The Journal of Poultry Science, 42 : 193–204, 2005

Effects of Age and Gonadal Steroids on the Localization of T Cell Subsets in the Epididymis of Male Chickens, *Gallus domesticus*

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The aim of this study was to examine the effects of age and gonadal steroids on the localization of T cell subsets in the epididymis of roosters in order to determine the factors affecting their populations. Immature and matured White Leghorn male birds were used, and some of the immature birds were i.m. injected daily with 1 mg testosterone propionate (TP), 1 mg estradiol benzoate (EB) or 100 μl of sesame oil (control) for 3 or 6 days. Cryostat sections of their epididymis were immunostained for CD4 or CD8 antigens. Many of the $CD4^+$ and $CD8^+$ T cells were found in the subepithelial layer of efferent ductules and epididymal duct and also in the interstitum in matured and immature birds. The frequencies of $CD4^+$ and $CD8^+$ T cells in the subepithelial layer of efferent ductules and interstitum was significantly greater in matured birds than in immature ones. The ratios of $CD4^+/CD8^+$ T cells in the efferent ductules and interstitum were not different between matured and immature birds. In the subepithelial layer of efferent ductules of immature birds, the frequencies of $CD4^+$ and $CD8^+$ T cells were significantly increased by Day 3 of TP-injection, followed by a decrease to the level of Day 0 by Day 6. Their frequencies were increased by EB-injection on Day 3 and 6 compared with Day 0. In the interstitum of TP-injected birds, the frequency of $CD4^+$ T cells were not changed until Day 6, whereas $CD8^+$ T cells increased on Day 3 and Day 6 compared with Day 0. Injection with EB caused a significant increase of both $CD4^+$ and $CD8^+$ T cells on Day 3 and Day 6 compared with Day 0. The $CD4^+/CD8^+$ T cells ratio in the epididymal subepithelial layer and interstitum were not significantly different among treatment days within control, TP- and EB-injected groups. These results suggest that the populations of $CD4^+$ and $CD8^+$ T cells in the epididymis are increased in the matured birds compared with immature ones, and luminal contents and gonadal steroids may be responsible in the increase of those T cells.

Key words : age, epididymis, gonadal steroids, rooster, T cells

Introduction

The epididymis of male chickens contains highly convoluted ductus system that plays important roles in the storage and transportation of spermatozoa, disposal of unejaculated spermatozoa and absorption of testicular fluids (Hess and Thurston,

Received : December 8, 2004, Accepted : February 28, 2005
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1977 ; Stefanini *et al.,* 1999 ; Aire, 2000 ; Kirby and Froman, 2000). The epididymal tissues grow in association with testicular development during sexual maturation (Aire, 2002). Their growth and functions are probably regulated by gonadal steroids because the surface epithelium of efferent ductules and epididymal duct express receptors for progesterone, androgen and estrogen (Kwon *et al.,* 1997 ; Nishizawa *et al.,* 2002). These receptors may receive a supply of androgen and estrogen via testicular fluid and indirectly from the blood. Estrogen is produced by germ cells and spermatozoa in the epididymis (Kwon *et al.,* 1995). Macrophages and non-cilliated surface epithelium of the efferent ductules phagocytose spermatozoa, which may be responsible for disposal of unejaculated spermatozoa and degraded epithelial cells (Tingari and Lake, 1972 ; Nakai *et al.,* 1989 ; Nakai and Nasu, 1991 ; Aire, 2000) as suggested in mammals (Jones, 2004). Histological studies have shown that lymphocytes distributed in the surface epithelium and interstitum of epididymis in normal male chickens (Aire and Malmquist, 1979 ; Yoshimura *et al.,* 2004) as is reported in mammals (El-Demiry *et al.,* 1985 ; Flickinger *et al.,* 1997 ; Serre and Robaire, 1999 ; Yakirevich *et al.,* 2002). However, anti-sperm autoimmunity leading to decline of fertility has not been shown in the healthy male birds (Froman *et al.,* 1990), although anti-sperm antibody was raised by immunization with sperm (Kirby *et al.,* 1992).

