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Research Article

Immuno-Histopathologic Lesions in Organs other than the Thymus and Bone Marrow During the Course of Experimentally-Induced Chicken Infectious Anaemia (CIA) Disease*

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Abstract: Chicken Infectious Anemia (CIA) is present in various regions of the world in which poultry husbandry is common and birds of any age are susceptible. CIA is a viral infection characterized by aplastic anemia and is accompanied by atrophy of the thymus and bone marrow. This condition was first diagnosed in 1979 and since then a large number of investigations have been undertaken in order to characterize and further understand the pathology of this infection. Most of these studies emphasized either clinical findings or physiological transformation of the thymus and bone marrow. There are a limited number of studies that investigated the lesions in organs other than the bone marrow and thymus.

The objective of the present study was to examine the histopathologic lesions that develop in organs other than the thymus and bone marrow of 1-day-old chickens intramuscularly infected with the chicken anemia virus (CAV) and to detect the antigen density with the streptavidin-biotin immuno-peroxidase staining technique. Seventy-one 1-day-old SPF quality white Leghorn chickens, acquired from the Manisa Poultry Diseases Research Institute, were used in this study. On day 1 of the study, 52 of these chickens were injected intramuscularly with 0.5 ml $TCID_{50}$:100,000 CAVs (Cux-1 strain). The remaining 19 chickens were kept in a separate facility as a control group. Necropsies were performed immediately after the chickens were sacrificed. Sampling occurred on the days defined by the experimental study protocol. Organ samples were taken from each animal's bursa of Fabricius, cecal tonsils, spleen, liver, kidneys, pancreas, large and small intestines, proventriculus, lungs, heart, trachea, esophagus, brain, and cerebellum. These samples were fixed in 10% buffered formalin solution. Despite the antigenic-specific stains observed in different organ samples, such as the bursa of Fabricius, spleen, lung, intestine, cecal tonsils, and glandular stomach after the seventh day of inoculation, no significant microscopic lesions were found in any of these organs. The findings of the present study show that CAV did not cause any typical microscopic lesions in the organs we studied (organs other than the thymus and bone marrow), unlike previous reports of CAV settling in the thymus and bone marrow causing lesions to transfers to other organs; however, they did become infected with the virus, especially lymphocytes.

Key Words: Chicken infectious anemia, histopathology, immuno-histopathology, lesions, bursa of Fabricius, spleen

Tavuklarda Deneysel Oluşturulan Enfeksiyöz Tavuk Anemisi Hastalığında Timus ve Kemik İliği Dışındaki Organlarda İmmunohistopatolojik Lezyonlar

Özet: Tavuk Enfeksiyöz Anemi, tavukçuluk yapılan değişik bölgelerde var olan, her yaşta tavuğun duyarlı olduğu, aplastik anemi, kemik iliği ve timusta atrofi ile karakterize viral bir enfeksiyondur. İlk olarak teşhis edildiği 1979 yılından beri, enfeksiyona ilgili olarak çok sayıda çalışmalar yapılmıştır. Bu çalışmaların çoğunda klinik bulgular veya timus ve kemik iliğinde meydana gelen değişimler üzerinde durulmuştur. Hastalıkta kemik iliği ve timus dışındaki organlarda gelişen lezyonlara ilgili sınırlı çalışma vardır. Bu çalışmada, deneysel olarak Enfeksiyöz Tavuk Anemisi Virüsü'nün Cux-l türü ile enfekte edilen 1 günlük yaştaki SPF civcivlerde, timus ve kemik iliği dışındaki organlarda gelişen histopatolojik lezyonların ve StreptAvidin-biyotin peroksidaz yöntemi ile boyanıp, antijen yoğunluklarının tespiti amaçlandı. Bu amaçla 71 SPF beyaz Leghorn civciv kullanıldı. Bu civcivlerin 52'sine 1 günlük yaşta intramuskuler yolla CAV'nın $TCID_{50}$ = 100.000 / ml olan Cux-l suşundan 0,5 ml inokule edilerek deney grubu olarak ayrıldı, 19 adedi herhangi bir uygulamaya tabi tutulmaksızın kontrol grubunu oluşturdu. Yetmişbir civciv deney planına göre belirtilen günlerde sakrifiye edildi ve nekropsileri yapıldı. Nekropsilerde bursa Fabricius, dalak, sekal tonsiller, karaciger, böbrek, pankreas, duodenum, ince ve kalın bağısak, proventrikulus, akciğer kalp, trakhea, özefagus, beyin ve beyincikten doku örnekleri alınıp bunlar %10'luk tamponlu formol saline solüsyonunda tespit edildi ve hazırlanan preperatlar ışık mikroskobunda incelendi. Bu organlarda herhangi bir histopatolojik lezyon saptanmamasına ragmen, virus inokulasyonundan sonraki 7. günden itibaren bursa Fabricius, dalak, sekal tonsiller, akciğer ve proventrikülus gibi organlarda antijen spesifik boyanmalar saptandı.

