Ovarian Cell-Mediated Immune Response to *Salmonella enteritidis* Infection in Laying Hens (*Gallus domesticus*)[1]

A. Barua and Y. Yoshimura[2]

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

ABSTRACT The aim of this study was to examine the response of cell-mediated ovarian immunity against *Salmonella* infection in hens. Laying hens were injected intraperitoneally with PBS (control) or *Salmonella enteritidis* (SE). Ovarian stroma containing stromal follicles, small white follicles (SWF), and third largest (F3) and the largest (F1) follicles were collected 12 or 24 h after inoculation and fixed in periodate-lysine-paraformaldehyde. Frozen sections were stained first for CD3+, CD4+, or CD8+ T cells and then for SE by a double immunostaining method. Immunoreaction products for SE were detected in the ovarian stroma, theca of stromal follicles, SWF, F3, and F1 at 12 and 24 h after inoculation. Immunopositive T-cell subsets were localized in the stroma and theca of follicles in birds inoculated with or without SE. The populations of CD3+, CD4+, and CD8+ T cells were significantly greater in the stroma and the theca of follicles 12 h after SE inoculation than in those of control birds ($P < 0.01$). Their frequencies were further increased in those tissues 24 h after inoculation ($P < 0.01$). Injection of SE did not cause significant differences in the CD4+:CD8+ T-cell ratio as both subsets increased proportionately. The current results indicate that the population of T-cell subsets increases in the ovarian stroma and the follicular tissues in response to SE invasion within 12 h of inoculation. Thus, cell-mediated immune response against SE, their products, or both may be induced in the hen ovary.

(*Key words*: cell-mediated immunity, follicle, laying hen, ovary, *Salmonella enteritidis*)

2004 Poultry Science 83:997–1002

INTRODUCTION

Pathogenic agents may infect hen ovarian tissues and subsequently be transmitted to the eggs (Blaxland et al., 1982). *Salmonella enteritidis* (SE) is one such agent that can be transmitted to the eggs from infected hens and cause food poisoning in humans (Cowden et al., 1989; Natasi et al., 1998; Tansel et al., 2003). Eradication of SE contamination is one of the most important problems in the poultry industry worldwide (Hopper and Mawer, 1988; Lister, 1988; Barrow et al., 2003).

Eggs may be contaminated by SE during formation in the ovary and oviduct (in-egg contamination) or by fecal contamination on the shell surface (on-egg contamination). Although the route of on-egg SE contamination has been thoroughly reported (Gast and Beard, 1990), the routes of in-egg contamination have not yet been established. *Salmonella enteritidis* has been isolated from the reproductive tissues of infected hens in the absence of cecal colonization (Lister, 1988). We have reported that SE organisms could invade and colonize the ovary and oviduct from the peritoneal cavity in Japanese quail (Takata et al., 2003). These reports suggest that the ovary may be a source of egg contamination by SE that may infect ovarian tissues from peritoneal colonization. Therefore, induction of immunity against SE infection in the ovary is important for defending the tissues and reducing the transmission of these bacteria.

Cell-mediated immunity has been reported to play important roles against intracellular pathogens such as SE (Schat, 1994; Lillehoj and Okamura, 2003). T cells are the regulators of cell-mediated immunity. In chickens, T cells bear surface antigens, namely CD3, CD4, and CD8. The CD3 antigen is the common T-cell surface antigen (Vainio and Lassila, 1989) and there are 2 subsets of T cells including helper/inducer T cells with CD4 antigen, and cytotoxic/killer T cells with CD8 antigen (Chan et al., 1988). We have localized all the subsets of T cells in the hen ovarian stroma and theca layer of healthy follicles (Barua and Yoshimura, 1999; Yoshimura and Kitamura, 2002), but the response of these immunocompetent cells in the ovary of infected hens is not well known. There are only few reports that examined the responses of these immu-

Received for publication October 13, 2003.

Accepted for publication December 30, 2003.

[1]Supported by Japan Society for the Promotion of Science (JSPS), Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan and Sumitomo Chem. Co. to Y. Yoshimura.

[2]To whom correspondence should be addressed: yyosimu@hiroshima-u.ac.jp.

Abbreviation Key: F1 = large follicle; F3 = third largest follicle; SWF = small white follicles.

nocompetent cells against pathogenic agents in the ovary. Withanage et al. (1998) reported that T cells increased in the oviduct and ovary of hens at 7 d of intravenous inoculation with SE. McSorley et al. (2002) reported that infiltration of CD4+ T cells was induced within 3 h of *Salmonella* infection in mice. Thus, it remains unknown whether T cells in the hen ovary respond to SE at an earlier phase of infection.

