Distribution of T Cell Subsets in Chicken Lymphoid Tissues

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ABSTRACT. The distributions of T cell subsets in chicken lymphoid tissues were investigated immunohistochemically using monoclonal antibodies (Lc-6, Lc-4) with specificity for chicken CD4 and CD8, respectively. In thymic tissues, CD8+ cells were found only in the cortex, while CD4+ cells were detected not only in the cortex but also in the medulla. The cortex just below the capsule demonstrated no immunoreactivity to either antibody. In the cecal tonsils, CD8+ cells were found restrictively in the subepithelial lamina propria. It was noted that the germinal centers were clearly surrounded with many CD4+ cells in the mid and deep portions of the lamina propria. In the spleen, clusters of CD8+ cells were observed only in the red pulp. Most lymphocytes in the periarteriolar lymphatic tissue and periportal lymphatic tissue showed a CD4-positive reaction. No lymphocyte in the germinal centers reacted with these two monoclonal antibodies. No immunoreactivity for either CD8 or CD4 was detected anywhere else in the bone marrow or bursa of Fabricius. In the case of exposure to the protein antigen (alum-precipitated bovine serum albumin), CD4+ cells were demonstrated in some germinal centers, which were increased in size, while the areas expressing CD8 in the red pulp were decreased in size. These results suggest that the preferential distributions of T cell subsets are inherent in chicken lymphoid tissues.—KEY WORDS: chicken, immunohistochemistry, lymphoid tissue, monoclonal antibody, T cell.


Because of the anatomical separation of primary lymphoid tissues such as the thymus and the bursa of Fabricius, domestic fowls, especially chickens, have provided useful experimental models for study of the immune system. The function, development, differentiation and histological distribution of lymphocytes in these tissues have been investigated by using surgical thymectomy, bursectomy, X-ray irradiation and administration of immunosuppressive drugs [6, 9, 10, 20, 27].

In mammals, many reports on the lineage of lymphocytes have shown a constant distribution of T cell subsets in several lymphoid tissues [8, 11, 22, 25, 29]. However, the lack of detailed criteria in the classification of surface markers for T and B lymphocytes and their subsets in the chicken have limited extensive studies for immunological responses. Recently, the presence of avian homologues of mammalian CD4 (helper T cell) and CD8 (suppressor/cytotoxic T cell) antigens has been made clear [4]. Furthermore, several types monoclonal antibodies (mAbs) recognizing chicken lymphocyte subpopulations in detail have been made at our laboratory [14, 15].

In this paper, the localizations of T cell subsets in the chicken lymphoid tissues and their distributional changes of peripheral lymphoid tissues, especially in the spleen after the administration of T cell-dependent antigens have been investigated immunohistochemically using mAbs (Lc-6, Lc-4) with specificity for chicken CD4 and CD8 antigens, respectively [14, 15].

MATERIALS AND METHODS

Animals and samples: A total of 55 white Leghorn chickens, separated into three groups (control, primary immunized and secondary immunized groups) was used in this study.

In the first group, 17 chickens at 7 weeks of age were used. The thymus, bursa of Fabricius, cecal tonsil, bone marrow and spleen were removed immediately, immersed in OCT Compound (Miles, U.S.A.) and quickly frozen in liquid nitrogen. In the second group, crystalline bovine serum albumin (BSA) (Sigma, U.S.A.) aggregated with alum was administrated intravenously to 13 animals 7 weeks old (4 mg of BSA/100 g body weight). In the final group, an equal volume of BSA was first injected in the same manner into 25 chickens aged 5 weeks old, and then administrated again in the same volume 2
weeks later. BSA solutions administrated as antigen into the latter 2 groups were prepared by the method of Katsura [12]. The spleens, which were representative of peripheral lymphoid tissues in these 2 immunized groups, were sampled in the same manner as above at 2, 4, 7 and 14 days after final injections of antigens. Peripheral blood of some of them was also subjected to flow cytometric analysis.

**mAbs:** The details of the mAbs recognizing chicken T cell subsets have been described in previous reports [14, 15]. Lc-4 and Lc-6 mAbs raised as IgG1 and IgM have been known to recognize chicken homologues of mammalian CD8 and CD4 surface antigens, respectively. In order to avoid confusion with the terminology used in the present paper, Lc-4- and Lc-6-positive cells are herein described as CD8+ and CD4+ cells, respectively.

