FULL PAPER *Anatomy*

Distribution and Developmental Change of Lymphoid Tissues in the Chicken Proventriculus

Rei MATSUMOTO and Yoshiharu HASHIMOTO*

Laboratory of Anatomy, Department of Biomedical Science, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan

(Received 3 June 1999/Accepted 29 October 1999)

ABSTRACT. In the chicken proventricular mucosa, aggregations of lymphocytes were localized in three different sites of the lamina propria, namely, underneath the surface epithelium, near the duct orifice of the deep proventricular gland, and in the gland tissue itself. In the lymphoid masses underneath the surface epithelium and in those near the duct orifice, $CD4^+$ T lymphocytes and $TCR2^+$ T lymphocytes occupied their central part, and B lymphocytes were localized in the periphery. $CD8^+$ T lymphocytes and $TCR1^+$ lymphocytes were evenly distributed in the masses. Infiltration of lymphocytes into these sites was first observed on the 20th embryonic day. At 1 week after hatching, $CD3^+$ lymphocytes began to occupy the central area of the masses and $His\text{-}C1^+$ B lymphocytes tended to be located in the periphery. Ultrastructurally, M cells were found neither in the epithelium of the mucosa nor in that of the excretory duct close to the lymphoid masses. In the deep proventricular gland, the lymphoid masses had a germinal center consisting of B lymphocytes, surrounded by the T lymphocyte-rich periphery. These masses were first recognized at the 3rd post-hatching week, presumably being formed against possible antigens invading into the lumen of the proventricular gland. On the other hand, the lymphoid masses beneath the surface epithelium and those near the duct orifice existing before the hatching period were considered to be prepared to establish the local mucosal immune barriers against the expectant antigenic invasion.—KEY WORDS: chicken, GALT, peri- and post-hatching development, proventriculus, T cell subset.

J. Vet. Med. Sci. 62(2): 161–167, 2000

Avian species, except water birds like the duck, do not possess any lymph nodes, but develop various types of mucosa-associated lymphoid tissues (MALT) [1, 18]. MALT consists of aggregated lymphoid nodules, such as Peyer's patches and cecal tonsils, and subepithelial/intraepithelial lymphocytes. These lymphoid tissues are named as gut-associated lymphoid tissues (GALT), or duct-associated lymphoid tissues (DALT) in mammalian and avian species [21, 22], suggestive of their important role in local mucosal immunity.

Chicken T lymphocytes express the cluster of differentiation (CD) molecules which are comparable to those of mammalian T lymphocytes, and the monoclonal antibodies which recognize chicken CD3 [8], CD4 or CD8 [6] molecules have become applicable to immunohistochemistry. As regards the T cell receptors (TCR) which classify T lymphocytes into two subpopulations, $\alpha\beta$ type and $\gamma\delta$ type, chickens have three types of TCR, namely, TCR1 ($\gamma\delta$), TCR2 ($\alpha\beta$, $V\beta_1$-specific), and TCR3 ($\alpha\beta$, $V\beta_2$-specific). Furthermore, the respective monoclonal antibodies which identify the lymphocytes with TCR1 [23], TCR2 [9], or TCR3 molecules [7] have been developed. A His-C1 monoclonal antibody against B lymphocytes is also produced [13]. Application of these monoclonal antibodies make it possible to study immunohistochemically the localization and postnatal development of the T lymphocyte subpopulations in the chicken thymus, spleen [4, 5], and oviduct [15].

A number of studies on the lymphocyte localization and postnatal change in the chicken digestive tract have so far been performed [17, 20, 24], but with only a few studies dealing with the upper digestive tract including the stomach [1]. Accordingly, the present study was carried out to examine the localization of lymphocyte subpopulations and their peri- and post-hatching development in the chicken proventriculus.

MATERIALS AND METHODS

Animals: Twenty one white Leghorn (Dekalb strain) chickens from 20th embryonic day to 20 post-hatching weeks were used in the present study. Chickens were purchased from the Central Chicken Breeding Center (Unicho, Hokkaido, Japan). All the chickens were healthy without any gross anatomical or histological lesions.

Monoclonal antibodies: Monoclonal antibodies for T lymphocyte subsets used in this study were as follows: mouse anti-chicken CT-3 (anti-CD3), CT-4 (anti-CD4), CT-8 (anti-CD8), TCR1 ($\gamma\delta$-specific), and TCR2 ($\alpha\beta$, $V\beta_1$-specific). All the antibodies were purchased from Southern Biotechnology Associates, Birmingham, U.S.A. His-C1 antibody for B lymphocytes was kindly donated by Dr. S. H. M. Jeurissen, Central Veterinary Institute, Department of Virology, A. J. Lelystad, The Netherlands.