Generally, CD8+ cells kill the pathogens directly when presented by antigen-presenting cells, whereas CD4+ cells stimulate inflammatory responses and induce B-cell activation (Gobel, 1996; Davison, 1996). The relative involvement of different T-cell subsets against SE has been a matter of controversy in some mammalian species (Hughes and Galan, 2002). It remains unknown which T-cell subsets respond to SE in the early phase of infection in the hen ovary.

The aim of this study was to determine how the cellular immune responses occur in the ovary against SE during the early phase of infection and to examine which T-cell subsets respond to SE invasion. We have identified each subset of T cells and SE by double immunostaining.

MATERIALS AND METHODS

Birds

White Leghorn laying hens of approximately 71 to 74 wk of age 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 14L:10D and provided with feed and water provided ad libitum.

Bacterial Inoculation

Chickens were divided into 3 groups with 5 hens in each group. They were intraperitoneally injected within 30 min of oviposition with sterile PBS (control, 1 group) or live SE suspended in PBS at 5.0×10^9 bacteria/bird (2 groups). This dosage did not elicit physiological side effects in a preliminary experiment. The bacteria were donated by Hiroshima Prefectural Health and Environmental Center[3] and have been reported to be invasive in quail (Takata et al., 2003).

Tissue Preparation

All birds were euthanized by carbon dioxide inhalation 12 or 24 h after of injection with SE or PBS and dissected for tissue collection. The largest (F_1) and third largest (F_3) follicles, small white follicles (SWF, 5 from each hen), and the ovarian stroma containing stromal 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). Small specimens (approximately 1 to 2 cm in length) of F1 and F3 follicular tissues were embedded in cryoembedding medium, OCT compound[4] and snap frozen in a mixture of isopentane and solid carbon dioxide. Frozen sections (15 μm thick) were prepared using a cryostat[5] and air-dried on slides treated with 3-aminoprpyl-triethoxysilane.[6] Whole SWF (approximately 3 to 4 mm in diameter) and stromal tissues were processed in the same manner. Sections were further fixed with cold acetone and methanol for 10 min each.

Antibodies

Anti-chicken CD3, CD4, and CD8 (CD8-α) monoclonal antibodies[7] and anti-*Salmonella* O-9 monoclonal antibodies[8] were used as primary antibodies for the localization of T-cell subsets and SE, respectively. A Histofine SAB-PO (M)[9] immunodetection kit was used for the detection of immunoprecipitates of primary antibodies.

Double Immunostaining

Immunopositive CD3+, CD4+, or CD8+ T-cell subsets and SE bacteria were localized by double immunostaining methods. In the first staining, sections were immunostained for CD3+, CD4+, or CD8+ T-cell subsets as described previously (Barua and Yoshimura, 1999), and in the second staining, each section was immunostained again for the localization of SE. Five sections per tissue from each hen were used for immunostaining for each T-cell type. Briefly, sections were incubated in 1% (vol/vol) goat serum for 15 min to block the nonspecific bindings of antibodies. They were then incubated overnight with mouse anti-chicken CD3, CD4, or CD8 antibodies diluted with PBS containing 0.5% (wt/vol) BSA at 1:100. Immunoreaction products were identified using a Histofine SAB-PO (M) kit; 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 incubating the sections with a reaction mixture consisting of 0.02% (wt/vol) 3,3′-diaminobenzidine tetra hydrochloride[10] and 0.005% (vol/vol) H_2O_2 in 0.05 mol/L Tris-HCl buffer (pH 7.6). After the first sequence, sections were washed with PBS for 15 min (5 min × 3 times) followed by 0.1 mol/L glycine for 90 min (30 min × 3 times). They were then incubated with anti-*Salmonella* O-9 antibody for 2 h. 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.[11] Sections were then washed in distilled water for 10 min (5 min × 2 times), dehydrated with graded alcohol, thor-

[3]Hiroshima, Japan.
[4]Miles Inc., Elkart, IN.
[5]Bright Instrument Company Ltd, Huntingdon, UK.
[6]Nacalai Tesque, Kyoto, Japan.
[7]Southern Biotechnology Associates Inc., Birmingham, AL.
[8]HyTest, Turku, Finland.
[9]Nichirei, Tokyo, Japan.
[10]Sigma, St. Louis, MO.
[11]KPL, Gaithersburg, MD.

