Effects of Intravenous Injection of Hens with Salmonella Antigen on the Distribution of Major Histocompatibility Complex Class II Positive Cells in Ovarian Follicles

Yukinori YOSHIMURA and Tomomasa TAKATA

Faculty of Applied Biological Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

Abstract The goal of this study was to determine whether the population of MHC class II+ cells was affected in response to foreign agents in the blood circulation. White Leghorn laying hens were i.v. injected with or without formalin fixed *Salmonella paratyphi* (SP), and the population of major histocompatibility complex (MHC) class II positive cells in the ovarian follicles including the largest and third largest follicles(F1, F3), white follicles (WF) and cortical follicles were examined by immunocytochemistry. The immunoreaction products for MHC class II were observed in the cells of theca layer of all follicles in both treated and control birds. Injection of birds with SP antigen caused a significant increase in the distribution of MHC class II+ cells in the theca of the F1, F3 and WF, but not in the cortical follicles. These results suggest that the MHC class II+ cells in the theca increase in response to circulating foreign agents, and play a significant role in ovarian local immunity.
Key words: ovarian follicle, MHC class II, salmonella, chicken

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The hen ovary contains numerous cortical follicles embedded in the stroma, prehierarchal white follicles, and several hierarchal preovulatory follicles growing rapidly (Bahr, 1991; Johnson, 1996). This organ is a susceptible site for many disease agents that can harm ovarian tissues as well as be transmitted to the eggs, such as *salmonella*, avian encephalomyelitis and lymphoid leucosis (Darbyshire et al., 1976; Blaxland et al., 1982; Poppe, 1994; Keller et al., 1995). Bacterial infection of laying hen's reproductive system causes contamination of the contents of eggs. The salmonella organisms could be isolated from the ovaries and oviducts of infected hens, and the clearance of them is one of the most important subjects in the current poultry industry. (Hopper and Mawer, 1988; Lister, 1988).

The immune system plays an essential role in the protection of ovarian tissues from the infections. Antigen presenting cells that express major histocompatibility complex (MHC) class II plays a primary role in immune response to foreign agents (Benacerraf, 1981). They present antigen to T cells expressing CD4 antigen, namely helper/inducer T ells (Maccubbin and Schierman, 1986; Vainio et al., 1987, 1988). Our recent study identified MHC class II in the ovary (Barua and Yoshimura, 1999b; Barua et al., 2001) and oviduct (Zheng et al., 2001). Also other immunocompetent cells including macrophages, T cell subsets, B cells were found in these organs (Zettergren , 1995; Barua et al., 1988a, b, c; Barua and Yoshimura, 1999a; Zheng et al., 1998, 2000, 2001). These results suggest that local immune response can be induced in the ovary.

The thecal cells of ovarian follicles expressed MHC class II peptide that increased in association with follicular growth (Barua et al., 2001). The population of MHC class II-expressing cells (MHC class II+ cells) was increased with sexual maturation and by the stimulation with estrogen (Barua and Yoshimura, 199b), suggesting that their population in the ovary was highly affected by reproductive activity. Although the presence of MHC class II+ cells and endocrine factors to regulate their population has been shown in the ovary, it remains unknown whether they respond to foreign agents. Thus, the goal of this study was to determine whether the population of MHC class II+ cells was

changed in response to foreign agents in the blood circulation. Salmonella antigen was used to stimulate them in this study.

Materials and Methods

Birds and treatment

The birds used in this study were White Leghorn hens of approximately 270-300d old, regularly laying 7 or more eggs in a sequence. They 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*. They were i.v. injected with *Salmonella paratyphi* (SP) in PBS at a dose of 5.0 X 10^7 CFU/ 250 μl/bird 5 to 8 h after oviposition. Injection of birds with this amount of SP antigen did not cause follicular atresia. The SP sample was kindly provided by Serum Research Institute, Chiba prefecture (Ichikawa, Japan), and had been fixed with formalin, followed by washing with PBS. Control birds were i.v. injected with 250 μl of PBS. Three birds were used in each SP antigen-injected and control groups.

Tissue preparation

The birds were killed by decapitation 12 h after injection of AP antigen or PBS. The largest (F1) and third largest follicles (F3), white follicles (WF), and ovarian stroma were collected. They were embedded in OCT compound (Tissue-Tek, Sakura Finetek Inc., CA) and snap-frozen in a mixture of isopentane and solid carbon dioxide. Cryostat sections (15 μm thick) of them were air-dried on slides treated with 3-aminopropyl-triethoxysiline (Van Prooijen-knegt, 1982), and fixed with acetone and methanol on ice for 10 min each.

