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Chicken Peripheral Blood CD3⁺CD4⁻CD8⁻ Cells are Regulated by Endocrine and Nerve Systems

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ABSTRACT. The existence of CD3⁺CD4⁻CD8⁻ T cells in thymus and spleen has already been known. However, because of the presence of large amounts of thrombocytes in peripheral blood (PB), the proportion of CD3⁺CD4⁻CD8⁻ T cells in PB has yet to be investigated. Therefore, the proportion of peripheral T cell subsets was investigated in 6-week-old chickens. The percentage of CD3⁺ cells, CD4⁺ cells, CD8⁺ cells, CD8α⁺ cells, CD8β⁺, and CD3⁺CD4⁺CD8⁻ cells was 76%, 41%, 14%, 5%, and 15%, respectively. The proportion of CD3⁺CD4⁺CD8⁻ cells in PB increased during egg-laying periods and in chickens treated with an analog of estrogen, while it decreased with age and in response to restraint stress. All of the CD3⁺CD4⁺CD8⁻ cells expressed TCRα, and did not have NK activity. CD3⁺CD4⁺CD8⁻ cells represent about 60% of peripheral TCRα⁺ cells. These findings indicate that the proportion of CD3⁺CD4⁺CD8⁻ cells is regulated by the endocrine and nerve systems.

KEY WORDS: aging, avian CD3⁺CD4⁻CD8⁻ cell, endocrine, restraint stress.

Mammalian lymphoid cells can be distinguished according to their phenotype. T cells can be divided into subsets according to their antigen receptors; αβ-type TCR⁺ cells and γδ-type TCR⁺ cells. Mature αβ-T cells and γδ-T cells each fall into two subsets that are distinguished by the presence or absence of CD4 and CD8 molecules. These subsets are CD4⁺CD8⁻ and CD4⁻CD8⁺ cells, and CD4⁺CD8⁺ and CD4⁻CD8⁻ cells, respectively.

The chicken immune system has the same T cell subsets as mammalian systems [8]. The proportion of T cell subsets in chicken thymus and spleen has already been established [6]. However, because of the presence of large amounts of thrombocytes in peripheral blood (PB), the proportion of T cell subsets in PB remains uncertain. In the present study, we first analyzed the true proportions of T cell subsets in PB lymphocytes (PBL) using antibodies against thrombocytes and T cell subsets, and then confirmed the presence of CD3⁺CD4⁺CD8⁻ cells in PB. The literature contains little information on CD3⁺CD4⁺CD8⁻ cells in PBL [11, 19]. Therefore, we characterized CD3⁺CD4⁺CD8⁻ cells in PBL in this study.

Both the nerve and endocrine systems influence immune function, and these systems make up a well-organized homeostatic network. It is reported that the chicken immune system is enhanced during sexual maturation, and that treatment with estrogen increased chicken immune function [2, 3]. Therefore, changes in the proportion of CD3⁺CD4⁺CD8⁻ cells during sexual maturation and after treatment with diethylstilbestrol (DES), which is an estrogen analogue, were observed in this study.

We previously reported that serum corticosterone levels increased in response to restraint stress [13]. This result indicates that restraint stress provokes nervous stimulation in the chicken. Restraint stress acts as a stressor to the chicken, and modifies the immune system [13]. Therefore, changes in the proportion of CD3⁺CD4⁺CD8⁻ cells in response to restraint stress were also observed in this study.

NK activity plays an important role in innate immunity, and thus we also evaluated the NK activity of CD3⁺CD4⁺CD8⁻ cells.

MATERIALS AND METHODS

Animals: Congenic chickens [16] (H-B15 White Leghorn; Bu-1⁺) were used in this study. Chickens were bred in our animal facilities and provided with feed and chlorinated water ad libitum. Determination of sex in chickens of various ages was performed using polymerase chain reaction with a set of primers specific for the W chromosome [15].

Antibodies: Monoclonal antibody, HUKT (mouse IgG1, κ), against chicken thrombocytes was made by us [9]. FITC-labeled anti-CD3, PE-labeled anti-CD3, biotin-labeled anti-CD3, FITC-labeled anti-CD4, PE-labeled anti-CD4, FITC-labeled anti-CD8, PE-labeled anti-CD8, PE-labeled anti-CD8α, PE-labeled anti-CD8β, PE-labeled anti-TCRα, PE-labeled anti-TCRβ, PE-labeled anti-TCRγ, and PE-labeled anti-TCRδ antibodies were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL, U.S.A.).

