Effects of Repeated Lipopolysaccharide Stimulation on the Development of Antigen-presenting Cells and T Cells Pool in Hen Vagina

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The aim of this study was to determine how the mucosal immunity mediated by T cells is developed in the vagina. Antigen-presenting cells and T cells play important roles in the early process of host defense. Mature T cells and immature dendritic cells expressing CC chemokine receptor (CCR) 6. In contrast, immature T cells and mature dendritic cells express CCR7. It was examined whether repeated antigen stimulation is effective for development of the pool of antigen-presenting cells and T cells in the vagina. White Leghorn hens were intravaginally injected 1 (single injection group) or 5 times (repeated injection group) in 10 days with saline (control) or lipopolysaccharide (LPS). The vagina was collected 1 day after the final injection. Frozen sections of them were immunostained for CD4, CD8, TCRγδ T cells and major histocompatibility complex (MHC) class II+ cells. Expressions of CCR6, CCR7 and MHC class II were analyzed by quantitative RT-PCR. The frequency of CD4+, CD8+, TCRγδ T cells were significantly increased in LPS-repeated injection group, however that of MHC class II+ cells was not changed significantly. The expression of CCR6 was significantly upregulated in LPS-repeated injection group, however, the expression of CCR7 and MHC class II was not affected. The densities of the each T cell subset and MHC class II+ cells, as well as expressions of chemokine receptor, MHC class II were not affected by single LPS injection or saline injection. These results suggest that the repeated antigen stimulation may not affect antigen-presenting ability by antigen-presenting cells, whereas it may lead influx of T cell subsets in the vagina. These accumulated T cells pool may contribute to form prequiescence of host immunity in the vaginal mucosa.

Key words: chemokine, MHC class II, repeated antigen stimulation, T cell, vagina

Introduction

Hen oviduct is susceptible to pathogenic microorganisms colonizing in the cloaca. Pathogenic microbial infection of hen oviduct leads to functional disorder for egg formation and production of contaminated eggs. CD4+ helper T cells directly or indirectly activate macrophages and B cells in response to antigen presentation by major histocompatibility complex (MHC) class II expressed on the antigen-presenting cells (APCs). CD8+ T cells kill the infected cells by releasing cytotoxic substances in response to antigens presented by MHC class I. Although biological function of chicken TCR-γδ T cells have not been established, they have cytotoxic activity and most of them in the intestinal epithelium express CD8 (Chen et al., 1994). TCR-γδ T cells may play role in early phase of the immune response to Salmonella infection (Born et al., 2007).

Immature dendritic cells do not have ability of antigen presentation, whereas they capture antigens. Then, they differentiate into mature cells expressing MHC class II and acquire the ability of antigen presentation to T cells (Winzler et al., 1997; Granucci et al., 1999). CC chemokine receptor (CCR) 6 is expressed on matured effector T cells (Liao et al., 1999) and immature dendritic cells to the infected site (Lukacs et al., 2001). In contrast, CCR7 is expressed on the immature T cells and matured dendritic cells (Sallusto et al., 1999; Wu et al., 2011). Thus MHC class II and CCR are important for antigen presentation process.

The density of CD4+ and CD8+ T cells was higher in the vagina than in the other part of oviduct in laying hens (Yoshimura et al., 1997; Withanage et al., 1997). In our recent study, the ability to recruit CD8+ T cells was kept in the vagina, but it was decreased in the other oviductal segments in molting hens (Nii et al., 2011). These results may suggest that the vagina maintains the immunoresponse ability even in molting phases because that segment is exposed to antigens colonizing in the cloaca, whereas the ability may be declined in the other segments where less antigens enter. We hypothesized that exposure to proper antigens may play role in
enhancing or maintaining the immune functions of the oviduct.

The goal of this study was to determine how the mucosal immunity mediated by T cells is developed in the vagina. It was examined whether repeated antigen stimulation is effective for development of the pool of APCs and T cell subsets in the oviductal vagina. We examined the effects of repeated stimulation by lipopolysaccharide (LPS), an antigenic agent, on the densities of APCs and T cell subsets as well as expression of molecules related to these cells in the vagina.