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Saptanan bulgulara bağlı olarak, çoğu çalışmalarda timus ve kemik iliğine yerleştiği ve orada lezyonlar oluşturduğu bildirilen tavuk anemi virusunun, enfeksiyonlar sırasında bu iki organ dışındaki organlara gittiği ve oralarda tipik mikroskobik lezyonlar oluşturmasa da özelikle lenfositleri enfekte ettikleri ortaya konuldu.

Anahtar Sözcükler: Tavuk enfeksiyöz anemi, histopatoloji, immunohistopatoloji, lezyon, bursa Fabricius, dalak

Introduction

Chicken Infectious Anemia (CIA) is present in various regions of the world in which poultry husbandry is common and birds of any age are susceptible. CIA is a viral infection characterized by aplastic anemia and is accompanied by atrophy of the thymus and bone marrow (1-6). This condition was first diagnosed in 1979 and since then a large number of investigations have been undertaken in order to characterize and further understand the pathology of this infection (3,7-11). Most of these studies emphasized either clinical findings or physiological transformation of the thymus and bone marrow (2,5,7,10-12). There are a limited number of studies that investigated lesions in organs other than the bone marrow and thymus (5,10,12-14). Necropsies of those CIA infections revealed atrophy in the bursa of Fabricius, hemorrhages in mucosa of the proventriculus and muscles, and mottled and enlarged livers. In microscopic investigations, lymphocytic depletions in the lymphoid center of organs, especially in the bursa of Fabricius and spleen, swelling in hepatocytes, and dilatation in liver sinusoids were noted. Nonetheless, because those gross and histopathologic findings were reported in field cases it is suggested that those findings could have been due to secondary infections (6,12,14,15). It was also reported that during immunohistochemical dying, different densities of antigenic staining were detected in those organs (12,14,15).

In the present experimental study, which was performed based on those conditions, the objective was to examine histopathologic lesions that develop in organs other than the thymus and bone marrow of 1-day-old chickens intramuscularly infected with the chicken anemia virus (CAV), and to detect the antigen density with the streptavidin-biotin immuno-peroxidase staining technique. In addition to identification of the target organs other than the thymus and bone marrow, the precise day of transmission of the viral agent to those organs and its density were confirmed.

Materials and Methods

Seventy-one 1-day-old SPF quality white Leghorn chickens, acquired from the Manisa Poultry Diseases Research Institute, were used in this study. On day 1 of the study 52 of these chickens were injected intramuscularly with 0.5 ml $TCID_{50}$:100.000 CAVs (Cux-1 strain). The remaining 19 chickens were kept in a separate facility as a control group. Throughout the study all the animals were given commercially prepared layers starter-diets, as well as boiled and cooled water ad libitum.

Necropsies were performed immediately after the chickens were sacrificed. Sampling occurred on the days defined by the experimental study protocol given in Table 1. Organ samples were taken from each animal's bursa of Fabricius, cecal tonsils, spleen, liver, kidneys, pancreas, large and small intestines, proventriculus, lungs, heart, trachea, esophagus, brain, and cerebellum. These samples were put in a 10% buffered formalin solution for a maximum of 18-24 h. Routine preparation processes were applied and samples were embedded in paraffin blocks. At least 2 different sections measuring 3 and 5 m were then taken from each of these blocks (16). The 5-m sections were stained with Harris's hematoxylin and eosin stain for histopathological examination, and the 3-m sections were mounted on poly-L-lysine slides and then stained using the labeled streptavidin-biotin peroxidase technique (LSAB). All sections were then examined with a compound light microscope.

