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Organ-specificity of Estrogen Effects on the Induction of Immunocompetent Cells in the Chicken

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Injection of immature hens with estrogen increases the population of antigen-presenting cells, T cells and Ig-containing cells in the ovary and oviduct. The aim of this study was to determine the organ-specificity of estrogen effects on the localization of immunocompetent cells in the chicken. Immature hens were injected with or without diethylstilbestrol, which is an estrogenic compound, for 3 or 6 days and their vagina, ovary, liver, duodenum and lung were collected. Paraffin or frozen sections of them were immunostained for major histocompatibility complex (MHC) class II, CD3 (T cell antigen), Bu-1 (premature B cell antigen) and IgG, respectively. All four types of immunocompetent cells, namely MHC class II^+, $CD3^+$, $Bu\text{-}1^+$ and IgG^+ cells, were observed in the sections of each tissue. Treatment with diethylstilbestrol increased the population of these immunocompetent cells in the vagina, ovary and liver that are estrogen target organs, but not in the duodenum and lung. We suggest that estrogen increases immunocompetent cells in an organ-specific manner in the chicken.

Key words : diethylstilbestrol, immunocompetent cells, organ-specificity, chicken

Introduction

The immune functions in the reproductive organs are essential for the defense to foreign agents and production of hygienic eggs in chickens. The oviduct contains estrogen receptors (Kusuhara and Ohashi, 1991), and its growth and functions are stimulated by estrogen (Burke, 1984). The hen ovary is also one of the estrogen target sites that contain estrogen receptors (Yoshimura *et al.*, 1995). Recently we found that treatment of immature hens with diethylstilbestrol (DES), which is an estrogenic compound, increased the population of major histocompatibility complex class II-positive (MHC class II^+) cells in the oviduct and ovary (Zheng *et al.*, 1998 ; Barua and Yoshimura, 1999b). Stimulation of chicken by DES also increased T cells and Ig-containing cells in both the oviduct (Khan *et al.*, 1996 ; Zheng *et al.*, 1997, 1998) and the ovary (Barua *et al.*, 1998 ; Barua and Yoshimura, 1999a). These results suggest that estrogen stimulates the induction of immunocompetent cells in the repro-

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ductive organs. However, it is unknown whether the increase of immunocompetent cells by estrogen occurs specifically in the reproductive organs. The purpose of this study was to determine whether there is an organ-specificity in estrogen effects on the localization of immunocompetent cells in the chicken. Changes in the immunocompetent cell frequencies in the vagina, ovary and liver, which are rich in estrogen receptors (Yoshimura *et al.*, 1995 ; Isola, 1990 ; Wolfson, 1981-82), and the duodenum and lung, which are considered to be non-target organs of estrogen, were examined in the immature hens stimulated with DES.

Materials and Methods

Treatment of birds and collection of samples

A total of 25 White Leghorn immature hens, approximately 110 d old, were used in this study. They were kept in individual cages under a lighting condition of 14 h light : 10 h dark, and were provided with *ad libitum* access to water and a commercial grower ration. The birds were injected *i.m.* daily with 1 mg DES (Nacalai Tesque. Inc., Kyoto) or 0.1 m*l* vehicle for 3 or 6 d. DES was dissolved in sesame oil (vehicle) at a concentration of 10 mg/m*l*. The vagina, ovary, liver, duodenum and lung were collected from the birds killed by decapitation before or 24 h after the Day 3 or Day 6 injection. A part of each organ was fixed in 10% formalin in PBS for the preparation of paraffin sections for the detection of IgG-containing (IgG^+) cells. The other part was embedded in OCT compound (Miles Inc., Elkhart, IN), snap-frozen in a mixture of isopentane and solid carbon dioxide, and stored at −80℃ until preparing cryostat sections for the immunocytochemistry of MHC class II^+ cells, T and B cells.

