Effects of sexual maturation and gonadal steroids on the localization of IgG-, IgM- and IgA-positive cells in the chicken oviduct

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The effects of sexual maturation and gonadal steroids on the localization of immunoglobulin-positive cells in chicken oviducts were studied. Oviductal tissues were collected from laying hens and chicks treated with stilboestrol (DES, an analogue of oestrogen) or progesterone. Paraffin wax sections of the tissues were immunostained for IgG, IgM and IgA, and the frequency of cells staining positive was examined using an image analysis system. Some of the cells in the mucosal epithelium and plasma cell-like cells in the stroma of the oviduct stained positive for IgG, IgM or IgA. In the mucosal epithelium of laying hens, there was a significantly greater number of IgG-positive (IgG⁺) cells in the shell gland than in the infundibulum, magnum and isthmus, more IgM⁺ cells in the magnum than in the infundibulum, and more IgA⁺ cells in the magnum than in the other segments of the oviduct with the exception of the vagina. The frequency of IgG⁺ and IgM⁺ cells in the mucosal epithelium of all oviductal segments and IgA⁺ cells in the magnum, isthmus and vagina was significantly higher in laying hens than in immature birds. In the subepithelial stroma of laying hens, there was a significantly greater population of IgG⁺ cells in the infundibulum and vagina than in the magnum and isthmus, more IgM⁺ cells in the infundibulum than in the magnum, and more IgA⁺ cells in the uterovaginal junction and vagina than in the magnum and isthmus. The frequency of IgG⁺, IgM⁺ and IgA⁺ cells in the subepithelium of infundibulum, uterovaginal junction and vagina was significantly greater in laying hens than in immature birds. The number of IgM⁺ cells in all oviductal segments and of IgA⁺ cells in the magnum of the mucosal epithelium of the chicks treated with DES increased significantly compared with those of control chicks. In addition, the number of IgG⁺ cells in the shell gland and vagina and of IgM⁺ cells in the vagina of the stroma of DES-treated birds were increased. Treatment of immature birds with progesterone had no effect on the localization of Ig⁺ cells in the oviduct except for a decrease in the number of IgM⁺ cells in the shell gland. These results suggest that the local immunity in the oviduct develops during sexual maturation, possibly under the control of oestrogen.

Introduction

The chicken oviduct consists of the infundibulum, magnum, isthmus, shell gland, uterovaginal junction (UVJ) and the vagina. In the oviduct, egg components that surround the yolk, including albumin, the shell membrane and the egg shell are formed. In addition, spermatozoa entering via the vagina are stored in the sperm-storage tubules in the UVJ (Fujii, 1963; Fujii and Tamura, 1963) before taking part in fertilization which occurs in the infundibulum. The local immune system in the chicken oviduct may play an important role in preventing infection as the vagina opens to the cloaca which contains numerous exogenous pathogens (Shivaprasad *et al.*, 1990; Poppe *et al.*, 1992). The oviductal immune system may also affect the selection, survival and the fertilizing ability of

spermatozoa (Steele and Wishart, 1992; Bakst *et al.*, 1994). It was reported that incubation of spermatozoa with anti-sperm antisera in turkeys drastically reduces the fertilizing ability of the sperm cells (Burke and Rieser, 1972; Burke and Yu, 1979). In the oviduct of laying hens, plasma cells (Van Krey *et al.*, 1987) and Ig-containing cells (Lebacqz-Verheyden *et al.*, 1972; Kirk *et al.*, 1989; Kimijima *et al.*, 1990) that may have a role in local immunity have been demonstrated. Yoshimura *et al.* (1996) reported that immunocompetent cells, including major histocompatibility complex class II positive cells, T and B cells and Ig positive (Ig⁺) cells, are numerous in the mucosal surface of the oviduct in laying hens, whereas, in moulting hens, they are reduced.