Tissue preparation: Three or 4 chickens each on the 20th embryonic day, on the 1st post-hatching day and at the 1st, 3rd, 8th and 20th week were sacrificed by exsanguination under adequete anesthesia. The proventriculus was

* CORRESPONDENCE TO: HASHIMOTO, Y., Laboratory of Anatomy, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060–0818, Japan.

immediately removed and pieces of tissues including proventricular glands were either fixed in 10% neutral buffered formalin, or snap frozen in liquid nitrogen and stored with OCT compound (Sakura Fine Technical, Tokyo, Japan) at −20°C for immunohistochemistry. The formalin-fixed tissues were embedded in paraffin according to a conventional method. Paraffin sections, 3 μm thick, were cut and stained with hematoxylin and eosin (H & E) for histological survey of the lymphoid tissues.

Immunohistochemistry: Sections, 6 μm thick, were prepared in a cryostat and placed on slides precoated with 0.5% Neopren W (Nisshin EM, Tokyo, Japan). They were fixed immediately in ice-cold 100% ethanol for 5 min and kept at −20°C until staining. The sections were placed in a moisture chamber and preincubated with 10% normal rabbit serum in 0.01 M phosphate-buffered saline for 30 min, followed by incubation with monoclonal antibodies (CD3, CD4, and CD8 were used at a dilution of 1:200, 1:100, and 1:100, respectively; TCR1, TCR2, and His-C1 were used at a dilution of 1:500, 1:500, and 1:20, respectively) for overnight at 4°C. The sections were treated with 1% biotin-conjugated goat anti-mouse Igs (Histofine SAB-PO (M) kit, Nichirei, Tokyo, Japan) for 1 hr, followed by incubation with ABC solution (Vectastain ABC kit, Vector laboratories Inc., Burlingame, U.S.A.) for 1 hr. The incubated sections were finally colored with 0.2 mg 3, 3'-diaminobenzidine-tetrahydrochloride dehydrate (Wako, Pure Chemical Ind., Osaka, Japan) per m*l* TRIS-HCl buffer (0.05 M, pH 7.6) containing 0.03% H_2O_2, and slightly counterstained with hematoxylin. Control sections were stained with a procedure as described above, but without the monoclonal antibodies.

Electron microscopic study: Tissues of 8-week-old chickens' proventriculus were taken and fixed in 2.5% glutaraldehyde solution in a phosphate-buffer, pH 7.4. Then, they were rinsed in 0.1 M phosphate-buffered saline (5 min × 3 times) and postfixed in 1% osmium tetroxide solution. These tissues were embedded in Quetol 812 resin and cut with an ultramicrotome (Leica ULTRACUT UTC). The sections were double stained with uranyl acetate and lead citrate, and examined with a JEM-1210 transmission electron microscope (JEOL, Akishima, Japan).

RESULTS

In H & E stained sections of the proventriculus at 3 weeks, lymphoid masses were found in the following three different sites of the lamina propria. 1) In the propria underneath the surface epithelium and close to the superficial proventricular gland (Fig. 1a). 2) In the deep proventricular gland where lymphocyte aggregations with a germinal center existed (Fig. 1b). 3) Around the orifice of the excretory duct of the deep proventricular gland where intraepithelial lymphocytes and lymphoid masses were located (Fig. 1c, d)

CD-3 and His-C1 positive lymphocytes were almost localized in lymphoid masses in the lamina propria underneath the surface epithelium and in those near the orifice of the duct. In addition, an unique distributional pattern of lymphocytes was noted in the lymphoid masses; $CD3^+$ lymphocytes occupied the central area of the masses and His-$C1^+$ B lymphocytes were located in the periphery (Fig. 2a, b). As to T lymphocyte subpopulations, a number of $CD4^+$ lymphocytes and $TCR2^+$ lymphocytes occupied the central part of the masses (Fig. 3a, d), while $CD8^+$ lymphocytes were scattered throughout the masses (Fig. 3b) and a few $TCR1^+$ lymphocytes were dispersed in the periphery (Fig. 3c). In the masses occurring in the areas of the deep proventricular gland, $CD4^+$ lymphocytes and $TCR2^+$ lymphocytes surrounded the germinal center mainly consisting of B lymphocytes. The epithelium of the excretory duct was infiltrated by some $TCR1^+$ lymphocytes.

On the 20th embryonic day, a few T and B lymphocytes appeared in minute clusters dispersed in the lamina propria. Near the duct orifice, several T lymphocytes and a few B lymphocytes were recognized close to the epithelium (Fig. 4a, b). No lymphocytes were distributed within the proventricular gland.

