Antibody production against a T cell-dependent antigen (goat red blood cell) and a T cell-independent antigen (killed *Brucella abortus*) was examined in chicks treated with estrogen in which eggs were dipped into ethyl alcohol solution containing β-estradiol. Relative weights (organ weights/body weight) of the primary lymphoid organs of chicks were also determined. Secondary antibody production against goat red blood cells (GRBC) was significantly increased by treating chick embryos with high doses of estrogen (1.0% solution) on the 4th day of embryogenesis. Primary and secondary antibody responses against killed *Brucella abortus* (BA) were dose-dependently increased with estrogen treatment on the 4th day of embryogenesis. In contrast, primary and secondary responses against BA of chicks treated with estrogen on the 14th day of embryogenesis were significantly suppressed. Thymus weight significantly increased in chicks treated with estrogen on the 4th day of embryogenesis. The rate of CD4-positive cells in the chick thymocytes significantly increased with estrogen treatment on the 4th day of embryogenesis. On the other hand, the weight of the bursa of Fabricius significantly decreased when treating chick embryos with high doses of estrogen (1.0%) on the 14th day of embryogenesis. Results from the present study indicated that estrogen treated at different stages of embryogenesis exerts discrete effects on B cell differentiation in the bursa and helper T cell differentiation in the thymus of chick embryos.

**Key words**: estrogen, chick embryo, antibody production, bursa of Fabricius, helper T cells

**Introduction**

It is well known that sex steroid hormones and glucocorticoids affect on immune functions. Of the steroid hormones, androgen has been reported to suppress B cell differentiation, production of immunoglobulins and T cell differentiation and maturation (Lahita, 1990). In chickens, disappearance or marked involution of the bursa of Fabricius and suppression of antibody production were induced in chicks by treating them with androgen during their embryonic period (Glick and Sadler, 1961). Injection of testosterone into chicks induced a decrease of lymphocytes at the cell differentiation stage in the bursa (Kondo et al., 1987).

On the other hand, it has been reported that the estrogen influence on the immune systems of animals varies. For example, estrogen enhanced rat humoral immunity.
(Erbach and Bahr, 1991), differentiation, and cytotoxic activity of human NK cells (Sorachi et al., 1993), but suppressed cell-mediated immunity in mice (Luster et al., 1984) and thymus epithelial cell differentiation in rats (Sakabe et al., 1994). The effect of estrogen differed depended on its concentrations. Phytohemagglutinin (PHA)-induced activation of chicken peripheral blood lymphocyte (PBL) in vitro was enhanced by low concentrations of estrogen, while high concentrations suppressed PBL activation by PHA (Kondo et al., 1994). The effect of estrogen on T cell differentiation in the mouse thymus is reported to be different depending on the differentiation stage of thymocytes and the administration periods of estrogen (Screpanti et al., 1989). These results indicate that the effects of estrogen to the immune system are complex, and a concrete conclusion regarding these effects can not be made.

The aim of this study is to establish the effect of estrogen treatment before (the 4th day of embryogenesis) or after entrance and colonization of lymphoid stem cells into the bursal primordium (14th day of embryogenesis) (Houssaint et al., 1976) on chick antibody production against T cell-dependent antigen and T cell-independent antigen.

Materials and Methods

Estrogen treatment: Fertilized eggs of the White Leghorn Shaver strain were incubated at 38°C and 80% humidity. Eggs were separated into 2 groups. Estrogen treatment was executed by dipping for 5 sec one group of eggs in their 4th day of incubation into respective containers of 100 ml of ethyl alcohol and 0.05 g, 0.1 g, 0.25 g, 0.5 g or 1.0 g β-estradiol (Sigma Chemical Co.). Another group of eggs were treated with estrogen on the 14th day of incubation by dipping the eggs for 5 sec into respective containers of 100 ml ethyl alcohol and 0.25 g, 0.5 g or 1.0 g β-estradiol. Control eggs were treated only with ethyl alcohol. It has been reported that testosterone (Glick and Sadler, 1961) and estrogen (Verheul et al., 1986) were successfully administered into chick embryos by dipping embryonated eggs for a few sec. Eggs were re-incubated after treatment, and hatched chicks were used for subsequent experiments. Chicks and experimental conduct were in accordance with the Guidelines for Animal Experiments of Okayama University Advanced Science Research Center.

Immunization, antibody titration and histological examination: Two weeks after hatching, chicks were weighed and injected intravenously with primary immunization of 0.2 ml of 4% goat red blood cells (GRBC)/100 g body weight (BW) and 0.1 ml of 1% killed Brucella abortus (BA)/100 g BW. One week after primary immunization, chicks were weighed and blood samples were collected. Then, GRBC and BA were injected intravenously. A week after the second immunization, blood samples were collected and chicks were anesthetized with 0.4 ml/100 g BW of pentobarbital sodium (Nembutal, Dainippon Pharmaceutical) and killed by exsanguination. Each chick’s bursa of Fabricius and thymus were sampled and weighed. The organs were fixed in Bouin fixative and embedded in paraffin. Sections were cut at 5 μm and stained with hematoxylin and eosin.