Materials and Methods

Experimental Birds

White Leghorn laying hens approximately 160-d-old were kept in individual cages under a daily light regimen of 14 h light: 10 h dark, and provided with feed and water ad libitum. They were divided into four experimental groups (n=5 each), and the birds of each group were intravaginally injected from cloaca 1 or 5 times at intervals of two days with 1 ml saline (Otsuka Pharmaceutical Co., Tokyo, Japan) with or without 10 μg LPS. The saline-single and LPS-single injection groups were injected with saline or LPS only one time. The saline-repeated and LPS-repeated injection groups were injected with saline or LPS 5 times. Stock solution of LPS was prepared by dissolving LPS from Salmonella minnesota (InvivoGen, San Diego, CA, USA) in saline at a concentration of 10 μg/ml. Approximately one third of total length of the vagina of cloaca side was collected 1 day after the final injection. It had been confirmed in preliminary experiments using a toluidine blue solution that the intravaginally injected solution reached approximately a half length of the vagina. Handling of birds was done in accordance with the regulations of Hiroshima University for animal experiments.

Histology and Immunohistochemistry for Immune Cells

The vagina tissues were fixed with 10% (v/v) formalin in phosphate buffered saline (PBS) and processed for paraffin sections (4 μm in thickness), or embedded in OCT compound (Sakura Co., Tokyo, Japan) and snap-frozen in isopentane and solid CO2 mixture to prepare frozen sections (10 μm in thickness). The paraffin sections were stained with Hansen’s hematoxylin and eosin for histological observation. Frozen sections were air-dried on slides and fixed with acetone on ice for 30 min. Then, they were washed with PBS, and incubated with 10% (v/v) normal rabbit serum (blocking solution) for 30 min. They were incubated overnight with mouse monoclonal antibodies to chicken CD4 (Santa Cruz Biotech., Inc, Santa Cruz, CA,USA), chicken CD8 (Santa Cruz Biotech., Inc), chicken TCR-γδ (Southern Biotech, Birmingham, AL, USA) or chicken MHC class II (Southern Biotech) diluted at a concentration of 1 μg/ml with PBS. After washing the sections with PBS (3×5 min), the immunoreaction products were detected using Histofine SAB-PO (M) kit (Nichirei Co., Tokyo, Japan). Briefly, the sections were incubated with biotinylated anti-mouse IgG+IgA+IgM and streptavidin-peroxidase for 1 h each with washing in PBS (3×5 min) after each step. Immunoprecipitates were visualized by incubating the sections with a reaction mixture of 0.02% (w/v) 3′,3′-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H2O2 in 0.05 M Tris-HCl buffer (pH 7.6). The sections were counter stained with Hansen’s hematoxylin.

Image Analysis for Immune Cell Frequencies

Sections were examined under a light microscope (Eclipse E400, Nikon, Tokyo, Japan) with image analysis software (NIS-Elements, Nikon). The numbers of three types of T cells within the mucosal epithelium (1.5×10⁵−4.6×10⁵ μm²) and lamina propria (2×10⁵−8×10⁵ μm²) were counted.

The concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). Then, RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co., Ltd, Osaka, Japan) according to the manufacturer’s instructions. The reaction mixture (10 μl) consisted of 1 μg of the total RNA, 1 × RT buffer, 1 mM dNTP mixture, 20 U of RNase inhibitor, 0.5 μg of oligo (dt) 20, and 50 U of ReverTra Ace. The reverse transcription was performed at 42 °C for 30 min, followed by heat inactivation for 5 min at 99 °C using the PTC-100 programmable thermal controller (MJ Research, Waltham, MA, USA), programmed at 37 °C for 45 min and 65 °C for 10 min. The concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). The extracted total RNA samples were dried and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA). They were treated with 1 U of RNase-Free DNase (Promega Co., Madison, WI, USA) on a PTC-100 programmable thermal controller (MJ Research, Waltham, MA, USA), programmed at 37 °C for 45 min and 65 °C for 10 min. The concentration of RNA in each sample was measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). Then, RNA samples were reverse-transcribed using ReverTra Ace (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer’s instructions. The reaction mixture (10 μl) consisted of 1 μg of the total RNA, 1 × RT buffer, 1 mM dNTP mixture, 20 U of RNase inhibitor, 0.5 μg of oligo (dt) 20, and 50 U of ReverTra Ace. The reverse transcription was performed at 42 °C for 30 min, followed by heat inactivation for 5 min at 99 °C using the PTC-100 programmable thermal controller (MJ Research). The real-time PCR was performed using the Roche Light Cycler system (Roche Applied Science, Indianapolis, IN, USA). The reaction mixture consisting of 20 μl buffer containing 1 μl of cDNA, 1 × SYBR Premix EX Taq (Takara, Tokyo, Japan), 0.4 μM of each primer was taken into 20 μl capillaries (Roche Diagnostics GmbH, Mannheim, Germany). Table 1 shows the primers used for PCR. The cycle parameters for PCR reaction program were denaturation at 95 °C for 5 s and annealing at 60 °C for 20 s. Real-time PCR data were analyzed by the 2−ΔΔCT method to calculate the relative level of mRNA in each sample and expressed as a ratio relative to RPS17 housekeeping gene (Livak and Schmittgen, 2001). A sample of vagina of non-treated hen was used for the standard.
Statistical Analysis

