# Lesions in the thymus and bone marrow in chicks with experimentally induced chicken infectious anemia disease

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One-day-old SPF chicks were inoculated with the Cux-l strain of chicken infectious anemia virus (CIAV), and the clinical development of disease and its macroscopic and microscopic alterations in the thymus and bone marrow, were observed. Tissue sections of thymus and bone marrow were stained using the streptavidin-biotin peroxidase method and examined under light microscope for evaluation of antigenic intensities in tissues. Those findings were then compared with blood parameters and ELISA results obtained through collected sera during sacrifice procedures. We sought to determine: the localization of viral antigens in thymus and bone marrow tissues after inoculation, the correlation between antigen intensities and hematologic, serologic and histopathologic findings, definitive diagnostic criteria using histopathologic and immunoperoxidase methods, and the reliability of these methods in the diagnosis of CIAV infection. For this purpose, 83, one-day-old SPF chicks were used. The birds were divided into experimental (n = 52) and control (n = 26) groups. A virus dose of TCID50 of 100,000/ml was administered intramuscularly to every bird in the experimental group. Based on the results of this study, we have suggested that clinical examination, along with macroscopic and microscopic evaluation of the thymus and bone marrow, maybe undertaken starting from day 7 post-inoculation (PI). ELISA, might be of value, as it might give consistent results starting from day 14 PI. However, the most reliable results were obtained through examination of thymus and bone marrow sections from infected birds stained by immunoperoxidase technique, as early as day 4 PI.

**Keywords:** bone marrow, chicken infectious anemia, histopathology, immunohistopathology, thymus

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## Introduction

The causative agent of chicken infectious anemia (CIA), which was first isolated in Japanese poultry by Yuasa *et al.* [28], is not considered to be ubiquitous all over the world. The causative agent is a circular, single-stranded DNA virus: chicken infectious anemia virus (CIAV) [23]. Packed cell volumes (PCV) of less than 25% have been detected in this disease [16,17,23,28]. The disease may pose a significant economic threat to the broiler industry [2]. Although it has not yet been officially confirmed, the Turkish poultry industry also seems to be affected by CIA, based on the results of two reports published independently, by Ergün *et al.* [4] and Yilmaz *et al.* [27].

The aim of this study was to observe the clinical, microscopic, and macroscopic changes caused by CIAV and to detect CIAV antigens immunohistochemically by using the streptavidin-biotin peroxidase technique in thymus and bone marrow tissues of one-day-old chicks infected with CIAV. We additionally sought to find out the first localization sites of the viral antigens and to clarify the correlation between the histopathologic observations made by routine light microscopy and staining intensity revealed by streptavidin-biotin peroxidase technique. The most important goal of the study was to frame diagnostic criteria and evaluate the reliability of these techniques in the diagnosis of CIA.

## Materials and Methods

### Animals

Eighty-three, one-day-old SPF white leghorn chicks were used in this study. Five randomly selected chicks were sacrificed before virus inoculation, and their blood sera were analyzed using ELISA to confirm the absence of antibodies against CIAV. Fifty-two of the chicks (experimental group) were inoculated with CIAV and placed in a floor pen, while the remaining 26 chicks were used as the control group.

## Experimental design

Cux-l isolate of CIAV with $TCID_{50} = 100,000/ml$ (obtained from the Veterinary Medical Research Institute of the Hungarian Academy of Sciences) was used in this study. Each bird was inoculated with 0.5 ml of virus suspension. Both groups of birds were fed *ad libitum* by a commercial layer starter feed. Pre-boiled and re-cooled tap water was supplied *ad libitum* to both groups. Two blood samples were obtained during the sacrifice procedure, according to the experimental design shown in Table 1, separately from each bird respectively, for hemogram and ELISA tests. A first blood sample from each bird was collected in a tube containing a sufficient amount of EDTA, while a second sample was collected in a sterile centrifuge tube. Samples for hemogram tests were evaluated using an electronic cell counter, within one hour after sampling. The sera samples, which were collected in sterile tubes, were kept at −20°C for the ELISA test.