In 1-day-old chickens, the minute clusters developed larger in size by infiltration of T lymphocytes with a few B lymphocytes in the periphery. Similar small T lymphocyte masses were also recognized close to the duct orifice (Fig. 4c, d). In the deep proventricular gland, a few T and B lymphocytes appeared but did not form aggregated masses.

In 3-week-old chickens, the lymphocyte masses both beneath the surface epithelium and near the duct orifice increased in size, displaying an accumulation pattern of the T and B lymphocytes more characteristic than that in 1-week-old chickens (Fig. 4e, f). In contrast, the lymphoid masses with B lymphocyte-rich germinal center surrounded by $CD3^+$ cells was first observed in the deep proventricular gland, (Fig. 5a, b).

In the lamina propria of the 8- or 20-week-old chickens, some lymphoid masses developed to have a germinal center. The masses near the duct orifice region also increased in size and encircled the ducts. In the deep proventricular gland region, some large lymphocyte masses with a germinal center were still encountered.

By TEM observation, intraepithelial lymphocytes with some cytoplasmic granules were located in the apical region of duct epithelium. Some macrophages and dendritic cells were localized in the propria underneath the duct epithelium. No M cells were recognized in the duct epithelium. All epithelia consisted of epithelial cells of columnar type.

DISCUSSION

The present study first clarified the developing lymphoid tissues and T lymphocyte subsets in the chicken proventriculus. Aggregations of lymphocytes were found in three different regions of the proventricular mucosa. Among them, the lymphoid masses within the deep proventricular gland presumably occurred against invading possible antigenic substances, because they possessed a