Previous studies in mammals have indicated that oestrogen enhances humoral immune responses (Trawick and Bahr, 1986; Erbach and Bahr, 1988, 1991) and that the local immune system in the reproductive tract is controlled by gonadal steroids (Grossman, 1984). Therefore, it is possible that the immune

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system of the hen's oviduct is affected by the reproductive cycle and influenced by gonadal steroids. However, it is not known whether the presence of Ig⁺ cells in the oviductal wall is under the control of gonadal steroids in hens. The aim of the present study was to determine the changes in the localization of IgG⁺, IgM⁺ and IgA⁺ cells in the oviduct of the hen during sexual maturation and the effects of oestrogen and progesterone on these Ig⁺ cells.

Materials and Methods

Treatment of birds

Immature and laying White Leghorn hens 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*. The chicks (50 days old, $n = 5$ birds) and mature hens (approximately 260 days old, $n = 5$ birds) that were laying more than five eggs in a sequence were used in Expt 1. The laying hens were used 6 h after oviposition, that is, when there was an egg in the shell gland. In Expt 2, immature chickens (36 days old) were injected i.m. daily with 1 mg stilboestrol (DES, Nacalai Tesque, Inc., Kyoto,) or 1 mg progesterone (Sigma Co., St Louis, MO) for 7 days. These steroids were dissolved in sesame oil (vehicle) at a concentration of 10 mg ml⁻¹. Control birds received 0.1 ml vehicle. Six birds were used in each group.

All birds were killed by decapitation. The oviduct was pre-fixed in 10% (v/v) formalin solution for approximately 15 h, and small pieces (approximately 3–5 mm long) were cut from the middle part of the infundibulum, magnum, isthmus, shell gland, UVJ and vagina, and re-fixed for another 36 h in the same fixative. Sections were embedded in paraffin wax (6 µm thick) and immunostained for Igs. Some of the sections were stained with haematoxylin and eosin for histological studies.

Immunocytochemistry

Antibodies. The first antibodies used for the staining of IgG⁺, IgM⁺ and IgA⁺ cells were rabbit anti-chicken IgG (O.E.M. Concepts Inc., Tomas River, NJ), goat anti-chicken IgM (Bethyl Lab. Inc., Montgomery, TX) and goat anti-chicken IgA (Bethyl Lab. Inc., Montgomery, TX), respectively. These first antibodies were diluted to a concentration of 1:500 with PBS containing 0.05% (w/v) BSA. Vector-staining ABC-PO kits (Vector Lab. Inc., Burlingame, CA) for the detection of rabbit IgG (in the case of detection of IgG⁺ cells) and goat IgG (for detection of IgM⁺ and IgA⁺ cells) were used to detect the immunoreaction products of the first antibodies.

Immunostaining. Sections were deparaffinized and washed with PBS for 15 min (5 min × 3 times). They were then incubated with normal horse serum (dilution 1:150) for 15 min followed by incubation with the first antibody for 2 h. After washing in PBS (5 min × 3 times), they were incubated with the second antibody for 1 h. Sections were then washed in PBS

(5 min × 3 times) and incubated with avidin–biotin–peroxidase complex for 1 h. After washing with PBS (5 min × 3 times), the immunoreaction products were revealed by incubating with a mixture of 0.02% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H₂O₂ in 0.05 mol Tris–HCl l⁻¹ buffer, pH 7.6. Sections were then rinsed with water and counterstained with haematoxylin. Control sections were prepared by the same procedure except that the first antibody with normal rabbit (in case of staining for IgG⁺ cells) or goat (in case of staining for IgM⁺ and IgA⁺ cells) serum, and no positive staining was observed in these sections. All steps were carried out at room temperature.

