Effects of ageing and oestrogen on the localization of immunoglobulin-containing cells in the chicken ovary

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Immunoglobulins in the chicken ovary are important for transfer of immunity to chicks through the egg and for protection of the ovary from infection. The aim of this study was to examine the effects of ageing and oestrogen on the population of Ig-containing cells in the chicken ovary. The ovarian tissue of immature, young laying and old laying hens and that of immature birds treated with diethylstilboestrol (DES), progesterone or sesame oil (vehicle) was processed for paraffin wax sections. The sections were stained for IgG, IgM and IgA by an indirect immunostaining method and the population of cells positive for each Ig was analysed under a light microscope. The number of cells positive for IgG, IgM and IgA was significantly greater in the ovarian stromal tissue of young laying hens than in immature or old laying hens ($P < 0.01$). The number of IgG- and IgM-positive cells in the thecal layer of primary follicles of young laying hens was significantly greater than that in immature and old laying hens ($P < 0.01$) and there were significantly more ($P < 0.05$) IgA-positive cells in young laying hens than in immature birds. The number of IgG-, IgM- and IgA-positive cells was significantly ($P < 0.01$) greater in both the stromal tissue and the thecal layer of DES-treated birds than in the vehicle-treated birds. Progesterone had no significant effect ($P < 0.05$) on the population of Ig-positive cells. These results indicate that the number of Ig-positive cells increases as chickens mature and decreases with ageing, and that oestrogen may be involved in this process.

Introduction

The avian ovary contains numerous small undifferentiated follicles in the stromal tissue before sexual maturation, and as birds mature, the follicles grow rapidly and a hierarchy of yellow preovulatory follicles is established (Gilbert and Wood-Gush, 1979; Etches, 1990). Yolk absorption by the oocyte and active cell proliferation in the follicular wall are responsible for the rapid follicular growth (Perry and Gilbert, 1979; Yoshimura *et al.*, 1996). Follicular growth as well as steroidogenesis in the ovary is primarily regulated by gonadotrophins (Johnson, 1986; Bahr, 1991). Intraovarian factors such as sex steroids (Yoshimura and Tamura, 1986; Tanaka *et al.*, 1987; Yoshimura *et al.*, 1993a; Nakada *et al.*, 1994) and growth factors (Pulley and Marrone, 1986; Yoshimura and Tamura, 1988; Li and Tsang, 1995) are also thought to play important roles in local regulation of ovarian functions. Localization of receptors for oestrogen (Kamiyoshi *et al.*, 1986; Yoshimura *et al.*, 1995), androgen (Yoshimura *et al.*, 1993b) and progesterone (Yoshimura and Bahr, 1991) in the ovary suggests that it is a target for sex steroids.

In hens, it has been reported that the transmission of immunity from mother to egg is mediated through the

transport of IgG into the egg yolk of the developing follicle (Heller, 1975; Rose and Orlans, 1981; Larsson *et al.*, 1993). There are reports that a newly laid egg can contain 10–13 mg IgG per ml of yolk (Akita and Nakai, 1992; Sunwoo *et al.*, 1996). It is also possible that immune responses affect folliculogenesis because a mild immune deficiency or autoimmunity can predispose the ovary to premature follicular depletion in mammals (Friedman *et al.*, 1984). Furthermore, the immune system may be responsible for protection of the ovary from infection by pathogenic organisms (Blaxland *et al.*, 1982; Keller *et al.*, 1995). Therefore, it is assumed that lymphoid cells in the ovary play an important role in both oogenesis and defence against pathogens in birds. Zettergren (1995) localized IgM-containing cells in the ovarian stroma and subcapsular areas and suggested that these tissues are sites of abundant Ig-containing cells in mature hens. In contrast, Withanage *et al.* (1997) reported a low population of Ig-positive lymphocytes in the hen ovary. The factors affecting the localization of ovarian Ig-containing cells are not known. It is possible that ageing and sex steroids affect localization of Ig-containing cells because oogenesis activity decreases during ageing in hens (Palmer and Bahr, 1992) and the ovarian tissues are a target for sex steroids (Kamiyoshi *et al.*, 1986; Yoshimura and Bahr, 1991; Yoshimura *et al.*, 1993b; Yoshimura *et al.*, 1995).

