Changes in the Localization of MHC Class II Positive Cells in Response to *Salmonella enteritidis* Invasion in the Ovary of Laying Hens

Animesh Barua and Yukinori Yoshimura*

Graduate School of Biosphere Science, Hiroshima University,
Higashi-Hiroshima 739-8528, Japan

Major histocompatibility complex class II (MHC-II) molecules play essential roles in the initiation of immune responses against pathogenic agents. *Salmonella enteritidis* (*S. enteritidis*) bacteria may contaminate hen eggs during their formation in the ovary. The goal of this study was to determine whether ovarian MHC-II⁺ cells respond to *S. enteritidis*. Laying hens were intraperitoneally injected with or without PBS (control) or *S. enteritidis* (5.0×10^9 bacteria/bird) and examined 12 h after inoculation. Cryostat sections of ovarian stroma, small white follicles and preovulatory follicles were double-immunostained sequentially for the localization of MHC-II⁺ cells and *S. enteritidis*. The MHC-II⁺ cells were localized in the ovarian stroma and the theca layer of the stromal, small white and preovulatory follicles. *S. enteritidis* was detected in the stroma and the theca layer of all types of follicles 12h after inoculation. The frequency of MHC-II⁺ cells was significantly increased in the ovarian stroma and the theca of all types of follicles by *S. enteritidis* inoculation ($P < 0.01$) but not by PBS injection. These results suggest that the population of MHC-II⁺ cells in ovarian tissues increases in response to *S. enteritidis* invasion for defending against them.

Key words : follicles, ovary, MHC-II⁺ cells, *Salmonella enteritidis*

Introduction

Ovarian tissues are the target sites of various pathogenic agents, such as *Salmonella*, avian encephalomyelitis and lymphoid leucosis virus which may be transmitted to the eggs (Poppe, 1994 ; Keller *et al.*, 1995). In recent years *Salmonella enteritidis* (*S. enteritidis*), one of the causal agents of human food poisoning, has been suggested to contaminate eggs from the infected ovary (Keller *et al.*, 1995 ; Thiagarajan *et al.*, 1994 ; Gast and Beard, 1990). Previous reports have suggested the possibility of ovarian invasion either through blood-borne *S. enteritidis* following oral inoculation (Thiagarajan *et al.*, 1996) or colonization of peritoneum by the organism (Timoney *et al.*, 1989 ; Shivaprasad *et al.*, 1990). Therefore, initiation of immune responses against *S. enteritidis* infection in the ovary is essential to reduce the potential of disease transmission.

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*Correspondence to : Yukinori Yoshimura, Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

Tel & Fax : +81-824-24-7958 E-mail : yyosimu@hiroshima-ac.jp

An immune response against a pathogen requires a series of cellular interactions. One of these events is the processing and presentation of antigens by the cells containing MHC molecules to T cells (Maccubbin and Schierman, 1986 ; Vainio *et al.*, 1987). The MHC-I molecules present peptides of endogenous antigens synthesized within the infected cells, which are recognized by killer/cytotoxic T cells (Heemels and Ploegh, 1995). In contrast, exogenous antigens such as bacteria are internalized by antigen-presenting cells and the antigen is presented to helper/inducer T cells by MHC-II molecules (Wolf and Ploegh, 1995). *S. enteritidis* bacteria are the invasive exogenous agents, therefore, MHC-II molecules on the antigen presenting cells may play important roles in the initiation of immune responses against these pathogens in the ovary. In previous studies we observed that MHC-II⁺ cells were present in the ovary of healthy hens, and their population was increased with sexual maturation and stimulation by estrogen (Barua and Yoshimura, 1999).

Information on the responses of the ovarian MHC-II⁺ cells against specific pathogens such as *S. enteritidis* is very limited. Recently, we have observed that intravenous injection of dead *Salmonella paratyphi* antigens increased the population of MHC-II⁺ cells in the follicular tissues (Yoshimura and Takata, 2002). The patterns of live *S. enteritidis* invasion and response of MHC-II⁺ cells in the *S. enteritidis* -invaded ovary is unknown. Thus the aim of this study was to examine whether MHC-II⁺ cells respond to live *S. enteritidis* during the early phase of ovarian invasion.

Materials and methods

Birds

White Leghorn laying hens of approximately 71- to 74-wk-old laying 6 or more eggs in a sequence were used in this experiment. Handling of chickens was done in accordance with the Hiroshima University regulations for the conduct of animal experiments. Birds were kept in individual cages under a light : dark regimen of 14 h : 10 h and provided with feed and water *ad libitum*.

Bacterial Inoculation

Laying hens were divided into 3 groups with 5 hens in each group. Two groups were intraperitoneally (i.p.) injected within 30 minutes of oviposition with sterile PBS (control) or live *S. enteritidis* suspended in PBS at a dose of 5.0×10^9 bacteria/bird, whereas, another group was untreated. This dose of inoculation did not elicit any side effect in a preliminary experiment. These bacteria were donated by Hiroshima Prefectural Health and Environmental Center (Hiroshima, Japan) and were reported to be invasive in quail ovary and oviduct (Takata *et al.*, 2003).