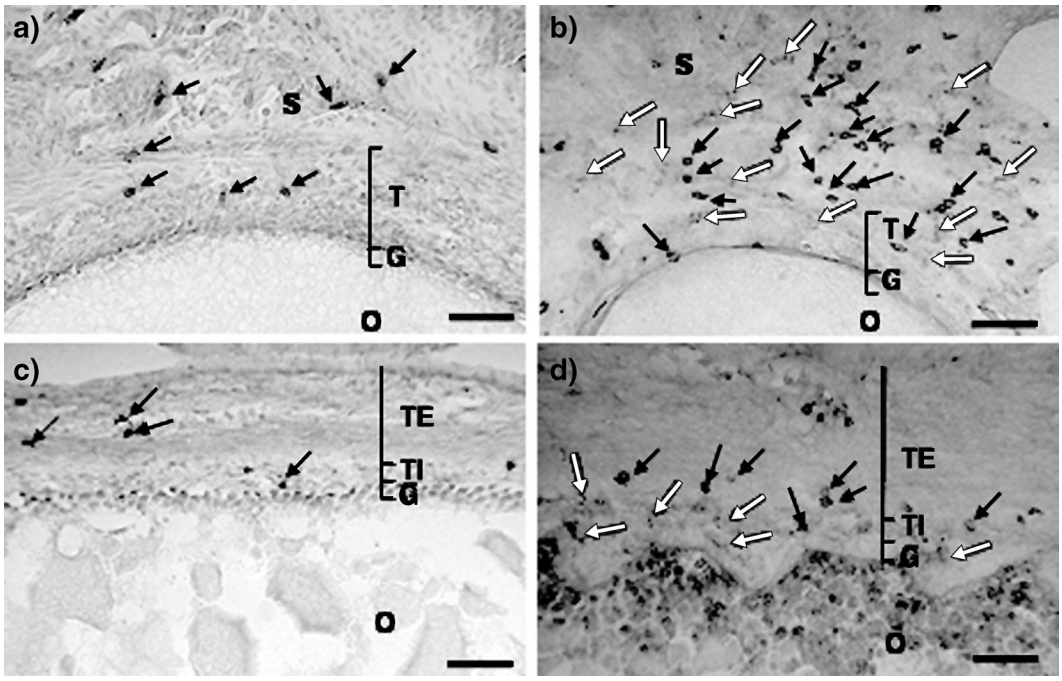

FIGURE 1. Sections of ovaries of hens (*Gallus domesticus*) inoculated i.p. with or without 5×10^9 *Salmonella enteritidis* (SE). Sections were double immunostained for CD3+ T cells and SE. White arrows and black arrows indicate the examples of SE and CD3+ T cells, respectively. G = granulosa layer; O = oocyte; T = theca layer; TI = theca interna; TE = theca externa; S = stroma. Scale bar = 20 μm. a) Ovarian stroma of control (PBS-treated) hens. Few CD3+ T cells are shown in the stroma and in the theca layer of stromal follicles. b) Ovarian stroma of birds 24 h after SE inoculation. Multiple SE have invaded stroma and theca layer of stromal follicles. Many CD3+ T cells are also shown in these tissues. c) Largest follicle of control hens. Few CD3+ T cells can be observed in the theca externa and interna layers. d) Largest follicle of birds 24 h after SE inoculation. Multiple SE have invaded in the theca interna layer. In comparison to the control birds many CD3+ T cells are shown in the theca layer.

oughly air-dried, and covered. Control staining was carried out simultaneously in which primary antibodies were replaced with normal mouse IgG. Positive staining was not observed on the control slides.

Counting of T Cells

All sections were examined under a light microscope with image analysis software,[12] and immunopositive cells were counted as described previously (Barua and Yoshimura, 1999). The numbers of immunopositive T-cell subsets were determined by observing 3 different areas of the theca of each follicle and the ovarian stroma on a section. Then the cell number was calculated to be the number of cells within 1×10^4 μm^2. The average of the 3 counts was expressed as the cell number in 1×10^4 μm^2 area in one tissue of a bird.

Statistical Analysis

The significance of differences in the immunopositive T cells within 1×10^4 μm^2 among the control and SE-injected birds (after 12 and 24 h of injection) was determined by one-way ANOVA (Snedecor and Cochran, 1967) followed by Duncan's multiple range test (Duncan, 1955). Differences were considered significant when $P < 0.05$.