Flow cytometric analysis: Chicken PB (1 ml) was taken from the wing vein into a heparinized syringe. Live peripheral blood mononuclear leukocytes (PBML) were isolated using a Ficoll-paque (Amersham Pharmacia Biotech, J. Vet. Med. Sci. 66(2): 143–148, 2004
Purification of T cell subsets: Chicken PB, taken from the wing vein, was collected into heparinized syringes. Live PBML were isolated using a Ficoll-paque with a density of 1.077 g/ml. PBML were incubated with PE-labeled anti-CD3, PE-labeled anti-CD4, PE-labeled anti-CD8β, or PE-labeled anti-CD8α antibodies on ice for 30 min. After washing, analysis of the double or the triple staining of CD3 + CD4 – CD8 – cells was measured as described above.

Treatment with restraint stress: Six chickens (age, 6 months) were housed individually in wire cages, placed in a temperature-controlled room (25°C) and light was provided from 8 am to 10 pm as previously described [13]. One day prior to restraint stress, blood (1 ml) was collected from each chicken at 3 pm. For restraint stress, both legs of each chicken were fixed with elastic string from 9 am until 3 pm. Changes in the lymphocyte subpopulation in PB at 3 pm were analyzed by flow cytometry. Experimental design on the treatment of stress was followed the Guideline for the Regulation of Animal Experimentation in Hiroshima University.

NK assay: NK assay was performed as previously described [13]. The LSCC-RP9 B lymphoblast cell line, which is derived from a tumor induced by the Rous-associated virus 2 [20], served as target cells. Target cells were labeled at 10⁶ cells/ml with FITC (50 μg/ml), suspended in 10% FBS-IMDM, and incubated for 30 min at 38°C. After washing three times with 10% FBS-IMDM, cell concentration was adjusted to 10⁵ cells/ml in 10% FBS-IMDM, including 5 × 10⁻⁶ M of 2-mercapto ethanol. Effector cells (5 × 10⁴ cells - 1 × 10⁶ cells/100 μl/well) were mixed in 96 wells of a round bottom plate at E/T ratios of 5:1, 25:1, or 50:1 with propidium iodide (PI; 200 ng/μl) to detect dead cells. After centrifugation at 50 × g for 3 min, the cells were incubated for 1 hr at 38°C. Flow cytometric analysis was carried out with the FACSCalibur™. Three thousand target cells (gating fraction of target cells) per sample were examined as previously described [13]. Maximum lysis and spontaneous lysis were carried out using target cells with and without Con A sup stimulation, respectively. Dead target cells (PI+ cells) were detected at FL3. Specific cytotoxicity (%) was calculated as follows,

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\text{Specific cytotoxicity} (%) = \left[\frac{\text{(% of dead target cells in the test} - \text{(% of spontaneous lysis)}}{\text{(% of maximum lysis} - \text{(% of spontaneous lysis)}}\right] \times 100
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RESULTS

Proportion of lymphocytes in chicken peripheral blood: Blood was drawn from 6-week-old and 20-month-old chickens. The PBML were gated as the HUK− fraction, and the proportions of CD3+, CD4+, CD8α+, CD8β+ and CD3−CD4−CD8− cells were measured using double or triple staining with HUKT. The percentages of HUKT−CD3+ cells, HUKT−CD4+ cells, HUKT−CD8α+ cells, HUKT−CD8β+ cells and HUKT−CD3+CD4+CD8− cells in 6-week-old chickens were 80.5%, 48.6%, 13.1%, 11.6%, and 14.0%, respectively, while those in 20-month-old chickens were 88.5%, 66.7%, 6.8%, 3.9%, and 5%, respectively (Table 1).

Changes in proportion of peripheral CD3+CD4−CD8− cells with age: Changes in the proportion of peripheral CD3+CD4−CD8− cells with age were observed using 3-week-old, 6-week-old, 6-month-old, 18-month-old, and 30-month-old female chickens. The percentage of CD3+CD4−CD8− cells gradually increased in sexually maturated female chickens (at 6-week-old and 6-month-old), but decreased in aged female chickens (at 18-month-old and 30-month-old) (Fig. 1).