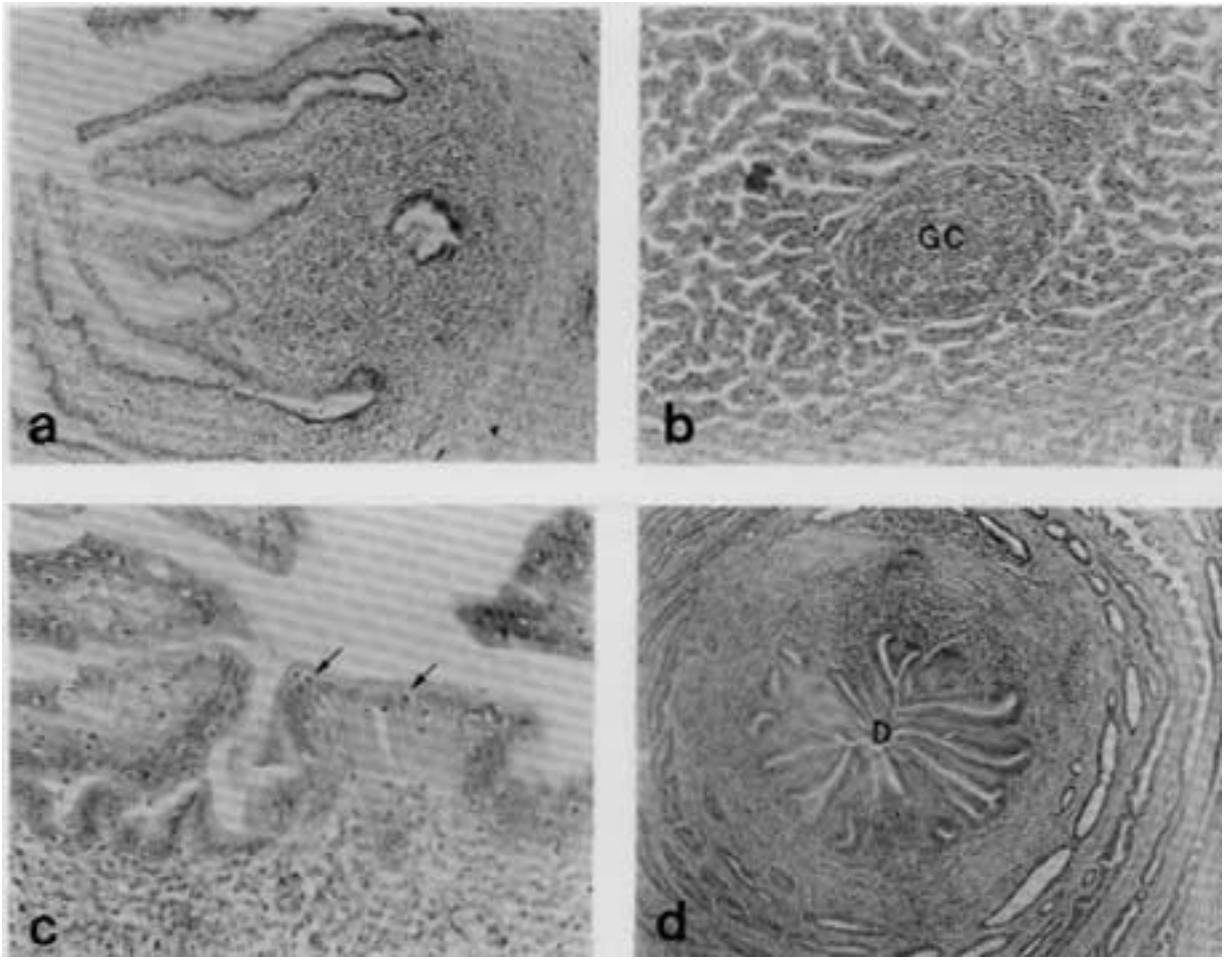

Fig. 1. Chicken proventriculus. 3-week-old. H & E stain. (a) Lymphocyte masses can be found beneath the epithelium. × 24. (b) A large lymphocyte mass with a germinal center (GC) exists in a glandular area of the proventricular gland. × 24. (c) A few lymphocytes (arrows) infiltrate in the epithelium of the duct. × 97. (d) Lymphocyte masses are always located in the lamina propria of the duct (D) of the deep proventricular gland. × 10.

conspicuous germinal center. Such lymphoid masses appeared at and after the 3rd week, so that the mucosal immune mechanism in the proventriculus may function actively from 3 weeks of post-hatching life.

Data concerning localization of lymphocyte subsets in the mammalian stomach are relatively few. Kolbjørnsen *et al*. [16] reported in the dog stomach the existence of lymphoid follicles with a B cell-rich germinal center surrounded by T cell-rich parafollicular areas. On the other hand, in the chicken, unique lymphoid masses with a T cell-rich central region were also observed in the vitelline diverticulum [14]. Therefore, the observed characteristic localization pattern of lymphocytes in the present study may be common to the lymphoid masses in the chicken digestivetracts.

As to the post-hatching development of these characteristic lymphoid masses, T lymphocytes and B lymphocytes first infiltrated on day 1 after hatching. Their localization became clear 1 week after hatching, and the masses increased in size with age up to 8 weeks. The observed developmental change of the lymphoid masses in the proventricular lamina propria underneath the surface epithelium and near the duct orifice suggests that the local mucosal immune mechanism develops primarily with a dominant participation of T lymphocytes in the early post-hatching period, and that the development of B lymphocytes takes place against the invasion of the antigens with food intake, owing to immunological information from prerequisite T lymphocytes.

The lymphoid masses occurring near the duct orifice of the deep proventricular gland are needed to be discussed because of their particular sites. Nair and Schroeder [21] reported in the monkey the lymphoid follicles around the duct orifice of minor salivary glands, designating them as DALT, and showed that these masses had germinal centers which composed of B lymphocytes and follicular dendritic

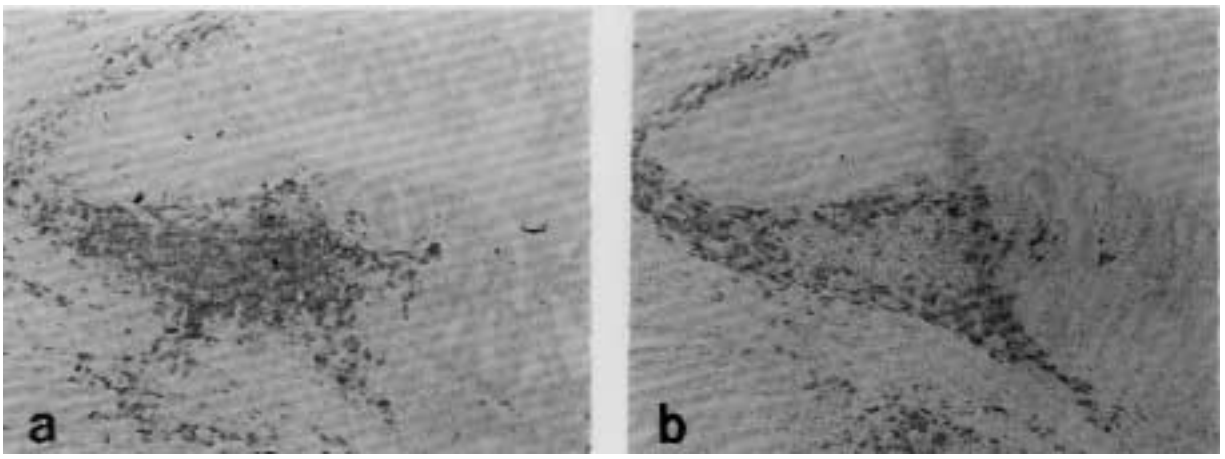

Fig. 2. Immunohistochemical staining with CD3 and His-C1 antibodies. 3-week-old. $CD3^+$ lymphocytes occupy the central part of the mass (a) and B lymphocytes are located in its periphery (b). × 48.

