depletion of CD4⁺ and CD8⁺ T cells in the thymus cortex (immunosuppression); however, neither reduction of PCV nor bone marrow depletion was observed. The effect of CAV infection in older chickens on the T-cell subsets of the thymus and spleen had not been previously demonstrated.

In the present study, the pathological lesions in 4-week-old SPF chickens were milder and the course of the disease was shorter and faster than those reported in 1-day-old chickens [6, 7]. This may be due to the rapid elimination of the virus by the rapidly developing neutralizing antibodies [34]. In chickens with intact immune systems that were inoculated with CAV at 4 and 7 weeks of age, neutralizing antibodies were produced faster and to higher titers than those inoculated at 1 day of age [34]. In contrast, embryonal bursectomy with cyclophosphamide treatment at hatching abrogated the age-related resistance. The bursectomized chickens developed signs and lesions of chicken infectious anemia when inoculated with CAV at 2, 3 or 5 weeks of age [10, 32]. Moreover, in the present study, both the thymocytes and splenocytes were infected with CAV in the first two weeks p.i., indicating that age-related resistance and subclinical infection depend on the maturation of the humoral immune response and not on the disappearance of the target cells as proposed by Jeurissen *et al.* [11].

The hematocrit value of the CAV and control groups had a similar range of PCV. Besides, subclinical infection with CAV is characterized by the absence of anemia and bone marrow lesions [6, 10, 15]. The absence of CAV inclusions in hematocytoblasts in bone marrow coincided with the absence of anemia, and this strongly supports the suggestion that CAV is directly cytotoxic for bone marrow hematopoietic precursor cells in young chickens [7] rather than the suggestion that anemia is induced by inhibition of the hematopoietic functions of the bone marrow caused by destruction of thymic lymphocytes [26].

The histopathological picture revealed that CAV replicated in the thymic cortex was associated with substantial lymphocytic depletion. Adverse effect of CAV on the functions of lymphocytes and macrophages such as decreases in lymphocyte transformation responses, T-cell growth factor production and interferon production have been reported [15]. In the present study, lymphoid depletion in the thymus cortex and CAV inclusions and antigens were observed within the first two weeks p.i. of CAV; however, one chicken still showed lymphoid depletion at 3 weeks p.i. Similarly, in a 29-day-old CAV-inoculated group, no lesion was recorded, except in one chicken that had lymphoid depletion in the thymus when euthanized at 45 days of age [6]. Enlarged lymphocytes, apoptotic bodies and intranuclear CAV inclusions in macrophages of the thymus cortex accompanied by the absence of bone marrow lesions and CAV inclusions in hematocytoblasts were observed in the CAV group. Similarly, lymphocyte depletion by pyknosis and karyorrhexis was observed in the thymus at 21 days p.i. of CAV in 21- and 38-day-old SPF chickens in spite of CAV antigen not being detected in the bone marrow [10].

In the present work, the lymphoid depletion in the thymus cortex was mild to moderate. The severity of the depletion varied in previous studies [10, 14, 22, 28]. The degree of thymus atrophy in older chickens may vary with the CAV strains, dose and routes of inoculation and age of chickens [6, 21, 28]. Although comparison of eleven isolates of CAV revealed no difference in their pathogenicity and antigenic-

ity when inoculated into 1-day-old SPF chicks [31], Takagi *et al.* [25] and Spackman *et al.* [24] suggested a difference in pathogenicity and antigenicity, respectively, among CAV isolates. The pathogenicity studies of CAV strains in older SPF chickens suggested differences in virulence and age-related resistance among CAV strains. Further studies are required for investigation of the pathogenicity difference among CAV strains in old maternal antibody-free chickens.

The detection of CAV inclusions in the epithelioreticular cells and lymphoblasts with karyomegaly in the thymus cortex has been described in CAV infection [7, 13, 22]. Apoptotic bodies were infrequently observed in the present experiment after 14 days p.i. Rare apoptotic bodies were also observed after 13 days p.i. of CAV [13]. However, Smyth *et al.* [22] detect apoptotic bodies from 15 and 17 days up to 22 days p.i. The differences in the time course of detection of apoptotic bodies is likely due to the difference in route of inoculation, as oral infection usually delays delivery of a virus compared with intramuscular injection [29].

In the bursa of Fabricius, the mild to moderate lesions observed at days 7 and 14 p.i. were related to CAV infection, and similar lesions were previously observed [7, 28]. However, the severe lesions at 21 and 28 days p.i. may be due to secondary infection after immunosuppression with CAV in the first two weeks p.i. This is supported by the appearance of a granulomatous reaction in the cecal tonsils. Van Santen *et al.* [29] detected CAV genome in the cecal tonsils, but histopathological changes were not observed.

Immunohistochemically, depletion of both CD4⁺ and CD8⁺ T cells was demonstrated in the thymus cortex and the splenic tissue in 4-week-old chickens inoculated with CAV. Reports concerning immunohistochemistry of the affected cells in CAV infection in 1-day-old chicks had revealed that transient severe depletion of CD4⁺ and CD8⁺ T lymphocytes is responsible for CAV-induced immunosuppression [1, 12]. However, a few immunological studies concerning CAV infection in 3-week-old chickens revealed a decrease in lymphocyte and macrophage functions [15]. The mild to moderate depletion of CD4⁺ and CD8⁺ T lymphocytes in the thymus cortex and spleen in our present study, besides the reduction in the cellular immune functions [15, 22, 28], was a characteristic of subclinical infection with CAV. Subclinical infection had an adverse effect on the immune status of the affected chickens that likely impaired productivity [3, 8, 17].

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