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The aim of this study was to determine the control of localization of Ig-containing cells in the hen ovary which may be responsible for immunity transfer during oogenesis and for the protection of the ovary from infection. Immunoglobulin-containing cells were identified by immunocytochemistry and the effects of ageing and sex steroids on their localization was examined.

Materials and Methods

Birds

Mature and immature White Leghorn chickens were kept under a photoperiod of 14 h light:10 h dark and provided with food and water *ad libitum*. In the experiment to examine the effect of ageing on the Ig-containing cell population, birds were divided into three groups: immature hens (approximately 50 days of age; $n=5$), young laying hens, laying more than five eggs in a sequence (approximately 180 days of age; $n=5$); and old laying hens, laying two–three eggs in a sequence (approximately 450 days of age; $n=5$). Immature birds (30 days of age) were divided into three groups ($n=5$ in each group) and were treated with sesame oil (vehicle; Nacalai Tesque, Kyoto), diethylstilboestrol (DES, an analogue of oestrogen; Nacalai Tesque, Kyoto), or progesterone (Sigma, St Louis, MO) to examine the effect of steroids on the population of Ig-containing cells. Diethylstilboestrol and progesterone were dissolved in sesame oil at a concentration of 10 mg ml⁻¹. Immature birds were injected i.m. with DES (1 mg day⁻¹), progesterone (1 mg day⁻¹) or sesame oil (0.1 ml day⁻¹) for 7 days.

Tissue preparation

Birds were killed by decapitation and ovarian tissue was collected and immediately fixed with Bouin's fixative for 1 day. The tissue was then processed for paraffin wax sections of 5 µm thickness.

Antibodies

Rabbit polyclonal antibody to chicken IgG (OEM Concepts Inc., Thomas River, NJ), and goat polyclonal antibodies to chicken IgM and chicken IgA (Bethyl Lab. Inc., Montgomery, TX) were used as primary antibodies. Vector staining ABC-peroxidase kits (Vector Lab. Inc., Burlingame, CA) were used for the detection of immunoreaction of the primary antibodies.

Immunostaining for IgG, IgM and IgA

Paraffin wax was removed from the sections and they were then incubated with methanol for 30 min to block endogenous peroxidase activity. The sections were washed with PBS for 15 min (3 × 5 min) and incubated with 1% (v/v) normal horse serum for 15 min to block the non-specific

binding of antibodies. Sections were then incubated for 2 h with antibodies to chicken IgG, IgM or IgA (diluted with PBS containing 0.5% (w/v) BSA at concentrations of 1:500, 1:500 and 1:200, respectively). Immunoreactions were detected by using a vector staining ABC-peroxidase kit according to the manufacturer's instructions. Briefly, after rinsing with PBS for 15 min (3 × 5 min) sections were incubated for 1 h with biotinylated anti-rabbit (for detection of chicken IgG) or anti-goat IgG (for detection of chicken IgM and IgA). After washing with PBS for 15 min (3 × 5 min) sections were incubated with avidin–biotin–peroxidase complex for 1 h. Sections were again washed in PBS for 15 min (3 × 5 min) and immunoprecipitates were visualized by incubation with a mixture of 0.02% (w/v) 3,3'-diaminobenzidine-4 HCl (Sigma, St Louis, MO) and 0.001% H₂O₂ in 0.05 mol Tris-HCl l⁻¹ (pH 7.6). After immunostaining, sections were stained with haematoxylin, dehydrated and covered. Control staining was carried out simultaneously in which the first antibodies were omitted and normal rabbit or goat serum was used. No staining was found in these control slides. Some sections were also stained with haematoxylin and eosin for the observation of general tissue structure.