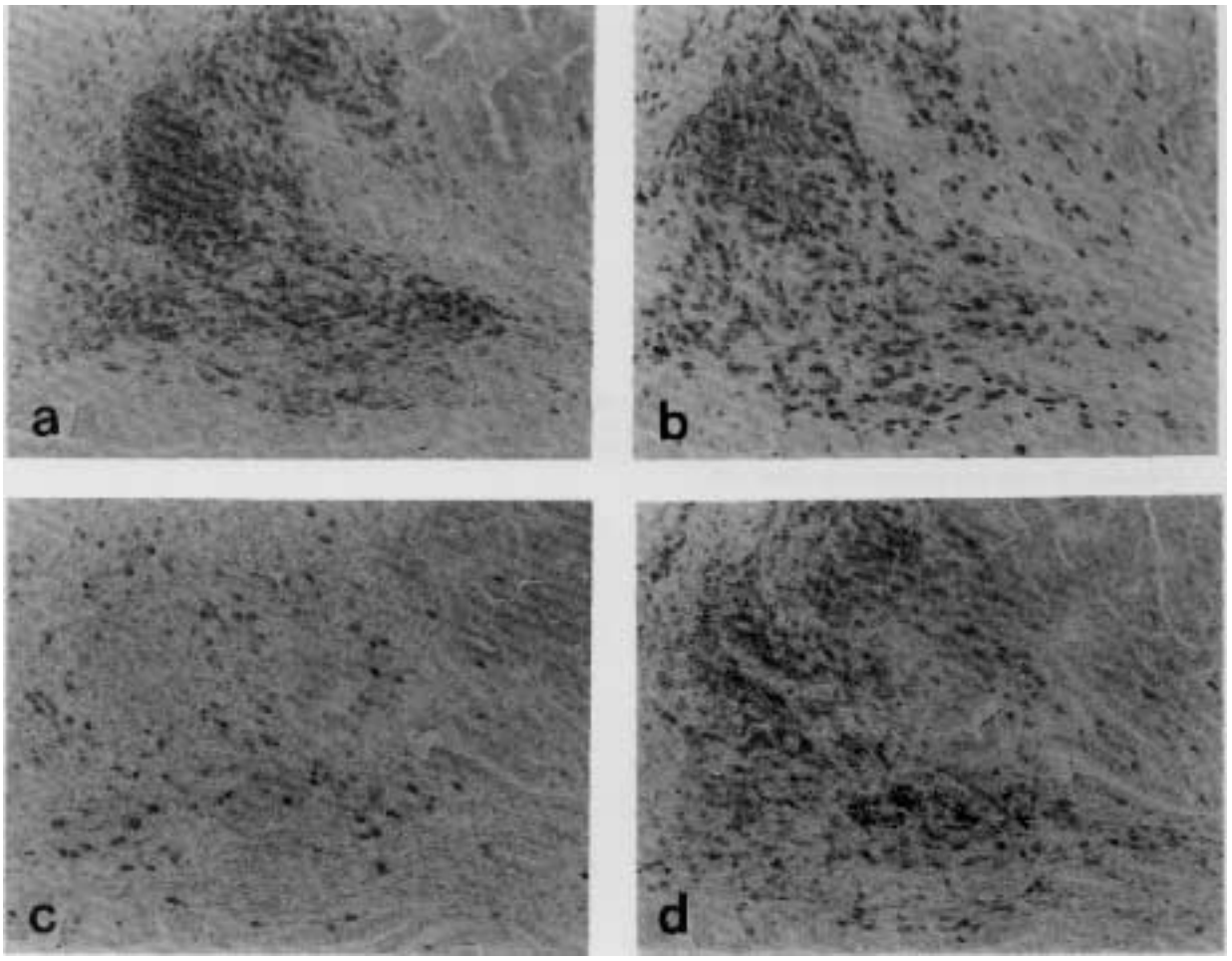

Fig. 3. Immunohistochemical staining of a mass near the duct orifice. 3-week-old. $CD4^+$ (a) lymphocytes and $TCR2^+$ (d) lymphocytes occupy the central part of the mass, while $CD8^+$ lymphocytes are scattered around the mass (b). $TCR1^+$ lymphocytes are also scattered in the periphery (c). × 48.

