FULL PAPER *Immunology*

Development of Peyer's Patch and Cecal Tonsil in Gut-Associated Lymphoid Tissues in the Chicken Embryo

Eiji KAJIWARA[1)], Akiko SHIGETA[1)], Hiroyuki HORIUCHI[1)], Haruo MATSUDA[1)] and Shuichi FURUSAWA[1)]*

[1)]*Laboratory of Immunobiology, Department of Molecular and Applied Bioscience, Graduate School of Biosphere Science, Hiroshima University, 1–4–4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739–8528, Japan*

(Received 13 December 2002/Accepted 5 February 2003)

ABSTRACT. It is well known that chicken B cells develop in the bursa of Fabricius (BF), which is categorized as gut-associated lymphoid tissue (GALT). Chicken GALT also includes Peyer's patch (PP) and cecal tonsil (CT). The relationship between these tissues in GALT during B cell development is currently unknown. In this study, we conducted comparative examination of PP, CT and BF development during embryogenesis using immunohistochemical staining. On day 13 of embryogenesis (E13), accumulation of MHC class II$^+$ cells was observed in the intestine. Thereafter, Bu-1$^+$ cells and IgM$^+$ cells appeared, and their number continuously increased at the same sites where MHC class II$^+$ cells were present. Similar results were obtained in the CT. The locations of embryonic PP were limited to two sites; near the Meckel's diverticulum and the ileocecal junction. Anlage of bursal follicles first appeared at E13 and developed thereafter. Immigration of Bu-1$^+$ cells to bursal follicles began at E13, and the number of Bu-1$^+$ cell subsequently increased. When the follicle of BF was eliminated from the embryo by treatment with testosterone, development of PP and CT were observed. We concluded therefore that the development of PP and CT start during late embryogenesis at the same time as the follicle of BF, and that appearance of surface IgM$^+$ cells in PP and CT is independent form the development of the follicle of BF.

KEY WORDS: bursa of Fabricius, cecal tonsil, chicken, embryo, Peyer's patch.

J. Vet. Med. Sci. 65(5): 607–614, 2003

Chicken B cells are known to develop in the bursa of Fabricius (BF), which is categorized as gut-associated lymphoid tissue (GALT). Bu-1 antigen is a surface marker of B cell except plasma cell in the chicken. Following their development at days 13–15 of embryogenesis (E13–15) [11], Bu-1$^+$ cells migrate in a single wave within the bursal follicles [15]. The follicle provides a microenvironment for differentiation of B cells, which undergo somatic diversification of the rearranged Ig V region by gene conversion [18, 26]. On the other hand, murine B cells develop in the fetal liver or adult bone marrow [19, 23]. In some mammals such as sheep, B cells develop in the ileal Peyer's patch (PP), which is categorized as GALT [6, 28]. PP in sheep is thought to be a bursa equivalent tissue, both morphologically and functionally [24]. These species also possess another type of PP, jejunal PP, which plays an important role in mucosal immunity as a type of peripheral immunity [10].

Chicken GALT also includes PP and CT. Histological analysis revealed the presence of a high number of endothelial venules and germinal centers in PP [3] and in CT [14]. These findings suggest that chicken PP and CT work as the peripheral lymphoid tissues. However, the role of PP and CT as GALT in B cell development is not currently understood. In the present study, we used immunohistochemical staining to conduct a comparative study on the development of PP, CT, and the follicle of BF during embryogenesis.

* CORRESPONDENCE TO: FURUSAWA, S., Laboratory of Immunobiology, Department of Applied Bioscience, Graduate School of Biosphere Science, Hiroshima University, 1–4–4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739–8528, Japan.

MATERIALS AND METHODS

Animals: Partially inbred chickens (H-B15 White Leghorn; Bu-1^a) were used in this study. These chickens, originally supplied by Dr. Vainio (Turk University, Finland) were bred in our animal facilities and provided with feed and chlorinated water *ad libitum*. Eggs derived from the chickens (10–12 months old) were incubated in a humidified incubator at 38.5°C.

Antibodies: Anti-chicken MHC class II (P2M11; mouse IgG2a, k) [17] and anti-Bu-1^a (L22; mouse IgG1, k) [16, 25] were kindly supplied by Dr. H. Lillehoj (USDA, ML, U.S.A.), and by Dr. O. Vainio, respectively. There are two types of phenotype in Bu-1 antigen; Bu-1^a and Bu-1^b. Because our H-B15 chicken express Bu-1^a phenotype, anti-Bu-1^a antibody was used in this study. Anti-chicken IgM (4μ, mouse IgG1, k) was produced as previously described [20].

Immunohistochemical staining: Intestine and BF were removed from normal embryos and chickens at developmental stages E11, 13, 15, 17, 19 and day of hatching (Incubation time of embryogenesis is according to the description by Hamburgery and Hamilton [11]), and from TP-treated embryos at E17. BF and CT were embedded in OCT compound (Miles Inc., IN). Intestine was rolled like "Swiss roll" for the preparation of serial sections. Cryosections (thickness: 6–10 μm) of BF and of whole intestine were taken at intervals of about 100 μm, air-dried on slides treated with 0.0001% poly-L-lysine (Sigma, MO) in distilled water (DW), and fixed with 95% ethanol in DW for 10 min, or with cold acetone for 10 min before air-drying.