Counting Ig-positive cells

The sections were examined under a light microscope. The images were captured through a Victor TK-1270 RGB colour video camera (Victor, Tokyo) and analysed by an image analyser computer program, Mac Aspect/PPC version 2.2 (Mitani Co., Fukui) and immunopositive cells were counted. The number of positive cells in the stromal tissue and in the thecal layer of primary follicles (400–600 µm in diameter) in each bird was obtained by observing two different regions of the stroma and thecal layer in one section (3–6 × 10⁴ µm² of stroma and 0.5–1.0 × 10⁴ µm² of thecal layer). The average of the two counts was expressed as the number of positive cells in 1 × 10⁴ µm² of stromal tissue or thecal layer for each bird. The significance of difference in positive cell number among different groups of birds was determined by one-way ANOVA followed by Duncan's multiple-range test.

Results

Primordial and primary follicles were observed in the ovarian stromal tissue of immature and mature hens. The oocyte was surrounded by a granulosa layer in primordial follicles and by granulosa and thecal layers in primary follicles (Fig. 1). In addition to primordial and primary follicles in the stroma, numerous white follicles and several yellow follicles protruded from the ovarian surface in young and old laying hens. Atretic follicles were occasionally observed in the stromal tissue of old laying hens. No significant histological changes were observed in the ovaries of DES- and progesterone-treated immature birds compared with immature vehicle-treated birds. Cells showing strong immunoreactions for IgG, IgM and IgA in their cytoplasm were localized in both the thecal layer of primary follicles and the stromal tissue of all hens examined (Fig. 1).

Fig. 1. Sections of chicken ovary immunostained for IgG. Small and large arrowheads indicate examples of IgG-positive cells in the thecal layer and stromal tissue, respectively. O, oocyte; S, stromal tissue; T, thecal layer. (a) Ovarian stroma of immature hen. A few cells stained positive for IgG in the stroma. Inset shows a magnified view of an IgG-positive cell in the stroma. (b) Ovarian stroma of young laying hen. Many cells stained positive for IgG in the stroma and thecal layer. Inset shows a magnified view of an IgG-positive cell in the stroma. (c) Ovarian stroma of old laying hen. A few IgG-positive cells were observed in the stromal tissue and the thecal layer of the primary follicle. (d) Ovarian stroma of immature bird treated with vehicle (sesame oil). A few cells stained positive for IgG in both the stromal tissue and the thecal layer of the primary follicle. (e) Ovarian stroma of diethylstilboestrol-treated immature bird. Many IgG-positive cells were observed in both the stroma and thecal layer. Inset shows a magnified view of an IgG-positive cell in the stroma. (f) Ovarian stroma of progesterone-treated immature bird. A few IgG-positive cells were observed in both the stroma and thecal layer. Scale bars represent 50 μm in main photographs and 20 μm in insets.

Effects of age on the number of Ig-containing cells

The IgG-containing cells were localized in the stromal tissue of immature birds at a population of approximately four cells in $1 \times 10^4 \mu\text{m}^2$ (Fig. 2). The number of IgG-containing cells was significantly greater ($P < 0.01$) by approximately 2.5-fold in young laying hens compared with immature and old laying hens, which had the same number of IgG-containing cells. The number of IgM- and IgA-containing cells in the stromal tissue showed the same pattern of change with age as that observed for the IgG-

containing cells. There were approximately twice as many IgM- and IgA-containing cells in young laying hens compared with immature and old laying hens ($P < 0.01$). There was no significant difference in the population of IgG-, IgM- and IgA-containing cells between immature and old laying hens.

In the thecal layer of the primary follicles, cells positive for IgG and IgM were more abundant in young laying hens than in immature and old laying hens ($P < 0.01$) (Fig. 2). The IgA-containing cells were present in greater numbers in the thecal layer in young laying hens than in immature birds ($P < 0.05$).