T cells play essential roles in cell mediated immune response (Lowenthal *et al.,* 1994 ; Lillehoj and Okamura, 2003). There are two subsets of T cells, namely helper/inducer T cells expressing CD4 molecule on their surface and cytotoxic/suppressor T cells expressing CD8 (Chen *et al.,* 1994). The $CD4^+$ T cells stimulate B cells to produce antibodies and activate macrophages. The $CD8^+$ T cells are capable of cytotoxic activity to intracellularly infected cells and also involved in down-regulation of immune response. Thus the T cell subsets in the epididymis may play significant roles not only in protection from infection but in the control of immunity in the epididymis such as macrophage activity and immunological barrier to suppress the autoimmunity to spermatozoa as suggested in mammals (Serre and Robaire, 1999 ; Diekman *et al.,* 2000). Recently, we have identified $CD4^+$ and $CD8^+$ T cell subsets in the efferent ductules and epididymal ducts as well as in the interstitum of matured male chickens, and no significant differences were shown in their populations among the types of ductules (Yoshimura *et al.,* 2004). However, factors regulating the populations of these T cell subsets remain to be determined. In the hen ovary and oviduct, the populations of T cell subsets and B cells were increased during sexual maturation. Increase of their population may be controlled by estrogen because injection of immature birds with estrogen increased their population (Zheng *et al.,* 1998 ; Barua and Yoshimura, 1999 ; Yoshimura, 2004).

The goal of this study was to examine the factors affecting the populations of T cell subsets in the epididymis of male chickens. Specifically, it was examined whether their populations differ between immature and matured birds and whether androgen and estrogen, which may affect the development and functions of epididymis, affect their populations in immature birds.

Materials and Methods

Animals and Hormone Treatment

White Leghorn male birds were kept in individual cages under a light regimen of 14 h light and 10 h dark, and provided with feed and water *ad libitum*. Immature (approximately 70-days-old) and matured (approximately 520-days-old) birds were used for the examination of the effects of age on the T cell population in the epididymis (n＝4 birds each). All of the matured birds were confirmed to produce semen. To examine the effects of sex steroids, immature birds (approximately 70-days-old) were i.m. injected daily with 1 mg testosterone propionate (TP ; Nakalai Tesque Inc., Kyoto, Japan), 1 mg estradiol benzoate (EB ; Sigma Co., St Louis, MO) or 100 μl of sesame oil (control) for 3 or 6 days (n＝5 birds each). The stock solutions of the hormone reagents were prepared by dissolving TP or EB in sesame oil at a concentration of 10 mg/ml. Our previous studies had shown that injection of immature hens with 1 mg diethylstilbestrol for 6 days caused development of the oviduct and increased the influx of the T and B cells in the ovary and oviduct (Zheng *et al.,* 1998 ; Barua and Yoshimura, 1999).

Tissue Preparations and Immunostaining

The epididymis attached to the testis was collected after euthanization of birds by decapitation. Handling of chickens was done in accordance with the Hiroshima University regulations for the conduct of animal experiments. The tissues of left-side epididymis was embedded in cryo-embedding medium (Sakura Co., Tokyo, Japan) and snap-frozen in isopentane and solid CO_2 mixture. Frozen sections of them were air-dried on slides and fixed with aceton on ice for 10 min. Then the sections were immunostained for CD4 and CD8 as described by Zheng and Yoshimura (2001). Briefly, sections were incubated with mouse monoclonal antibodies to chicken CD4 or CD8 (Southern Biotech Associates Inc., Birmingham, USA) diluted to 1 : 100 in phosphate buffered saline (PBS) containing 1% (w/v) bovine serum albumin in for 2 h. After washing with PBS for 15 min (5×3 times), the immunoreaction products were identified using Histofine SAB-PO (M) kit (Nichirei Co., Tokyo, Japan), namely sections were incubated with biotinylated anti-mouse IgG＋IgM＋IgA, and avidin-peroxidase complex for 1 h each. They were then incubated with a reaction mixture consisting of 0.02% (w/v) 3, 3'-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6. Finally, the sections were counterstained with hematoxylin, dehydrated and covered. For the control staining the first antibodies were replaced with normal mouse IgG (Santa Cruz Biotechnology Inc., Santacruz, CA, USA), and no specific staining was observed on these sections.

Observations and Counting of Cells

The sections were examined under a light microscope with image analysis software (Image-Pro Plus, Media Cybemetics, Silver Spring, MD, USA). Counting of the immunopositive cells were performed in the subepithelial tissues (subepithelial stroma and fibromuscular layer) of proximal efferent ductules. Three sections were examined in one tissue of a bird, and more than two different ductules and interstitum were

analyzed on each section. The average number of cells was calculated to be the cell number in $5\times10^4\mu m^2$ area of a tissue.

