Hormonal Regulation of T-Cell Subsets in the Oviduct: An Immunohistochemical Study Using Sex-Hormone-Treated Chicken

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ABSTRACT. The present immunohistochemical study deals with dynamic alteration of T-cell subsets in the oviduct in sex-hormone-treated chickens. Monoclonal antibodies (CT3, CT4, and CT8) specific for the chicken homologues of CD3, CD4, and CD8 were used in estrogen- or progesterone-treated chickens. In control animals, no lymphocytes appeared throughout the oviduct until 4 weeks of age. When 7-day-old chickens were injected with either diethylstilbestrol (DES) or DES plus progesterone, T cells immunoreactive for CT3 first infiltrated the oviduct at 12 hr after the hormone treatment. Their frequency of occurrence rose from 48 to 96 hr. Subsequently, CT3+ cells in the magnum declined in number per area coincident with the proliferation of albuminous glands in the lamina propria, while in the vagina no decline of T cells was observed. The population of T-cell subsets in the lamina propria of both the magnum and vagina was significantly higher in the DES-treated chickens than in DES plus progesterone-treated chickens. Among T-cell subsets, CT8+ cells were more numerous than CT4+ cells throughout the study, this relative frequency being shared by normal adults. Depopulation of lymphocytes from the thymus, spleen and cecal tonsil, their mobilization to the circulating blood, and subsequent dynamic infiltration into the oviduct suggested that the sex hormones induced the traffic of T cells from the lymphoid organs into the oviduct. — KEY WORDS: chicken, immunohistochemistry, oviduct, sex hormone, T lymphocyte.


The presence of T-cell subpopulations and their functional roles have been studied in different lymphoid and mucosal organs of the chicken [3, 4, 10, 19]. The chicken oviduct, like the intestine, is known to be rich in T cells which are localized in both the epithelium and lamina propria. The occurrence of T cells in the chicken has been reported to depend upon the host age and genetic background [14], the exposure to antigens [6–7] and the different segments of the organ [19]. In a previous study we reported that the development of T-cell subsets in the chicken oviduct depends on different anatomical regions of the oviduct and the age of the animals [12]. However, their dependence on sex hormones in the oviduct is still unknown.

For studies on hormonal regulation of immunocompetent cells, ovariectomy is a common method. In the chicken, the ovary lies in close association with the aorta and the caudal vena cava, and receives branches from them [17]. Because of its complex vascular supply the ovariectomy technique has become successful only in day-old chickens, although their mortality rate is high (30.0%). Moreover, residual ovarian tissues were found in 37.5% of the operated female chickens [15]. To avoid these problems, we tried to use the oviducts of immature female chickens 7 to 12 day-old for the study of hormonal effects on T-cell subsets. Chickens of these ages are suitable, because the oviduct is completely free from lymphocytes until 4 weeks of age [12] and the plasma level of sex hormones is very low [18].

In the present study the dynamical changes of T cells induced by sex hormones were investigated in the immature chicken oviduct. A monoclonal antibody, CT3, was used as a pan T-cell marker. Immunohistochemistry with CT4 and CT8 were also carried out in order to identify most predominant T-cell subsets.

MATERIALS AND METHODS

Animals: White Leghorn (Dekalb-strain) chickens of 1 day old were purchased from Central Chicken Breeding Center, Uni-cho, Hokkaido, Japan. The chickens were reared in the departmental poultry house under identical (provided with same commercial food and drinking water, and with sufficient air and light throughout the period of study) hygienic conditions.

Monoclonal antibodies: The mouse monoclonal antibodies (mAbs) used in this study were raised against chicken CT3, CT4 and CT8, which are equivalent to CD3, CD4 and CD8, respectively, in mammals. All these antibodies were purchased from Southern Biotechnology Associates, U.S.A.