[12]Image-ProPlus, Media Cybernetics, Silver Spring, MD.

RESULTS

The ovarian stroma consisted of the loose connective tissue in which stromal follicles were embedded. The small white follicles and preovulatory follicles protruded from the ovarian surface. The theca layer was differentiated into the theca interna and theca externa in SWF, F_3, and F_1 follicles but was undifferentiated in the stromal follicles.

Localization of T-Cell Subsets and SE in the Ovary

The CD3+ T cells were localized in the stroma and the theca of the stromal follicles of control and SE-injected birds (Figure 1, a and b). The CD4+ and CD8+ T cells were similarly observed in these tissues (data not shown). Immunoreaction products for SE were detected in the stroma of birds 12 h after SE injection, whereas they were identified in the stroma and theca of stromal follicles 24 h after SE injection (Figure 1b). However, an influx of T cells at the site of SE invasion was not observed. Figure 2 shows the frequencies of T-cell subsets in the ovarian stroma and the theca layer of stromal follicles of control and SE-injected birds. The frequencies of CD3+, CD4+, and CD8+ T cells increased significantly in the stroma and theca layer of stromal follicles 12 h after SE injection as compared with control birds ($P < 0.01$). Furthermore, their frequencies were greater in the birds 24 h after SE injection than 12 h after injection ($P < 0.01$).

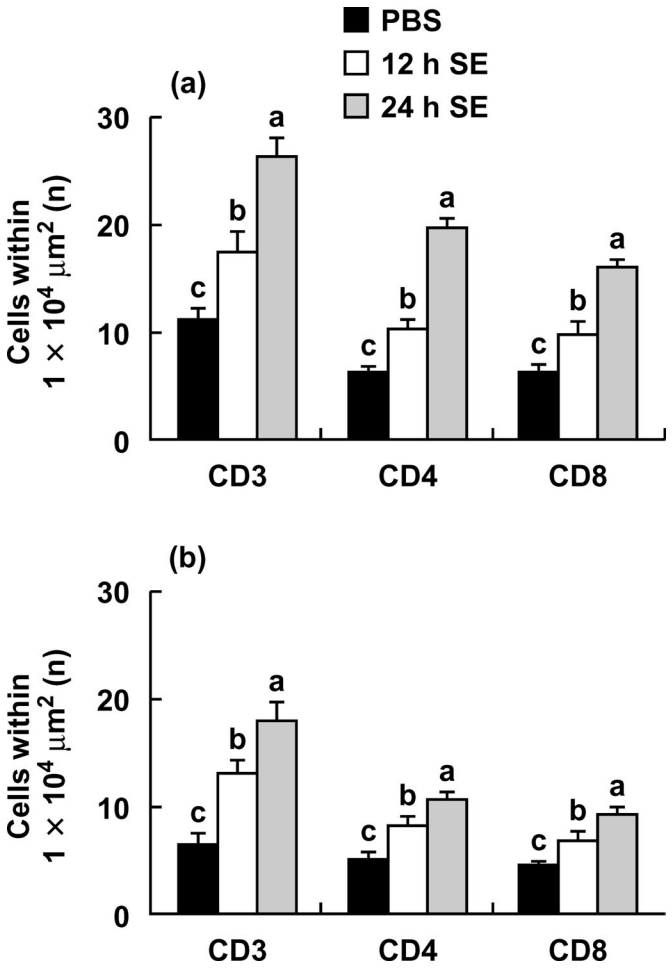

FIGURE 2. Changes in the frequency of T-cell subsets in the a) ovarian stroma and b) the theca layer of stromal follicles in response to *Salmonella enteritidis* (SE) invasion. Hens were examined 24 h after PBS injection and 12 and 24 h after i.p. inoculation with 5×10^9 SE. Each value shows mean ± SEM of the number of T cells within 1×10^4 μm^2 (n = 5 hens in each group). [a–c]Bars with different letters are significantly different ($P < 0.01$).