Fig. 2. Population of Ig-containing cells in (a) the ovarian stroma and (b) the thecal layer of primary follicles of immature (■), young laying (□) and old laying hens (▨). Each bar represents the mean $\pm$ SEM of the number of Ig-positive cells in $1 \times 10^4 \mu\text{m}^2$ tissue ($n = 5$ birds for each bar). Within the stromal tissue and thecal layer and within each class of immunoglobulin, bars with different letters are significantly different ($P < 0.01$ for IgG-, IgM- and IgA-containing cells in the stromal tissue, and IgG- and IgM-containing cells in the thecal layer; $P < 0.05$ for IgA-containing cells in the thecal layer).

but not in old laying hens. There was no significant difference in the number of Ig-positive cells in the thecal layer of immature and old laying hens. In both the stromal tissue and the thecal layer of young laying hens, IgG-positive cells were present in significantly greater numbers than other Ig-positive cells ($P < 0.01$).

Effects of sex steroids on the number of Ig-containing cells

The number of IgG-positive cells increased approximately seven-fold and the numbers of IgM- and IgA-positive cells increased three-fold in the stromal tissue of DES-treated birds compared with vehicle-treated birds ($P < 0.01$) (Fig. 3). In the thecal layer of primary follicles, IgG-positive cells were five times more abundant and IgM- and IgA-positive cells were 2.5 times more abundant in DES-treated birds than in vehicle-treated birds ($P < 0.01$) (Fig. 3). Progesterone had no significant effect on the population of Ig-containing cells in either the stromal tissue or the thecal layer.

Discussion

We are the first to report that there is a significant increase in the number of Ig-containing cells in the chicken ovary as birds mature and a significant decrease in the number of these cells in old laying hens. In addition, the number of Ig-containing cells increases significantly in immature birds injected with DES (an analogue of oestrogen) but not with progesterone. Ig-positive cells were considered to be plasma cells containing Igs; their features resembled plasma cells containing Igs found in the chicken oviduct (Kimijima *et al.*, 1990).

In hens, Igs are classified into three major classes (isotypes), namely IgG, IgM and IgA (Benedict and Berestecky, 1987). Zettergren (1995) localized IgM-containing cells in the ovarian stroma and subcapsular areas in hens, whereas Withanage *et al.* (1997) reported that the hen ovary was relatively sparsely

populated with Ig-containing lymphocytes. In the present study, cells containing three classes of Igs, namely IgG, IgM and IgA, were identified in the hen ovary. In both the stromal tissue and the thecal layer, IgG-positive cells were predominant among the three Ig classes. A higher amount of IgG compared with IgM and IgA was reported in the follicular fluid of the mammalian ovary (Hogarth, 1982). Therefore, the predominance of IgG may be a common feature in both avian and mammalian ovaries.

The population of cells positive for each class of Ig in the stromal tissue and in the thecal layer of primary follicles was found to be significantly higher in young laying hens than in immature birds, suggesting that the population of each class of Ig increases when hens start laying. Previous reports have shown that IgG in the blood circulation is incorporated into the yolk during the process of oogenesis to provide maternal antibodies (Patterson *et al.*, 1962; Heller, 1975; Rose and Orlans, 1981; Larsson *et al.*, 1993). Furthermore, as the egg passes down the oviduct both IgM and IgA are added to the egg-white enclosing the yolk (Yamamoto *et al.*, 1975). We suggest that in addition to serum IgG, IgG secreted from ovarian IgG-containing cells may also contribute to IgG in the yolk. Immunoglobulins may also play an essential role in protecting ovarian tissues from infection by pathogens including *Salmonella*, avian encephalomyelitis virus and lymphoid leukosis virus (Darbyshire *et al.*, 1976; Blaxland *et al.*, 1982; Poppe, 1994; Keller *et al.*, 1995). However, in the absence of information on pathogens in the tissues of the birds used in the present study, this cannot be confirmed. Increased numbers of Ig-containing cells in the ovaries of young laying hens compared with immature hens suggests that these cells may be associated with egg laying. In old laying hens, the population of cells positive for each class of Ig was lower in the stromal tissue and the thecal layer compared with those of young laying hens. This decrease in Ig-containing cells may be associated with a decrease in egg production during ageing. The number of preovulatory follicles decreases constantly in chickens during the course of ageing (Bahr and Palmer, 1989).