**Immunohistochemistry:** Cryosections cut 6–8 µm thick were fixed in acetone at 4°C for 5 min and OCT Compound was removed by rinsing with three changes of phosphate-buffered saline (PBS). Immunohistochemistry for T cell subsets was performed by the following procedure. Briefly, endogenous peroxidase activity was eliminated by the incubation of sections with methanol containing 3% H2O2 for 30 min. After washing carefully with running water, distilled water and PBS, the sections were incubated with 1% BSA at room temperature for 30 min, and then with primary mAbs overnight at 4°C. In mAb for CD8, the incubating fluid was applied directly for primary mAb, while in mAb for CD4 ascitic fluid was used with a dilution of 1:20. After rinsing with PBS 3 times, the sections were subsequently incubated with goat anti-mouse IgG (Cappel, U.S.A.) diluted at 1:100 or IgM (American Qualex, U.S.A.) diluted at 1:50 antiserum at room temperature for 1 hr, and then with peroxidase mouse anti-peroxidase complex (Serotec, U.K.) at room temperature for 1 hr. Finally the sections were reacted with 0.05% diaminobenzidine tetrahydrochloride containing 0.03% H2O2 in 0.01 M Tris-HCl buffer (pH 7.6), and slightly counterstained with aqueous methyl green. Control sections were incubated with PBS or non-immune ascitic fluid (Cedarlane, Canada) substituted to primary mAbs.

**Flow cytometric analysis:** In this analysis, 5 animals in the control group, 3–5 animals of the primary immunized group after immunization at 4 days and 2 weeks 3–5 animals of the secondary immunized group 2 days and 3 weeks after immunization were used. Single cell suspensions from spleen and peripheral blood of each animal were prepared following previous reports [16]. They were then reacted by the indirect immunofluorescence method and analyzed with a flow cytometer. Briefly, the samples were incubated with each primary antibody at 4°C for 30 min, and then with goat anti-mouse IgG, IgA and IgM mixed antisera conjugated with fluorescein isothiocyanate (Cappel, U.S.A.) diluted at 1:60 for 30 min at 4°C. After washing with minimum essential medium, they were analyzed with an EPICS-752 flow cytometer (Coulter, Miami, U.S.A.). The results were shown as the ratio of the number of positive cells against the total number of lymphocytes examined. Monoclonal anti-hemagglutinin-neuraminidase (HN) antibody recognizing HN protein of Newcastle disease virus was used for control or non-specific reactions. The obtained data were statistically analyzed by Student's t-test.

**RESULTS**

**Distribution of T cell subset positive cells in intact conditions:** In the thymic tissues, almost all CD8+ cells were found only in the cortex, while CD4+ cells were detected not only in the cortex but also in the medulla (Figs. 1a, 1b). The distributional density of CD4+ cells in the cortical region was less than that in CD8+ cells. No immunoreactivity for either mAb was demonstrated in some cell layers of the cortex just below the capsule (Figs. 1c, 1d).

In the cecal tonsils, CD8+ cells were scattered in the subepithelial and villous regions of the lamina propria. The germinal centers (GCs) were predominantly surrounded with many CD4+ cells in the mid and deep portions of the lamina propria (Figs. 2a, 2b). In the villous region, a few CD4+ cells existed but were lesser in number than CD8+ cells. As a whole, many CD4+ cells were predominantly distributed in the deep lamina propria of the cecal tonsils, while a few CD8+ cells were scattered only in the subepithelial lamina propria. No immunoreactivity for either mAb was demonstrated in the intraepithelial lymphocytes as far as examined.

In the spleen, clusters of CD8+ cells were observed in the red pulp, especially surrounding the periarterial lymphatic tissue (PA). Most lymphocytes distributed in the PA and perivenous lymphatic tissue (PV) were demonstrative with CD4 stain-
Fig. 1. Immunohistochemical detection of CD8⁺ and CD4⁺ cells in chicken thymus. 1a: The cortex (C) and the medulla (M) stained with CD8. Many CD8⁺ cells are observed only in the cortex. × 270. 1b: The neighboring section of “1a” stained with CD4. Immunoreactive cells are distributed in both cortex (C) and medulla (M). × 270. 1c, 1d: The cortex just below the capsule reacted with neither CD8 (c) nor CD4 (d) (arrows). × 270.