Sections were washed in DW and phosphate-buffered

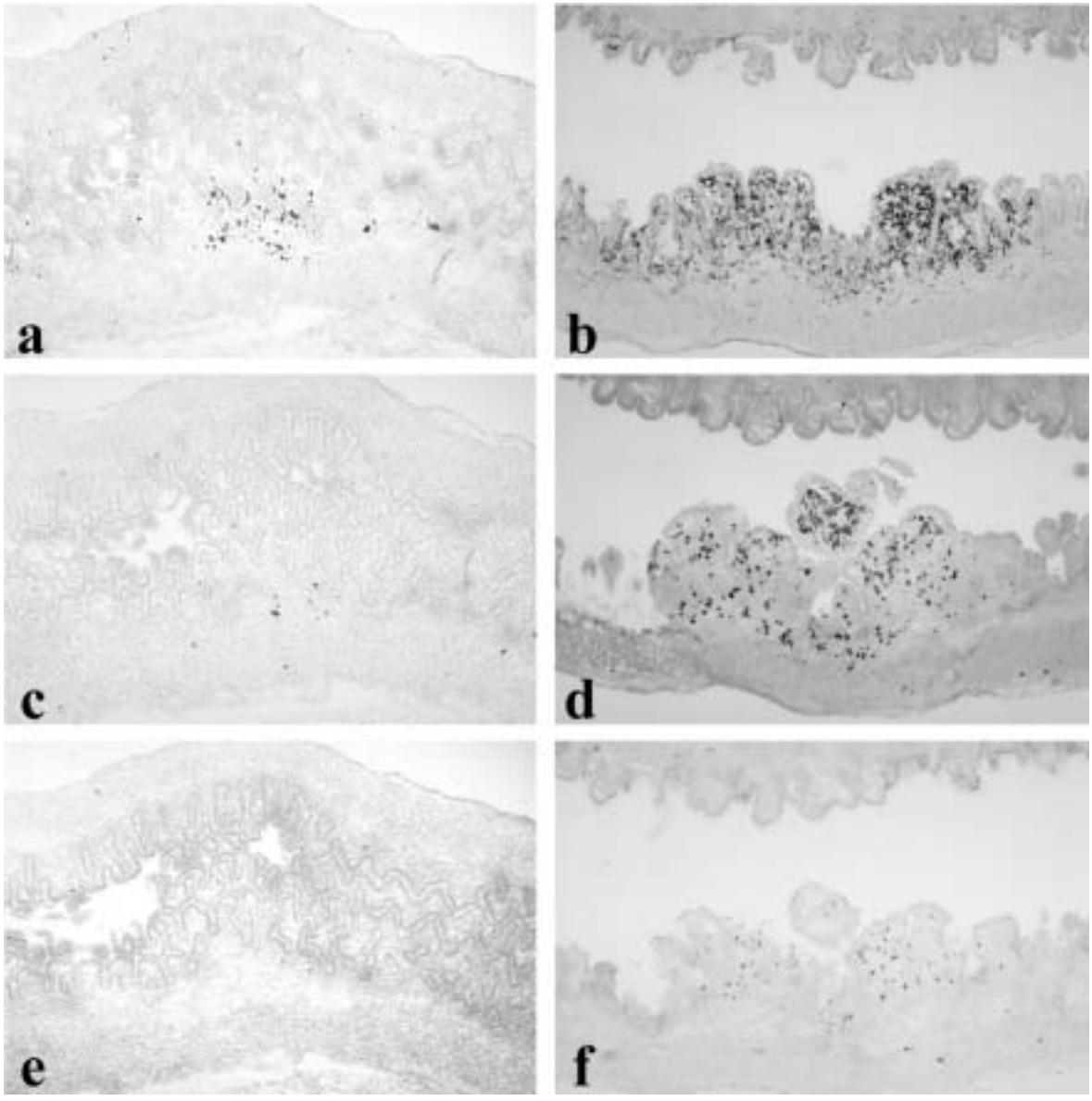

Fig. 1. Development of PP in the embryo. Serial sections of whole intestine taken at E13 and E15 were stained immunohistologically with P2M11, L22 or 4 μ. At E13, clusters of MHC class II^+ cells (a), a few scattered Bu-1^+ cells (c) and IgM^+ cells (e) were observed in the lamina propria (25 ×). At E15, the clusters of MHC class II^+ cells had developed more widely and densely than those at E13 (b). Bu-1^+ (d) and IgM^+ cell (f) density had increased in the lamina propria when compared to E13 (25 ×).

saline (PBS), then incubated with 10% normal horse serum in PBS for 20 min in order to block non-specific binding. Sections were then incubated for 1 hr with monoclonal antibodies as described above. Sections were washed three times with PBS (5 min × 3) and incubated with biotinylated horse anti-mouse IgG (Vector Lab., CA), diluted with 1% bovine serum albumin PBS for 30 min, and washed. Endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol for 30 min, followed by incubation with ABC complex (Vector Lab., CA) for 30 min and washing. The sections were developed using Vector NovaRED substrate kit (Vector Lab., CA) or DAB Substrate kit (Vector Lab., CA), and were counterstained in hematoxylin or methyl green. All incubations were performed at room temperature in a moist chamber. Control staining, in which the first antibody was replaced with normal mouse IgG, was simultaneously carried out. No specific staining was observed in the control slides.

Testosterone treatment: For hormonal bursectomy, 3.5 mg of testosterone propionate (TP) dissolved in 0.1 m*l* corn oil or an equal volume of corn oil alone was injected into the chorioallantoic fluid at E11.