Statistical Analysis

The significance of differences in the number of positive cells was analyzed by one-way ANOVA, followed by Duncan's multiple range test or Student's t-test. Significance was assigned to differences of $P<0.05$.

Results

The epididymis of immature birds contained many small ductules, whereas some of the proximal efferent ductules were identified by the feature of small folds (Fig. 1 a, b). In the epididymis of matured birds well developed efferent ductules and epididymal duct were observed (Fig. 1 c-f). The proximal efferent ductules formed many folds with wide lumen, whereas the epididymal duct showed smooth mucosal surface. The mucosal epithelium of efferent ductules and epididymal ducts was surrounded by subepithelial layer composed of narrow subepithelial stroma and circulating fibromuscular layer in both immature and matured birds. The interstitum among the ductules were fibrous connective tissue.

Many of the $CD4^+$ and $CD8^+$ T cells were found in the subepithelial layer of efferent ductules and epididymal duct in immature and matured birds (Fig. 1). Both types of T cells were occationally found in the mucosal epithelium and luminal cavity in efferent ductules and epididymal duct of matured birds, however, no positive cells were located in those tissues of immature birds. The $CD4^+$ and $CD8^+$ T cells were also found in the interstitum of both immature and matured birds.

The frequency of $CD4^+$ T cells in the subepithelial layer of efferent ductules and interstitum was significantly greater in matured birds than in immature ones. The frequency of $CD8^+$ T cells was also significantly greater in those tissues of matured birds (Fig. 2). The ratios of $CD4^+/CD8^+$ T cells in the efferent ductules and interstitum were not different between immature and matured birds (Fig. 3).

Significant structural differences or degenerative features were not observed in control, TP- or EB-injected birds. $CD4^+$ and $CD8^+$ T cells were observed in the subepithelial layer of efferent ductules and interstitum, but not in the mucosal epithelium and luminal cavity in all groups (Figs. 4, 5). In the subepithelial layer of efferent ductules, the frequencies of $CD4^+$ in control birds were not changed until Day 6 (Fig. 6 a), whereas those of $CD8^+$ T cells were greater on Day 6 compared with Day 0 and Day 3 (Fig. 6 b). The frequencies of $CD4^+$ and $CD8^+$ T cells were significantly increased by Day 3 of TP-injection, followed by a decrease to the level of Day 0 by Day 6 (Fig. 6 a, b). Their frequencies were increased by Day 3 of EB-injection and then kept greater than Day 0 until Day 6 of injection (Fig. 6 a, b). The frequencies of each T cell subset in the subepithelial layer were significantly greater in TP- or EB-injected birds than control on Day 3 of injection, and were significantly greater in EB-injected birds than control or TP-injected birds on Day 6 (Fig. 6 a, b).

In the interstitum, the frequency of $CD4^+$ and $CD8^+$ T cells were not changed until Day 6 in control (Fig. 6 c, d). In the TP-injected birds, the frequency of $CD4^+$ T