## Immunostaining procedure

The primary antibody, which is cell supernatant of a monoclonal antibody specific for VP3, was obtained from the Institute for Animal Science and Health (ID-DLO; Netherlands), and was used for the streptavidin-biotin peroxidase technique. The antibody was intended for use at a dilution of 1∶100. A commercially available test kit (LSAB-2 kit, Dako, USA) containing both goat anti-mouse and anti-rabbit IgG conjugated with biotin, was used in this study. Standard techniques were used for the streptavidin-biotin staining procedures [20,26]. Each staining lot included at least one known positive and one known negative tissue. Antigen retrieval was achieved using the microwave-based antigen retrieval technique [11,24] with a working dilution (1∶10) of a commercially available antigen retrieval Citra solution (pH 6.0; BioGenex, USA) and a kitchen type microwave oven (Arçelik, Turkey). Endogenous peroxidase activity was quenched by incubating the tissues 20 min with a methanol solution containing 0.1 ml of 3% $H_2O_2$. All washing steps were performed by using Tris buffer solution (TBS; Dako, USA), and all incubations were done in room temperature conditions. The tissues were incubated with 1∶20 dilution of normal goat serum in TBS for 20 min in order to prevent non-specific binding of the secondary antibody. Each slide was incubated with the anti-CIAV monoclonal antibody for 30 min. The link solution (biotin conjugated anti-mouse and anti-rabbit IgG) and peroxidase conjugated streptavidin, which were commercially supported within the kit (LSAB-2 kit; Dako, USA), were applied for 15 min. In order to obtain a brown colored reaction product on antigenic sites, a chromogen/substrate solution containing 0.02 % $H_2O_2$ and 1 mg of diaminobenzidine per ml was applied to the tissues and incubated for 10 min. The slides were counterstained with Mayer's hematoxylin (Dako, USA) and mounted with Glycer-Gel (Dako, USA).

## Methods

Together with the 5 birds sacrificed on day 1 prior to virus inoculation procedures, thymus and bilateral femoral bone samples were collected from all birds at various time intervals, as shown in Table 1. The tissues were then fixed in 10% formalin solution for 18 to 24 h. All tissue specimens, except femoral bone samples, were routinely processed. Femoral bone samples were decalcified in a 17% EDTA solution for an additional period of 18 to 24 h. 3 and 5 μm thick sections were prepared from each organ sample, using a rotary microtome. 3 μm thick sections were used in streptavidin-biotin staining technique and 5 μm thick sections were stained with hematoxylin and eosin.

## Statistical analyses

The mean, standard deviation, and significant values of the data obtained were determined by Duncan's test [3], as groups comprising the days 1-7, 8-10, 12-14, 17-20, 25-28, and 30.

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

| Days | Sacrificed/ sampled | |
|---|---|---|
| | Experimental group | Control group |
| 1* | | 5 |
| 3 | 2 | 1 |
| 4 | 2 | 1 |
| 5 | 4 | 2 |
| 7 | 4 | 2 |
| 8 | 4 | 2 |
| 10 | 4 | 2 |
| 12 | 4 | 2 |
| 13 | 4 | 2 |
| 14 | 4 | 2 |
| 17 | 4 | 2 |
| 20 | 4 | 2 |
| 25 | 4 | 2 |
| 28 | 4 | 2 |
| 30 | 4 | 2 |
| Total | 52 | 31 |

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

## Results

### Clinical findings

Clinical signs, including anorexia, lethargy, and listlessness characterized by drooping of both wings, were observed in only two of the birds in the experimental group, one at the fourteenth day and the other at the seventeenth day of the experiment. The symptoms progressed over a 24 h period, and these birds were sacrificed for examination (in their own groups, before a natural death occurred). Although the live weights of the groups, which were determined every day before the sacrifice, were different, the difference between groups was statistically insignificant. Weight values were found to be 4.46, 6.69, 3.57, 1.44, 15.57, and 9.09 percentage points lower in experimental group animals sacrificed on day intervals 1-7, 8-10, 12-14, 17-20, 25-28 and 30, respectively (Table 2).