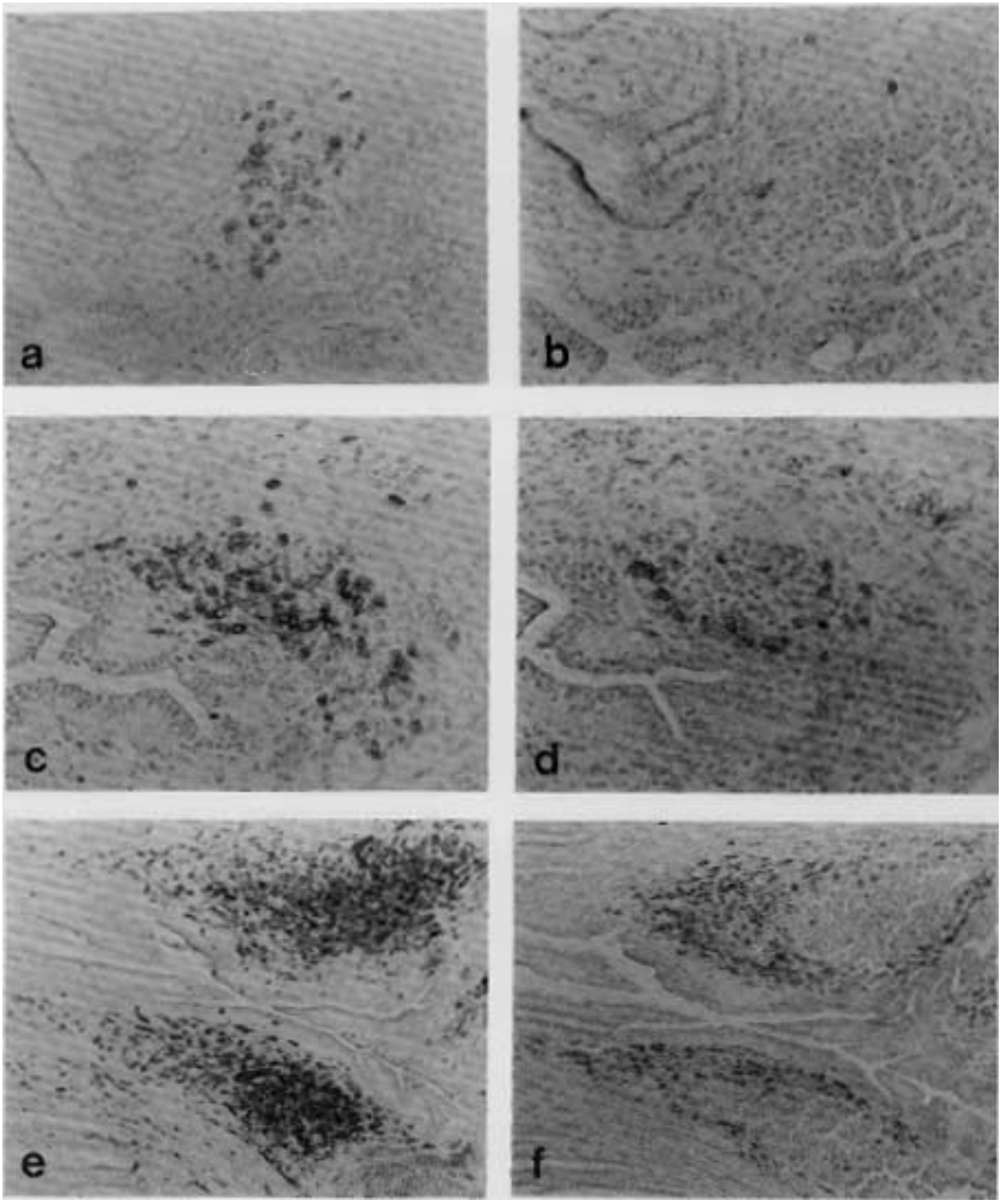

Fig. 4. Developmental changes of T ($CD3^+$) and B (His-$C1^+$) lymphocytes. T lymphocytes (a, c, e) and B lymphocytes (b, d, f) in the masses near the duct orifice on the 20th embryonic day (a, b) × 97, 1-day-old (c, d) × 97, 3-week-old (e, f) × 48.

cells and surrounded by a T cell layer.