The primary antibody (VP3-specific anti-CAV monoclonal antibody) necessary for streptavidin-biotin staining was obtained in cell supernatant form from the Institute for Animal Science and Health (ID- DLO) in Holland, and this antibody was used in a 1:100 dilution for immunohistochemical studies. Additionally, commercial test kits (Dako, LSAB 2 Kit, peroxidase-universal code no. K675), which included goat anti-mouse and goat anti-rabbit immunoglobulins conjugated with biotin, were employed.

Post-mortem blood samples were taken from all animals in order to conduct ELISA tests. The sera were

separated, put into Eppendorf tubes, and kept at –20 °C until testing could be performed.

Results

No differences in macroscopic appearance were observed between the organs sampled from the experimental and control groups. Statistically insignificant ($P > 0.05$) differences in the mean weights of the bursa of Fabricius and spleen were observed between the experiment and control groups (Tables 2 and 3). The liver samples and spleen samples from the experimental group were slightly faded between the 14th and 25th days, and between the 10th and 20th days, respectively, compared to the control group. The first positive results of the ELISA tests were detected in 2 birds from the experimental group that were sacrificed on day 3 and in 1 bird from the experimental group that was sacrificed on day 7; however, the most reliable results for both groups were obtained on day 14 and thereafter.

Histopathological examination of each bursa of Fabricius of the experimental group birds revealed a definitive decrease in cell number in the region close to the corticomedular border in a few follicles of 1 animal on day 25 and in another animal on day 20. A decrease in peri-arterial lymphoid cells was observed in spleen sections taken from experimental group birds on days 13-17; these areas were filled with reticulum cells and some secondary lymphoid follicle formations. Finally, a decrease in lymphoid cells in the lymphoid follicles was detected in some of the glandular stomach and intestine sections sampled between day 10 and day 30.

Table 1. Sacrifice and sampling dates, and number of animals before and after virus inoculation.

DAY	Sacrificed/Sampled	
	Experimental	Control
1 *	-	5
3	2	1
4	2	1
5	4	1
7	4	1
8	4	1
10	4	1
12	4	1
13	4	1
14	4	1
17	4	1
20	4	1
25	4	1
28	4	1
30	4	1
Number of birds in the experimental and control groups	52	19
Total number of birds	71	

*Only on day 1 sacrifice procedures were performed before virus inoculation.

In the bursa of Fabricius sections stained with LSAB, both intranuclear and intracytoplasmic antigenic-specific staining were observed only in the experimental group between day 7 and day 30, especially in the lymphoid cells in the interfollicular area; however, this type of stained cell

Table 2. bursa of Fabricius mean weight according to day.

Days	Groups			
	Experimental		Control	
	n	mean ± SD	n	mean ± SD
1-7	12	$0.094 + 0.0173^{cA}$	9	$0.170 + 0.0380^{cA}$
8-10	8	$0.129 + 0.0332^{cA}$	2	$0.099 + 0.0264^{cA}$
12-14	12	$0.171 + 0.0288^{bA}$	3	$0.143 + 0.0428^{bA}$
17-20	8	$0.217 + 0.0952^{bA}$	2	$0.181 + 0.0544^{bA}$
25-28	8	$0.234 + 0.0434^{bA}$	2	$0.267 + 0.0352^{bA}$
30	4	$0.406 + 0.0268^{aA}$	1	$0.416 + 0.0212^{aA}$

a,b,c: Differences between the groups with different letters are statistically significant ($P < 0.05$).
A: Differences between the experimental and control groups are not statistically significant ($P > 0,05$).

Table 3. Spleen mean weight according to day.

Days	Groups			
	Experimental		Control	
	n	mean ± SD	n	mean ± SD
1-7	12	0.027 + 0.0101^{bA}	9	0.022 + 0.0071^{bA}
8-10	8	0.029 + 0.0106^{bA}	2	0.032 + 0.0050^{bA}
12-14	12	0.034 + 0.0116^{bA}	3	0.035 + 0.0098^{bA}
17-20	8	0.059 + 0.0163^{aA}	2	0.066 + 0.0212^{aA}
25-28	8	0.078 + 0.0184^{aA}	2	0.081 + 0.0196^{aA}
30	4	0.108 + 0.0216^{aA}	1	0.122 + 0.0208^{aA}

a,b,c: Differences between the groups with different letters are statistically significant ($P < 0.05$).
A: Differences between the experimental and control groups are not statistically significant ($P > 0.05$).

was not observed to occur in numbers greater than 3 or 4 (Figure 1).