Immunocytochemistry

For the immunostaining of IgG^+ cells, deparaffinized sections (6μm thick) were washed in running water for 10 min and in PBS for 15 min (5 min×3 times). Cryosections (6μm thick) were air-dried on the slides pretreated with 2% (vol/vol) 3 -aminopropyl triethoxysilane in acetone and fixed with acetone at 4℃ for 10 min, and air-dried again. They were then rinsed with PBS for 5 min, immersed in 0.3% (vol/vol) H_2O_2 in methanol for 30 min to block the endogenous peroxidase activity, followed by washing in PBS for 15 min (5 min×3 time). All the sections were incubated with 1% casein milk (Boehringer Mannheim GmbH, Germany) for 30 min to block the non-specific bindings of antibodies. Then they were incubated at room temperature for 2 h with the specific primary antibodies, namely, mouse anti-chicken MHC class II (1 : 100 dilution, Veromaa *et al.*, 1988 b), mouse anti-chicken CD 3 (1 : 500 dilution, Southern Biotech Associates Ins., Birmingham, AL, USA), mouse anti-chicken Bu-1 that recognizes premature B cells but not plasma cells (1 : 500 dilution, Veromaa *et al.*, 1988 a), and mouse anti-chicken IgG (1 : 200 dilution, Nishinaka *et al.*, 1996), respectively. After washing in PBS (5 min×3 times), the immunoreactions were detected by using a S-HRP staining kit for the detection of mouse IgG, IgA and IgM (Seikagakukougyo Co., Tokyo) according to the manufacturer's instruction. Finally, the sections were visualized by incubation with a mixture of 0.02% (wt/vol) 3, 3′-diaminobenzidine tetrahydrochloride and 0.005% (vol/vol) H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6.

Slides were counterstained with haematoxylin, dehydrated with alcohol, cleared with xylene and covered. The control sections were prepared by the same procedure except that the primary antibodies were replaced with normal mouse IgG. No positive staining was observed in control slides.

Observations and analysis of data

The population of MHC class II^+ cells, CD 3-positive ($CD3^+$) cells, Bu-1-positive ($Bu\text{-}1^+$) cells and IgG^+ cells was analyzed by an image analysis computer system, MacAspect (Mitani Co., Fukui, Japan) under a light microscope as described previously (Zheng *et al.*, 1998). The number of positive cells was counted within the subepithelial stroma in the vagina and within the stroma in the ovary. Since sections of the chicken liver showed a homogeneous structure, the positive cell number was counted in the parenchyma. The number of positive cells in the duodenum sections was counted in the lamina propria (within 50μm of the boundary of the absorptive epithelium), and that in the lung was done in the parabronchus wall in the tertiary bronchi region, which formed the main mass of the lung. In each section, the number of positive cells was counted in two different areas (approximately 15,000–20,000μm^2 each). The average of the two counts was calculated and expressed as the number of cells in 10,000μm^2 of one section. The significance of difference in numbers of cells among treatment days was examined by one-way ANOVA followed by Duncan's multiple t test, and that between the control and DES-treated birds was examined by Student's t test. $P<0.05$ was considered to be significant.

Results

The oviduct weight of the control birds injected with oil showed a small increase during the experiment period, which was possibly a spontaneous oviducal growth because the age of the birds was 110–117 days old. In contrast, treatment of immature

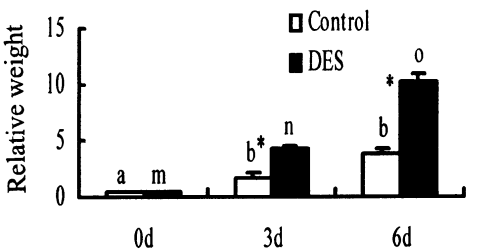

Fig. 1. Relative weight of the oviduct of immature hens treated with or without diethylstilbestrol (DES). Each bar represents the mean±SEM of the relative weight per 1,000 g body weight (n=5 birds for each bar). Within the same treatment, bars with different grouped superscripts ($^{a/b}$ for control birds, $^{m/n/o}$ for DES-treated birds) are significantly different ($P<0.05$). An asterisk indicates that the difference between the control and DES-treated birds is significant ($P<0.05$). 0 d : before treatment ; 3 d : treated for 3 days ; 6 d : treated for 6 days.

hens with DES for three or six days caused a much greater increase in the weight of the oviduct, and there were significant differences between the control and DES-treated birds on Days 3 and 6 (Fig. 1). Histological examination showed that the mucosal tissue of the vagina was undeveloped in the control birds on Day 6, whereas it was well developed with the appearance of many secondary folds in the DES-treated birds on Day 6 (data not shown). These results indicate that the DES injection in the current study exerted a significant estrogenic effect.