Fig. 2. CD8⁺ (2a) and CD4⁺ (2b) cells in the lamina propria of the cecal tonsil. 2a: A few CD8⁺ cells are observed at the subepithelial lamina propria (arrows). × 270. 2b: Many CD4⁺ cells surrounding germinal centers (GC) are distributed in the mid and deep lamina propria. × 225.
Fig. 3. The distribution of CD8⁺ and CD4⁺ cells in the spleen. 3a: The clusters of CD8⁺ cells are scattered in the red pulp surrounding periarteriolar lymphatic tissue (PA). × 270. 3b, 3c: CD4⁺ cells are detected at the PA and the perivenous lymphatic tissue (PV). No lymphocyte in the germinal center (GC) reacted with two monoclonal antibodies. × 135.

Fig. 4. CD8⁺ cells in the spleen after 3 weeks in the primary immunized group. No positive cell is detected in the red pulp. PA: Periarteriolar lymphatic tissue × 270.

Fig. 5. CD4⁺ cells in the spleen after 2 days in the secondary immunized group. They are demonstrated in the germinal center (GC) surrounded by arrows. × 270.

ing (Figs. 3a, 3b, 3c). None of the lymphocytes in the GCs reacted with either mAb.

No immunoreactivity for either mAb was detected anywhere in the bone marrow and the bursa of Fabricius to the extent examined.

*Distribution of positive cells after BSA immunization*: Only histological and immunohistochemical changes of the spleen were examined. In the primary immunized group, primary nodules at PA and PV were observed from 4 days after the injection, and they were then organized into GCs 1 week later in all cases. The GCs tended to increase in number and in size at 3 weeks after primary immunization. Immunohistochemically, no or a very small number of CD8⁺ cells organized into small clusters or were isolated and scattered in the red pulp (Fig. 4). For the CD4⁺ cells, there was no difference in intrasplenic distribution between the
Table 1. The results of flow cytometrical analysis. The statistical values are shown as average (％)± standard error. In the peripheral blood, no difference among all cases is demonstrated, while a significant decrease of CD8⁺ cells in the spleen, especially from 29％ (control group) to 20％ (after 2 days in the secondary immunized group) is detected.

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<tr>
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<th>Peripheral blood</th>
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<tr>
<td>Control</td>
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<td></td>
<td>CD4⁺ 16.15±4.46</td>
<td>CD8⁺</td>
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<td>Four days after</td>
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<td>1st immunization</td>
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<td>17.32±2.27</td>
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<td>Two weeks after</td>
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<td>1st immunization</td>
<td>28.42±3.64</td>
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<td>Two days after</td>
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<td>2nd immunization</td>
<td>31.90±2.21</td>
<td>22.40±7.82</td>
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<td>Three weeks after</td>
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<td>26.40±4.60</td>
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* : differ significantly from control (p<0.05).

Control and primary immunized groups.

In the secondary immunized group, GCs showing degeneration and regeneration were observed. The number and size of GCs increased at 4 days after secondary injection, and these tendencies continued throughout the experimental period. Many phagocytic islets in the red pulp were observed in all cases. Many GCs larger in size were organized at PA and PV regions after the secondary antigen administration. No immunoreactivity for CD8 was detected in the red pulp throughout the experimental period. Many CD4⁺ cells were observed in PA and PV as well as in the control and primary immunized groups. It was noted that a number of CD4⁺ cells were present in some GCs at 2 and 4 days after the final immunization (Fig. 5).

Flow cytometric analysis: In the quantitative analysis of T lymphocyte subsets in the peripheral blood samples, there was not any difference among the values of the control and the two experimental groups. In the splenic lymphocytes, distinct decreases of CD8⁺ cells were demonstrated throughout the experimental period, especially at 2 days after immunization of the secondary immunized group (Table 1).

DISCUSSION

The preferential distributions of T cell subsets in mammals have been reported by many investigators. For example, it has been studied in thymi [8], spleens [11, 29], tonsils [25], lymph nodes [22] and Peyer's patches [26] in humans, and in gut associated lymphoid tissues (GALT) containing Peyer's patches in mice [7, 19], rats [17], sheep [1] and minipigs [24]. In fowls, although the existence of T cell subsets and T cell receptors similar to those in mammals has been reported in several lymphoid tissues by Bucy et al. [3], the histological division of these subsets has not been clarified until now. In the present study, the first clarification of the preferential distribution of chicken T cell subsets was clearly emphasized. In such a discussion, the similarities and the differences between mammals and fowls should be discussed for each lymphoid tissue.