Observations and analysis of data

Sections were examined under a light microscope. Populations of cells positive for immunoreactions in the mucosal epithelium and in the stroma were analysed by an image analysis computer system, MacAspect (Mitani Co., Fukui). The subepithelial stroma of 50 µm depth from the basal boundary of the mucosal epithelium covered the whole stromal tissue of each oviductal segment in immature chickens regardless of treatment. However, in laying hens, it did not cover the middle part of stroma because the tubular glands had developed there. Therefore, the frequency of positive cells in the stroma was examined in the subepithelial stroma (the area 50 µm in depth from the boundary of the mucosal epithelium) of immature hens and in the subepithelial and middle part of the stroma of laying hens. The number of positive cells was counted in two different areas (approximately 5000–15 000 µm² each) in one oviductal segment of a bird and the values were calculated to be the number of cells in 5000 µm². The average of the two counts was then used as the number of cells in one segment of a bird. The significance of difference in the number of cells among treatments or segments was examined by one-way ANOVA followed by Duncan's multiple *t* test.

Results

Experiment 1: changes in the Ig⁺ cell population in the oviduct before and after sexual maturation

The mucosal tissue of the oviduct consisted of the mucosal epithelium and stroma in both immature and laying hens. Tubular glands were well developed in the magnum, isthmus and shell gland in laying hens. Plasma cell-like cells in the stromal connective tissues in each oviductal segment stained positive for IgG (Fig. 1a–f), IgM and IgA in both immature and laying hens. Some mucosal epithelial cells also stained positive for IgG (Fig. 1b, d, f), IgM and IgA in laying hens, whereas few of these cells stained in immature hens.

The frequency of Ig⁺ cells in the oviductal mucosal epithelium in laying and immature hens is shown (Fig. 2). In the mucosal epithelium of laying hens, the population of IgG⁺ cells in the shell gland was significantly higher than that in the infundibulum, magnum or isthmus. The frequency of IgM⁺ cells in the magnum was significantly higher than that in the infundibulum, and the frequency of IgA⁺ cells in the magnum

Fig. 1. Sections of the oviducts of immature and laying hens immunostained for IgG. Arrows and arrowheads indicate examples of positively stained cells in the stroma and mucosal epithelium, respectively. E: mucosal epithelium; S: stroma; T: tubular gland in the stroma; L: lumen of oviduct. Scale bars represent 20 μ m. (a) The magnum of an immature hen. There are very few IgG⁺ cells in the stroma and none in the epithelium. (b) The magnum of a laying hen. Tubular glands have developed in the stroma and positive cells are observed in the mucosal epithelium and stromal connective tissue. (c) The shell gland of an immature hen. There are very few IgG⁺ cells in the stroma and none in the epithelium. (d) The shell gland of a laying hen. There are many positive cells in the mucosal epithelium and in the stromal connective tissue. (e) The vagina of an immature hen. There are a few IgG⁺ cells in the stroma and none in the epithelium. (f) The vagina of a laying hen. Many IgG⁺ cells can be seen in the mucosal epithelium and in the stroma.

was significantly higher than that of the other oviductal segments with the exception of the vagina. In immature hens, there was no significant difference in the frequency of each kind

of Ig⁺ cells in the mucosal epithelium among the oviductal segments. The frequency of IgG⁺ and IgM⁺ cells in the mucosal epithelium of all oviductal segments and IgA⁺ cells in

Fig. 2. Frequency of the cells positive for immunoglobulins (a) IgG, (b) IgM and (c) IgA in the mucosal epithelium of the oviduct in (□) immature and (■) laying hens. Each bar represents the mean $\pm$ SEM of the number of positive cells in 5000 μm^2 tissue ($n = 5$ birds for each bar). Bars with different superscripts are significantly different ($P < 0.05$) for laying hens. There were no significant differences among the segments from immature hens. An asterisk indicates a significant difference between laying and immature birds ($P < 0.05$). In: infundibulum; Ma: magnum; Is: isthmus; Sg: shell gland; Uv: uterovaginal junction; Va: vagina.

the magnum, isthmus and vagina was significantly higher in laying hens than in immature birds.