Figure 2 shows the changes in immunocompetent cell population in the vagina of immature hens treated with or without DES. MHC class II^+, $CD3^+$, $Bu\text{-}1^+$ and IgG^+ cells were mainly localized in the mucosal tissues, with a greater population in the

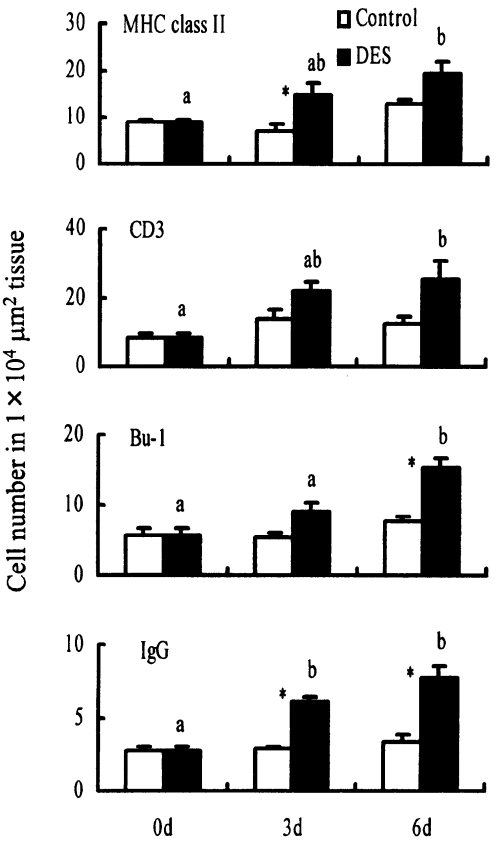

Fig. 2. Frequencies of immunocompetent cells in the vagina of immature hens treated with or without diethylstilbestrol (DES). Each bar represents the mean ±SEM of the positive cells in 10,000μm^2 tissue (n=5 birds in each bar). Bars with different superscripts are significantly different ($P<0.05$). An asterisk indicates the significant difference between the control and the DES-treated birds ($P<0.05$). 0d : before treatment ; 3d : treated for 3 days ; 6d : treated for 6 days.

subepithelial stroma. The treatment of immature hens with DES for 3 days significantly increased the number of IgG^+ cells, and that for 6 days significantly increased all four types of immunocompetent cells compared with the birds before treatment (Day 0). There was also a significant difference in the population of MHC class II^+ and IgG^+ cells between the control and the DES-treated birds on Day 3, and in that of $Bu\text{-}1^+$ and IgG^+ cells on Day 6.

The ovarian stroma consisted of connective tissue in which primary follicles were embedded. MHC class II^+, $CD3^+$, $Bu\text{-}1^+$ and IgG^+ cells were localized in the ovarian stroma and the thecal layer of primary follicles. Changes in the population of ovarian immunocompetent cells are present in Fig. 3. As compared with the birds before treatment, the population of $CD3^+$ cells was increased by DES treatment for 3 days, and that of MHC class II^+ cells was increased by DES treatment for 6 days. There was also significant difference in the population of $CD3^+$ and IgG^+ cells between the control and the DES-treated birds on Day 3, and in that of MHC class II^+ and $CD3^+$ cells on

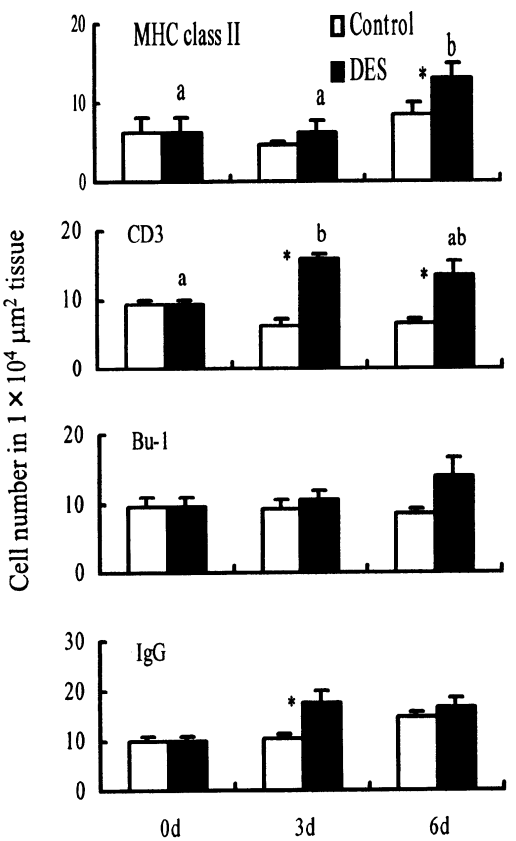

Fig. 3. Frequencies of immunocompetent cells in the ovary of immature hens treated with or without diethylstilbestrol (DES). Bars, superscripts and asterisks represent the same as in Fig. 2.

