学 位 論 文 の 要 旨

論文題目　Studies on Eggshell Malformation through Cytotoxic Immune Factors Induced by Bacterial and Viral Antigens in the Hen Oviduct (ニワトリ卵管における細菌およびウイルス抗原誘導性の細胞傷害性免疫関連因子による卵殻形成不全に関する研究)

広島大学大学院生物圏科学研究科 生物資源科学　専攻
学生番号　D126120
氏　　名　新居 隆浩

Infection by pathogenic bacteria and virus in the oviduct causes not only the problem in eggshell formation, but also debility of host bird, pollution of egg and chicks, and food poisoning for human. The regulatory mechanism of immune function in the oviduct is of importance to enhance the defence function against infection, and it is also important for host animal health and safe and efficient egg production. The cytotoxic cells such as NK cells and $CD8^+$ T cells with their chemokines and cytokines may have primary role for the defense systems against infection by invasive bacteria and virus. The aim of this study was to determine the immunoresponse process including recruitment of cytotoxic cells induced by bacterial and virus infection, and the effects of the immune factors on the eggshell formation in hen oviducts.

1. Effects of lipopolysaccharide on the recruitment of immunocomponent cells in the hen oviduct

The infection risk of pathogenic bacteria is reported to be higher in the molting phase than laying phase. Understanding the factors regulating the local immune functions in the oviduct are expected to contribute to enhance host health and safe egg production. Therefore, the aim of the study in Chapter 2 was to know why the susceptibility is high in hen oviduct during the molting phase. In Experiment 1, the influx of T cells and the expression of cytokines to regulate their influx were examined in the laying and molting hens inoculated with lipopolysaccharide (LPS). White Leghorn laying and molting hens were intravenously injected with saline (control) or LPS. After 3 or 6 h of injection, expression of *IL-1β*, *IL-6*, and *IL-8*, and the localization of $CD4^+$ and $CD8^+$ T cells in the uterus and vagina were examined by real-time PCR and immunohistochemistry. The expressions of *IL-1β*, *IL-6*, and *IL-8,* and the frequency of $CD4^+$ T cells of both laying and molting hens and $CD8^+$ T cells of laying hens in the uterus and vagina were upregulated 3 or 6 h after LPS injection. However, in the molting hens, LPS stimulation increased $CD8^+$ T cell in the vagina, but not in the uterus. In Experiment 2, it was examined whether exposure to proper continuous antigen stimulation enhances the immune functions in the oviduct. The LPS (10 μg) or saline (control) were repeatedly injected (1 or 5 times for 10 days) in the vagina lumen, and the density of T cells ($CD4^+$, $CD8^+$, $TCR\gamma\delta^+$ T cells) and antigen presenting cells (APCs) as well as the expression of molecules related to those immunocompetent cells (*CCR6*, *CCR7* and *MHC class II*) were examined**.** The frequency of T cells and the expression of *CCR6*, but not the frequency of MHC class II^+ cells and the expression of *CCR7* and *MHC class II* were significantly increased in LPS-repeated injection group. The densities of the each immune cells, and expressions of all gene were not affected by single LPS injection. These results suggest that the expression of *IL-1β*, *IL-6* and *IL-8* were up-regulated in association with $CD4^+$ and $CD8^+$ T cells recruitment in response to LPS in the oviduct of the laying hens, but the ability to recruit $CD8^+$ T cells may be depressed during the molting phase. The lesser recruitment of $CD8^+$ T cells may be one of the reasons why the oviducts are more high susceptible during the molting phase. Because T cell pool was more

developed in the vagina of LPS-repeated stimulation group than control, the lesser antigen stimulation in the vagina during molting may result in the reduction of T cell pool, and it may be also one of the reason for the higher susceptibility in the oviduct during the molting phase.