The frequencies of Ig⁺ cells in the stroma of oviductal tissues are shown (Figs 3 and 4). The populations of IgG⁺ cells in the subepithelial stroma in the infundibulum and in the vagina were significantly higher than those in the magnum and the isthmus in the case of laying hens. In addition, the frequency of IgM⁺ cells in the infundibulum was significantly higher than that in the magnum, and the frequencies of IgA⁺

Fig. 3. Frequency of the cells positive for immunoglobulins (a) IgG, (b) IgM and (c) IgA in the subepithelial stroma of the oviduct in (□) immature and (■) laying hens. Each bar represents the mean $\pm$ SEM of the number of positive cells in 5000 μm^2 tissue ($n = 5$ birds for each bar). For each type of immunoglobulin, bars with different superscripts are significantly different for laying hens ($P < 0.05$). There was no significant difference among the segments from immature chickens. An asterisk indicates that the difference between laying and immature birds is significant ($P < 0.05$). In: infundibulum; Ma: magnum; Is: isthmus; Sg: shell gland; Uv: uterovaginal junction; Va: vagina.

cells in the UVJ and vagina were significantly higher than those in the magnum and isthmus (Fig. 3). In the middle part of the stroma of laying hens, there were greater numbers of IgG⁺ and IgM⁺ cells in the infundibulum and vagina and a greater number of IgA⁺ cells in the vagina than in the magnum and isthmus (Fig. 4). In immature chickens, no significant differences were detected in the frequency of each kind of Ig⁺ cells in the subepithelial stroma among oviductal segments (Fig. 3). The frequencies of IgG⁺, IgM⁺ and IgA⁺ cells in the subepithelial stroma of infundibulum, UVJ and vagina and also that of IgG⁺ cells in the shell gland were significantly greater in laying hens than in immature birds (Fig. 3).

Fig. 4. Frequency of the cells positive for immunoglobulins (□) IgG, (▨) IgM and (■) IgA in the middle part of oviductal stroma of laying hens. Each bar represents the mean $\pm$ SEM of the number of positive cells in 5000 μm^2 tissue ($n = 5$ birds for each bar). Within each type of immunoglobulin, bars with different grouped superscripts (^{a/b/c} for IgG, ^{m/n/o} for IgM, ^{u/v/w} for IgA) are significantly different ($P < 0.05$). In: infundibulum; Ma: magnum; Is: isthmus; Sg: shell gland; Uv: uterovaginal junction; Va: vagina.

Experiment 2: effects of gonadal steroids on the localization of oviductal Ig⁺ cells in immature birds

The mucosal tissue of the oviduct in control and progesterone-treated chicks showed an undifferentiated feature, which was similar to that seen in normal immature chickens, whereas in DES-treated chicks, the mucosal tissue showed a developed structure including the formation of tubular glands in the magnum and shell gland (Fig. 5). Plasma cell-like cells positive for IgG, IgM and IgA were observed in the stromal connective tissue of all oviductal segments in chickens treated with oil, DES or progesterone (Fig. 5a–f), and a few of the mucosal epithelial cells also stained positive for IgG, IgM and IgA.

The frequencies of IgG⁺, IgM⁺ and IgA⁺ cells in the oviductal mucosal epithelium and stroma of chickens treated with oil, DES or progesterone are shown (Figs 6 and 7). In the mucosal epithelium, the effect of treatment with DES on the population of IgG⁺ cells was not significant. However, the frequency of IgM⁺ cells in the mucosal epithelium of all oviductal segments and that of IgA⁺ cells of the magnum were significantly increased by DES treatment. Progesterone treatment had no significant effect on the frequency of Ig⁺ cells in the mucosal epithelium. The population of IgA⁺ cells in the mucosal epithelium of the DES-treated group was significantly greater in the magnum than in the shell gland and vagina (Fig. 6).

A significant increase in the population of IgG⁺ cells was observed in the oviductal stroma of the shell gland and vagina, and of IgM⁺ cells in the vagina as a result of treatment with DES (Fig. 7). Treatment of chickens with progesterone had no significant effect on the populations of Ig⁺ cells in the oviductal stroma with the exception of the population of IgM⁺ cells in the shell gland which was significantly decreased by treatment. In DES-treated birds, the population of IgG⁺ cells was approximately 2–4 times higher than those of IgM⁺ and IgA⁺ cells in the magnum, shell gland and vagina. In addition, the populations of IgG⁺ cells were not significantly

different among the magnum, shell gland and vagina, whereas the population of IgM⁺ cells in the vagina was significantly higher than in the magnum and shell gland, and that of IgA⁺ cells was significantly higher in the shell gland and vagina than in the magnum.