Functionally, DALT would play an immunological defensive role by secreting Ig with saliva into the oral cavity [21]. In the monkey, M cells were recognized in the duct epithelium of major salivary glands [19]. It was suggested that M cells were engaged in the uptake of antigenic substances invading from oral cavity into the duct. Then, the antigenic substances would be transported to

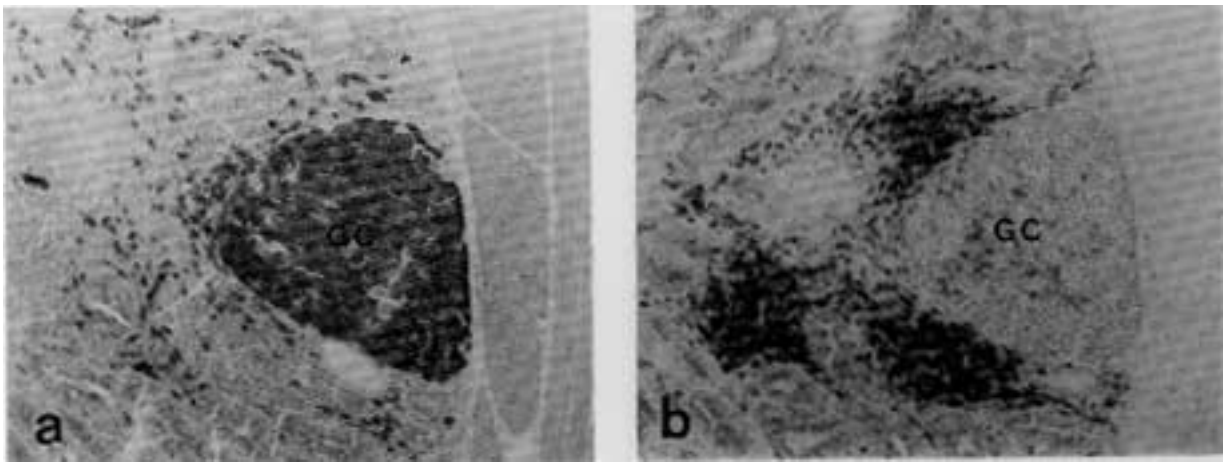

Fig. 5. In an area of the deep proventricular gland, B (His-C1$^+$) lymphocyte-rich germinal center (GC) (a) and surrounding T lymphocyte (CD3$^+$) masses (b) are observed at 3 weeks. × 48.

lymphocytes in the epithelium or in the subepithelial lymphoid masses, and thereafter, the lymphocytes would set about producing and secreting immunoglobulins on the mucosa.

The intestinal epithelium is rich in the infiltrated lymphocytes [2, 25], and the lymphocytes are mainly $\gamma\delta$ type in the mammalian intestine, [10, 11, 25]. They may play a role in the surveillance of invading antigens in addition to removal of abnormal worn-out epithelial cells [12, 25], and further participate in epithelial cell cytodieresis [3].

Intraepithelial lymphocytes in the chicken intestine consist of $\gamma\delta$ T lymphocytes and B lymphocytes [14]. In the present study, chicken lymphocytes infiltrating in the duct epithelium of the deep proventricular gland were mainly $\gamma\delta$ T lymphocytes which would play important roles both in recognition of antigenic substances invading into the epithelium and in renovation of damaged epithelial cells. In the present TEM survey in the chicken proventriculus, M-like cells could be demonstrated neither in the epithelium overlying the lymphoid masses in the lamina propria nor near the duct orifice, except for the rich intraepithelial lymphocytes. The lymphoid masses underneath the epithelium are not active to form germinal centers, but those near the duct orifice of the deep proventricular gland are active to form them, showing a characteristic localization pattern of lymphocyte subsets. Both the masses may start to develop from the 20th embryonic day, appearing as small infiltrations of a few T lymphocytes and B lymphocytes in the beginning. Based on the observed immunohistochemical characteristics, these masses and intraepithelial lymphocytes may have a possible function as a DALT-like lymphoid tissue. In the chicken proventriculus mucosa, it would be further required to demonstrate some routes possible for the uptake of intraluminal antigens other than the route associated with M cells.