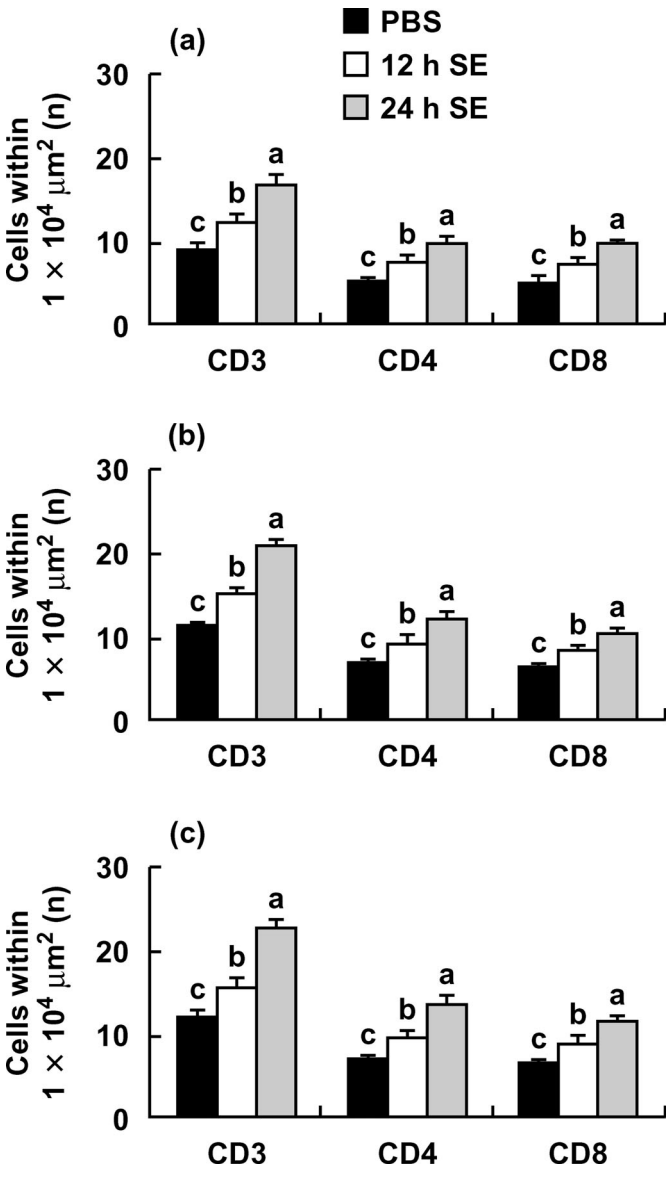

FIGURE 3. Changes in the population of T-cell subsets in the theca layer of a) small white follicle, b) third largest follicle and c) largest follicle in response to *Salmonella enteritidis* (SE) invasion. Hens were examined 24 h after PBS injection and 12 and 24 h after i.p. inoculation with 5×10^9 SE. Each value shows mean ± SEM of the number of T cells within 1×10^4 μm^2 (n = 5 hens in each group). [a–c]Bars with different letters are significantly different ($P < 0.01$).

Localization of T-Cell Subsets and SE in Follicular Tissues

Immunopositive CD3+ cells were localized in the theca interna and externa layers in the F1 (Figure 1, c and d), F3, and SWF of the control and SE-injected birds. The CD4+ and CD8+ T cells were similarly observed in these tissues (data not shown). Many SE were detected in the theca interna of F1 follicles 24 h after injection (Figure 1d). Similar patterns of SE invasion were also detected in the SWF and F3 follicles 24 h after SE inoculation. As in the stroma, the influx of T cells at the site of SE invasion was not observed. The frequencies of CD3+, CD4+, and CD8+ T cells were greater in the theca layer of 12 or 24 h after SE injection than that of control birds ($P < 0.01$) (Figure 3). In comparison to the birds 12 h after SE injection, the frequencies of T-cell subsets in the theca layer increased in birds 24 h after SE injection ($P < 0.01$).

Injection of birds with SE did not change the ratio between CD4+ and CD8+ T cells in the ovarian stroma or follicular theca (Figure 4). This result was due to the proportionate increase in the population of the T-cell subsets in each group of SE-injected birds.

DISCUSSION

To our knowledge this is the first report in which SE and T-cell subsets were localized in the same tissues in hens. The significant finding was that the population of CD3+, CD4+, and CD8+ T cells increased with the invasion of SE in the ovarian stroma and follicular theca within 12 h of the i.p. inoculation, suggesting that cell-mediated immunity to SE could be induced in the ovary for defending against these pathogens.