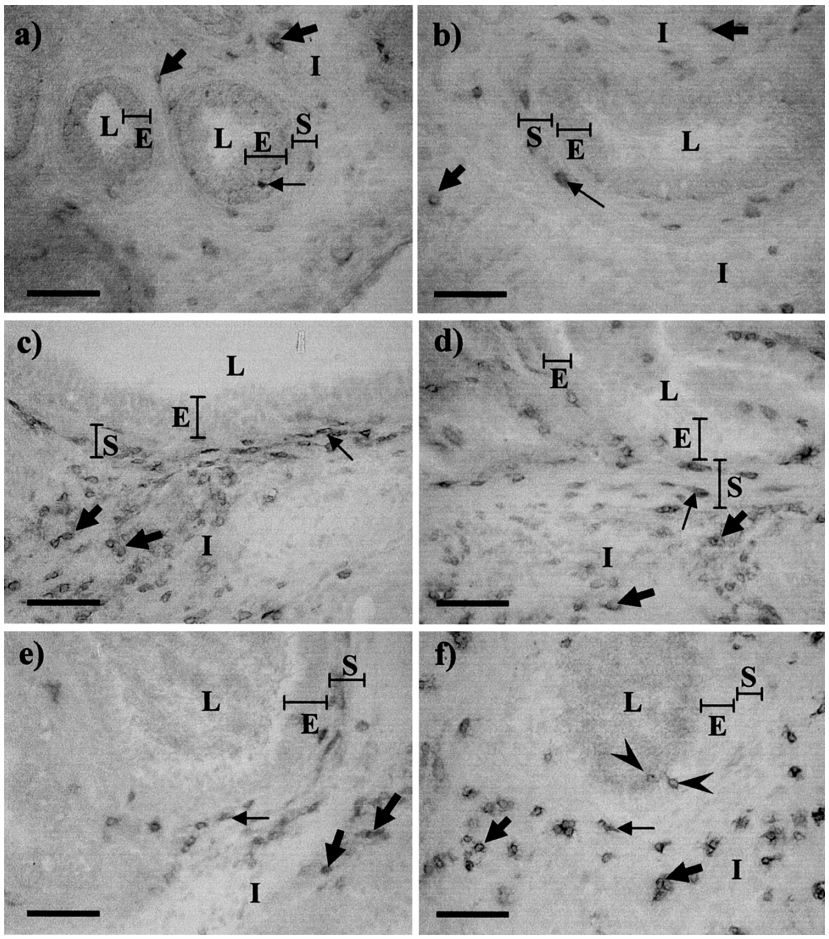

Fig. 1. Sections of epididymis of immature and matured male chickens immunostained for CD4 (a, c and e) and CD8 (b, d and f).
a and b) $CD4^+$ and $CD8^+$ cells in the efferent ductules of an immature bird. c and d) $CD4^+$ and $CD8^+$ cells in the efferent ductules of matured birds. e and f) $CD4^+$ and $CD8^+$ cells in the epididymal ducts of matured birds. Small and large arrows indicate the $CD4^+$ or $CD8^+$ cells in the subepithelial layer and interstitum, respectively. Arrow heads show the $CD8^+$ cells in the mucosal epithelium and lumen of epididymal duct. E=mucosal epithelum, I =interstitum, L=lumen of duct, S=subepithelial layer. Scale bar=50μm.

cells were not changed until Day 6 (Fig. 6c), whereas that of $CD8^+$ T cells was greater on Day 3 and Day 6 compared with Day 0 (Fig. 6d). Injection with EB caused a significant increase of both $CD4^+$ and $CD8^+$ T cells on Day 3 compared with Day 0, which were kept greater until Day 6 (Fig. 6c, d). There were no significant differences in the frequency of $CD4^+$ T cells among control, TP- and EB-injected birds within Day 3 or Day 6 (Fig. 6c). The frequency of $CD8^+$ T cells was significantly greater in TP- or EB-injected birds than control on Day 3, and was greater in EB-injected birds than control birds on Day 6 (Fig. 6d). The $CD4^+/CD8^+$ T cells ratio in the epididymmal

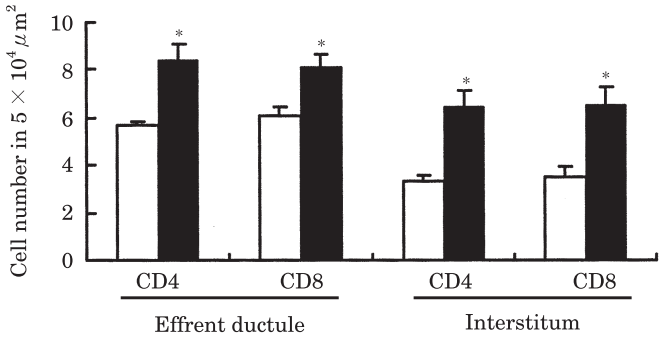

Fig. 2. Frequencies of $CD4^+$ and $CD8^+$ cells in the epididymal efferent ductules and interstitum of immature (□) and matured (■) male chickens.
Values are mean±SEM of positive cell number in $5 \times 10^4 \mu m^2$ area (n=4).
*Significantly different between immature and matured groups ($P < 0.05$).