Intestinal sections were taken from the experimental group on day 10 and thereafter. Both intracytoplasmic- and intranuclear-specific staining in the lymphocytes of the lymphoid centers on the propria mucous layer and cecal tonsils, and on the villus of the intestine sections were observed; however, the densities of the staining were different. Between day 7 and day 20 of the study, specific antigenic staining (similarly located) was also detected in lung sections of the experimental group animals, especially in the lymphocytes that filled the air capillaries (Figure 2). Specific staining was also detected in the lymphocytes within the glandular stomach sections of the experimental group animals on different days, beginning on day 10 of the experiment and until the end of the study (Figure 3). This specific staining was observed to develop more often in the esophagus-glandular stomach transit area and in the lymphocytes on the propria mucous layer of the glandular stomach.

Antigenic-specific staining was abundant in the spleen sections, especially between day 5 and day 20 of the

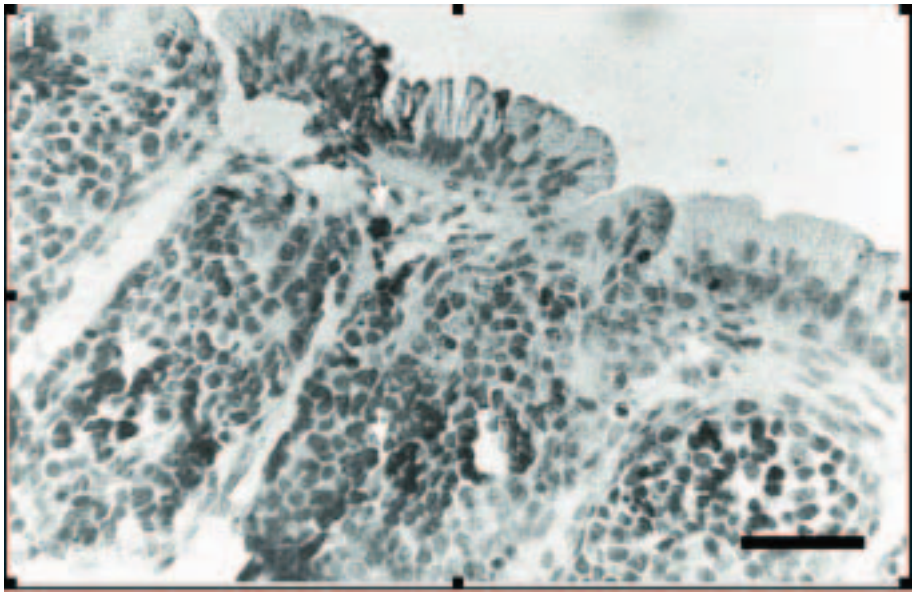

Figure 1. Intranuclear or intracytoplasmic antigen-specific staining in the bursa of Fabricius sections of an experimental group animal stained with LSAB (400×).

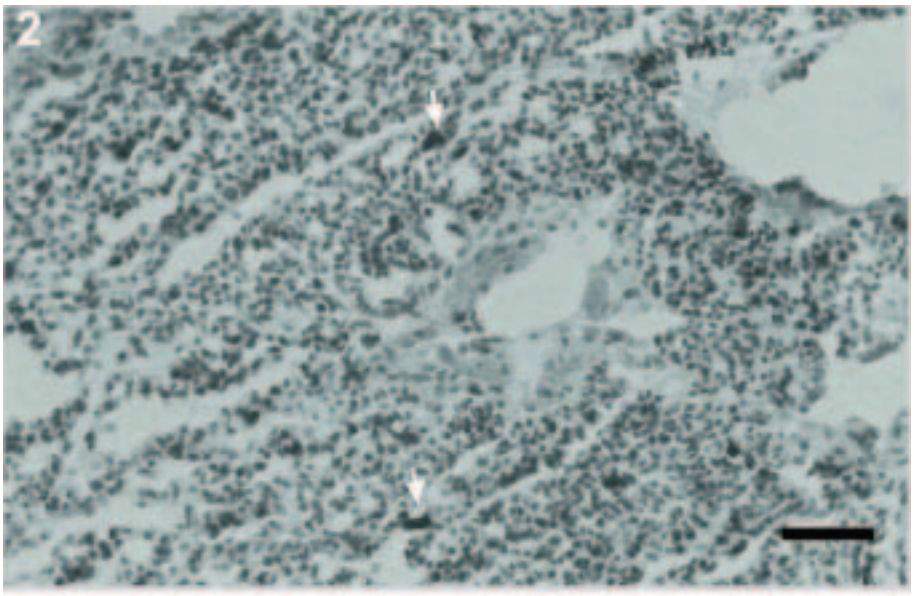

Figure 2. Antigen-specific staining in lymphoid cells filling the area between air capillaries in lung section of an experimental group animal stained with LSAB (400×).