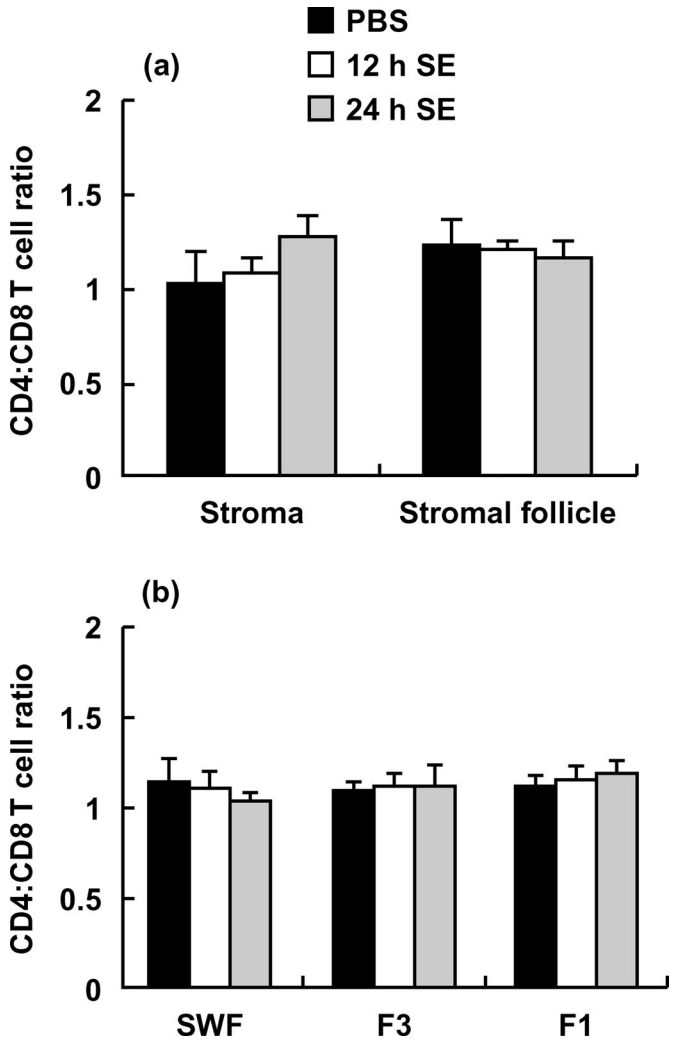

FIGURE 4. Ratio of CD4+:CD8+ in the ovarian stroma and the theca layer of follicles of hens inoculated with or without *Salmonella enteritidis*. Hens were examined 24 h after PBS injection and 12 and 24 h after i.p. inoculation with 5×10^9 SE. a) ovarian stroma and the theca layer of stromal follicles. b) small white follicle (SWF), third largest follicle (F3), the largest follicle (F1). Each value shows mean ± SEM of the CD4+:CD8+ T cell ratio within 1×10^4 μm^2 (n = 5 hens in each group).

There are reports that SE bacteria were isolated from ovaries and egg yolk following oral and intravenous experimental inoculation (Shivaprasad et al., 1990; Barrow and Lovell, 1991; Keller et al., 1995, 1997; Withanage et al., 1998, Okamura, 2001). Isolation studies of *Salmonella* by organ culture have suggested the possibility of infection of ovary through colonization of the peritoneum by SE (Timoney et al., 1989). In the present study, after i.p. inoculation bacteria were detected in the ovarian stroma and in the follicular theca of infected hens that exhibited no significant side effects, including ovulation and fecal appearance. This finding supports those of Takata et al. (2003), who examined SE infection in Japanese quail.

The cellular immune response is considered more important for the development of a protective immune response to SE infection than antibodies (Desmidt et al., 1998). The present study supports our earlier findings in which T cells were present in the ovarian stroma and the theca of follicles of healthy hens (Barua and Yoshimura, 1999; Yoshimura and Kitamura, 2002). In the present study, no consistent co-localization of SE and T cells was observed in the tissues, and correlation between the SE and the population of T cells was not tested. However, the frequency of T cells was significantly greater in the ovarian tissues of hens 12 h after SE injection than those of control hens, and further increased in hens 24 h after SE injection. These results suggest that tissue invasion of SE might be one of the reasons for the increase in T-cell population in these tissues. Substances released from the damaged cells and bacterial products are powerful chemotactic factors for immune cells (Mosser, 1994). Bacterial toxins secreted from *Salmonella* are active participants in the induction of host responses and, therefore, have the potential to modulate the immune response (Hughes and Galan, 2002). Therefore, comparisons of T-cell populations in the ovarian tissues between control and SE-injected birds indicated that the influx of T cells in the hen ovarian tissues might be caused in response to the invasion of SE bacteria and their products.