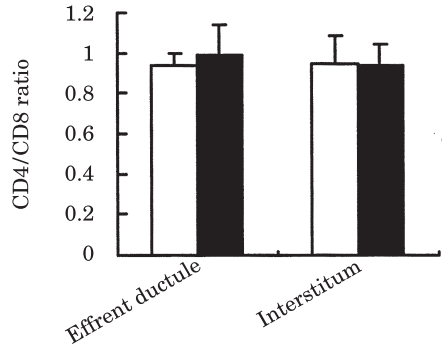

Fig. 3. Ratio of CD4/CD8 T cells in the epididymal efferent ductules and interstitum of immature (□) and matured (■) male chickens.
Values are mean±SEM (n=4). Significant differences are not observed.

subepithelial layer and interstitum were not significantly different among treatment days within control, TP- and EB-injected groups (Fig. 7).

Discussion

We are reporting that age and gonadal steroids affect the populations of $CD4^+$ and $CD8^+$ T cells in the epididymis of roosters. The significant findings were (1) their populations were greater in matured birds than immature ones, and (2) testosterone and estrogen significantly increased their populations, although the effect of testosterone appeared only early phase of stimulation.

In the epididymis of matured birds, the subepithelial layer of ductules and interstitum were the major site where both $CD4^+$ and $CD8^+$ T cells located, whereas those T cell subsets appeared occasionally in the mucosal epithelium and luminal cavity of the ductules. These results support our previous reports that localized T cells in the reproductive organs of matured roosters (Yoshimura *et al.,* 2004). Both T cell subsets

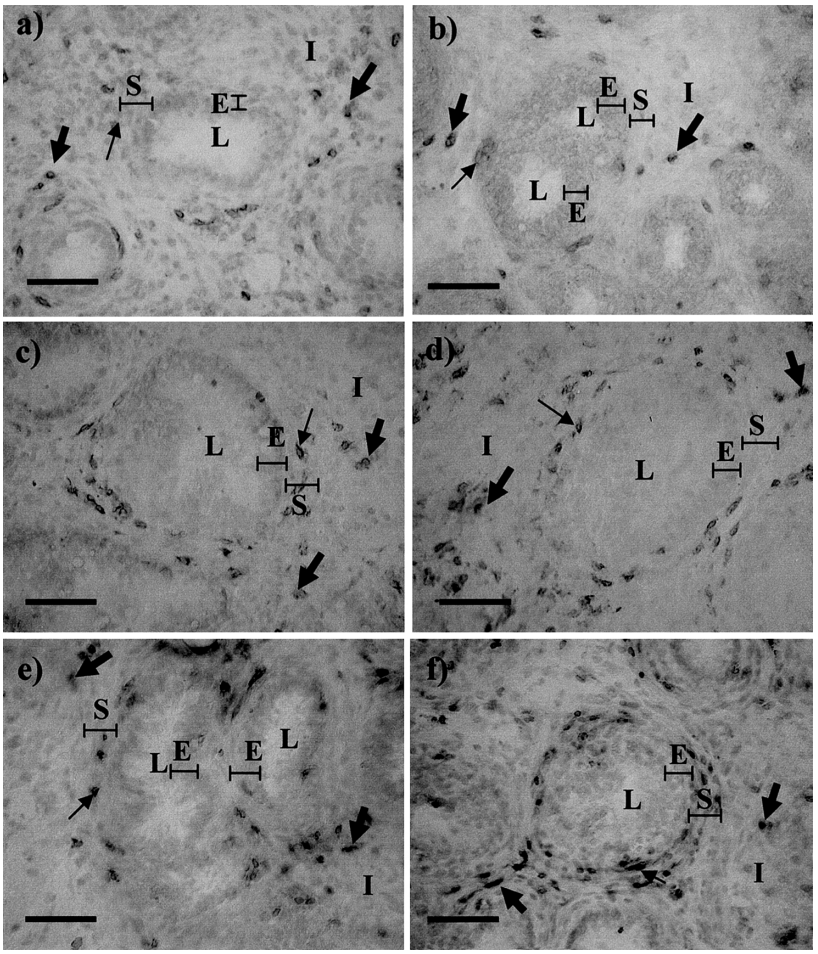

Fig. 4. Sections of epididymis immunostained for CD4 (a, c and e) and CD8 (b, d and f) in immature male chickens injected with testosterone propionate or estradiol benzoate for 3 days.
a and b) $CD4^+$ and $CD8^+$ cells in the epididymis of an immature bird injected with oil for 3 days. c and d) $CD4^+$ and $CD8^+$ cells in the epididymis of immature birds inject ed with testosterone propionate for 3 days. e and f) $CD4^+$ and $CD8^+$ cells in the epididymis of immature birds injected with estradiol bensoate for 3 days. Small and large arrows indicate the $CD4^+$ or $CD8^+$ cells in the subepithelial layer and interstitum, respectively.
See Fig. 1. for explanations of abbreviations. Scale bar=50μm.