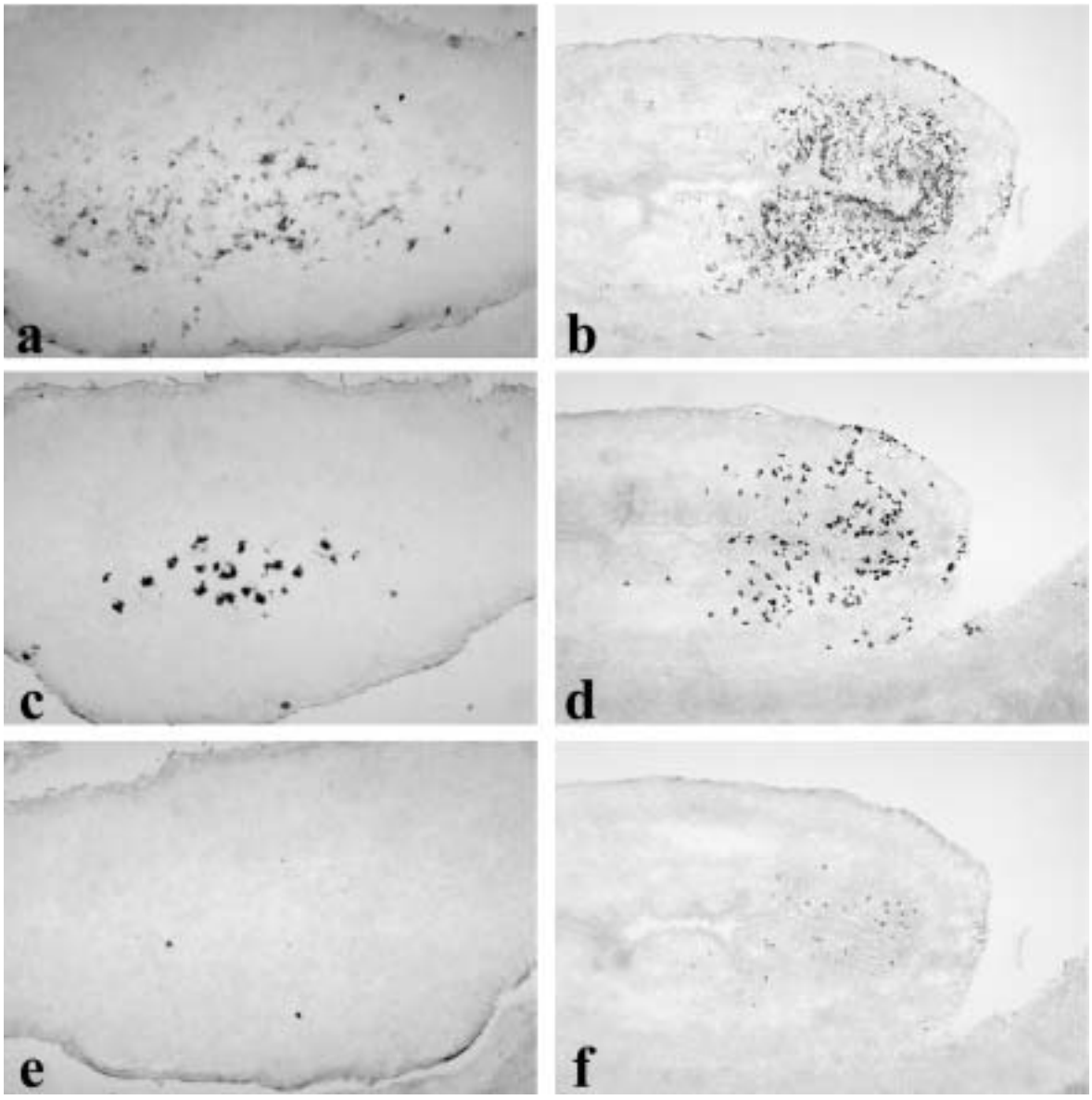

Fig. 2. Development of CT in the embryo. Serial sections of the cecum proximate the colocecal junction taken at E13 and E15, were stained immunohistologically with P2M11, L22 or 4 μ. At E13, Clusters of MHC class II$^+$ cells (a), a few scattered Bu-1$^+$ cells (c) and IgM$^+$ cells (e) were observed in this area (50 ×). At E15, clusters of MHC class II$^+$ cells had expanded more widely and densely than those at E13 (b). Bu-1$^+$ (d) and IgM$^+$ cell (f) density had increased compared with E13 (25 ×).

RESULTS

Development of PP, CT and follicle of BF during embryogenesis: In order to observe the appearance of GALT in chicken embryo, immunohistochemical staining was performed. Clusters of MHC class II$^+$ cells were present in the intestine at E13 (Fig. 1a), but not at E11 (data not shown). These cells were located in the lamina propria. Few Bu-1$^+$ cells (Fig. 1c) and IgM$^+$ cells (Fig. 1e) were observed in the same area in the serial sections. At E15, the clusters of MHC class II$^+$ cells expanded within the same area (Fig. 1b). The number of Bu-1$^+$ cells (Fig. 1d) and IgM$^+$ cells also increased in this area (Fig. 1f). Near the ileocecal junction, which is considered to be the presumptive site of the CT, clusters of MHC class II$^+$ cells (Fig. 2a) and fewer Bu-1$^+$ (Fig. 2c) and IgM$^+$ cells (Fig. 2e) were also present in addition to those in PP on E13. As in PP, the number of MHC class II$^+$ cells (Fig. 2b), Bu-1$^+$ cells (Fig. 2d) and IgM$^+$ cells (Fig. 2f) had increased in CT on E15. These results indicate that the appearance of Bu-1$^+$ cells and IgM$^+$ cells in PP and in CT occur prior to hatching at E13 and that this appearance of B cells takes place after MHC class II$^+$ cells cluster in the