The relative contribution of each of the T-cell subsets in the mediation of immunity against SE infection is not well known in hens. The populations of the CD4+ and CD8+ T cells were significantly increased by SE invasion, whereas their ratio (CD4+ T:CD8+ T) was not affected (Figure 4). These results indicate that the CD4+ and CD8+ T-cell subsets increased proportionally in response to SE invasion. CD4+ T cells induce immune responses when presented by antigen-presenting cells containing major histocompatibility complex class II, leading to the activation of humoral immunity (Gobel, 1996). On the other hand, CD8+ T cells induce cytotoxic affects when presented by major histocompatibility complex class I (Davison, 1996). Therefore, present results suggest that both pathways of cell-mediated immunity are active in response to SE invasion in hens. McSorley et al. (2002) reported that intestinal mucosal CD4+ T cells were increased in response to oral inoculation of *Salmonella* organism in mice. In contrast, CD8+ T cells have also been reported to play important roles in SE clearance from the spleen in mice (Nauciel, 1990). A balanced CD4+ T:CD8+ T ratio is essential for normal immune function, and imbalance in this ratio may increase susceptibility to disease (Michael, 1983; Hirokawa, 1992). It is assumed that the proportional increase of the CD4+ and CD8+ T cells may be necessary to defend hen ovary against SE infection.

In conclusion, the present study showed that the population of T-cell subsets in the ovarian stroma and follicular tissues increased in response to SE invasion. It is suggested that cell-mediated immune functions become active in the hen ovary in response to SE bacteria, their products, or both.

ACKNOWLEDGMENTS

The authors to thank H. Nakano, Graduate School for Biosphere Science, Hiroshima University, for his kind technical help regarding the bacterial culture. This work

was supported by the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Barrow, P. A., and M. A. Lovell. 1991. Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. Avian Pathol. 42:194–199.

Barrow, P. A., G. C. Mead, C. Wray, and M. Duchet-Suchaux. 2003. Control of food-poisoning salmonella in poultry-biological options. Worlds Poult. Sci. J. 59:373–383.

Barua, A., and Y. Yoshimura. 1999. Effects of aging and sex steroids on the localization of T cell subsets in the ovary of chicken, *Gallus domesticus*. Gen. Comp. Endocrinol. 114:28–35.

Blaxland, J. D., G. A. Gullen, R. F. Gordon, and F. T. W. Jordon. 1982. Diseases caused by bacteria, mycoplasmas and Chlamydia. Pages 9–75 in Poultry Diseases. R. F. Gordon, ed. Bailliere Tindal, London, England.

Chan, M. M., C-L. H. Chen, L. L. Ager, and M. D. Cooper. 1988. Identification of the avian homologues of mammalian CD4 and CD8 antigens. J. Immunol. 140:2133–2138.

Cowden, J. M., D. Lynch, C. A. Joseph, M. O'Mahony, S. L. Mawer, B. Rowe, and C. L. R. Bartlett. 1989. Case control study of infections with *Salmonella enteritidis* phage type 4 in England. Br. Med. J. 299:771–773.

Davison, T. F. 1996. Cell-mediated immunity: Effector functions. Pages 115–134 in Poultry Immunology. T. F. Davison, T. R. Morris, and L. N. Payne, eds. Carfax Pub, London, England.

Desmidt, M., R. Ducatelle, J. Mast, B. M. Goddeerise, B. Kaspers, and F. Haesebrouck. 1998. Role of the humoral immune system in *Salmonella enteritidis* phage type four infection in chickens. Vet. Immunol. Immunopathol. 63:355–367.

Duncan, D. B. 1955. Multiple range and F test. Biometrics. 11:1–42.

Gast, R. K., and C. W. Beard. 1990. Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. Avian Dis. 34:991–993.

Gobel, T. W. F. 1996. The T-dependent immune system. Pages 32–45 in Poultry Immunology. T. F. Davison, T. R. Morris, and L. N. Payne, eds. Carfax Pub, London, England.

Hirokawa, K. 1992. Understanding the mechanism of the age related decline in immune function. Nutr. Rev. 50:361–366.

Hopper, S. A., and S. Mawer. 1988. *Salmonella enteritidis* in a commercial layer flock. Vet. Rec. 123:351.

Hughes, E. A., and J. E. Galan. 2002. Immune response to *Salmonella*: Location, location, location. Immunity. 16:325–328.