GRBC-agglutination antibody titers and BA-agglutination antibody titers in the sera sampled at a week after each immunization were measured by micro-titration.
Antibody titers were shown as dilution rates of the sera.

Measurement of CD4- and CD8-positive cell rates in the thymocytes of chicks: The thymus was collected from 4-week old chicks that were treated for 5 sec on the 4th day of incubation with the 100 ml ethyl alcohol containing 0.25 g or 1.0 g β-estradiol. Lymphocytes were isolated from the thymus as described previously (Kondo et al., 1987). Rates of CD4-positive and CD8 positive lymphocyte were determined by immunofluorescence using mouse anti-chicken CD4 antibody and mouse anti-chicken CD8 antibody (Southern Biotechnology Associates, Inc.) as the primary antibodies, and FITC-labeled goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc.) as the secondary antibody.

Statistical analysis: Nine to 14 chicks were used in each experimental group. Variance analysis was used to determine the significance between mean values of estrogen-treated chicks and control chicks. Significance was determined at the 5% level. Results are given as means and standard errors.

Results

1. Antibody titers

Antibody titers of chicks treated with estrogen on the 4th day of embryogenesis are shown in Fig. 1 and Fig. 2. Estrogen treatment did not exert any influence on primary responses against GRBC. Secondary responses tended increase with estrogen, and the titers of chicks treated with 1.0% estrogen solution were significantly higher than the control titers (p < 0.05). Primary and secondary responses against BA increased with estrogen treatment in a dose-related fashion (Fig. 2). Anti-BA titers significantly

![Graph](image-url)

Fig. 1. Anti-goat red blood cell (GRBC) antibody titers in the sera of chicks treated with β-estradiol on the 4th day of embryogenesis. Antibody titers were shown as dilution rates of the sera. Open columns and shadowed columns represent titers in primary responses and secondary responses, respectively. * = Mean significantly differs from the control mean (p < 0.05).
increased in chicks treated with estrogen solutions above 0.25% (0.25% : p<0.05, 0.5% and 1.0% : p<0.01).

Antibody titers of chicks treated with estrogen on the 14th day of embryogenesis are shown in Fig. 3 and Fig. 4. Antibody titers against GRBC in the primary and secondary responses did not change with estrogen treatment (Fig. 3). In contrast to chicks treated with estrogen on the 4th day of embryogenesis, antibody titers against BA

Fig. 2. Anti-Brucella abortus (BA) antibody titers in the sera of chicks treated with β-estradiol on the 4th day of embryogenesis. Antibody titers were shown as dilution rates of the sera. Open columns and shadowed columns represent titers in primary responses and secondary responses, respectively. ** = Mean significantly differs from the control means (p<0.01), * = p<0.05.

Fig. 3. Anti-GRBC antibody titers in the sera of chicks treated with β-estradiol on the 14th day of embryogenesis. Antibody titers were shown as dilution rates of the sera. Open columns and shadowed columns represent titers in primary responses and secondary responses, respectively.
decreased in chicks treated with estrogen on the 14th day of embryogenesis (Fig. 4). The primary response of chicks treated with 0.25% estradiol solution (p < 0.05) and the secondary response of chicks treated with 0.25% and 1.0% estradiol solution (p < 0.05) were significantly lower than the associated controls.

2. Organ weight and histological change

Relative weights (mg/100 g BW) of the bursa and thymus from estrogen-treated chicks are shown in Fig. 5. Bursa weights did not change in chicks treated with estrogen on the 4th day of embryogenesis. On the other hand, thymus weights significantly increased (0.05% and 1.0% : p < 0.05, 0.1%, 0.25% and 0.5% : p < 0.01) with estrogen treatment on the 4th day of embryogenesis (Fig. 5-A). In chicks treated with estrogen on the 14th day of embryogenesis, bursa weights decreased and the mean value of chicks treated with 1.0% estrogen solution differed significantly (p < 0.05) from those in the controls (Fig. 5-B). On the other hand, thymus weights did not change (Fig. 5-B). Any histological changes due to estrogen treatment in the bursa and thymus were not observed (data not shown). Throughout the experiment, differences in body weights between estrogen-treated chicks and control chicks were not observed throughout the experiment (data not shown).

3. Rates of CD4-positive cells and CD8-positive cells in thymocytes

CD4-positive cell rates in thymocytes were elevated significantly (p < 0.05) with estrogen treatment on the 4th day of embryogenesis, as shown in Fig. 6. On the other hand, CD8-positive cell rates decreased. The mean value of 1.0% estradiol solution-treated chicks differed significantly (p < 0.05) than the control value (Fig. 6). The sum of CD4-positive rates and CD8-positive rates was more than 100% in all groups. This

![Anti-BA antibody titers in the sera of chicks treated with β-estradiol on the 14th day of embryogenesis. Antibody titers were shown as dilution rates of the sera. Open columns and shadowed columns represent titers in primary responses and secondary responses, respectively. * = Mean significantly differs from the control mean (p < 0.05).](image)
indicates the presence of CD4- · CD8- double positive cells in the thymus of chicks (Chan et al., 1988).