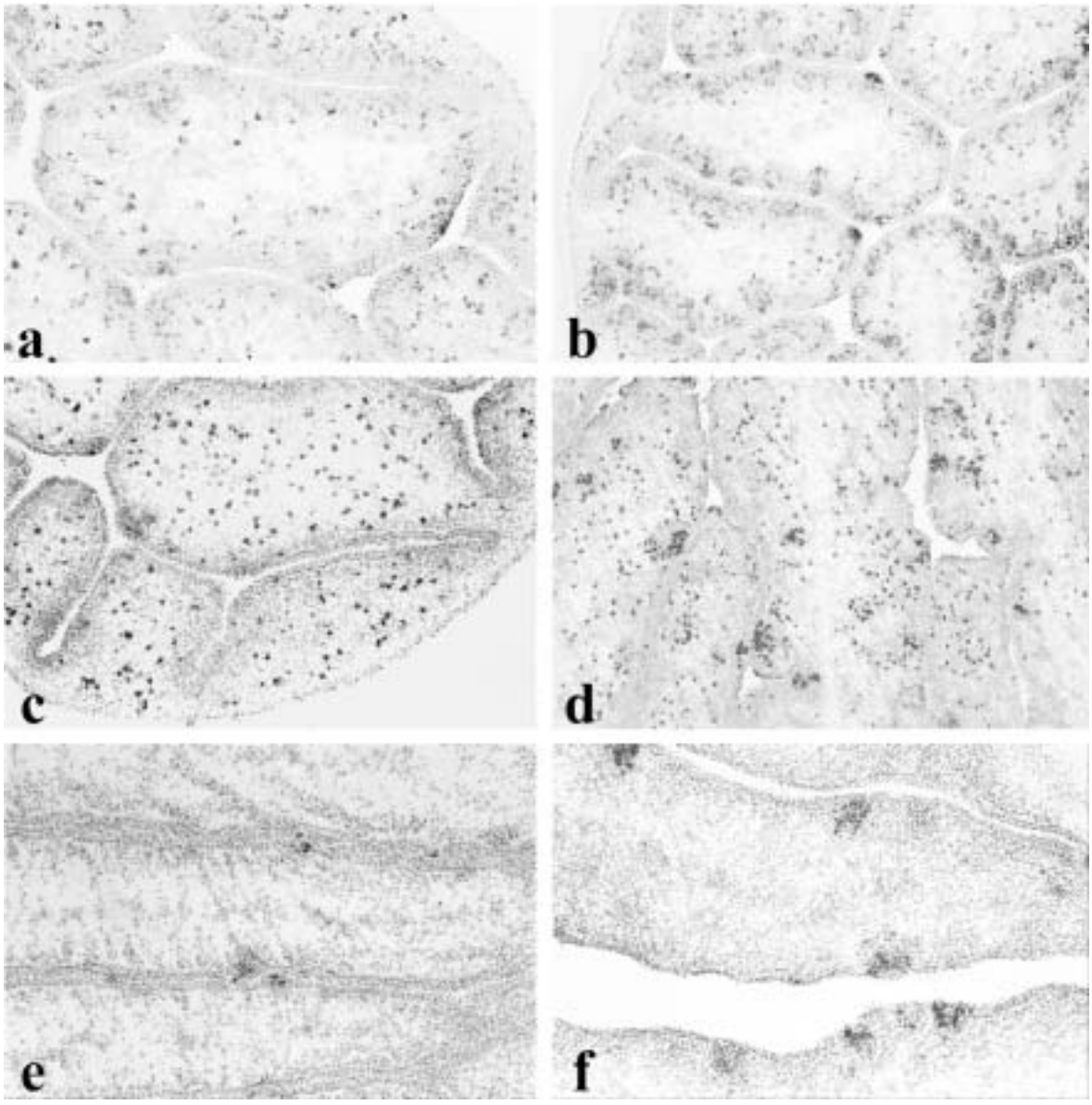

Fig. 3. Immunohistochemical staining of MHC class II^+, Bu-1^+ and IgM^+ cells in the ontogeny of bursal follicle. Bursal sections taken from E13 and E15 were stained with P2M11, L22 or 4μ. At E13, migration of MHC class II^+ cells within lymphoid buds was clearly observed (a)(33 ×). Thereafter, at E15, increased migration of those cells was observed (b)(33 ×). At E13, few Bu-1^+ cells migrated into the lymphoid buds (c)(33 ×), but were abundant at E15 (d)(25 ×), and were also observed in the mesenchyme. In the BF, IgM^+ cells first appeared at E13 (e)(33 ×), and were observed at E15 (f) within the lymphoid buds (33 ×).

lamina propria of the intestine.

The ontogeny of PP and CT was compared with that of the bursal follicles by immunohistologically staining BF removed from the same embryo with anti-MHC class II, anti-Bu-1 or anti-IgM. Migration of MHC class II^+ cells within the lymphoid buds was first observed at E13 (Fig. 3a), while few Bu-1^+ cells and IgM^+ cells were present in the buds (Fig. 3c, e). Thereafter at E15, MHC class II^+ cells in the developed bursal follicles increased (Fig. 3b), and migration of Bu-1^+ cells and IgM^+ cells had progressed (Fig. 3d, f).