### Necropsy findings

Between days 12 and 25 of the study, in the experimental group, some of the birds' beaks, wattles, and conjunctivae were observed to have pale and yellowish color. Some of the thymuses in the experimental group were observed to be slightly hyperemic on day 8, and this seemed to be more severe between days 10 and 14. Starting from day 14, this hyperemia was observed to lose its severity, and slight atrophy of the organs was observed. This atrophy was quite prominent in most of the animals in the experimental group until day 28. Starting from day 8, some of the bone marrows of the birds in the experimental group kept their pale, yellowish-pink color and fatty appearance, while the marrows of the control group turned from a pink to a dark red color. These findings were still detectable at day 17, and the yellowish appearance was recognized only in the diaphyseal zones of the bones of the experimental group on day 20.

### Hematologic and serologic findings

The most prominent hematologic findings were recorded in PCV and RBC values, especially on the seventeenth to

**Table 2.** Comparison of average live weight at different days after CIAV infection

| Days | Groups | |
|------|--------------------------------|--------------------------------|
| | Experiment group (n)[†] | Control group (n) |
| 1-7 | *46.84 ± 3,049[d,A] (12) | 49.03 ± 2,530[d,A] (11) |
| 8-10 | 51.60 ± 3,606[d,A] (8) | 55.30 ± 5,281[c,d,A] (4) |
| 12-14 | 65.78 ± 6,225[c,A] (12) | 68.22 ± 13,374[c,d,A] (6) |
| 17-20 | 71.73 ± 10,253[c,A] (8) | 72.78 ± 12,194[c,A] (4) |
| 25-28 | 80.75 ± 12,824[b,A] (8) | 95.65 ± 6,859[b,A] (4) |
| 30 | 135.20 ± 11,703[a,A] (4) | 148.65 ± 20,435[a,A] (2) |

*Mean ±SD, [a, b, c, d]Each group designed with different letters are statistically significant ($p < 0.05$). [A]Difference between trial and control groups are statistically insignificant ($p > 0.05$). [†]n = number.

**Table 3.** Comparison of the optic density (O.D.) and sample / negative (S/N) value of the sacrificed chicken

| Days | Experiment group | | | | | | | | | | | | Control Group | | | | | |
|------|------|-------|--------|------|-------|--------|------|-------|--------|------|-------|--------|------|-------|--------|------|-------|--------|
| | 1 | | | 2 | | | 3 | | | 4 | | | 1 | | | 2 | | |
| | O.D. | S/N | Result | O.D. | S/N | Result | O.D. | S/N | Result | O.D. | S/N | Result | O.D. | S/N | Result | O.D. | S/N | Result |
| 3 | 0.212 | 0.314 | + | 0.373 | 0.552 | + | | | | | | | 0.461 | 0.682 | − | | | |
| 4 | 0.503 | 0.744 | − | 0.472 | 0.698 | − | | | | | | | 0.515 | 0.762 | − | | | |
| 5 | 0.473 | 0.700 | − | 0.450 | 0.666 | − | 0.510 | 0.754 | − | 0.598 | 0.885 | − | 0.334 | 0.509 | + | 0.398 | 0.589 | + |
| 7 | 0.832 | 1.231 | − | 0.459 | 0.679 | − | 0.342 | 0.506 | + | 0.350 | 0.518 | + | 0.500 | 0.740 | − | 0.595 | 0.880 | − |
| 8 | 1.392 | 2.059 | − | 0.580 | 0.858 | − | 0.481 | 0.712 | − | 0.562 | 0.831 | − | 0.610 | 0.902 | − | 0.617 | 0.913 | − |
| 10 | 0.477 | 0.706 | − | 0.461 | 0.682 | − | 0.643 | 0.951 | − | 0.507 | 0.750 | − | 1.720 | 2.544 | − | 0.528 | 0.782 | − |
| 12 | 0.525 | 0.777 | − | 0.207 | 0.306 | + | 0.582 | 0.861 | − | 0.610 | 0.902 | − | 0.542 | 0.802 | − | 0.527 | 0.780 | − |
| 13 | 0.882 | 1.305 | − | 0.502 | 0.743 | − | 1.279 | 1.892 | − | 0.858 | 1.269 | − | 0.939 | 1.389 | − | 1.262 | 1.867 | − |
| 14 | 0.685 | 1.013 | − | 0.390 | 0.577 | + | 0.317 | 0.469 | + | 0.298 | 0.441 | + | 1.105 | 1.635 | − | 0.509 | 0.753 | − |
| 17 | 0.286 | 0.423 | + | 0.168 | 0.249 | + | 0.221 | 0.327 | + | 0.308 | 0.456 | + | 0.749 | 1.108 | − | 0.559 | 0.827 | − |
| 20 | 0.258 | 0.382 | + | 0.255 | 0.377 | + | 0.336 | 0.497 | + | 0.182 | 0.269 | + | 0.973 | 1.439 | − | 0.433 | 0.641 | − |
| 25 | 0.185 | 0.274 | + | 0.589 | 0.871 | − | 0.257 | 0.380 | + | 0.297 | 0.439 | + | 0.585 | 0.865 | − | 0.598 | 0.885 | − |
| 28 | 0.149 | 0.220 | + | 0.336 | 0.497 | + | 0.502 | 0.743 | − | 0.109 | 0.161 | + | 0.555 | 0.821 | − | 0.544 | 0.805 | − |
| 30 | 0.138 | 0.204 | + | 0.169 | 0.250 | + | 0.333 | 0.439 | + | 1.051 | 1.555 | − | 0.514 | 0.760 | − | 0.435 | 0.643 | − |