Fig. 3. Population of Ig-containing cells in (a) the ovarian stroma and (b) the thecal layer of primary follicles of immature birds treated with vehicle (sesame oil) (■), diethylstilboestrol (□) or progesterone (▨). Each bar represents the mean $\pm$ SEM of the number of Ig-positive cells in $1 \times 10^4 \mu\text{m}^2$ tissue ($n=5$ birds for each bar). Within the stromal tissue and thecal layer and within each class of immunoglobulin, bars with different letters are significantly different ($P < 0.01$).

Previous reports have shown that oestrogen receptors are localized in the bursa of Fabricius, suggesting that oestrogen has a role in the proliferation of B lymphocytes in this tissue (Grossman, 1984). Trawick and Bahr (1986) observed a significant linear relationship between serum oestrogen concentration and antibody titre, and concluded that oestrogen is immunoenhancing in rats. These results suggest a possible link between oestrogen and lymphocyte functions. The present study showed that injection of immature birds with DES but not with progesterone increased the population of Ig-positive cells in the ovarian stromal tissue and thecal layer. In addition, IgG-positive cells were predominant among the cells positive for three classes of Igs in laying hens. Therefore, it is likely that changes in oestrogen concentrations affect the population of Ig-containing cells in the chicken ovary. The role of sex steroids in the control of Ig-containing cell migration has been suggested in the mammalian uterus. Oestrogen, but not progesterone, increased Ig-containing cells in the uterus of mammals (Wira and Sandoe, 1980) which contained progesterone and oestrogen receptors (Toft and Gorski, 1966; Brenner *et al.*, 1990; Koji and Brenner, 1993). The presence of receptors for oestrogen (Yoshimura *et al.*, 1995), androgen (Yoshimura *et al.*, 1993b) and progesterone (Yoshimura and Bahr, 1991) in the chicken ovary indicates that ovarian tissues are target sites for sex steroids. Therefore, it is possible that oestrogen concentrations modify the release of factors in the hen ovary that affect the migration of Ig-containing cells as has been suggested in the mammalian uterus (Wira and Sandoe, 1980). Further studies are required to identify the presence of such factors.

Increases in the number of ovarian Ig-containing cells by injection with DES suggests that oestrogen may be one of the factors that increases the Ig-containing cell population in young laying hens. Furthermore, decreases in oestrogen production may be responsible in part for the decrease in the number of Ig-positive cells in old laying hens. Johnson *et al.* (1986) reported that in the thecal layer of old hens laying

short sequences, there was a significantly lower concentration of oestradiol than in the thecal layer of young laying hens.

In hens, immunoresponsive cells have a positive effect on adrenal steroidogenesis (Siegel *et al.*, 1985), whereas adrenocorticoids suppress immune functions (Trout and Mashaly, 1995). IgG was reported to stimulate steroidogenesis in the adrenal cortex in rats (Zaichik *et al.*, 1985). In contrast, a modulatory effect of immunocompetent cells on the interaction of gonadotrophins with steroidogenic cells and on steroidogenesis in the mammalian ovary has been suggested (Mori, 1990). Further studies are required to determine the effect of Igs on ovarian steroidogenesis in hens.

In conclusion, it was found that the number of Ig-containing cells in the chicken ovary increased when hens became reproductively active and later decreased with age. The potential involvement of oestrogen in increasing the infiltration of Ig-containing cells into the ovary is also suggested by our results. Previous reports have identified lymphoid cells in the mammalian ovary, such as major histocompatibility complex class II-positive cells, macrophages, and T and B lymphocytes, and have suggested their role in the control of ovarian functions (Adashi, 1990; Mori, 1990). Further studies of lymphoid cells in the hen ovary are required to establish the interaction between the immune system and the ovary.

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