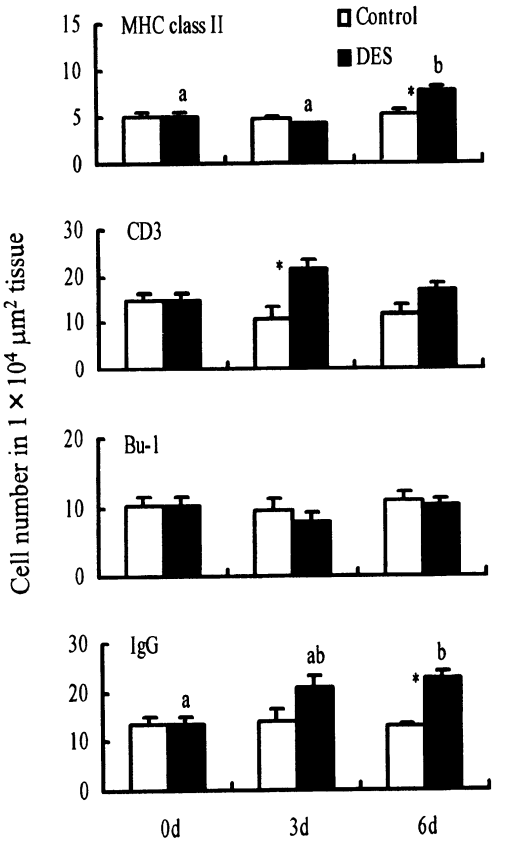

Fig. 4. Frequencies of immunocompetent cells in the liver of immature hens treated with or without diethylstilbestrol (DES). See Fig. 2 for further explanations.

Day 6.

MHC class II$^+$, CD 3$^+$, Bu-1$^+$ and IgG$^+$ cells were all localized in the liver sections from the immature hens treated with or without DES. Treatment of immature hens with DES for 6 days significantly increased the population of MHC class II$^+$ and IgG$^+$ cells compared with that before the treatment (Fig. 4). There was also significant difference in the population of CD 3$^+$ cells between the control and the DES-treated birds on Day 3, and in that of MHC class II$^+$ and IgG$^+$ cells on Day 6 (Fig. 4).

In the duodenum and lung, histological differences were not observed between the control and DES-treated birds. MHC class II$^+$, CD 3$^+$, Bu-1$^+$ and IgG$^+$ cells were found mainly in absorptive mucosal region, especially in the lamina propria, and they were also frequently observed in the mucosal epithelium of the villi. No significant difference was found in the population of immunocompetent cells in the lamina propria of the duodenum in the DES-treated birds, or between the control and DES-treated

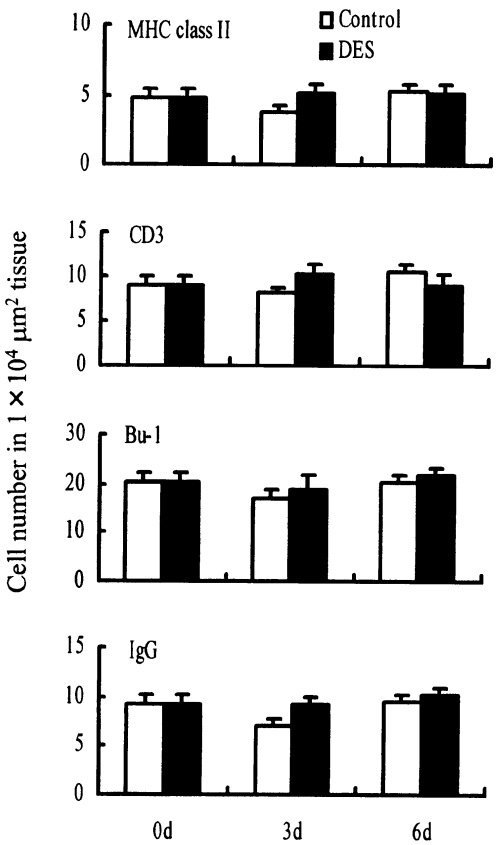

Fig. 5. Frequencies of immunocompetent cells in the duodenum of immature hens treated with or without diethylstilbestrol. Asterisks, bars and superscripts represent the same as in Fig. 2.