The fold differences in the mRNA expression and frequencies of immunopositive cells were expressed as the mean±SEM. Significance of differences between the control and LPS groups within single or repeated injection groups and between single and repeated injection groups within control or LPS groups were examined by t-test. Differences were considered significant when P value was < 0.05.

Results

The lamina propria of the vagina consisted of loose connective tissue, and the mucosal surface was lined by ciliated pseudostratified epithelium. No histological abnormality was found in all birds (Data not shown). Figure 1 shows the results of immunostaining for CD4+, CD8+, TCRγδ+ T cells and MHC class II+ APCs in the vagina of LPS-repeated injection group. The CD4+ T cells and MHC class II+ cells were localized in the lamina propria and seldom observed in the mucosal epithelium (Fig. 1a and d), whereas CD8+ and TCRγδ+ T cells were localized in both mucosal epithelium and connective tissues of the lamina propria (Fig. 1b and c).

The frequency of CD4+ T cells in the lamina propria was not different between saline-single and LPS-single injection groups. However, their frequencies in the LPS-repeated injection group were greater than LPS-single injection and saline-repeated injection groups (Fig. 2a).

The frequency of CD8+ T cells in the lamina propria did not show difference between saline-single and LPS-single injection groups. Their frequency was greater in the LPS-
repeated injection group than saline-repeated injection group (Fig. 2b). The frequencies of CD8+ T cells were 3.0–4.7 cells in $1 \times 10^5 \mu m^2$ in the mucosal epithelium, and there was no significant difference among each group (Data not shown).

Figure 2c shows the frequency of TCRγδ+ in the lamina propria. Their frequency was not different between saline-single and LPS-single injection groups. However, the frequency was significantly greater in LPS-repeated injection group than LPS-single injection and saline-repeated injection groups. The frequencies of TCRγδ+ T cells were 0.17–0.27 cells in $1 \times 10^5 \mu m^2$ area in the mucosal epithelium in each group, and there was no significance of difference among them (data not shown).

The density of MHC class II+ cells in the lamina propria was approximately 9–12% in all the experimental groups. No significant difference was found among each group (Fig. 2d).

Figure 3 shows the effects of LPS injection on expression of CCR6, CCR7 and MHC class II. The CCR6 expression was not different between saline-single and LPS-single injection groups (Fig. 3a). However, its expression was significantly greater in the LPS-repeated injection group than in the saline-repeated injection group (Fig. 3a). The expressions of CCR7 and MHC class II were not significantly different among each experimental group (Fig. 3b–d).

Discussion

We have examined whether the repeated injection with LPS into the vagina affects the frequency of T cell subsets and APCs, and mRNA expressions of molecules related to them. Significant findings were (1) the densities of CD4+, CD8+ and TCRγδ+ T cells in the lamina propria of the vagina were increased by repeated LPS injection, however that of MHC class II+ cells was not affected, and (2) the expression of CCR6 was increased by repeated LPS injection, although expression of CCR7 and MHC class II was not changed. The CD8+ and TCRγδ+ T cells were localized in both mucosal epithelium and lamina propria, whereas CD4+ T cells and MHC class II+ APCs were mainly in the lamina propria. Thus, it is assumed that elimination of infected cells by CD8+ and TCRγδ+ T cells occurs in both mucosal epithelium and lamina propria. Antigen presentation by APCs and stimulation
of B cells and macrophages by CD4⁺ T cells may occur in the lamina propria in response to pathogenic agents invading that tissue.