Development of PP and CT in the bursectomized chicken: In an attempt to examine whether the development of PP and CT are related to those of BF follicle, the appearance of PP and CT was observed in bursectomized chicken. Depletion of bursal follicle development in the TP-treated embryo (Fig. 4a & c) but not in the normal embryo (Fig. 4b & d) was confirmed by immunohistochemical staining with anti-epithelial antibody (HU1) [24]. Three TP-treated embryos were prepared, and the presence of the follicle in BF was

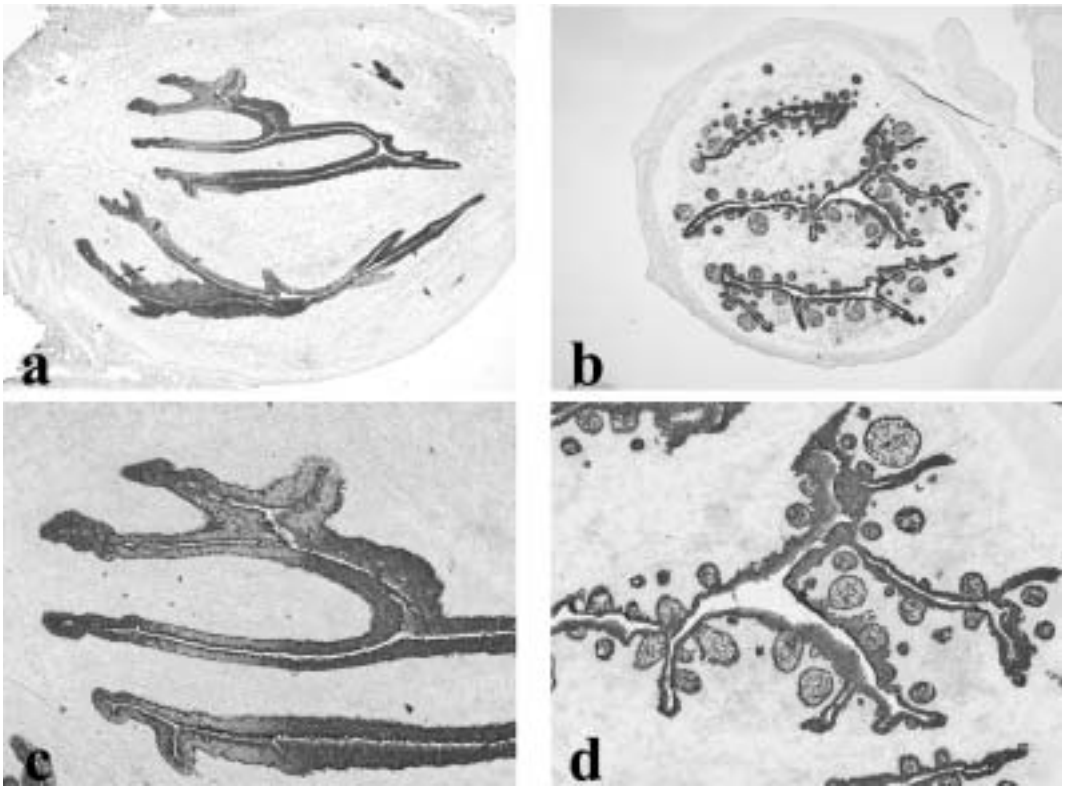

Fig. 4. Depletion of bursal follicle development in the TP-treated embryo. Sections of normal embryo (b; 10 × and d; 25×) and the TP-treated embryo (a; 10 × and c; 25×) at E17 were stained with HU-1. Depletion of bursal follicle development was observed in the TP-treated embryo, but not in the normal.

observed. The follicle in BF was not observed in all three TP-treated at E17 (embryo #1 was used for Fig. 4).

Sections from these embryo at E17 treated with TP at E11 were stained with anti-class II molecule, anti-Bu-1, or anti-IgM at E17. MHC class II^+ cells (Fig. 5a), Bu-1^+ cells (Fig. 5c), and IgM^+ cells (Fig. 5e) were present in the lamina propria of the intestine (the PP). The same observation was noted in CT (Fig. 5b, 5d and 5f, respectively) at E17. These data indicate that the appearance of PP and CT is independent of bursal follicle development during embryogenesis.

Location of embryonic PP in the intestine: The location where primary PP develops in the intestine was determined at various embryo stages. As shown in Fig. 6, PP was observed in the embryonic intestine at two sites. The development of PP occurred near Meckel's diverticulum (MD), and upstream from the ileocecal junction at E13 and E15. During the embryogenesis, the PP was not observed at any other sites.

DISCUSSION

Several reports have been studied on the ontogeny of the PP and CT in chicken. The appearance of CT near hatching has been observed [9, 14]. The appearance of PP after hatching is also observed as lymphoid cell infiltration in presumptive sites on microscopic examination [3]. However, the development of PP and CT during embryogenesis has not been described in detail. We indicated here that Bu-1^+ cells and IgM^+ cells aggregated at particular sites in the embryonic intestine as the anlage of PP and CT composed of MHC class II^+ cells. On the other hand, it has been reported in murine system that embryonic PP development occurs [2], and that expression of VCAM-1 and interleukin-7 receptor alpha are critical for the induction of PP formation [1]. It has not been reported the presence of chicken VCAM-1 and interleukin-7 receptor alpha. However, the steps occurring prior to the migration of MHC class II^+ cells, as mentioned above, might exist in the chicken system.