**Table 4.** ELISA test results and scoring of histopathologic lesions and immunoperoxidase results in the bone marrow and thymus

| Days | Bird No. | ELISA test results | Histopathologic lesions | | Immunoperoxidase staining results | |
|------|----------|-------------------|-------------|--------|-------------|--------|
| | | | Bone marrow | Thymus | Bone marrow | Thymus |
| 1 | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| | C3 | N | − | − | − | − |
| | C4 | N | − | − | − | − |
| | C5 | N | − | − | − | − |
| 3 | E1 | P | − | − | − | − |
| | E2 | P | − | − | − | − |
| | C1 | N | − | − | − | − |
| 4 | E1 | N | − | − | − | − |
| | E2 | N | − | + | + | + |
| | C1 | N | − | − | − | − |
| 5 | E1 | N | − | − | − | − |
| | E2 | N | − | + | + | + |
| | E3 | N | + | + | + | + |
| | E4 | N | − | − | − | − |
| | C1 | P | − | − | − | − |
| | C2 | P | − | − | − | − |
| 7 | E1 | N | − | + | ++ | + |
| | E2 | N | + | + | ++ | + |
| | E3 | N | + | + | + | + |
| | E4 | P | + | + | + | + |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 8 | E1 | N | ++ | ++ | +++ | ++ |
| | E2 | N | ++ | ++ | +++ | ++ |
| | E3 | N | ++ | + | ++ | + |
| | E4 | N | ++ | ++ | +++ | ++ |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 10 | E1 | N | +++ | ++ | +++ | +++ |
| | E2 | N | +++ | +++ | +++ | +++ |
| | E3 | N | +++ | +++ | +++ | +++ |
| | E4 | N | +++ | ++ | +++ | +++ |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 12 | E1 | N | +++ | +++ | +++ | +++ |
| | E2 | P | +++ | +++ | +++ | +++ |
| | E3 | N | +++ | +++ | +++ | +++ |
| | E4 | N | +++ | +++ | +++ | +++ |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 13 | E1 | N | +++ | +++ | +++ | +++ |
| | E2 | N | ++ | +++ | ++ | +++ |
| | E3 | N | ++ | +++ | ++ | +++ |
| | E4 | N | ++ | +++ | ++ | ++ |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |

**Table 4.** Continued

| Days | Bird No. | ELISA test results | Histopathologic lesions | | Immunoperoxidase staining results | |
|---|---|---|---|---|---|---|
| | | | Bone marrow | Thymus | Bone marrow | Thymus |
| 14 | E1 | N | +++ | +++ | ++ | +++ |
| | E2 | P | ++ | +++ | ++ | ++ |
| | E3 | P | +++ | +++ | ++ | + |
| | E4 | P | ++ | +++ | + | + |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 17 | E1 | P | ++ | +++ | + | ++ |
| | E2 | P | + | ++ | + | ++ |
| | E3 | P | + | ++ | + | ++ |
| | E4 | P | ++ | +++ | + | + |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 20 | E1 | P | + | ++ | − | − |
| | E2 | P | + | ++ | + | + |
| | E3 | P | + | ++ | + | + |
| | E4 | P | + | ++ | − | − |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 25 | E1 | P | + | + | − | − |
| | E2 | N | − | +* | + | + |
| | E3 | P | − | + | − | − |
| | E4 | P | +* | +* | − | − |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 28 | E1 | P | +* | +* | − | − |
| | E2 | P | +* | − | − | − |
| | E3 | N | +* | +* | − | − |
| | E4 | P | +* | − | − | − |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |
| 30 | E1 | P | − | − | − | − |
| | E2 | P | +* | − | − | − |
| | E3 | P | +* | − | − | − |
| | E4 | N | − | − | − | − |
| | C1 | N | − | − | − | − |
| | C2 | N | − | − | − | − |

C: control, e: experimental, n: negative, p: positive, −: normal (or no antigen specific staining), +: slight, ++: moderate, +++: severe, *Hyperplasia.

twentieth day interval in the experimental group. In this period, PCV values were found to be 8.65%, and RBC values were found to be 7.9% lower than the control group of birds examined. The differences were found to be statistically insignificant ($p > 0.05$). Although the first positive results of the ELISA were detected in two experimental group birds sacrificed on day 3 and in two control birds sacrificed on day 5, the most consistent results from both groups were obtained on day 14 and later (Tables 3 & 4).

**Histopathologic findings**
The first histopathologic changes noted in the thymuses of the experimental group's birds were seen in a single bird on day 4 and in two birds on day 5. All of the thymuses of the experimental group's birds showed minor changes

starting from day 7. Although, on day 4 and 5, those thymic lesions were confined to small foci in the thymic cortex which were defined by a few scattered cells staining paler and showing marginal hyperchromasia in their nuclei, those lesions seemed to extend, and some of these cells showed large, eosinophilic inclusions in their nuclei on day 7. During the same time interval, microfocal areas of coagulative necrosis were also prominent in the thymic cortex of some of the experimental birds, and some of the destructive areas were observed to be replaced by reticular cells. Those lesions seemed to progress, develop and were more significant and diffuse between days 8 and 10. It was still possible to observe intranuclear eosinophilic inclusions in these enlarged cells and in reticular cells situated both in the medullar and the cortical areas. Starting from day 7, hemorrhage was observed, especially in the subcapsular areas of the thymic cortex, and its appearance seemed to be more significant and diffuse between days 10 and 12. One of the most interesting findings recorded in the thymuses of the animals in the experimental group was the reduction in the size of the thymic cortex due to depletion of cortical thymocytes, resembling the medullar zone, between days 10 and 25. This finding was most prominent between days 12 -14 (Figs. 1A & B). The first signs of regeneration were observed on day 20, and mitotic activity resulted in repopulation of cortical thymocytes until day 30 of the study (Table 4).
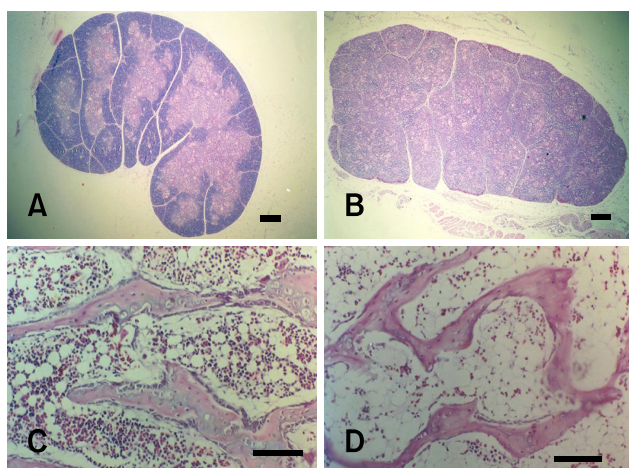
The first histopathologic changes detected in the bone