The APCs such as dendritic cells express MHC class II to present antigens to CD4⁺ T cells. The chicken immature dendritic cells express CCR6, whereas mature dendritic cells that have acquired antigen-presenting ability express CCR7 (Wu et al., 2011). The current results showed the localization of MHC class II⁺ cells (Fig. 1) and expression of CCR6, CCR7 and MHC class II (Fig. 3) in the vaginal mucosa. These results suggest the presence of immature and mature dendritic cells in that tissue. Our previous study showed that expression of MHC class II in the follicular theca was increased after 4 h post intraperitoneal injection of vial Salmonella enteritidis (Barua and Yoshimura, 2004), suggesting that the expression of MHC class II was upregulated within a short time after antigen exposure. However, in the current study, in which the expression of MHC class II was examined after 24 h post LPS-single or repeated injection, significant increase of MHC class II was not identified. This result suggests that stimulation by antigen may not affect the density of APCs and their basal level of MHC class II expression even after repeated injection.

The densities of T cell subsets including CD4⁺, CD8⁺ and TCRγδ⁺ T cells in the lamina propria of the vagina were increased in the LPS-repeated injection group, but not in the LPS-single injection group. The CCR6 expression was also higher in the LPS-repeated injection group than saline-repeated injection groups. Since it is known that those three T cell subsets express CCR6 (Liao et al., 1999), the higher CCR6 expression in the LPS-repeated injection groups may reflect the increase of T cell subsets. The macrophage inflammatory protein (MIP)-3α is a chemokine that affects the CCR6⁺ cells (Baba et al., 1997). Toll-like receptors (TLRs) are a member of pathogen-associated molecular pattern receptors that recognize microbial agents. The interaction of TLRs and their ligands induces cellular response such as synthesis of proinflammatory cytokines, chemokines and antimicrobial peptides (Kogut et al., 2006; Berndt et al., 2007; Cheeseman et al., 2008). Expression of TLR4 that recognizes LPS of Gram-negative bacteria has been identified in chicken ovary (Takeuchi et al., 1999; Li et al., 2009; Ozoe et al., 2009). Human intestinal epithelial cells express various cytokines and chemokines including MIP-3α by interaction of TLR4 with LPS of Salmonella (Eckmann and Kagnoff, 2001). We also showed chemokine (C-X-C Motif) Ligand 2 (CXCL12), a member of CXC chemokine that may attract T cells, was expressed in the hen vagina and its expression was upregulated by LPS stimulation (Nii et al., 2011). Thus, increase in T cell frequencies by repeated LPS stimulation may be caused by chemokines attracting T cells synthesized by LPS stimulation. Significantly higher den-

![Fig. 3. Effects of lipopolysaccharide (LPS) on the mRNA expression of CC chemokine receptors (a, b), and MHC class II (c) in the mucosal tissues of the vagina. Hens were intravaginally injected with saline or LPS as described in Fig. 2. The values are the mean±SEM of fold change in the expression. **Significantly different (P<0.01).](image-url)
sities of T cells were observed in repeated LPS injection group, but not in single injection group. It is assumed that T cells densities in the vagina are increased by single LPS injection and they returned to the original level within 24 h. However, frequent increase of T cells by repeated LPS injection may lead to the accumulation of T cell subsets in the lamina propria, which eventually formed a pool of T cells in the mucosa. In hen ovary, CD4^+ T cells, but not CD8^+ T cells, were increased by 6 h after single LPS injection, followed by decreasing tendency by 12 h (Abdelsalam et al., 2011). Consequently, it is suggested that the repeated LPS injection in the vagina may lead the accumulation of T cell subsets in the lamina propria, probably under regulation of chemokines.

T cell subsets play essential roles in the defense against viral and bacterial infection (Janse et al., 1994; Berndt and Methner, 2001; Beal et al., 2004), and CD4^+ and CD8^+ T cells were increased in hen oviduct infected by Salmonella enteritidis (Withange et al., 1998, 2003). The current study showed that repeated stimulation by antigen increased the density of CD4^+, CD8^+ and TCRγδ^+ T cells in the mucosa of vagina. The immunoresponse to infectious agents by T cells may occur quickly if the mucosa tissue contains a greater T cells pool. Previous study by Zheng et al. (1998) showed that migration of T cells into oviductal mucosa was stimulated by estrogen. The current results suggest that adequate antigen stimulation has also a benefit to enhance the cellular immunity by forming T cells pool in the vaginal mucosa of laying hens.

In conclusion, we suggest that stimulation by repeated LPS injection results in formation of T cell subset pool in the vaginal mucosa, although it may not affect the antigen-presenting ability. The accumulated T cells pool may contribute to form prequiescence of host immunity. Thus, proper antigen stimulation may be effective for the development of mucosal immunity mediated by T cells in hen vagina.

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