Hormonal treatment: A total of 55 chickens were used in this study. Seven-day-old chickens were divided into three groups. Group 1 consisted of 25 chickens each receiving 1mg of diethylstilbestrol (DES, Kishida, Japan), and Group 2 consisted of 25 chickens receiving both 1 mg of DES and 1 mg of progesterone (Sigma, U.S.A.). The hormones used were well dissolved in sesame oil (Kanto Chemical, Japan) and injected subcutaneously for 5 consecutive days. For control, 5 chickens of Group 3 were given 0.2 ml of sesame oil in the manner mentioned above.

Tissue preparations: The chickens were killed by anesthesia followed by subluxation of cervical vertebrae. The magnum and vaginal part of the oviduct, and lymphoid
organs, including the thymus, spleen, and cecal tonsil, were collected from Groups 1 and 2 at 12 hr, 48 hr, 72 hr, 96 hr, and 120 hr after injection; in Group 3 the same organs were collected only at 120 hr after sesame oil treatment. All the materials were fixed in Bouin's fluid and embedded in paraffin according to a conventional method. Longitudinal sections 3 μm thick were stained with hematoxylin and cosin (HE stain) for general histological studies. For immunohistochemistry the fresh pieces of the magnum and vagina were snap frozen in liquid nitrogen and stored at −20 °C.

**Immunohistochemistry:** Frozen sections of the magnum and vagina were stained by a standard avidin-biotin-complex (ABC) method as described previously [12]. In brief, longitudinal frozen sections 4 μm thick were dried for 30 min, fixed in ice-cold acetone for 15 min and kept at −20 °C until use. The sections were incubated for 3 hr with mAbs diluted with 0.01 M phosphate-buffered saline (PBS) at the appropriate working dilution. After rinsing with PBS, the sections were overlaid with 1% biotin-conjugated goat anti-mouse immunoglobulin (Tago Immunologicals, U.S.A.) for 1 hr, followed by incubation with ABC solution (Vector Lab., U.S.A.) for 1 hr. The antigen-antibody reaction was visualized with 0.05 M Tris HCl buffer (pH 7.6) containing 0.02% 3, 3' diaminobenzidine-tetrahydrochloride dihydrate (Kanto Chemical, Japan) and 0.03% H2O2, and counterstained slightly with hematoxylin. Control sections were stained with the usual method without use of the mAbs.

**Point-counting method:** We selected the lamina propria of the magnum and vagina for counting of T-cell subsets per area because of the following reasons: (1) Significant growth of the magnum and vagina was noticed after the sex-hormone treatment. The other parts of the oviduct were not large enough to obtain sufficient tissues for histological examination. It might be significant to compare the dynamical changes of T-cell infiltration between the magnum as a glandular part and the vagina as an aglandular part. (2) Our preliminary examination showed that the kinetics of T cells (CT3+ cells) and the ratio of T-cell subsets (CT8+ cells versus CT4+ cells) were similar between the lamina propria and epithelium. Therefore, we omitted counting of intraepithelial lymphocytes.

Counting of T-cell subsets was done randomly in 20 fields (at 400 magnification) where lymphocytes were evenly and diffusely distributed in the lamina propria, and their relative frequency per 0.1 mm2 area was calculated [20]. The areas containing the nodular aggregates of lymphocytes were excluded from counting.

**Evaluation of peripheral blood lymphocytes:** Blood samples were collected from all groups at 120 hr after the hormone or sesame oil administration. Smear preparations were stained by routine Giemsa staining. Totally, 40 fields each consisting of 0.158 mm2 area were selected at a magnification of 400 under a light microscope for counting.

**Statistical analysis:** Student's t-test [21] was used to compare the infiltration of T-cell subsets in the lamina propria of the oviduct between Groups 1 and 2. The same test was also done to compare the peripheral blood lymphocytes between hormone-treated and control groups. P<0.05 was accepted as the level of significance.