Our data indicated that early PP development during embryogenesis is limited to two sites near the MD and near the ileocecal junction. However, lymphoid cell infiltration in lymphoid tissues was morphologically detected with the naked eye in intestine scattered after hatching (data not shown). PPs are observed at not only the above two sites but also at other sites in adult chicken (data not shown), in agreement with a previous report [3]. Because PP development occurs at two sites independently of foreign antigens, those at other sites may develop as the result of interaction with foreign antigens. It is still unknown whether these tissues in adult and embryo immunologically and histologi-

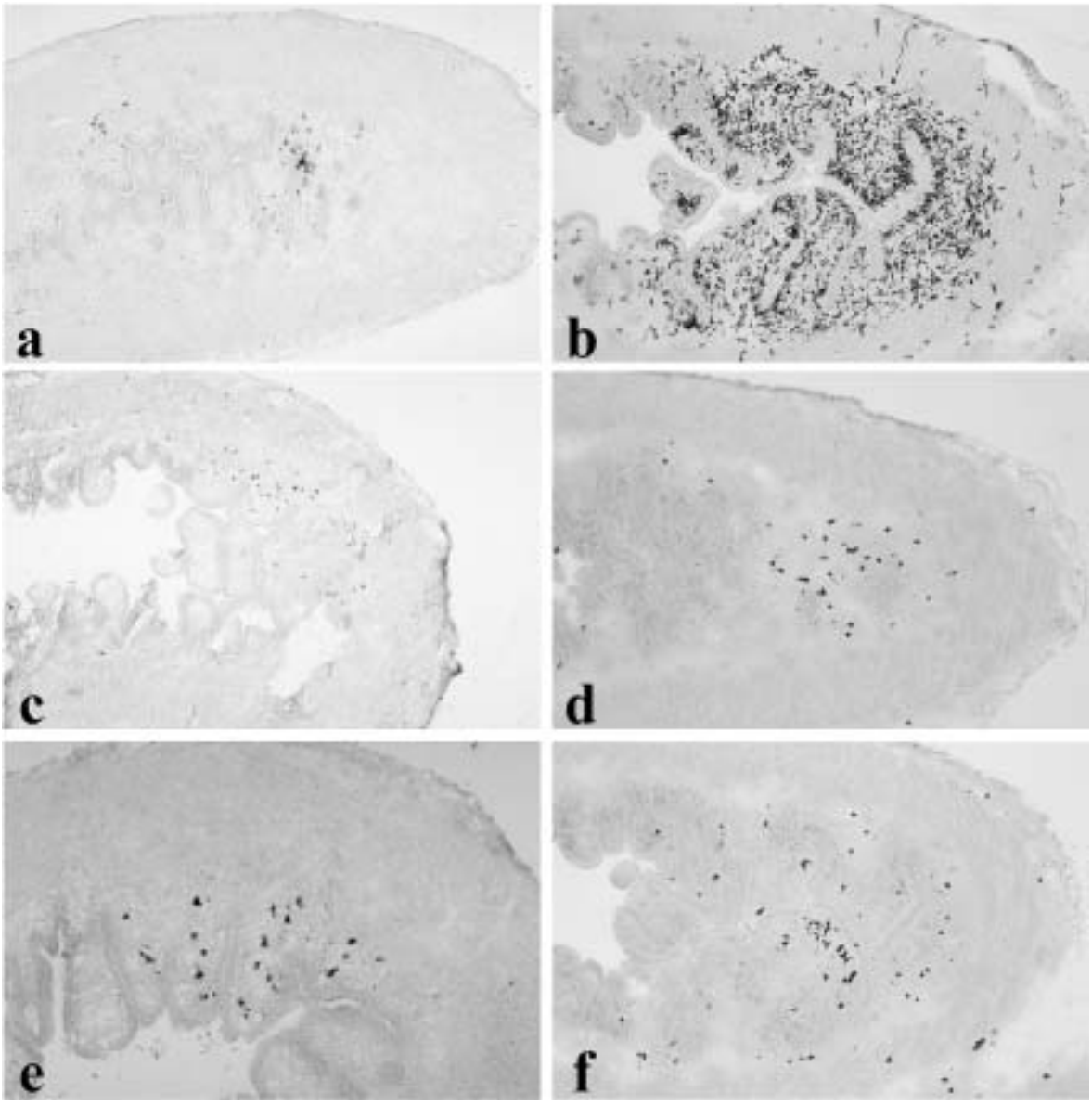

Fig. 5. Immunohistochemical staining of MHC class II$^+$, Bu-1$^+$ and IgM$^+$ cells in embryos treated with TP. Serial sections of intestine at E17 were stained immunohistologically with P2M11, L22 or 4 μ. MHC class II$^+$ cells (a), Bu-1$^+$ cells (c), and IgM$^+$ cells (e) were present in the lamina propria of the intestine (25 ×). MHC class II$^+$ cells (b), Bu-1$^+$ cells (d), and IgM$^+$ cells (f) were present in lamina propria at the presumptive sites of CT (25 ×).

cally differ from one another.

It has been reported that the precursors of dendritic cells, which are considered to express CL-1 (leucocyte common antigen) and MHC class II [15], migrate into the bursal epithelium and form the lymphoid bud at E12, and that these cells provide a microenvironment for differentiation of B cells [8, 15]. These finding are consistent with the present data as well as our previous observation that HU1 recognizes the appearance of lymphoid buds in BF at E13 [17]. In this study, the anlages of PP and CT were observed as clusters of MHC class II$^+$ cells at E13. This suggests that MHC class II$^+$ cells are dendritic cells and that these cells would provide a microenvironment for lymphocytes.