Changes in proportion of peripheral CD3+CD4+CD8− cells after treatment with DES: Chicken PB were taken from the young female and male chickens, injected with DES. The proportions of CD3+CD4+CD8− cells were measured at days 0, 1, 2, 7 and 14 after the first injection. The percentage of the CD3+CD4+CD8− cells in female chickens was elevated at 2 days after treatment with DES (Fig. 2a), but no change was seen in male chickens (Fig. 2b).

Proportion of CD3+CD4+CD8− cells in the TCR1+ fraction: TCR1+ cells were purified, and the proportion of CD3+CD4+CD8− cells in those was observed. The percentage of CD3+CD4+CD8− cells among TCR1+ cells was 62.2% (Fig. 3).

Proportion of TCR1+ cells in the CD3+CD4−CD8− fraction: CD3+CD4−CD8− cells were purified, and the proportions of TCR1+, TCR2+, and TCR3+ cells in those were observed. The percentages of TCR1+, TCR2+, and TCR3+ cells were 95.2%, 0.4%, and 0.6%, respectively (Table 2).

Changes in proportion of peripheral CD3+CD4+CD8− cells after restraint stress: Chickens were housed individually in wire cages, and were treated with restraint stress. After restraint stress, the percentages of CD4+ cells (39%), CD8α+ cells (14%), and CD8β+ cells (7%) decreased to 29%, 7%, 3%, respectively. The percentages of CD3+ cells (77%) and CD3−CD4−CD8− cells (29%) decreased dramatically to 39% and 9%, respectively (p<0.05). Therefore, the decrease in the proportion of CD3+ cells after restraint stress seems to be the result of the decrease in CD3+CD4−CD8− cells (Fig. 4).

NK activity of CD3+CD4−CD8− cells: Thrombocytes, CD8− cells, CD4+ cells and CD3+CD4−CD8− cells were puri-
fied using the MACS system and their NK activities were measured (Fig. 5). The purification levels of thrombocytes, CD8⁺, and CD4⁺ cells were >99%, >98%, >98%, respectively. Chicken peripheral CD8⁺ cells had strong NK activity, while thrombocytes did not. Peripheral CD3⁺CD4⁻CD8⁻ cells had little cytotoxic activity at E/T 50.

**NK activity of CD3⁺CD4⁺CD8⁻ cells after stimulation with ConA-sup:** To evaluate the stimulation of NK activity by the cytokine IL-2, Con A-sup, in place of IL-2, was added to the CD8⁺ and CD3⁺CD4⁺CD8⁻ cell cultures. The supernatant of Con A-activated splenocytes enhanced the NK activity of PB CD8⁺ cells, but had no effect on the NK activity of CD3⁺CD4⁺CD8⁻ cells (Fig. 6).

**DISCUSSION**

The proportion of T cell subsets in chicken thymus and spleen has already been established [6]. However, because large amounts of thrombocytes are present in PB, the proportion of T cell subsets in PB remains uncertain. The proportion of peripheral CD3⁺ cells in aged chickens was...
higher than that in egg-laying chickens. The proportion of peripheral CD4$^+$ cells in aged chickens also tended to be high. The proportions of peripheral CD8$^+$ cells and CD8$^{αβ}$ cells in aged chickens tended to be low. It is well understood that proportion of peripheral CD8$^+$ cells in aged chickens is low. The percentages of T cell subsets observed in this study were higher than those reported previously [6, 16]. This discrepancy may be due to the effects of mixed thymocytes.