Immunostaining for MHC class II

Sections were washed in PBS for 10 min and incubated with 1 % (w/v) casein milk for 30 min. Sections were incubated overnight with mouse anti-chicken MHC class II monoclonal antibody (Veromaa et al., 1988) diluted with PBS containing 0.5 % (w/v) BSA at a dilution of 1:100. Sections were then washed with PBS for 15 min (3 X 5 min). Immunoreactions of the first antibody on the sections were detected by S-HRP immunostaining kit (Nichirei Co., Tokyo) according to the manufacturer's instructions. Briefly, the sections were incubated with the biotinylated secondary antibody and with avidin-peroxidase complex for 1 h each. The immunoreaction products were visualized by incubation with a mixture of 0.02 % (w/v) 3',3'-diaminobenzidine and 0.001 % (w/v) H2O2 in 0.05 M Tris-HCl (pH 7.6). Sections were counter-stained with hematoxylin, dehydrated and covered. Control staining were carried out simultaneously in which the first antibody was replaced with normal mouse IgG. No specific staining was observed in the control slides. Some sections were stained with hematoxilin and eosin for histology.

Analysis of MHC class II+ cell population

The sections were examined under a light microscope with an image analysis software (Image-ProPlus, Media Cybernetics, Silver Spring, MD). The immunopositive areas were measured in four different areas of the theca. Then the positive area in 100 μm^2 was calculated, and the mean of the four counts was expressed as the positive area in one tissue.

Statistical analysis

The significance of differences of positive area between SP antigen-injected and control groups was determined by Student's t tests. Differences of the positive area among follicles within control and SP

antigen-injected birds were examined one-way ANOVA (Snedecor and Cochran, 1967), followed by Duncan's multiple t test (Duncan, 1955). Differences were considered significant at $P<0.05$.

Results

The follicular wall consisted of the granulosa and theca layers, and loose connective tissue coat. The theca layer was differentiated into the theca interna and externa in WF, F3 and F1, whereas that of cortical follicles showed undifferentiated appearance. Capillary network distributed in the theca interna, and arterial and venous vessels were observed in the theca externa. The immunoreaction products for MHC class II were observed in the thecal layer of all follicles in both SP antigen-injected and control birds. Specifically, some of the theca interna cells around the capillaries and the fibroblast-like cells in the theca externa were positive for MHC class II immunoreaction (Fig. 1a-d). The MHC class II+ area was significantly greater in the F3 and F1 follicles than in WF and cortial follicles in control hens (Fig. 1a and c; Fig. 2). Also it was larger in WF, F3 and F1 than control follicles in SP antigen-injected birds (Fig. 1b and d; Fig. 2). The immunopositive areas for MHC class II in the theca of F1, F3 and WF were significantly greater in SP antigen-injected birds than control, whereas such difference was not observed in the cortical follicles (Fig. 2).

Discussion

We have examined whether the distribution of MHC class II+ cells in the theca of follicles was affected by an injection of hens with foreign antigen. Some of the theca interna cells and fibroblast-like cells in the theca externa were positive for MHC class II immunoreaction in control birds, and their population was greater in the F1 and F3 than WF and cortical follicles. These results support our previous report that MHC class II+ cells were increased in association with follicular growth, postovulatory regression and atresia (Barua et al., 2001).

Injection of birds with SP antigen significantly increased the distribution of thecal MHC class II+ cells in F1, F3 and WF, but not in cortical follicles. The area of MHC class II+ cells was greater in WF, F3 and F1 than cortical follicles in these birds. These results suggest that thecal MHC class II+ cells increase in response to circulating antigens, and play a significant role in the ovarian local immunity. Our previous report showed the fibroblast-like cells in the theca interna could phagocytose carbon particles injected intravenously into laying hens (Yoshimura and Okamoto, 1998). The population of cells that phagocytose the particles was greater in the larger follicles than smaller ones. Antigen presenting cells phagocytose foreign agents to present the antigen by MHC class II (Benacerraf, 1981; Bourlet, 1988). Thus it is likely that both the phagocytic activity and expression of MHC class II in response to foreign agents are greater in the larger follicles than smaller ones.

Antigen presenting cells expressing MHC class II stimulate helper/inducer T cells that express CD4 on their surface(Maccubbin and Schierman, 1986; Vainio et al., 1987, 1988), leading to enhance immunoglobulin production by B cells and macrophage activity (Arstila, 1994). We have localized CD4 T cells, which increase with sexual maturation and stimulation by estrogen, in the follicular tissues (Barua and Yoshimura, 1999a). Presence of macrophages and immunoglobulin containing cells in hen ovarian follicles and stroma has also been reported (Zettergren , 1995; Withange et al., 1997; Barua and Yoshimura, 1998a, b, c). It is assumed that increased thecal MHC class II cells in response to antigen stimulate more strongly CD4 T cells. In conclusion, it is suggested that the MHC class II+ cells in the theca increase in response to circulating foreign agents in ovarian follicles, and play a significant role in ovarian local immunity.