**Fig. 1.** (A) Normal appearance of the thymus of a bird from the control group sacrificed on day 12. Scale bar = 200 μm. (B) Depletion of the cortical thymocytes and medulla-like appearance of the thymic cortex in the thymus section of a bird from the experimental group sacrificed on day 12. Scale bar = 200 μm. (C) Normal appearance of the bone marrow of a bird from the control group sacrificed on day 10. Scale bar = 100 μm. (D) A prominent decrease in the number of cells in the bone marrow of a bird from the experimental group sacrificed on day 10. Scale bar = 100 μm. H&E stain.

marrow sections of the experimental group's animals was characterized by the presence of a few, larger hemocytoblasts on day 7. These cells and intranuclear eosinophilic inclusions were observed occasionally between days 8 and 10. In the mean time, the number of cells of the erythrocytic and granulocytic series was found to be decreased, and the empty spaces left behind were filled by adipose tissue (Figs. 1C & D). Abundant osteoclasts and macrophages were also found quite frequently around the degenerative areas. Hematopoietic activity seemed to return to normal levels starting from day 17. This condition was detectable until the end of the experimental period (Table 4).

**Immunoperoxidase findings**

The first antigen-specific staining in the experimental group's birds' thymus sections was recorded in one bird sacrificed on day 4. Specifically stained cells were identified as a few large thymocytes that were closely situated to each other, on the subcapsular zone of the thymic cortex, within only one of the thymic lobules. The staining pattern was quite similar to that of the birds which were sacrificed on day 5, which was still limited to only a single lobe. While all thymus sections were stained specifically in all the birds sacrificed between days 7 and 14, only some cells in different areas were found to be specifically stained in experimental group animals sacrificed between days 17 and 25. Specifically stained sections were encountered to a lesser degree in the cortex, and only a few stained cells could be found in the medulla between days 7 and day 8. A prominent increase in the number of specifically stained cells was detected, and stained cells were also found to be more abundant in the medullar zone. However, they did not reach a frequency equal to the number present in the cortex on day 10 (Fig. 2A). These specifically stained cells showed a homogenous distribution in the medulla and were indistinguishable in the cortex on days 12 (Fig. 2B). Specifically stained cells could only be detected in the thymus section of one bird in the experimental group on day 25.