Chicken GALT includes BF, PP, CT, MD and the diffusely infiltrated area (DIA) of BF. Histological studies on PP and MD have been described by Befus, *et al.* [3] and by Olah I and Glick [22], respectively. CT has been also examined histologically [9, 21] and immunologically [5]. These

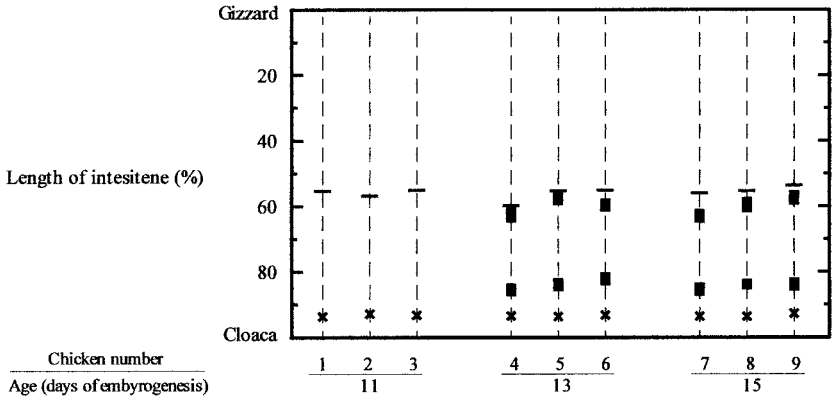

Fig. 6. Number and distribution of PPs in embryonic intestine. Only two PPs were observed and their distribution was limited to regions near the Meckel's diverticulum and near the ileocecal junction of individuals at E13 (n=3) and 15 (n=3). Square indicates a single PP. Dashed line indicates the gut of a single animal. Cross bar and cross indicate the MD and the ileocecal junction, respectively.

reports indicated that PP and CT work as secondary lymphoid tissue, similar to the spleen (SP). On the other hand, the proportion of Bu-1$^+$ cells in the embryonic SP reaches a peak at E14, and then decreases to undetectable levels at the near-hatching stage [16]. The decline in Bu-1$^+$ cells is inconsistent with our result that Bu-1$^+$ cells were present in PP and CT in the later stages of embryogenesis. These results suggest that PP and CT are different from SP in secondary systemic lymphoid tissue. Furthermore, the DIA of BF starts to develop after hatching, which does not include lymphocytes at E20 [4]. This indicates that DIA is different from PP in GALT. Bu-1$^+$ cells are first detected in SP and bone marrow, independently of BF, at around E9 [16]. Bu-1$^+$ cells migrate to the bud in BF [7, 15], which agrees with the data shown in Fig. 3. We observed that surface IgM$^+$ B cells are present and increase in number at the anlages of PP and CT where MHC class II$^+$ cells are present at E12–13. These data indicate that the development of PP, CT and the follicle of BF occur simultaneously during chicken embryogenesis.

TP treatment of embryos at E11 impairs the development of bursal follicles. These animals still possess B cells, but their immunological function is impaired [12], which suggests that B precursors appear before the bursal stage [13, 29]. We demonstrated here that PP and CT developed at E17 in TP-treated embryos (Fig. 4). During ontogeny, the emigration of B cells from BF is not detected prior to E18 [30]. These findings exclude the possibility that early development of PP and CT is dependent on bursal follicle-derived B cells. However, it strongly indicates the presence of the extra-bursal differentiation of B lineage cells. These cells would differentiate independently of BF, and acquire the rearranged variable region of the Ig gene through their intrinsic programs [27]. We believe that B precursors, which have a productive Ig gene, in part migrate to the anlage of PP and CT as well as bursal follicle, concomitantly. However, whether the VH and VL repertory of B cell immunoglobulins in embryonic PP and CT differ from those in B cells in the BF is not yet known.

Based on our observations, we conclude that PP and CT develop by signals from clusters containing MHC class II$^+$ cells at E13, independently of the BF follicle, and that few Bu-1$^+$ and IgM$^+$ cells emerge at E13. Development of PP and CT can be classified into two steps; (1) MHC class II$^+$ cells accumulate, and (2) lymphocytes migrate into the anlage of PP or CT. MHC class II$^+$ cells may provide a microenvironment for lymphocytes concomitantly with the comparable process in BF.

ACKNOWLEDGMENTS. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Agriculture, Forestry and Fisheries of Japan (Special Scientific Research and Pioneering Research Project in Biotechnology), as well as from the Bio-oriented Technology Research Advancement Institution [BRAIN].

REFERENCES

1. Adachi, S., Yoshida, H., Honda, K., Maki, K., Saijo, K., Ikuta, K., Saito, T. and Nishikawa, S.I. 1998. Essential role of IL-7 receptor α in the formation of Peyer's patch anlage. *Int. Immunol.* **10**: 1–6.
2. Adachi, S., Yoshida, H., Kataoka, H. and Nishikawa, S. 1997. Three distinctive steps in Peyer's patch formation of murine embryo. *Int. Immunol.* **9**: 507–514.
3. Befus, A.D., Johnston, N., Leslie, G.A. and Bienenstock, J. 1980. Gut-associated lymphoid tissue in the chicken. I. Morphology, ontogeny, and some functional characteristics of Peyer's patches. *J. Immunol.* **125**: 2626–2632.
4. Cortes, A., Fonfria, J., Vicente, A., Varas, A., Moreno, J. and Zapata, A.G. 1995. T-dependent areas in the chicken bursa of Fabricius: an immunohistological study. *Anat. Rec.* **242**: 91–95.
5. Gallego, M., del Cacho, E. and Bascuas, J.A. 1995. Antigen-