In the thymus of mammals, some lymphocytes located just below the capsule have been known as CD4⁺ 8⁻ double-negative immature T cells newly derived from bone marrow [8, 21, 23]. They have been thought to become either CD4⁺ 8⁻ or CD4⁺ 8⁺ single-positive cells via CD4⁺ 8⁺ double-positive cells in the thymic cortex. Recently, it has been reported in vitro that many double-positive cells existed in the thymus of the chicken [5, 15]. In the present study, because the cell layers just below the capsule were composed not only of non-CD8, CD4 cells, but also the cortex without just below the capsule contained many CD8⁺ and CD4⁺ (possibly double-positive) cells, the developmental procedure in chicken T cells is suggested to be similar to that in mammals.

Peyer's patches representative of the GALT of mammals, have clearly been shown to be divided into B cell-dependent follicular and GC areas, a parafollicular area that is T cell dependent and a dome area that is both T and B dependent [1, 7, 17, 19]. In the chicken, the cecal tonsils corresponding to mammalian Peyer's patches have been subdivided
by surgical thymectomy and/or bursectomy into the mid and deep layers which are T dependent areas and the superficial layer and GCs, which are B-dependent areas [2, 10]. In the present study, the so-called T-dependent area in previous report adjacent to GCs was demonstrated to correspond to the CD4 positive region. These results suggest that CD4+ cells may be associated with the development of antibody-producing cells in the GCs.

In mammals, CD4+ cells have been detected in GCs [1, 7, 17, 24–26], whereas in the chicken no such cells were demonstrated in them. It was supposed in mammals, because of the histological separation between GCs and the parafollicular area, a T-dependent area by the follicular area, CD4+ cells were needed in the GCs for direct activation of B cells. It has been confirmed by many investigations that in mammals the intraepithelial lymphocytes (IEL) express CD8 positivity [13, 17]. In the present study, neither CD4 nor CD8 positivity was demonstrated in IEL of the cecal tonsils. Further studies are required to analyze the immunological nature of chicken IEL.

The immunohistochemical results obtained from the normal spleen are summarized in Fig. 6a. In chicken spleen, lymphatic areas have been classified into four types: PA, PV, GC and periplapoidal lymphatic tissue (PE) of white pulp [27, 28]. In the present study, both PA and PV, known as T-dependent areas, were CD4-positive zones surrounding GCs as well as the cecal tonsils, while CD8+ cells were observed only in the red pulp as noted in a previous report [3]. In the human, it is known that the periarterial lymphatic sheath (PALS) is CD4 positive and that the red pulp is a CD8 positive zone [11, 29]. It was thus demonstrated that chicken PA possessed similar function as mammalian PALS. In previous reports on domestic fowls, PV has been thought to be a T, B-independent zone as a result of surgical thymectomy and/or bursectomy, administration of immunosuppressive drugs and X-ray irradiation [27, 28]. However, it was suggested in the present study that PV was functionally equal to PA, because PV was the location forming GCs and a CD4 positive zone.

In the administration of alum-precipitated BSA as a T-dependent antigen, the changes of general histology were similar as those noted in previous reports by Ogata et al. [18]. However, the following two differences from the control group can be emphasized as summarized in Fig. 6b. First is the appearance of CD4+ cells in some GCs, and second the decrease of the number of CD8+ cells in the red pulp.

In mammalian peripheral lymphoid tissues, it has been accepted that the CD4+ cells appearing in GCs are involved in the activation of B lymphoblasts, transforming into plasma cell series even in conventional conditions [22]. However, in normal chickens CD4+ cells could not be detected in GCs, and after
immunization they were demonstrated for the first time in a few GCs. In the formation of chicken GCs, it has been suggested that B lymphoblasts originating from PE first migrate into PA and PV as helper T cell areas, proliferate in these areas, and then push away the reticulum cells organizing the nodular capsules [18]. During these processes, some antigen-presenting cells such as follicular dendritic cells migrate into GCs. It could be suggested from our results and previous reports that the antigen presentation from antigen-presenting cells to helper T cells was performed at the beginning of the process of GC organization or just before it, and that lymphokines secreted from helper T cells surrounding and/or in GCs then activate the differentiation and proliferation of B cells and the formation of GCs.

After the antigen administration in the present study, the number of splenic CD8+ cells decreased immediately and remained low throughout the experimental period. These immunohistochemical findings also corresponded to the results demonstrated by flow cytometry. However, the ratio of CD8+ cells in peripheral blood lymphocytes did not show any distinct changes. It is possible that because of enlargement of the lymphocytic pool in peripheral blood no significant change was demonstrated in the flow cytometrical analysis.

In conclusion, this study clarified that the distribution of T cell subsets had a constant pattern in chicken lymphoid tissues as in those of mammals.

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