Tissue Preparation

Treated birds were euthanized 12 h after injection with *S. enteritidis* or PBS and untreated birds were euthanized just before use through carbon dioxide inhalation and dissected for tissue collection. Ovarian stroma containing stromal follicles, small white follicles (SWF, 3–4 mm in diameter), third largest (F₃) and the largest (F₁) follicles were collected from each bird ($n = 5$ for each tissue in all groups of hens). Tissues were immediately fixed in periodate-lysine-paraformaldehyde solution overnight as described

by Mclean and Nakane (1974). Samples were then embedded in cryoembedding medium (OCT compound, Tissue-Tek, Sakura Finetek Inc. CA) and snap frozen in a mixture of isopentane and solid carbondioxide. Cryostat sections of 15 μ m thickness were air-dried on slides treated with 2% 3-aminopropyl-triethoxysilane.

Immunohistochemistry

Immunopositive MHC-II cells and *S. enteritidis* bacteria were co-localized sequentially on the tissue sections by double-immunostaining method. In the first staining sections were immunostained for MHC-II⁺ cell as described previously (Barua and Yoshimura, 1999). Briefly, sections were incubated with 1% (v/v) goat serum for 15 min, followed by overnight incubation with mouse anti-chicken Ia antibody (Southern Biotechnology Associates Inc., Birmingham, AL) diluted with PBS containing 0.5% (w/v) bovine serum albumin (BSA) at a concentration of 1 : 200. Immunoreaction products were identified using Histofine SAB-PO (M) kit (Nichirei, Tokyo, Japan), that is, sections were incubated with biotinylated anti-mouse IgG + IgM + IgA, and avidin-biotin-peroxidase complex for 1 h each. Immunoreactions were visualized by DAB-H₂O₂ mixture. After the first sequence, sections were washed with PBS for 15 minutes (5 min $\times$ 3 times), followed by 0.1M glycine for 90 minutes (30 min $\times$ 3 times). They were then incubated with anti-*Salmonella* O-9 antibody (HyTest, Turku, Finland) for 2 h. The immunoreaction products were identified by using Histofine SAB-PO (M) as mentioned above except for the color development, which was performed with TrueBlue peroxidase substrates (KPL, Gaithersburg, MD). Sections were then washed in distilled water, dehydrated with graded alcohol, and covered. Control staining was carried out simultaneously in which primary antibodies were replaced with normal mouse IgG. No positive staining was observed on the control slides.

Counting of the number of MHC-II⁺ cells

The sections were examined under a light microscope with an image analysis software (Image-ProPlus, Media cybernetics, Silver Spring, MD) as described previously (Barua and Yoshimura, 1999). The number of MHC-II⁺ cells was determined by observing three different areas of the theca of each follicle or the stroma within one ovary. Then the cell number was calculated to be the cell number in $1 \times 10^4 \mu\text{m}^2$ area. The average of the three counts was expressed as the cell number in $1 \times 10^4 \mu\text{m}^2$ area in one tissue of a bird.

Statistical analysis

The significance of differences in the MHC-II⁺ cell numbers in $1 \times 10^4 \mu\text{m}^2$ area among the untreated, PBS- and *S. enteritidis*-injected birds was determined by one-way ANOVA (Snedecor and Cochran, 1967) followed by Duncan multiple range test (Duncan, 1955). Differences were considered significant when $P < 0.05$.

Results

Localization of MHC-II⁺ cells and *Salmonella* antigen in the stroma

The MHC-II⁺ cells were localized in the stroma and the theca of the stromal follicles of all birds (Fig. 1 A, B). Many of the MHC-II⁺ cells in the ovarian stroma were macrophage-like cells showing oval or irregular shapes. Some fibroblast-like cells

in the theca layer of stromal follicles were also immunopositive for MHC-II molecules. Immunoreaction products for the *S. enteritidis* were detected in the stroma and the theca of stromal follicles of birds 12 h after (Fig. 1B). However, no consistent correlation was observed between the sites of MHC-II⁺ cells and *S. enteritidis* invasion. Figure 2 shows the frequencies of MHC-II⁺ cells in the ovarian stroma and the theca layer of stromal follicles of before (untreated) and 12 h after PBS or *S. enteritidis* injection. Significant differences in the population of MHC-II⁺ cells were not observed between the untreated and PBS-injected birds. The frequency of MHC-II⁺ cells increased significantly in both the stroma and the theca layer of stromal follicles 12 h after *S. enteritidis* injection as compared to those before injection or 12 h after PBS injection ($P < 0.01$).