were localized only in the subepithelial layer of ductules and interstitum in the epididymis of immatured birds. Thus, the subepithelial layer and interstitum are likely to be the reservoir site of T cells in both immature and matured birds. Our previous study showed that the population of T cell subsets in the subepithelial layer was not different among the types of ductules in the epididymis, namely, proximal efferent ductules, distal ones and epididymal ducts (Yoshimura *et al.,* 2004). In the current study, the structures of ductules were not fully differentiated in immature birds

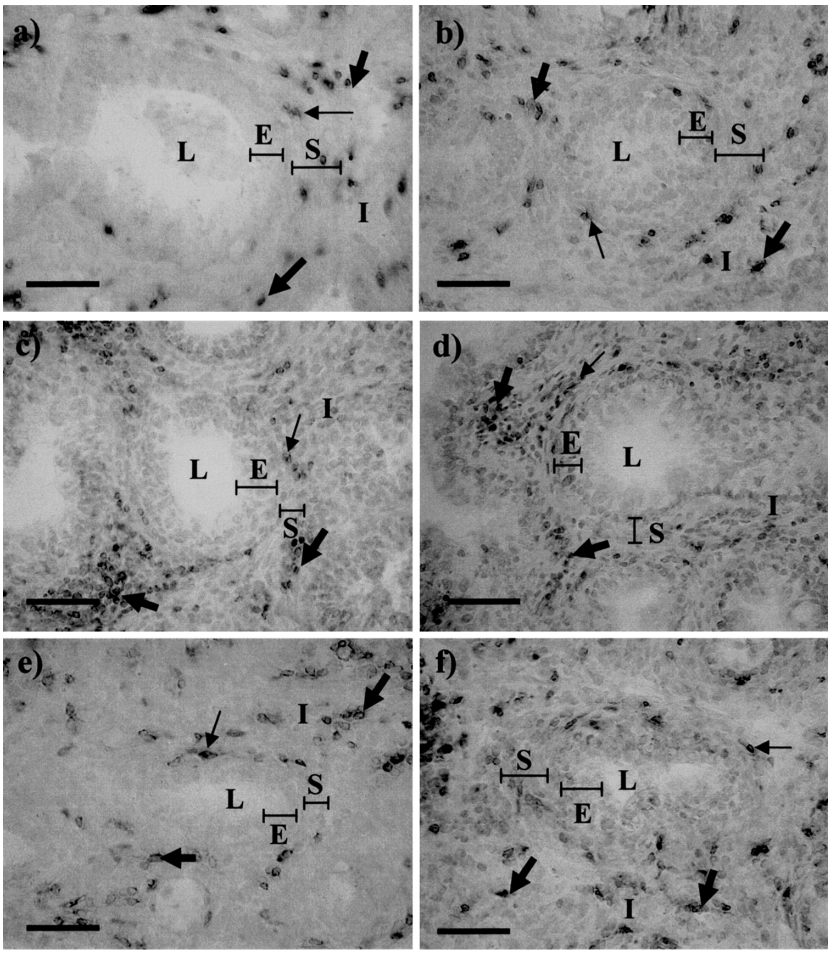

Fig. 5. Sections of epididymis immunostained for CD4 (a, c and e) and CD8 (b, d and f) in immature male chickens injected with testosterone propionate or estradiol benzoate for 6 days.
a and b) $CD4^+$ and $CD8^+$ cells in the epididymis of an immature bird injected with oil for 6 days. c and d) $CD4^+$ and $CD8^+$ cells in the epididymis of immature birds injected with testosterone propionate for 6 days. e and f) $CD4^+$ and $CD8^+$ cells in the epididymis of immature birds injected with estradiol bensoate for 6 days. Small and large arrows indicate the $CD4^+$ or $CD8^+$ cells in the subepithelial layer and interstitum, respectively.
See Fig. 1. for explanations of abbreviations. Scale bar=50μm.

although the proximal efferent ductules were identified based on the appearance of mucosal folds. Thus the image analysis to compare the T cell populations in immature and matured birds was performed in the proximal efferent ductules and interstitial tissue. The results revealed that the frequencies of $CD4^+$ and $CD8^+$ T cells in the subepithelial layer and interstitial tissues were greater in matured birds than immature ones. Thus it is suggested that the influx of T cell subsets increase with a development of epididymis and some of them may migrate in the mucosal epithelium and luminal