**RESULTS**

**Changes of the oviduct during hormone treatment:** Macroscopically, the oviduct in 7-day-old control chickens was thin and fiber-like; its segments did not develop enough to differentiate into each part from the infundibulum to the vagina. Histologically, villi in the mucosa of the oviduct were not prominent and the lamina propria was packed only with fibroblasts. The first morphological changes of the oviduct due to sex-hormone administration were recognizable at 12 hr after the hormone injection in both Groups 1 and 2, and characterized by the infiltration of lymphocytes (Figs. 1a, b). Its differentiation into the infundibulum, magnum, isthmus, uterus and vagina similar to the adult hen was noticed at 72 hr post-injection. The size and volume of the oviduct gradually increased with daily hormone treatments. The proliferation of the albuminous gland in the magnum appeared to start from the epithelial lining at 72 hr and had spread throughout the lamina propria by 120 hr post-injection. In contrast, no significant changes were observed in the oviduct in control animals up to 120 hr after injection.

**Infiltration of T lymphocytes in the oviduct:** T-cell subsets immunoreactive for CT3, which is a pan T-cell marker, first appeared in the lamina propria of the magnum at 12 hr after hormone injection. The number of CT3+ lymphocytes in the magnum sharply increased at 48 hr after the treatment with DES (Group 1) or DES plus progesterone (Group 2) (Figs. 2, 6a). After 48 hr, the frequency of occurrence of CT3+ cells in both groups remained to high, but significantly fell at 120 hr along with the abrupt growth of albuminous glandular cells in the lamina propria of the magnum (Fig. 6a). The infiltration rate of CT3+ lymphocytes was higher in DES-treated chickens than with DES plus progesterone. CT8+ cells were more predominant in the lamina propria of the magnum than CT4+ cells (Figs. 6b, c). Both T-cell subsets, CT4+ cells and CT8+ cells, were significantly more numerous in the DES-treated chickens than in the DES plus progesterone-treated chickens (Figs. 6b, c).

In the vagina, the first infiltration of CT3+ cells was also recognized at 12 hr after the injection of DES or DES plus progesterone. The DES treatment induced a gradual increase in the number of T cells from 12 to 120 hr, but the decline of infiltrated T cells which was observed in the magnum was not noticed up to 120 hr in the vagina (Fig. 7). CT3+ lymphocytes in DES plus progesterone-treated chickens were less numerous than those in the DES-treated chickens and did not significantly increase in number after 48 hr (Fig. 7). The nodular aggregates of CT3+ cells were dispersed in the villus core in the vagina when chickens were treated with only DES. The whole lamina propria in occasional villi was occupied by CT3+ cells (Fig. 3). CT4+ cells and CT8+ cells in the lamina propria of the vagina

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showed almost the same tendency in the frequency of occurrence with CT3+ cells (data are not shown).

The first infiltration of CT3+ cells into the epithelium of the magnum (Fig. 2) and vagina was found at 48 hr after the
Fig. 4. The chicken vagina at 5 days after hormones injection. (a) Severe infiltration of CT3+ cells is observed in the vaginal lamina propria (LP) of a DES-treated chicken. Positive cells also infiltrate into the epithelium to form a cluster (arrowhead). (b) In the vagina of a DES plus progesterone-treated chicken only a few lymphocytes are observed in the lamina propria (large arrowheads) and within the epithelium (small arrowhead). LP, lamina propria. × 320.

Fig. 5. The thymus obtained from a chicken receiving DES for 5 days (a) and a control chicken (b). There is a marked reduction in the thickness of the cortical zone (C) in the former. M, medulla. HE stain. × 160.
hormone injection, and the infiltration rate of CT3+ cells was not sufficient from 48 to 96 hr. At 120 hr after DES treatment a significant number of CT3+ cells infiltrated into the epithelium of the vagina (Fig. 4a). The intraepithelial T cells in the magnum and vagina were more numerous in the DES-treated chickens than in DES plus progesterone-treated chickens (Figs. 4a, b). CT4+ cells also predominated over CT4+ cells in the epithelium, like those in the lamina propria.

Changes in the lymphoid organs: After the hormone treatment, atrophy of the thymus, spleen and cecal tonsil was observed grossly. Microscopical observation of HE-stained sections revealed that in the thymus the reduction of the volume of the cortical zone, due to the loss of lymphocytes, was more prominent than that of the medulla (Figs. 5a, b). Similar depopulation of the lymphocytes was also noticed in the lymphatic nodules of the spleen and cecal tonsil.