Discussion

The significant findings of the present study are that there are significantly more Ig⁺ cells in both the mucosal epithelium and stroma of the oviducts of laying hens than in immature chickens, and that stilboestrol, an analogue of oestrogen, significantly increases the population of oviductal Ig⁺ cells in immature hens, while progesterone has no such effect.

The Ig⁺ cells are localized in the cells of mucosal epithelium and plasma cell-like cells in both the subepithelial and the middle part of stroma in laying hens. This observation supports previous reports which localized Ig⁺ cells in the oviductal stroma (Lebacqz-Verheyden *et al.*, 1972; Van Krey *et al.*, 1987; Kirk *et al.*, 1989; Kimijima *et al.*, 1990) and mucosal epithelium (Kimijima *et al.*, 1990) in laying hens. It is not known whether the mucosal epithelial cells produce immunoglobulins or if they absorb them from blood or plasma cell-like cells localized in the subepithelial stroma. Immunoglobulins secreted from the epithelium into the oviductal lumen may act locally. It has been reported that in laying hens infected with *Salmonella enteritidis* the level of infection was much lower in the oviduct than in the intestine, even though the vagina opens to the cloaca, and also that contamination of the egg components was much less than that on the surface of the eggshell (Shivaprasad *et al.*, 1990; Poppe *et al.*, 1992). The presence of many Ig⁺ cells in the stroma of both ends of the oviduct may protect the oviduct from infection by bacteria and other pathogens. Barr and Parr (1985) demonstrated that, in mammals, IgA, IgG and possibly IgM bind to many of the bacteria in the luminal contents of the

Fig. 5. Sections of the magnum, shell gland and vagina of immature chickens treated with stilboestrol and control sections immunostained for IgG. Arrows indicate examples of positive cells in the stroma. E: mucosal epithelium; S: stroma; T: tubular gland in the stroma; L: lumen of the oviduct. Scale bars represent 20 μm . (a) The magnum of a control chick. There are very few IgG positive (IgG^+) cells and no tubular glands have formed in the stroma. (b) The magnum of immature bird treated with stilboestrol. Note the presence of many positive cells and the formation of tubular glands in the stroma. (c) The shell gland of a control bird. Only a few positive cells are present. (d) The shell gland of the immature bird treated with stilboestrol. There are more positive cells compared with the control section and tubular glands have developed in the stroma. (e) The vagina of a control bird. Only a few positive cells are observed in the stroma. (f) The vagina of an immature bird treated with stilboestrol. Note the increased number of IgG^+ cells compared with the control section.

uterus. They also suggested that the secretory immune system in the reproductive tract of female mice may be involved in returning the uterus to an aseptic state by at least three

mechanisms, namely the blocking of the bacteria-binding sites of the mucosal epithelium, the agglutination of bacteria and the opsonization of bacteria in preparation for phagocytosis. The

Fig. 6. Frequency of the cells positive for immunoglobulins (a) IgG, (b) IgM and (c) IgA in the mucosal epithelium of the oviduct in immature chickens treated with (▨) stilboestrol (DES) or (■) progesterone. (□) Control. Each bar represents the mean $\pm$ SEM of the number of positive cells in 5000 μm^2 tissue ($n=6$ birds for each bar). Within each oviductal segment, bars with different superscripts are significantly different ($P < 0.05$). (c) In the DES-treated birds, bars with superscripts m/n are significantly different for the frequency of IgA⁺ cells ($P < 0.05$).