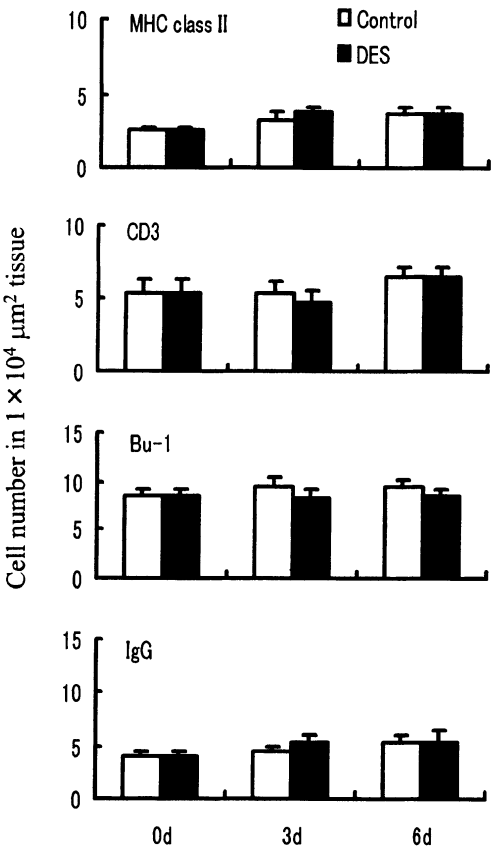

Fig. 6. Frequencies of immunocompetent cells in the lung of immature hens treated with or without diethylstilbestrol. See Fig. 2 for further explanations.

birds (Fig. 5). Also in the lung, the injection of immature hens with DES did not cause significant changes in the frequencies of MHC class II^+, $CD3^+$, Bu-1^+ and IgG^+ cells compared with the control birds (Fig. 6).

Discussion

The current results suggest that DES, an estrogenic compound, increased MHC class II^+, $CD3^+$, Bu-1^+ and IgG^+ cells in the vagina, ovary and liver, but not in the duodenum and lung in the chicken.

In the current study, MHC class II^+, $CD3^+$, Bu-1^+ and IgG^+ cells were localized in the vagina, ovary, liver, duodenum and lung, suggesting that immunocompetent cells play a role in the local defense functions in these organs. Injection of DES to immature hens increased the population of immunocompetent cells in the vagina and ovary, which supports our previous findings (Zheng *et al.*, 1997, 1998 ; Barua *et al.*, 1998 ; Barua and Yoshimura, 1999 a and b). Furthermore, significant increases in the population of MHC class II^+, $CD3^+$ and IgG^+ cells also occurred in the liver of DES-treated immature hens, which suggests that estrogen affects the localization of immunocompetent cells in the liver. On the other hand, no significant difference was observed in the population of immunocompetent cells in the duodenum and lung between the control and DES-treated birds in this study. Therefore, it is likely that estrogen increases the immunocompetent cell population in the tissues, but the rate of the increase is different among the organs.

There are reports that the ovary, oviduct and liver contain estrogen receptors (Yoshimura and Bahr, 1995 ; Yoshimura *et al.*, 1995 ; Isola, 1990 ; Kusuhara and Ohashi, 1991 ; Wolfson, 1981–82). In contrast, estrogen receptors have not been identified in the duodenum and lung, and also no significant structural changes were induced in these organs by DES injection in the current study. Therefore, it is likely that the vagina, ovary and the liver are the target organs for estrogen and the duodenum and lung are not. Thus, the current results suggest that DES increases immunocompetent cells in the estrogen target organs, but not in the non-target organs.

It was reported that MHC class II molecules are expressed in macrophages, fibroblast-like cells in the ovary and oviduct, activated T and B cells (Ewert *et al.*, 1980 ; Vainio *et al.*, 1987). In the estrogen target organs, DES might stimulate the expression of MHC class II molecules or the production of some factors, like macrophage colony-stimulating factors, that induced MHC class II^+ cells. MHC class II^+ cells may cause the influx of the activated T cells followed by an induction of B cell and/or plasma cell influx (Dietert *et al.*, 1987 ; Gottschall *et al.*, 1988 ; Benjamini and Leskowitz, 1991). Therefore, increased expression of MHC class II molecule or influx of MHC II^+ cells by the effect of DES might be one of the factors to increase the $CD3^+$ and Bu-1^+ cell influx in the estrogen target organs. Estrogen enhances IgG production by plasma cells (Grossman, 1984), whereas, it is unknown whether estrogen stimulates the proliferation of B cells or plasma cells. The current results suggest that the population of IgG^+ cells was increased specifically in the estrogen target organs.

In conclusion, the increase of immunocompetent cell population by estrogen may

occur with a tissue specific manner. We suggest that estrogen increases the population of immunocompetent cells, including MHC class II^+ cells, $CD3^+$ and $Bu\text{-}1^+$ cells as well as IgG^+ cells (plasma cells), specifically in estrogen target organs but not in the non-target organs.

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