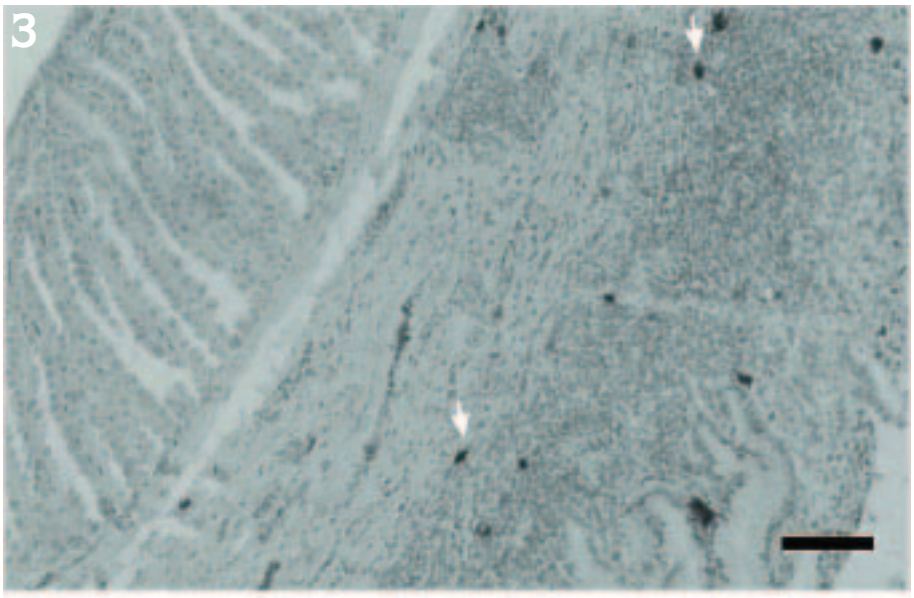

Figure 3. Antigen-specific staining in lymphoid cells at the propria mucosa of proventriculus stained with LSAB (200×).

experiment. This specific staining, either intranuclearly or intracytoplasmically located, was especially abundant in lymphocytes within the peri-arterial regions of the white pulp at the beginning of the experiment; however, after day 13 and day 14, this specific staining was observed in the cells located towards the periphery of the white pulp in larger quantities (Figure 4).

Discussion

During some outbreaks of CIA severe anemia in infected birds has occurred, in addition to hemorrhages in the proventriculus mucosa, sub-dermal layer, and muscular tissue, as well as atrophy of the bursa of Fabricius (2,4,5,17). In the present study no important differences