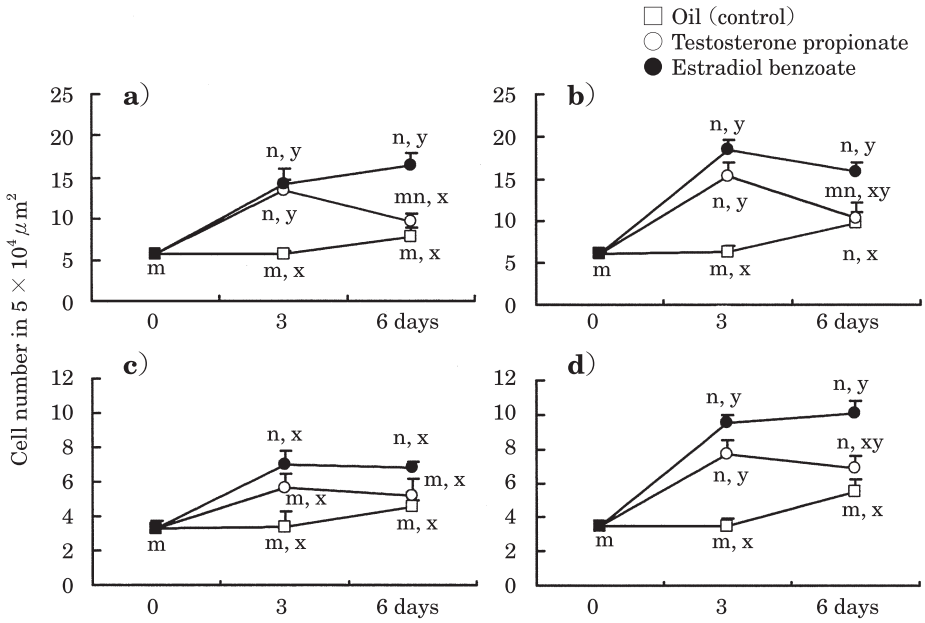

Fig. 6. Changes in the frequencies of $CD4^+$ and $CD8^+$ cells in the epididymal efferent ductules (a and b) and interstitum (c and d) of immature male chickens treated with testosterone propionate or estradiol benzoate.
a) $CD4^+$ cells in the subepithelial layer of efferent ductules. b) $CD8^+$ cells in the subepithelial layer of efferent ductules. c) $CD4^+$ cells in the interstitum. c) $CD8^+$ cells in the interstitum. Values are mean±SEM of positive cell number in $5\times10^4\mu m^2$ area (n=5). ^{m,n} Significantly different among treatment days within treatment group ($P<0.05$). ^{x,y} Significantly different among treatment groups within each treatment day ($P<0.05$).

cavity in matured birds.

The significant differences in the reproductive functions of matured birds compared with immature ones are the seminal production and active steroidogenesis. Therefore, one of the factors that caused the increase of $CD4^+$ and $CD8^+$ T cell population in the epididymis may be the seminal components which are produced only in the matured birds. The increase of immune cells in the epididymis in response to luminal contents, including accumulated damaged epithelial cells and antigens of germ cell origin, has been suggested in mammals (Serre and Robaire, 1999). Nakai *et al.* (1989) reported that macrophages together with mucosal epithelium of efferent ductules phagocytosed spermatozoa in the epididymis of roosters, which may be responsible in the disposal of unejaculated spermatozoa. Helper/inducer T cells expressing CD4 molecules play roles in activation of macrophages and stimulation of B cells to produce immunoglobulins (Chen *et al.,* 1994). Thus, the increased $CD4^+$ T cells may play roles in activation of macrophages that play a role in elimination of damaged cells and unejaculated cells. The population of B cells was very low in the epididymis of healthy matured roosters (Yoshimura *et al.,* 2004). This result suggested that production of autoantibody to