binding cells in the cecal tonsil and Peyer's patches of the chicken after bovine serum albumin administration. *Poult. Sci.* **74**: 472–479.
6. Gerber, H.A., Morris, B. and Trevella, W. 1986. The role of gut-associated lymphoid tissues in the generation of immunoglobulin-bearing lymphocytes in sheep. *Aust. J. Exp. Biol. Med. Sci.* **64**: 201–213.
7. Glick, B. 1995. Embryogenesis of the bursa of Fabricius: stem cell, microenvironment, and receptor-paracrine pathways. *Poult. Sci.* **74**: 419–426.
8. Glick, B. and Olah, I. 1993. Bursal secretory dendritic-like cell: a microenvironment issue. *Poult. Sci.* **72**: 1262–1266.
9. Gomez, Del, Moral, M., Fonfria, J., Varas, A., Jimenez, E., Moreno, J. and Zapata, A.G. 1998. Appearance and development of lymphoid cells in the chicken (Gallus gallus) caecal tonsil. *Anat. Rec.* **250**: 182–189.
10. Griebel, P.J. and Hein, W.R. 1996. Expanding the role of Peyer's patches in B-cell ontogeny. *Immunol. Today* **17**: 30–39.
11. Hamburgery, V. and Hamilton, H.L. 1951. A stages in the development of the chick embryo. *J. Morphol.* **88**: 44–92.
12. Hirota, Y., Suzuki, T. and Bito, Y. 1980. The development of unusual B-cell functions in the testosterone-propionate-treated chicken. *Immunology* **39**: 29–36.
13. Hirota, Y., Suzuki, T. and Bito, Y. 1980. The B-cell development independent of the bursa of Fabricius but dependent upon the thymus in chickens treated with testosterone propionate. *Immunology* **39**: 37–46.
14. Hoshi, H. and Mori, T. 1973. Identification of the bursa-dependent and thymus-dependent areas in the tonsilla caecalis of chickens. *Tohoku J. Exp. Med.* **111**: 309–322.
15. Houssaint, E. 1987. Cell lineage segregation during bursa of Fabricius ontogeny. *J. Immunol.* **138**: 3626–3634.
16. Houssaint, E., Diez, E. and Pink, J.R. 1987. Ontogeny and tissue distribution of the chicken Bu-1a antigen. *Immunology* **62**: 463–470.
17. Lillehoj, H.S., Kim, S., Lillehoj, E.P. and Bacon, L.D. 1988. Quantitative differences in Ia antigen expression in the spleens of 15I5-B congenic and inbred chickens as defined by a new monoclonal antibody. *Poult. Sci.* **67**: 1525–1535.
18. Masteller, E.L., Pharr, G.T., Funk, P.E. and Thompson, C.B. 1997. Avian B cell development. *Int. Rev. Immunol.* **15**: 185–206.
19. Melchers, F., Strasser, A., Bauer, S.R., Kudo, A., Thalmann, P. and Rolink, A. 1991. B cell development in fetal liver. *Adv. Exp. Med. Biol.* **292**: 201–205.
20. Nishinaka, S., Akiba, H., Nakamura, M., Suzuki, K., Suzuki, T., Tsubokura, K., Horiuchi, H., Furusawa, S. and Matsuda, H. 1996. Two chicken B cell lines resistant to ouabain for the production of chicken monoclonal antibodies. *J. Vet. Med. Sci.* **58**: 1053–1056.
21. Olah, I. and Glick, B. 1979. Structure of the germinal centers in the chicken caecal tonsil: light and electron microscopic and autoradiographic studies. *Poult. Sci.* **58**: 195–210.
22. Olah, I., Glick, B. and Taylor, R.L, Jr. 1984. Meckel's diverticulum. II. A novel lymphoepithelial organ in the chicken. *Anat. Rec*. **208**: 253–263.
23. Osmond, D.G. 1986. Population dynamics of bone marrow B lymphocytes. *Immunol. Rev*. **93**: 103–124.
24. Otsubo, Y., Chen, N., Kajiwara, E., Horiuchi, H., Matsuda, H. and Furusawa, S. 2001. Role of bursin in the development of B lymphocytes in chicken embryonic Bursa of Fabricius. *Dev. Comp. Immunol.* **25***:* 485–493.
25. Pink, J.R. and Rijnbeek, A.M. 1983. Monoclonal antibodies against chicken lymphocyte surface antigens. *Hybridoma* **2**: 287–296.
26. Ratcliffe, M.J. and Jacobsen, K.A. 1994. Rearrangement of immunoglobulin genes in chicken B cell development. *Semin. Immunol*. **6**: 175–184.
27. Reynaud, C.A., Imhof, B.A., Anquez, V. and Weill, J.C. 1992. Emergence of committed B lymphoid progenitors in the developing chicken embryo. *EMBO J.* **11**: 4349–4358.
28. Reynolds, J.D. 1987. Peyer's patches and the early development of B lymphocytes. *Curr. Top. Microbiol. Immunol*. **135**: 43–56.
29. Weber, W.T. and Foglia, L.M. 1980. Evidence for the presence of precursor B cells in normal and in hormonally bursectomized chick embryos. *Cell. Immunol.* **52**: 84–94.
30. Woods, R. and Linna, J. 1965. The transport of cells from the bursa of Fabricius to the spleen and the thymus. *Acta. Pathol. Microbiol. Scand*. **64**: 470–476.