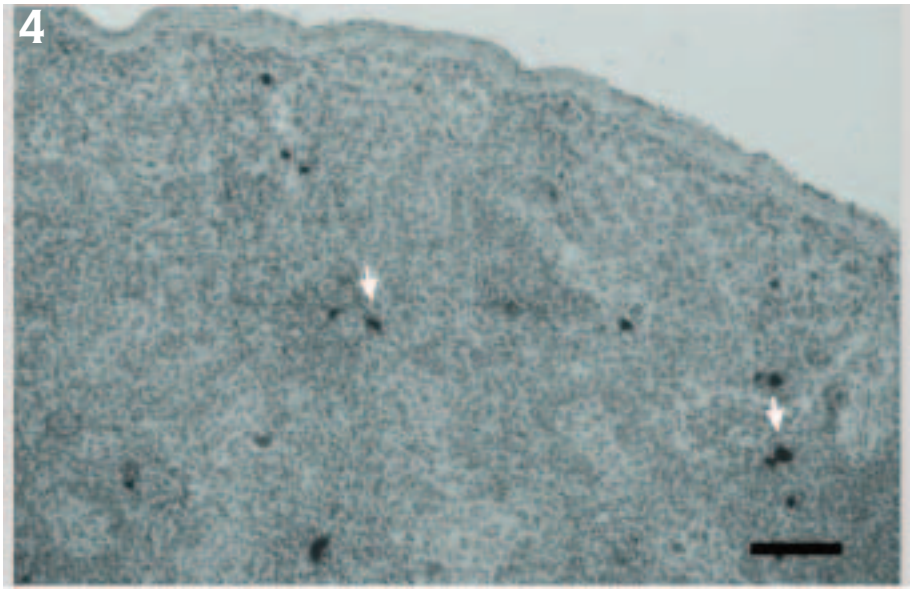

Figure 4. General view of antigen-specific staining in white pulp cells in the spleen stained with LSAB (200×).

between the experimental and control groups were visually detected in the bursa of Fabricius. There was some variation in weight between the birds in the experimental and control groups, but the difference was not statistically significant (Table 2). The hemorrhages previously reported in different organs were not observed during the present study. The enlargement and color change in the liver reported in previous studies (2,3,6) were also observed in the livers and spleens of the birds in the present study; this was especially true of the samples taken between day 14 and day 25.

In the present study the first positive results of ELISA obtained from birds in the experimental group that were sacrificed on day 3 and day 7 were considered to be non-specific false-positives. This conclusion was based on consistent negative findings, both in routine histopathological and immuno-peroxidase staining (Table 4). The most reliable results from both groups were obtained on day 14 and thereafter.

Drouin et al. (18) reported lymphoid depletion in follicles, pyknosis in lymphocytes, and intraepithelial cysts in the bursa of Fabricius during naturally occurring CIA infections. On the other hand, Weikel et al. (19) reported that during natural CIA infections the medullas of some follicles filled with macrophages, and some of the follicles had completely vanished and had been replaced with connective tissue. They also reported deep invaginations that developed on the epithelial layer. In contrast, Goryo et al. (2) reported that during experimentally induced CIA infection intranuclear inclusion bodies inside a few lymphocytes in some bursa of Fabricius sections were observed, especially after day 12 of the infection. Furthermore, some investigators reported that they did not observe cysts or necrosis in the bursa of Fabricius of the samples examined during the course of a natural infection (20). It is clear that during natural and experimentally induced infections, reports of lesions in the bursa of Fabricius are variable. Our observation that no change was detected in the bursa of Fabricius, other than a weak lymphoid depletion in 2 animals (1 on day 20 and the other on day 25) is similar to other experimental investigations (18,19).

Hoop and Reece (15) reported that during experimentally induced infections they did not observe any antigenic-specific stains in the bursa of Fabricius using immunochemical staining. On the other hand, Smyth et al. (14) observed antigenic-specific staining of variable densities in the bursa of Fabricius between day 7 and day 12 in animals experimentally infected with CAV. In the present study antigenic-specific stained cells were detected between day 7 and day 30. No significant lesions were detected in the bursa of Fabricius in our study, nor in many other studies of experimentally induced CIA infection; however, very clear lesions were reported in instances of

Table 4. ELISA test results, scoring of histopathological lesions, and immuno-peroxidase results in some organs.

			Histopathologic Lesions				Immuno-peroxidase Staining Results			
Day	Bird No.	ELISA Test Results	bursa of Fabricius	Lungs	Spleen	Proventriculus and intestine	bursa of Fabricius	Lungs	Spleen	Proventriculus and intestine
1	K_1	N	-	-	-	-	-	-	-	-
	K_2	N	-	-	-	-	-	-	-	-
	K_3	N	-	-	-	-	-	-	-	-
	K_4	N	-	-	-	-	-	-	-	-
	K_5	N	-	-	-	-	-	-	-	-
	D_1	P	-	-	-	-	-	-	-	-
3	D_2	P	-	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	-	-	-	-
4	D_2	N	-	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	-	-	-	-
	D_2	N	-	-	-	-	-	-	+	-
5	D_3	N	-	-	-	-	-	-	+	-
	D_4	N	-	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	+	+	+	-
	D_2	N	-	-	-	-	+	-	+	-
7	D_3	N	-	-	-	-	-	-	-	-
	D_4	P	-	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	-	+	+	-
	D_2	N	-	-	-	-	+	-	+	-
8	D_3	N	-	-	-	-	-	-	-	-
	D_4	N	-	-	-	-	+	-	+	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	-	-	-	-
	D_2	N	-	-	-	+	-	+	++	+
10	D_3	N	-	-	-	+	-	-	+	-
	D_4	N	-	-	-	-	-	+	++	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	-	-	+	-	+	-
	D_2	N	-	-	-	-	-	-	-	-
12	D_3	N	-	-	-	-	-	+	+	-
	D_4	N	-	-	-	+	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	N	-	-	+	-	+	+	++	+
	D_2	N	-	-	++	-	+	+	+++	+
13	D_3	N	-	-	++	+	+	+	+++	+
	D_4	N	-	-	++	-	-	-	++	+
	K_1	N	-	-	-	-	-	--	-	-