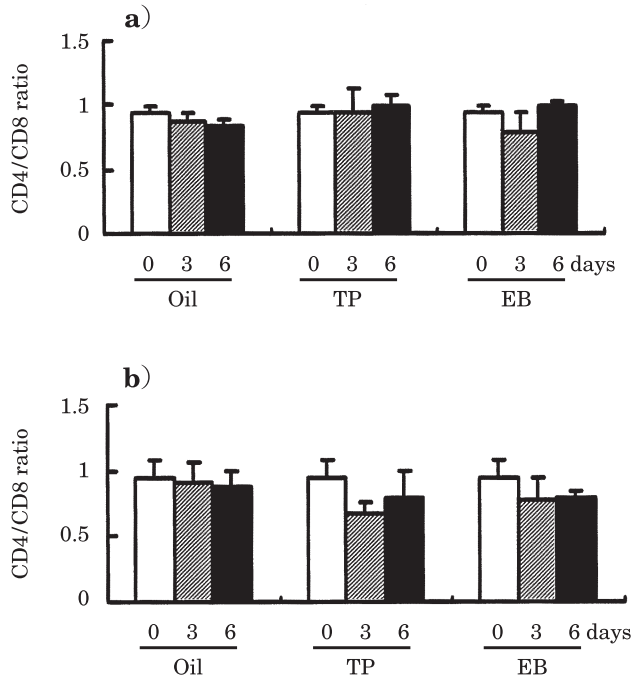

Fig. 7. Ratio of CD4/CD8 T cells in the epididymis of immature male chickens treated with testosterone propionate or estradiol benzoate for 6 days.
a) Subepithelial layer of efferent ductules. b) Interstitum. Values are mean ±SEM (n=5). Significant differences are not observed among treatment days within oil (control), TP (testosterone propionate) or EB (estradiol benzoate) group.

spermatozoa may be limited even though $CD4^+$ T cells were present in the epididymal tissues. The $CD8^+$ T cells, namely cytotoxic/suppressor T cells are capable of cytotoxic activity and may be involved in down regulation of immune responses (Chen *et al.* 1994). Thus, the $CD8^+$ T cells that were increased together with $CD4^+$ T cells in matured birds may play a role to down-regulate the immunoresponses in the epididymis including B cell migration and autoantibody to spermatozoa. It is suggested also in mammals that cytotoxic T cells may contribute to immunological barrier preventing the immune response to spermatozoa in testicular excurrent ducts (Yakirevich, 2002).

Testicular germ cells and epididymal sperm are sites for the synthesis of estrogen in roosters (Kwon *et al.,* 1995). Therefore, significant amount of estrogen and testosterone produced in the testis should appear in the epididymis in matured birds. Injection of immature birds with EB for 3 or 6 days significantly increased the populations of $CD4^+$ and $CD8^+$ T cells, whereas did TP injection 3 days after injection, followed by decreasing to the level of control on Day 6 of injection. It may be possible that T cells released from thymus might redistribute in peripheral tissues of the birds treated with these steroids. However, the effects of testosterone may appear only during early phase because the T cell populations were decreased by Day 6 of injection. In contrast,

estrogen may affect the population of them for prolonged period since their population was kept higher than control even on Day 6 of injection. It is reported that T cell subsets and B cells in hen ovary and oviduct were increased by estrogen (Barua and Yoshimura, 1999 ; Zheng *et al.,* 1998 ; Yoshimura, 2004). Zheng *et al.* (2001) described that there may be organ-specificity in such effects of estrogen because those lymphocytes were increased only in the target organ of estrogen including ovary, oviduct and liver but not in intestine and lung. The epithelial cells in the epididymis express not only androgen receptor but estrogen receptor (Nishizawa *et al.,* 2002 ; Kwon *et al.,* 1997). Thus, estrogen may be one of the significant endocrine factors to stimulate the infiltration of T cell subsets in the epididymis in matured birds that are active in spermatogenesis and steroidogenesis.

The $CD4^+/CD8^+$ T cells ratio was not different between immature and mature birds and among the treatment days with EB or TP in the subepithelial layer of efferent ductules and interstitum. These results suggest that the changes in the population with sexual maturation and steroids treatment occur in a parallel way between $CD4^+$ and $CD8^+$ T cells.

In conclusion, we suggest that the populations of $CD4^+$ and $CD8^+$ T cells in the epididymis are increased in the matured birds compared with immature ones, and luminal contents and gonadal steroids, especially estrogen, may be responsible in the increase of those T cells.

Acknowledgement

This work was supported by Grants-in-Aid for Scientific research from the Ministry of Education, Science, Sports and Culture, Japan to Y.Y.

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