The first antigen specific staining in the bone marrow was recorded in one bird from the experimental group sacrificed on day 4. Specifically stained cells were identified as large hemocytoblasts and reticulocytes situated in the extrasinusoidal or intrasinusoidal spaces. Moreover, stem cells that were in various stages of differentiation were also determined to be stained specifically. The number of stained cells showed an increase between days 8 and 10 (Figs. 2C & D). A decrease in the number of stained cells was recorded at day 14 and afterwards. Specific staining could be observed in only one experimental bird on day 25, and no stained cell was encountered in the bone marrow sections of birds sacrificed on day 28 and afterwards (Table 2).
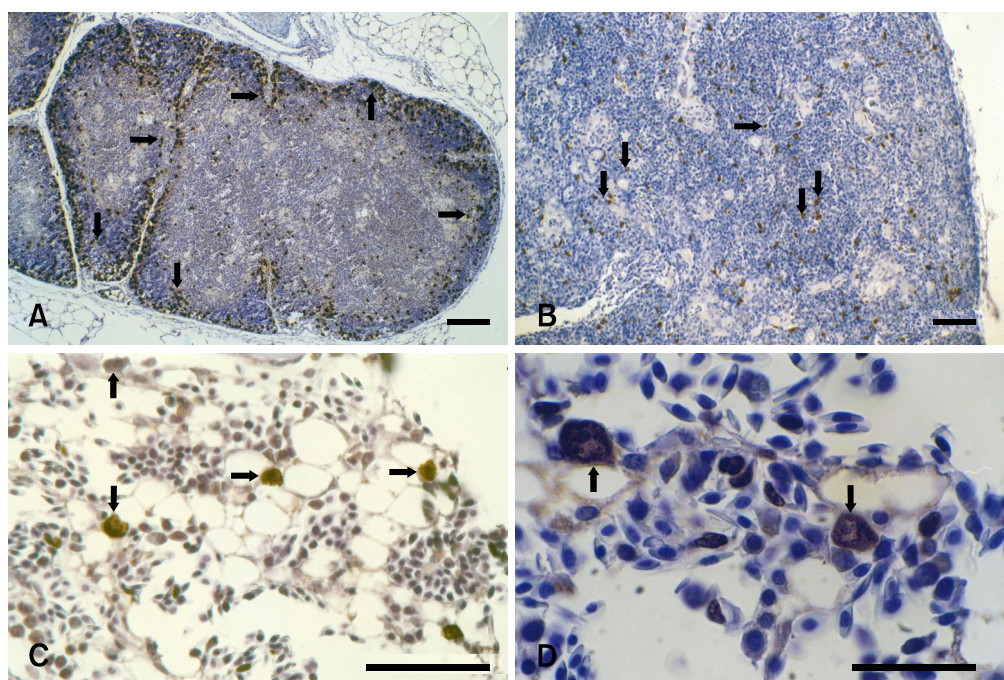
**Fig. 2.** (A) Abundant antigen-specific staining in the cortex and, to a lesser extent, in the medulla of the thymus section of an experimental bird sacrificed on day 10. Scale bar = 100 μm. (B) Antigen-specifically stained cells both in the depleted cortical and medullar zones of the thymus section of an experimental bird sacrificed on day 12. Scale bar = 200 μm. (C) Antigen-specifically stained large hemocytoblasts in the bone marrow section of an experimental bird sacrificed on day 8. Scale bar = 100 μm. (D) Antigen-specifically stained hemocytoblasts and stem cells at different stages of differentiation in the bone marrow section of an experimental bird sacrificed on day 10. Scale bar = 50 μm. Immunoperoxidase stain.

## Discussion

As stated in much of the literature, CIAV infections have become a worldwide economical problem, and their effect is most likely to be observed in 2- to 4-week-old broilers, as well as layers of the same age [2,16,18].

Although no significant change was found between the mean values of live weights of the animals in the control and the experimental groups, the mean live weight of the experimental group was found to be consistently lower throughout the entire experimental period, in comparison with the control group. The results of Hoop [12], obtained through inoculation of day-old Light Sussex chicks with $TCID_{50} = 10^6$ of a Scottish isolate of CIAV, were compatible with the current study.

Despite the fact that there was no antigenic difference between CIAV isolates in terms of cross-neutralization and cross-immunofluorescence tests, the incidence of anemia induced by different isolates of CIAV was reported to have ranged between 0 and 88% [1,28]. Considering the dose used in this study (50,000 $TCID_{50}$/chick), it was possible to induce the subclinical form of the disease. The incidence of anemia in the experimental group was found to be within the expected range. Although PCV values below 25-27% were accepted as clinical anemia, recent studies have

indicated that neonatal physiological anemia can be observed in avian species. The threshold of anemia for SPF leghorn chicks should be accepted as PCV values lower than 23, 25, 26, 28, 31, and 30% on days 3, 7, 14, 21, 28, and 35, respectively [6-8]. All data obtained from clinical observations, necropsies, and hemogram tests have illustrated the point that the pathological picture might be variable, even between the birds infected with the same dose of CIAV. It was difficult to identify the animals which were experiencing the subclinical form of the disease. Goodwin and Brown [5] indicated that anemia and atrophy of hematopoietic organs are not sufficient criteria for the diagnosis of CIAV infections. Practical methods are also needed to confirm the disease in a virological, serological, and pathological manner. Some authors have emphasized the influence of genetic constitution, nutritional state, and management conditions on the incidence of clinical manifestations and the severity of anemia in CIAV infections [1,7,9].