Table 4. (continues)

Day	Bird No.	ELISA Test Results	Histopathologic Lesions				Immuno-peroxidase Staining Results			
			bursa of Fabricius	Lungs	Spleen	Proventriculus and intestine	bursa of Fabricius	Lungs	Spleen	Proventriculus and intestine
	D_1	N	-	-	+	+	+	+	++	+
	D_2	P	-	-	++	-	-	-	+	-
14	D_3	P	-	-	+	+	+	-	+	+
	D_4	P	-	-	-	-	-	-	+	+
	K_1	N	-	-	-	-	-	-	-	-
	D_1	P	-	-	-	-	-	-	-	-
	D_2	P	-	-	-	+	-	-	-	-
17	D_3	P	-	-	-	-	-	-	-	-
	D_4	P	-	-	-	-	-	-	+	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	P	+	-	-	+	+	+	+	+
	D_2	P	-	-	-	-	-	-	-	-
20	D_3	P	+	-	-	-	-	-	-	-
	D_4	P	-	-	-	-	-	-	-	-
	K_1	N-	-	-	-	-	-	-	-	-
	D_1	P	+	-	-	-	-	-	-	-
	D_2	N	+	-	-	-	-	-	-	+
25	D_3	P	-	-	-	+	+	-	-	-
	D_4	P	+	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	P	-	-	-	-	+	-	-	+
	D_2	P	-	-	-	-	-	-	-	-
28	D_3	N	-	-	-	-	-	-	-	-
	D_4	P	-	-	-	-	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-
	D_1	P	-	-	-	+	-	-	-	-
30	D_2	P	-	-	-	-	+	-	-	-
	D_3	P	-	-	-	-	-	-	-	-
	D_4	N	-	-	-	+	-	-	-	-
	K_1	N	-	-	-	-	-	-	-	-

N: Negative; +: slight; ++: moderate; +++: severe; P: positive.

natural infection, giving the impression that these lesions developed due to secondary infections. On the other hand, the antigenic-specific stain observed in the immunohistochemically stained organs shows that the infecting agent was present in the bursa of Fabricius at some time during the infection. According to the literature and our findings, it can be suggested that important lesions do not develop in the bursa of Fabricius directly by the agent of infection and that the bursa of Fabricius is not an important organ used for the replication of the virus.

It has been reported that during natural and experimental infections, local necrosis and lymphoid depletion occurs inside the white pulp within the spleen (3,19). In the present study lymphoid depletion was observed to develop, particularly between the 4th and the 6th post-inoculation (PI) days. This was followed by the development of lesions in the thymus and bone marrow (3,9,11). In our study lesions in the spleens were apparent, especially on post-inoculation days 13 and 17. We agree with other investigators that suggest the lymphoid

depletion that develops in the spleens of infected animals might be related to necrosis and atrophy, which develop in the thymus and bone marrow.

Despite the antigenic-specific stains observed in different organs, such as the bursa of Fabricius, spleen, lung, intestine, cecal tonsils, and glandular stomach 7 days after inoculation, no significant microscopic lesions were found in any of these organs.

Our results and those published in the literature would suggest that the virus first settles in the thymus and bone marrow during the viremic period of infection and causes typical lesions in these tissues. Furthermore, after day 7 the virus is transferred from the thymus and bone marrow to other organs via the blood and settles in these other organs, especially the lymphocytes. When the virus reaches these other organs replication occurs.

The findings of the present study indicate that the CIA virus, which has been reported in may other studies to settle in the thymus and bone marrow, cause lesions, and transfers to other organs, did not cause any typical microscopic lesions in these organs (organs other than the thymus and bone marrow); however, they did become infected with the virus, especially the lymphocytes.

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