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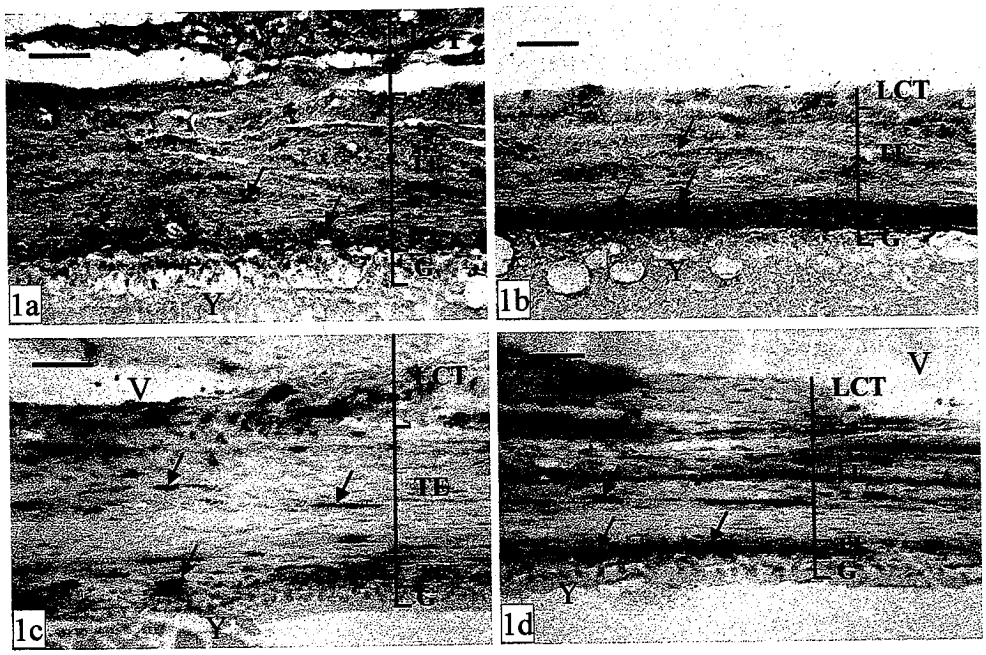
Explanation of Figures

Fig.1. Sections of ovarian follicles immunostained for major histocompatibility complex class II. (a) White follicle of a control bird. Immunoreactivities are observed in the theca. (b) White follicle of a bird injected with *Salmonella paratyphi* antigen. Note a increased population of immunopositive cells. (c) the largest follicle of a control bird. Immunopositive cells are located around the capillaries in the theca interna and in the connective tissue of the theca externa. (d) The largest follicle of a bird injected with *Salmonella paratyphi* antigen. Immunoreactive cells are increased in the theca layer. Arrows indicate the examples of immunopositive cells. G= granulosa layer, TI= theca interna, TE= theca externa, LCT= loose connective tissue coat, Y= yolk. Scale bar= 50 □m.

Fig.2. Differences in the immunopositive area for major histocompatibility complex class II in the follicles between *Salmonella paratyphi* antigen-injected and control birds.
Bars represent mean + SE of immunopositive area in 100 μm^2 (n=3 each). *Significant difference between control and antigen-injected hen groups ($P<0.05$). Bars with different letters are significantly different within control (a and b) and antigen-injected (m and n) birds ($P<0.05$).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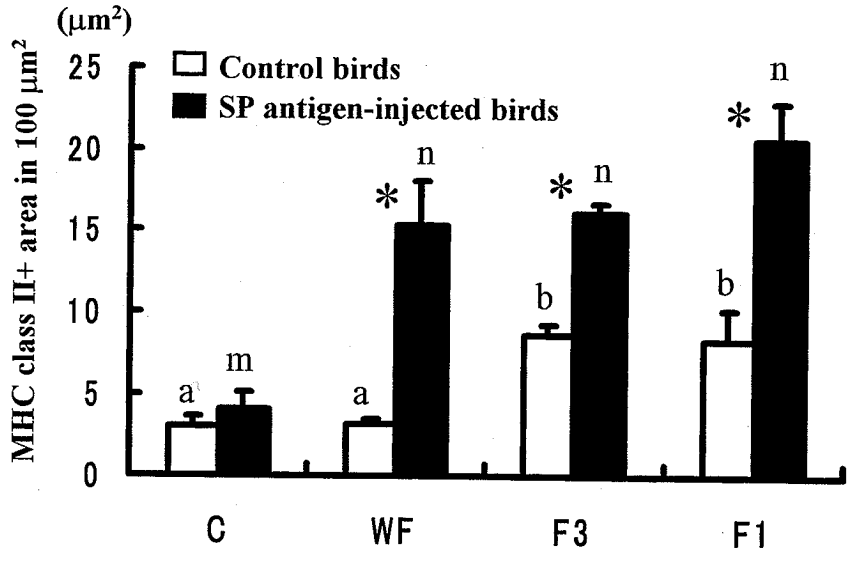
(μm²)
MHC class II+ area in 100 μm²
25
20
15
10
5
0
Control birds
SP antigen-injected birds
a
m
a
n
b
n
b
n
C
WF
F3
F1

Fig. 2