Keller, L. H., C. E. Benson, K. Krotec, and R. J. Ekroade. 1995. *Salmonella enteritidis* colonization of the reproductive tract and forming and freshly laid eggs of chickens. Infect. Immun. 63:2443–2449.

Keller, L. H., D. M. Schifferli, C. E. Benson, K. Krotec, and R. J. Ekroade. 1997. Invasion of chicken reproductive tissues and forming eggs is not unique to *Salmonella enteritidis*. Avian Dis. 41:535–539.

Lillehoj, H., and M. Okamura. 2003. Host immunity and vaccine development to coccidia and salmonella infections in chickens. J. Poult. Sci. 40:151–193.

Lister, S. A. 1988. *Salmonella enteritidis* infection in broilers and broiler breeders. Vet. Rec. 123:351.

McLean, I. W., and P. K. Nakane. 1974. Periodate lysine paraformaldehyde fixative: A new fixative for immunoelectron microscopy. J. Histochem. Cytochem. 22:1077–1083.

McSorley, S. J., S. Asch, M. Costalonga, R. L. Reinhardt, and M. K. Jenkins. 2002. Tracking *Salmonella*-specific CD4 T cells in vivo reveals a local mucosal response to a disseminated infection. Immunity 16:365–377.

Michael, S. D. 1983. Interaction of the thymus and the ovary. Pages 445–465 in Factors Regulating Ovarian Function. G. S. Greenwald, and P. F. Terranova, eds. Raven Press, New York.

Mosser, D. M. 1994. Receptors on phagocytic cells involved in microbial recognition. Pages 99–114 in Macrophage-Pathogen interactions. B. S. Zwilling, and T. K. Eisenstein, eds. Dekker, New York.

Natasi, A., C. Mammina, G. P. Piersante, M. Robertazzo, and P. Caruso. 1998. A foodborne outbreak of *Salmonella enteritidis* vehicled by duck and hen eggs in southern Italy. New Microbiol. 21:93–96.

Nauciel, C. 1990. Role of CD4+ T cells and T-independent mechanisms in acquired resistance to *Salmonella typhimurium* infection. J. Immunol. 145:1265–1269.

Okamura, M., Y. Kamijima, T. Miyamoto, H. Tani, K. Sasai, and E. Baba. 2001. Differences among six Salmonella serovars in abilities to colonize reproductive organs and to contaminate eggs in laying hens. Avian Dis. 45:61–69.

Schat, K. 1994. Cell-mediated immune effector function in chickens. Poult. Sci. 73:1077–1081.

Shivaprasad, H. L., J. F. Timoney, and S. Morales. 1990. Pathogenesis of *Salmonella enteritidis* infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serologic responses. Avian Dis. 34:548–557.

Snedecor, G. W., and W. G. Cochran. 1967. One-way classifications: Analysis of variance. Pages 258–298 in Statistical Methods. G. W. Snedecor, and W. G. Cochran, eds. Iowa State Univ. Press, Ames, IA.

Takata, T., J. Liang, H. Nakano, Y. Yoshimura. 2003. Invasion of *Salmonella enteritidis* in the Tissues of reproductive organs in laying Japanese quail: An immunocytochemical study. Poult. Sci. 82:1170–1173.

Tansel, O., G. Ekuklu, M. Otkun, M. T. Otkun, F. Akata, and M. Tugrul. 2003. A food-borne outbreak caused by *Salmonella enteritidis*. Yonsei Med. J. 44:198–202.

Timoney, J. F., H. L. Shivaprasad, R. C. Baker, and B. Rowe. 1989. Egg transmission after infection of hens with *Salmonella enteritidis* phage type 4. Vet. Rec. 125:600–601.

Vainio, O., and O. Lassila. 1989. Chicken T cells: Differentiation antigens and cell-cell interactions. Crit. Rev. Poult. Biol. 2:97–102.

Withanage, G. S. K., K. Sasai, T. Fukata, T. Miyamoto, E. Baba, and H. S. Lillehoj. 1998. T lymphocytes, B lymphocytes and Macrophages in the ovaries and oviducts of laying hens experimentally infected with *Salmonella enteritidis*. Vet. Immunol. Immunopathol. 66:173–184.

Yoshimura, Y., and A. Kitamura. 2002. Changes in the population of lymphocytes expressing CD4 and CD8 during the process of atresia of white follicles in hens. Anim. Sci. J. 73:457–463.