The results of this study confirm that histopathologic changes such as depletion of cells and increase in the number of reticulocytes and general atrophy in the bone marrow and thymus might be important evidences of CIAV infection, although they are not considered pathognomonic lesions, as stated by previous authors [10,15,25,28]. Thus,

we conclude that histopathologic changes might give a clue to the diagnosis of CIAV infections. For differential diagnosis, it is also important to consider sulfur intoxication, mycotoxicoses, trichloroethylene intoxication, and concurrent infection with infectious bursal disease (IBD) virus as well as adenovirus strains, some strains of Marek's Disease (MD) virus, and osteopetrosis in the etiology of aplastic anemia and pancytopenia. Moreover, it has reported that atrophy in the thymus cortex has been induced by some strains of IBD [14]. The presence of inclusions encountered in the reticulocytes and hemocytoblasts of the bone marrow and cortical thymocytes and reticulocytes in the thymus between post-inoculation days 8 and 10 were also reported by other authors [10]. In contrast, there was no evidence about the presence of inclusions in the reports from natural outbreaks. This condition might have been associated with the temporary character of those inclusions, before the onset of clinical signs. Thus, it is possible to say that clinical and histopathologic findings might have revealed important evidences, but inclusions were unable to be considered as an important finding in the diagnosis of naturally occurring diseases.

In our study, the first positive serum antibody titers in the experimental group were detected on day 3 and day 7, while consistent positive results in the experimental group were obtained starting from day 14. Results obtained from the experimental and control birds sacrificed on day 3 and day 5 were considered as non-specific false positives, based on our consistent negative findings on routine histopathologic and immunoperoxidase staining. On the basis of our own findings and some of the relevant literature [19,22], it has been suggested that in 1-day-old chicks inoculated with CIAV, seropositivity by ELISA might be obtained as early as day 7 or 8, while consistent positive results might be achieved starting at day 14.

CIAV genome is reported to have three different Open Reading Frames (ORF's), and these ORF's are responsible for the synthesis of three viral proteins designed as VP1, VP2, and VP3 [21]. VP1 is reported to be the capsid protein, and VP1 and VP2 together are said to determine the antigenic characteristics of the virus [21]. VP3 (also termed "apoptin") is reported to be only detectable in virus-infected cells and is the particular indication of apoptosis in the target cell [21,22]. The monoclonal antibody used in this study had the capability to specifically bind VP3, enabling us to label virus-infected cells in different stages of apoptosis. It also had the advantage of directly labeling the antigen, compared to ELISA, which can only give an idea about the antibody status against the disease, but cannot describe whether the antibodies present are maternally derived or acquired as a response to direct infection. In birds infected with CAV at one day of age, consistent results were observed in the examination of thymus sections obtained between the seventh and seventeenth days, and in bone marrow sections obtained between the thirteenth and seventeenth days. These results are supported by the reports of parallel studies [13,15,18,25] and also by our observations from routine histopathologic examinations.

The two main target organs affected by CIAV were determined to be the bone marrow and thymus, on the basis of our viral antigen detection studies by the streptavidin-biotin peroxidase technique. The severity of histopathologic lesions and antigen staining intensities detected by the immunoperoxidase technique used in this study showed a positive correlation, particularly for thymus and bone marrow sections.

It was determined that for birds infected via the intramuscular route on the day of hatch, histopathological alterations were detectable in the thymus and bone marrow starting from day 10, and labeling of CIAV antigens was possible between days 4 and 25. It is possible to say that the streptavidin-biotin peroxidase technique involving a monoclonal antibody like the one we used in this study enables early and specific diagnosis of CIAV infections. The earliest and most reliable results can be obtained by the immunoperoxidase technique using a monoclonal antibody like the one we used in this study, considering that immunoperoxidase techniques are quite practical and do not require expensive equipments such as fluorescent microscopes. Attempts to produce various monoclonal antibodies, not only against CIAV but also against other pathogens, would be of great help for pathologists, in order to enable the early and reliable diagnosis of various infections, even in poorly equipped laboratories.

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