infundibulum, UVJ and vagina are closely related to fertilization, storage and selection of spermatozoa in hens (Fujii, 1963; Fujii and Tamura, 1963; Bakst *et al.*, 1994). Recent reports have indicated that there is an increase in the number of leukocytes in the lumen of the vagina just after insemination and that phagocytosis of spermatozoa by oviductal cells occurs in UVJ (Higaki *et al.*, 1995), suggesting that an anti-sperm immunoreaction occurs in the oviduct of hens. Steele and Wishart (1992) reported that spermatozoa recovered from the vagina carried Igs, suggesting that Igs may affect the survival, or are involved in the immunological selection, of spermatozoa in the

Fig. 7. Frequency of the cells positive for immunoglobulins (a) IgG, (b) IgM and (c) IgA in the subepithelial stroma of the oviduct in immature chickens treated with (▨) stilboestrol (DES) or (■) progesterone. (□) Control. Each bar represents the mean $\pm$ SEM of the number of positive cells in 5000 μm^2 tissue ($n=6$ birds for each bar). Within each oviductal segment, bars with different superscripts are significantly different ($P < 0.05$). In birds treated with stilboestrol, bars with superscripts x/y are significantly different in the frequency of (b) IgM or (c) IgA positive cells ($P < 0.05$).

oviduct. Immunoglobulins in the vagina, UVJ and infundibulum may participate in these anti-sperm immunoreactions as most spermatozoa remain in these parts of the reproductive tract just after insemination (Bakst *et al.*, 1994; Higaki *et al.*, 1995). Therefore, we suggest that the infundibulum, UVJ and vagina contain greater numbers of Ig⁺ cells, and that these segments play an important role in the immune responses to both pathogenic agents and spermatozoa.

The present results show that there were more Ig⁺ cells in the infundibulum, shell gland, UVJ and vagina of laying hens than in these areas of immature birds. Treatment of immature

birds with DES increased the number of IgM⁺ cells in the mucosal epithelium in all oviductal segments, of IgA⁺ cells in the mucosal epithelium of the magnum, of IgG⁺ cells in the stroma of the shell gland and vagina, and of IgM⁺ cells in the stroma of the vagina. Progesterone had no effect. We previously reported that the population of IgG⁺ cells, as well as T cells (CD3 positive cells) and B cells (Bu-1b positive cells) was greater in laying hens than in moulting hens (Yoshimura *et al.*, 1996). These results indicate that the increase in Ig⁺ cells during sexual maturation is closely associated with oviductal growth which is possibly under the control of oestrogen. Leitner *et al.* (1996) demonstrated that the administration of oestrogen to hens significantly enhanced the humoral immune response to *Escherichia coli* and sheep erythrocytes, whereas treatment with an anti-oestrogen strongly inhibited these immune responses. Therefore, oestrogen may increase the humoral immune response and also local immunity in the specific organs that are the targets for oestrogen, like the oviduct. The population of IgM⁺ cells in the shell gland was lower in progesterone-treated birds than in control birds. Although we did not examine the effects of progesterone on the Ig⁺ cell population in the oviducts of laying hens or DES-treated birds, progesterone may suppress the infiltration of Ig⁺ cells into the oviduct. Studies in mammals have also provided evidence that gonadal steroids might play a significant role in immune function (Trawick and Bahr, 1986; Erbach and Bahr, 1988, 1991). Wira and Sandoe (1980) reported that oestrogen increased uterine IgA and IgG concentrations in ovariectomized or hypophysectomized rats and that the spontaneous increase of Ig concentrations during the oestrous cycle resulted from the action of oestrogen in the uterus. Wang *et al.* (1996) reported that the transport of polymer IgA from the blood into female mouse genital tissues decreased significantly after ovariectomy, and that this decline was rectified by oestradiol but not by progesterone. The effects of progesterone on the Ig⁺ cells in the hen's oviduct need to be examined further.

In conclusion, the present results suggest that the local immunity in the oviduct develops during sexual maturation and is under the control of oestrogen. The presence of relatively greater numbers of Ig⁺ cells in both ends of the oviduct suggests that the immune system in these tissues plays an important role in the immune response to pathogenic agents and spermatozoa.

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