Changes in the peripheral blood lymphocytes: The effect of sex-hormone treatment on the lymphocyte population in the peripheral blood is shown in Table 1. The number of circulating lymphocytes significantly rose in the groups treated with DES and with DES plus progesterone, though the tendency was more conspicuous in the former group.

DISCUSSION

In the present study the sex-hormone-induced changes of T-cell subsets in the chicken oviduct were clarified by an immunohistochemical examination. Our previous work [12] reported that there was no lymphocyte population in the chicken oviduct until the 4th postnatal week. The
administration of sex hormones in 7-day-old chickens was shown in the present study to induce massive infiltration of T-cell subsets as early as 12 hr after hormone injection. In the chickens treated with sex hormones, there were significantly more CT8 cells than CT4 cells in both the epithelium and the lamina propria. The comparative frequency of occurrence between these two T-cell subsets is similar to that in the normal adult chicken [12]. The frequency of occurrence of T cells infiltrating the lamina propria of the magnum decreased from 72 to 120 hr in spite of daily hormone injection, but did not in the vagina. This escape of lymphocytes from the lamina propria of the magnum may be due to heavy proliferation of glands [12]. Hormone-dependent changes of T lymphocytes have been demonstrated in various peripheral organs of mammals. For example, Dubois et al. [9] reported that the migration of lymphocytes in the bovine oviduct during the estrus cycle was due to hormonal changes.

The present study demonstrated, for the first time, the involution of lymphoid organs in sex-hormone-treated chickens. Earlier studies on the immunological effects of sex hormones have shown similar changes in lymphoid organs of the mouse and rat [1, 13]. More recently, Okuyama et al. [16] reported that estrogen has a suppressive effect on T cells in the thymus, while it can accelerate T-cell proliferation and differentiation in the liver of mouse. The chickens treated with either DES or DES plus progesterone displayed a significant rise in the number of lymphocytes in the peripheral blood as well as atrophic changes of all the lymphoid organs examined. On the other hand, cell division of lymphocytes in the oviduct was not frequently encountered in the hormone-treated groups. These findings suggested the sex-hormone-induced migration of T cells from lymphoid organs into the oviduct. Thus the question was raised as to the mechanism by which sex-hormones induced the infiltration of T cells in the oviduct. Brinsfield et al. [2] reported that leukocyte emigration in the sheep uterus is facilitated by increased vascular porosity in the endometrium. Danel et al. [8] demonstrated the presence of estrogen-binding proteins in mononuclear cells of the spleen and thymus in humans. Similarly, the presence of receptors for sex hormones has been shown in peripheral T cells in humans [5]. Although no studies are available on the presence of binding proteins or receptors for sex hormones in chicken T cells, the depopulation of lymphocytes in the thymus, spleen and cecal tonsil and their subsequent rise in the circulating blood indicate that chicken T cells probably possess binding sites for these hormones to migrate into the oviduct.

The present histologicometrical results showed the rich existence of lymphocytes in the DES-treated chickens in comparison with the DES plus progesterone-treated chickens. A similar phenomenon was recognized for lymphocytes in the peripheral blood. Moreover, our preliminary study showed that administration of only progesterone did not induce any infiltration of lymphocytes into the oviduct (Khan et al. 1995, unpublished data).

Therefore, it is supposed that progesterone does inhibit the migration of lymphocytes from lymphoid organs to the oviduct. The inhibitory effect of progesterone on leukocytic emigration has been shown in the sheep uterus [2].

In conclusion, sex hormones in female chickens cause the mobilization of lymphocytes from lymphoid organs to circulation, and their subsequent infiltration into the oviduct. The present study showed that the administration of sex hormones using immature chickens is useful for studies on sex-hormone-dependent kinetics of lymphocytes in the oviduct. Although the exact function of T cells in the chicken oviduct is not clear, data from our previous study [12] and others [11] suggested that T cells in the chicken oviduct are engaged in immunological surveillance of epithelial cells, and regulation of secretory IgA production in the lamina propria.

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REFERENCES

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