REFERENCES

1. Arai, N., Hashimoto, Y., Kitagawa, H., Kon, Y. and Kudo, N. 1988. Immunohistochemical study on the distribution of lymphoid tissues in the upper alimentary and respiratory tract of chickens. *Jpn. J. Vet. Sci.* 50: 183–192.
2. Beagley, K. W. and Husband, A. J. 1998. Intraepithelial lymphocytes: origins, distribution, and function. *Crit. Rev. Immunol.* 18: 237–254.
3. Boismenu, R. and Havran, W. L. 1994. Modulation of epithelial cell growth by intraepithelial $\gamma\delta$ cells. *Science* 266: 1253–1255.
4. Bucy, R. P., Chen, C. L., Cihak, J., Loesch, U. and Cooper, M. D. 1988. Avian T cells expressing $\gamma\delta$ receptors localize in the spleen sinusoids and the intestinal epithelium. *J. Immunol.* 141: 2200–2205.
5. Bucy, R. P., Chen, C. H. and Cooper, M. D. 1990. Ontogeny of T cell receptors in the chicken thymus. *J. Immunol.* 144: 1161–1168.
6. Chan, M. M., Chen, C. H., Ager, L. L. and Cooper, M. D. 1988. Identification of the avian homologues of mammalian CD4 and CD8 antigens. *J. Immunol.* 140: 2133–2138.
7. Char, D., Sanchez, P., Chen, C. H., Bucy, R. P. and Cooper, M. D. 1990. A third sublineage of avian T cells can be identified with a T cell receptor-3-specific antibody. *J. Immunol.* 145: 3547–3555.
8. Chen, C. H., Ager, L. L., Gartland, G. L. and Cooper, M. D. 1986. Identification of a T3/T cell receptor complex in chickens. *J. Exp. Med.* 164: 375–380.
9. Cihak, J., Ziegler-Heitbrock, H. W., Trainer, H., Schranner, I., Merkenschlager, M. and Loesch, U. 1988. Characterization and functional properties of a novel monoclonal antibody which identifies a T cell receptor in chickens. *Eur. J. Immunol.* 18: 533–537.
10. De Geus, B., Van den Enden, M., Coolen, C., Nagelkerken, L., Van der Heijden, P. and Rozing, J. 1990. Phenotype of intraepithelial lymphocytes in euthymic and athymic mice: implications for differentiation of cells bearing a CD3-associated $\gamma\delta$ T cell receptor. *Eur. J. Immunol.* 20: 291–298.
11. Guy-Grand, D., Malassis-Seris, M., Briottet, C. and Vassalli,

P. 1991. Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocyte is induced locally. Correration between functional assays, presence of Perforin and granzyme transcripts, and cytoplasmic granules. *J. Exp. Med.* 173: 1549–1552.

12. Guy-Grand, D., Cerf-Bensussan, N., Malissen, B., Malassis-Seris, M., Briottet, V. and Vassalli, P. 1991. Two gut intraepithelial $CD8^+$ lymphocyte populations with different T cell receptors: A role for the gut epithelium in T cell differentiation. *J. Exp. Med.* 173: 471–481.
13. Jeurissen, S. H., Janse, E. M., Ekino, S., Nieuwenhuis, P., Koch, G. and de Boer, G. F. 1988. Monoclonal antibodies as probes for defining cellular subsets in the bone marrow, thymus, bursa of Fabricius, and spleen of the chicken. *Vet. Immunol. Immunopathol.* 19: 225–238.
14. Jeurissen, S. H., Janse, E. M., Koch, G. and de Boer, G. F. 1989. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell Tissue Res.* 258: 119–124.
15. Khan, M. Z. I., Hashimoto, Y., Konno, A. and Iwanaga, T. 1996. Development of T-lymphocyte subpopulations in the postnatal chicken oviduct. *Cell Tissue Res.* 284: 317–325.
16. Kolbjørnsen, C. R., Press, C. M., Moore, P. F. and Landsverk, T. 1994. Lymphoid follicles in the gastric mucosa of dogs. Distribution and lymphocyte phenotypes. *Vet. Immunol. Immunopathol.* 40: 299–312.
17. Lillejoj, H. S. and Chung, K. S. 1992. Postnatal development of T-lymphocyte subpopulations in the intestinal intraepithelium and lamina propria in chickens. *Vet. Immunol. Immunopathol.* 31: 347–360.
18. Manabe, S. 1930. Studies on the avian lymphatic system. III. Histology and differentiation of duck lymph nodes. *Acta Anat. Nippon* 3: 282–297.
19. Matsuda, M., Ina, K., Kitamura, H., Fujikura, Y. and Shimada, T. 1997. Demonstration and organization of duct-associated lymphoid tissue (DALT) of the main excretory duct in the monkey parotid gland. *Arch. Histol. Cytol.* 60: 493–502.
20. 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.
21. Nair, P. N. and Schroeder, H. E. 1986. Duct-associated lymphoid tissue (DALT) of minor salivary glands and mucosal immunity. *Immunology* 57: 171–180.
22. Pabst, R. 1987. The anatomical basis for the immune function of the gut. *Anat. Embryol.* 176: 135–144.
23. Sowder, J. T., Chen, C. -L. H., Chan, M. M. and Cooper, M. D. 1988. A large subpopulation of the mammalian T γ/δ receptor. *J. Exp. Med.* 167: 315–322.
24. Vervelde, L. and Jeurissen, S. H. M. 1993. Postnatal development of intra-epithelial leukocytes in the chicken digestive tract: phenotypical characterization in situ. *Cell Tissue Res.* 274: 295–301.
25. Viney, J. L., Kilshaw, P. J. and MacDonald, T. T. 1990. Cytotoxic $\alpha\ \beta^+$ and $\gamma\ \delta^+$ cells in murine intestinal epithelium. *Eur. J. Immunol.* 20: 1623–1626.