2. Effects of estrogen on the cytotoxic response to avian infectious bronchitis (I**B) virus infection in hen oviduct**

Development of hen oviduct is regulated by gonadal steroid, therefore it may be possible that the local immunity is also regulated by these endocrinological factors. The aim of Chapter 3 was to determine whether the egg-laying phase and estrogen affect the induction of cytotoxic cells in response to IB virus infection in the oviduct. Attenuated IB virus (aIBV group) or its vehicle (control group) was introduced to the oviductal magnum lumen of the laying and molting hens, as well as molting hens injected with estradiol benzoate (M-EB hens) or corn oil (M-oil hens). Oviductal isthmus and uterus were collected 24 h after aIBV injection. The frequency of $CD8^+$ and TCR-$\gamma\delta^+$ T cells, and the gene expression of Toll-like receptor 7 (TLR7), natural killer cell receptor (B-NK), cytotoxic substances (granzyme, perforin), and cytokines (CXCL12, CX3CL1 and IFN-γ) were examined. The frequency of $CD8^+$ and TCR-$\gamma\delta^+$ T cells in the isthmus, and $CD8^+$ cells in the uterus was significantly higher in the aIBV group in the laying and M-EB hens, but not in the molting and M-oil hens. The expressions of all genes in the isthmus, and of CX3CL1 and IFN-γ in the uterus were higher in the aIBV group in the laying and M-EB hens, but not in the molting and M-oil hens. These results suggests that infection by IB virus causes the cytotoxic immunoresponse with up-regulation of cytokines in the isthmus and uterus. This response may be declined during the molting phase due to decrease in the circulating estrogen level.

3. Effects of IB virus antigen on eggshell formation in hen oviduct

IB virus infects oviduct, and leads to impair of eggshell formation, and disorder of egg production in hens. Thus, the effects of IB virus antigen on the immunoresponse factors and eggshell formation may be one of the models to understand the mechanism by which the eggshell formation is disrupted by virus infection. The aim of this study was to determine the mechanism by which the IB virus affects eggshell formation. Attenuated IB virus (aIBV group) or vehicle (control group) was injected into the magnum lumen. The changes in the expression of genes related to eggshell formation (collagen types I and V, and calbindin), densities of cytotoxic cells ($CD8^+$ cells and TCR$\gamma\delta^+$ T cells) as well as gene expression of molecules related to cytotoxic immunoreaction (B-NK, perforin and granzyme) and cytokines (IL-1β, IL-6, IFN-γ and IL-2) were examined in the isthmus and uterus collected after 24 or 48 h of aIBV injection. Gene expression of collagen type I, but not collagen type V, in the isthmus and calbindin in the uterus was decreased in the aIBV group. Whereas, the frequencies of T cells, and the expression of cytotoxic molecules and cytokines in the isthmus and uterus were significantly higher in the aIBV group than in the control group. These results suggest that IBV infection causes disorder of eggshell formation by disturbing gene expression of collagen type I in the isthmus and calbindin in the uterus, probably via the effects of substances from cytotoxic cells and proinflammatory cytokines.

4. Effects of IL-1β and IL-6 stimulation on the expression of the eggshell formation-related factors in the cultured uterus tissue

From the above results, it was hypothesized that pro-inflammatory cytokines possibly affect the function of the uterus glandular cells which are responsible for shell formation. The aim of this study was to determine whether the IL-1β and IL-6 affect the expression of the eggshell formation-related factors in the uterus mucosa. Uterine mucosa tissues collected from laying hens were cultured for 1.5 h or 3 h in TCM-199 medium with or without chicken recombinant IL-1β or IL-6 at concentrations of 0 to 1000 ng/ml. The expression of *IL-1β and IL-6 receptors* in the tubular gland cells and whole mucosa of the uterus was analyzed. Gene expression of *calbindin* (Ca^{2+} transporter), *PMCA1*, *PMCA2* (calcium pumps*)*, *CA2* (carbonic anhydrase) and *SLC26A9* (HCO_3^- transporter), and protein density of calbindin were examined. The expression of IL-6 receptor was identified in the tubular gland cells and whole tissues, but IL-1β receptor was not identified in the tubular gland

cells. Expression of *calbindin*, *PMCA1*, *PMCA2*, *CA2* and *SLC26A9* was significantly increased, whereas the density of immunoreactive calbindin was significantly decreased in the tissues incubated with IL-1β and IL-6 than in the control group. These results suggest that IL-1β and IL-6 temporarily upregulate the gene expression of eggshell formation-related genes, but down-regulate the protein density of calbindin in hen uterine mucosa at the early stage of stimulation. It is assumed that IL-1β and IL-6 affect the transportation of Ca^{2+} to cause eggshell malformation in hen uterus.

5. Conclusion

In conclusion, the cytokine production and attraction of the cytotoxic cells are important to protect the oviduct from infection by pathogenic bacteria and virus in hens. Estrogen is likely necessary for enhancement of cytotoxic immune reaction in the oviduct. It was also established by the current studies that the produced IL-1β and IL-6, and cytotoxic cells and cytotoxic factors induced by microbe infection are likely one of the factors of eggshell malformation caused by pathological microbe infection. These knowledge is expected to be useful for the basis of the technology development for the preventive hygiene in the oviduct and the safe egg production.