**Discussion**

This study indicates that the effect of estrogen treatment during embryogenesis on antibody production of hatched chicks is different depending on the day during embryogenesis that estrogen treatment is administered.

Because testosterone (1.0% solution) treatment on the 5th day of embryogenesis has been reported to induce bursa disappearance after hatching, the treatment is known as chemical bursectomy (Glick and Sadler, 1961). Antibody production was severely inhibited in these testosterone-treated chicks. In contrast, estrogen treatment on the 4th day of embryogenesis elevated in a dose-related manner antibody production against foreign erythrocytes and killed bacteria. These results indicate that estrogen and androgen treatment during the 4th to 5th days of embryogenesis exert reverse effects on
antibody production in hatched chicks.

Bursal estrogen receptors and androgen receptors have been found in chick embryos (Gasc and Stumpf, 1981) and in 1- to 17-week old chicks (Sullivan and Wira, 1979) through binding assay using radioactive ligands. Bursal sex steroid receptors and signal pathways through the receptors are believed to be involved in the sex steroid effect on B cell differentiation in the bursa, and the resulting changes in antibody production. Lymphoid cells are absent in the bursa of 4-day-old chick embryos, because entrance and colonization of lymphoid stem cells into the bursal primordium takes place in chick embryos during days 8 through 14 of incubation (Houssaint et al., 1976). Estrogen is believed to elevate antibody production in chicks treated with estrogen on the 4th day of embryogenesis through acting on cells other than lymphoid cells.

The network of reticuloepithelial cells in bursal lymphoid follicles plays essential role as a major component in the microenvironment for B cell differentiation in the bursa (Eerola et al., 1987). In addition to lymphoid cells, epithelial cells have been reported to be target cells for estrogen and androgen in the bursal basic components (Sullivan and Wira, 1979), although a presence of target cells in bursal epithelial components has not yet been detected. Thus, it is estimated that antibody production after hatching is influenced by sex steroid hormones affecting the reticuloepithelial network of the bursal lymphoid follicles.

Chick thymus weights were significantly increased by estrogen treatment on the 4th day of embryogenesis, although bursal weights were not changed. The increase in thymus weight may reflect augmentation of T cell proliferation in the chick thymus, because thymus histological features are common in both estrogen-treated chicks and
controls. CD4-positive cell rates in the thymus were significantly elevated with estrogen treatment. These results present 2 possibilities: 1) estrogen treatment on chick embryos during the 4th day of embryogenesis induced both elevations of B cell differentiation and activation, and 2) the treatment resulted in antibody production by increasing the number of helper T cells through estrogen influence on the thymus. Estrogen receptors were founded in human thymocytes (Daniel et al., 1983) and in the human thymus matrix (Stimson, 1988) and in epithelial cell components of the mouse thymus (Luster et al., 1984). However, estrogen receptors in the chicken thymus have not yet been reported. Entrance and colonization of lymphoid stem cells in the thymus of chick embryos are known to occur during restrictive periods of embryogenesis. The same is true for the bursa. Lymphoid stem cells first enter the thymus when chick embryos are 6.5 days old (Jotereau et al., 1980; Le Douarin et al., 1984). This suggests that estrogen treatments in 4 day-old chick embryos act on the thymus matrix such as its epithelial cell components. Thymic epithelial cells form a network of reticuloepithelial cells, as do lymphoid follicles of the bursa. The network contributes to T cell differentiation and proliferation (Frazer, 1973). In rats, estrogen elevated humoral immune reactions which are reported to be due to its effect on the thymus (Erbach and Bahr, 1991). An acceleration of helper T cell differentiation and proliferation in the thymus of 4-day old chick embryos treated with estrogen is a possible factor for the elevation of antibody production in the present study.

Estrogen treatment on the 14th day of embryogenesis significantly reduced chick antibody production against BA. However, anti-GRBC antibody production was not affected with estrogen treatment. Among the antigens used in this experiment, GRBC and BA are known as a T cell-dependent antigen and a T cell-independent antigen, respectively. The reduction of anti-BA antibody production suggests that estrogen treatment in this period of embryogenesis has a suppressive effect mainly on the bursa. A reduction in only the bursal weight supports this suggestion. Because entrance and colonization of lymphoid cells have already started in 14-day old chick embryos (Houssaint et al., 1976), lymphoid cells and BF matrix, such as epithelial components were present in the bursa. Estrogen receptors were identified in the bursa (Fassler et al., 1986) and bursal epithelial components (Sullivan and Wira, 1979), although bursal lymphoid cells have not yet been demonstrated to be positive for estrogen receptor. However, the presence of estrogen receptor in bursacytes is possible since the receptor was found in mouse B lymphocytes (Luster et al., 1984). Estrogen treatment on 14-day-old chick embryos probably influenced the bursal lymphoid cells to reduce proliferation activity and functional levels, which resulted in a reduction of antibody production in chicks.

It is of interest that a sex steroid hormone exerts discrete effects on humoral immune functions depending on the developmental stage of the chick embryo. Research is needed on the mechanism of estrogen’s discrete effects.

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