The presence of CD3$^+$ CD4$^-$ CD8$^-$ cells in the thymus and spleen has already been established [6, 7, 14]. In the present study, we demonstrated the proportion of peripheral these cells in PBL. Because PBL in this study did not contain thymocytes, the above proportion should be true. It is known that the mouse $αβ$ T-cell subset has a third phenotype, CD3$^+$ CD4$^-$ CD8$^-$ cells, and these cells are thought to be NK cells [12]. In chicken, however, Göbel reported that TCR1$^+$ CD3$^+$ cells ($γδ$-TCR) in PB may or may not have the CD8 antigen [6]. In our experiment, 62% of TCR1$^+$ cells in PBL were CD3$^+$ CD4$^-$ CD8$^-$, and almost all CD3$^+$ CD4$^-$ CD8$^-$ cells were TCR1$^+$. This suggests that there are two types of TCR1$^+$ cell in chicken PBL, about 40% of TCR1$^+$ cells express the CD8 antigen, and the other 60% are CD3$^+$ CD4$^-$ CD8$^-$ and are sensitive to stress.

In this study, we observed an increase in levels of CD3$^+$ CD4$^-$ CD8$^-$ cells in sexually mature or young DES (an analogue of estrogen)-treated female chickens, and a decrease in levels of these cells in chickens following restraint stress. The magnitude of this restraint stress gave rise to a transient increase in serum corticosterone levels [13]. These changes lead to a decrease in CD3$^+$ CD4$^-$ CD8$^-$ cells. Because levels of these cells were elevated in sexually mature female chickens, the cell population seems to be regulated by sex hormones. Arstila et al. reported that treatment with DES did not affect the proportion of T cell subsets in female chickens [1]. However, DES treatment of 6-week-old female chickens increased the proportion of this subset in PB in this study. The discrepancy between their results and ours may be explained by the purity of lymphocytes in PBL.

Stress, such as that induced by heat or restraint, disrupts the homeostatic network and down regulates the immune system [21]. It is well known that secretion of catecholamines and corticosterone is induced by stress [5, 10] and that corticosterone has an inhibitory effect on the immune system [4]. Heat, cold, overpopulation, handling, noise, and restraint act as stressors for chickens, and modify immune function [21]. In this study, chickens were subjected to restraint stress for 6 hr. The magnitude of this stress was enough to increase transient serum corticosterone and catecholamine levels [13]. When the chickens were released at the end of 6 hr, levels of the above indicators had deceased [13]. While this treatment was not harmful for the chickens, it was sufficient to induce stress in them.

Restraint stress dramatically suppresses NK activity in mice [10]. In this study, CD3$^+$ CD4$^-$ CD8$^-$ cells in chicken PB were sensitive to restraint stress. Therefore, determination of NK activity of CD3$^+$ CD4$^-$ CD8$^-$ cell was important in order to investigate the role of this subset. However, CD3$^+$ CD4$^-$ CD8$^-$ cells did not possess NK activity, even after treatment with Con-A sup.

CD3$^+$ CD4$^-$ CD8$^-$ cells are observed in Marek’s disease herpes virus-transformed lymphoblastoid tumor cell lines [18]. The reason for the appearance this subset is not yet understood. In humans, the proportion of CD3$^+$ CD4$^-$ CD8$^-$ cells is higher in patients with SLE [19]. Human CD3$^+$ CD4$^-$ CD8$^-$ cells express CD40L, produce IL-4 and IFN-γ, and assist in antibody production through the CD1c molecules on their surface [19]. In severely handicapped children, Kawano et al. reported increases in the number of CD3$^+$ CD4$^-$ CD8$^-$ cells in those with elevated serum IgG levels [11]. The two reports above indicate that CD3$^+$ CD4$^-$ CD8$^-$ cells might have a helper effect in the immune response. Chicken peripheral CD3$^+$ CD4$^-$ CD8$^-$ cells may also have this function. It should be noted that the proportion of CD3$^+$ CD4$^-$ CD8$^-$ cells in chicken with elevated serum levels of both IgG and IgM [17] was not high in our pilot study (n=2). The role of CD3$^+$ CD4$^-$ CD8$^-$ cells in chicken PB is not yet clear.

In conclusion, the proportion of peripheral T cell subsets was measured. We demonstrated that the proportion of peripheral CD8$^+$ cells in aged chickens is low. We also showed the presence of CD3$^+$ CD4$^-$ CD8$^-$ TCR1$^+$ cells in PB. Furthermore, changes in the proportion of these cells in PB appear to be regulated by the nerve and endocrine systems. The role of CD3$^+$ CD4$^-$ CD8$^-$ TCR1$^+$ cells remains to be elucidated.

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