Localization of MHC-II⁺ cells and *Salmonella* antigen in the follicular tissues

The theca layer was differentiated into the theca interna and theca externa in SWF, F3 and F1 follicles. Immunopositive MHC-II cells were localized in the theca interna

Fig. 1. Sections of ovarian stroma and the largest follicle of hens inoculated intraperitoneally with PBS or 5×10^9 *Salmonella enteritidis* (*S. enteritidis*). Sections were immunostained for MHC-II⁺ cells and *S. enteritidis*. Thin arrows and black arrows indicate the examples of *S. enteritidis* and MHC⁺ cells, respectively. G : granulosa layer, O : oocyte, T : theca layer, TI : theca interna, TE : theca externa, S : stroma. Scale bars represent 20 μ m. A) Ovarian stroma of control (PBS-treated) hen. MHC-II⁺ cells are seen in the stroma and in the theca layer of stromal follicles. B) Ovarian stroma of hen 12 h after *S. enteritidis* inoculation. Note the *S. enteritidis* in the stroma and theca layer of stromal follicle, and many MHC-II⁺ cells these tissues. C) The largest follicle of control hen. MHC-II⁺ cells are seen in the theca externa and interna layers. D) Largest follicle of hen 12 h after *S. enteritidis* inoculation. The *S. enteritidis* and many MHC-II⁺ cells are seen in the theca externa and interna layers.

Fig. 2. Changes in the frequency of MHC-II⁺ cells in the ovarian stroma and the theca layer of stromal follicles in response to *S. enteritidis* invasion. Hens were examined before (C) or 12 h after intraperitoneal injection with PBS (PBS) or 5×10^9 *S. enteritidis* (SE). Each value shows mean $\pm$ SEM of the number of MHC-II⁺ cells in $1 \times 10^4 \mu\text{m}^2$ area ($n = 5$ hens in each group). Bars with different letters are significantly different ($P < 0.01$).

and externa layers in F1 follicles of all birds. Some fibroblast-like cells in the theca layer were also immunopositive for MHC-II molecules (Fig. 1 C, D). *S. enteritidis* were localized in the theca externa and occasionally in the interna layer 12 h after injection. The patterns of MHC-II⁺ cells and *S. enteritidis* localization in SWF and F3 were similar to those in F1 follicles (figure not shown). As in the stroma, no consistent correlation was observed between the site of MHC-II⁺ cells and *S. enteritidis* invasion. Significant differences in the frequency of MHC-II⁺ cells in the theca layers of all types of follicles were not observed between the PBS-injected and untreated birds. The frequency of MHC-II⁺ cells was significantly greater in the theca layers 12 h after *S. enteritidis* injection than those before treatment or 12 h after of PBS treatment in SWF, F3 and F1 ($P < 0.01$) (Fig. 3).

Discussion

This is the first report that identified MHC-II⁺ cell and *S. enteritidis* in the same ovarian tissues. The significant finding was that the population of MHC-II⁺ cells in the ovarian stroma and follicles increased with the invasion of *S. enteritidis* within 12 h of the i.p. inoculation.

There are reports that *S. enteritidis* bacteria were isolated from the ovaries and egg yolk following oral infection by a culture system (Shivaprasad *et al.*, 1990, Barrow and Lovell, 1991). Isolation studies of *Salmonella* organism by organ culture have suggested the possibility of infection of ovary through a colonization of peritoneum by the *S. enteritidis* (Timoney *et al.*, 1989). In the present study, bacteria were detected in the ovarian stroma, SWF, F3 and F1 follicles 12 h after intraperitoneal inoculation. These results indicate that *S. enteritidis* could invade the ovarian and follicular tissues from the peritoneal cavity within 12 h of inoculation, supporting the results in Japanese

Fig. 3. Changes in the population of MHC-II⁺ cells in the theca layer of small white follicle (SWF), third largest follicle (F3) and the largest follicle (F1) in response to *S. enteritidis* invasion. Hens were examined before (C) or 12 h after intraperitoneal injection with PBS (PBS) or 5×10^9 *S. enteritidis* (SE). Each value shows mean $\pm$ SEM of the number of MHC-II⁺ cells in $1 \times 10^4 \mu\text{m}^2$ area ($n=5$ hens in each group). Bars with different letters are significantly different ($P < 0.01$).

quail reported by Takata *et al.* (2003).

In present study, the *S. enteritidis* and MHC-II⁺ cells in the ovarian tissues were localized in the same tissues by double-immunostaining. It was clearly demonstrated that the population of MHC-II⁺ cells in the ovarian stroma and all types of follicles was greater in *S. enteritidis*-injected group than pre-inoculation or PBS-injected group. Our previous study revealed an increase in the population of MHC-II⁺ cells in the ovarian follicles in response to intravenous injection of dead *S. paratyphi*, whereas the current study showed their increase following i.p. inoculation with live *S. enteritidis*. However, consistent correlation between the sites of MHC-II⁺ cells and *S. enteritidis* in the tissues was not observed. Bacterial toxins secreted from *Salmonella* are active participants in the induction of host responses and have the potential to modulate the immune response (Hughes and Galan, 2002). Therefore, present results suggest that the population of MHC-II⁺ cells is increased in response to the invasion of *Salmonella* bacteria and their products in the whole ovarian tissues.

In conclusion, we suggest that the population of MHC-II⁺ cells in the ovarian tissues increased in response to *S. enteritidis* within 12 h of invasion, which may play significant roles in defending against these pathogens.

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