

Immunohistochemical Analysis of Tissue Distribution of B and T Cells in Germfree and Conventional Chickens

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ABSTRACT. The distribution of lymphocytes in lymphoid organs of germfree (GF) and conventional (CV) chickens was compared immunohistochemically with several monoclonal antibodies. The lymphoid follicles in the cecal tonsils of GF chickens were more poorly developed than in those of CV chickens, and IgG and IgA positive cells were also not found in GF chickens. The number of both B and T cells in the villous region of the cecal tonsils in GF chickens was less than that in CV chickens. In the spleen of GF chickens, the distribution of B cells was similar, but T cells were distributed more widely in CV chickens than in GF chickens. There was no clear difference in the distribution of lymphoid cells in the bursa of Fabricius and the thymus. These results suggest that the development of gut-associated lymphoid tissues is affected by intestinal flora.—**KEY WORDS:** chicken, germfree, immunohistochemistry.

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The absence of low levels of natural antibodies, immunoglobulins and spontaneous plaque-forming cells is reported in germfree (GF) animals [2, 5, 7], because intestinal flora have an important influence on the immune system as well as physiology, metabolism and nutrition of the animal host.

As a useful laboratory animal, the chicken has been used for immunological studies because it possesses the bursa of Fabricius, a unique central lymphoid organ, primarily involved in the development of the B cell dependent immune system. Little is reported about the effect of intestinal flora on the development of lymphoid organs in the chicken. The purpose of the present study is to compare the distribution of B and T cells in the lymphoid organs of GF and conventional (CV) chickens immunohistochemically.

Inbred line P chickens (MHC; B²B²), kept at the National Institute of Animal Health, Japan, were used. GF chickens were produced according to the method described by Ishibashi *et al.* [4]. They were maintained in stainless isolators, and given an irradiated commercial diet (Nippon Formula Feed Manufacturing, Japan; Chick Food, 50 kGy) and sterilized water. Fresh fecal samples were collected for microbial contamination test [4]. CV chickens were reared under conventional conditions. Three GF and 6 CV chickens were euthanized at 4 weeks of age. Tissues of the bursa of Fabricius, thymus, spleen, and cecal tonsil were taken from GF and CV chickens. Fresh tissues were embedded in Tissue-Tek OCT compound (Miles, U.S.A.), snap frozen in liquid nitrogen, and stored at –80°C until use. The 4 µm frozen sections were cut, immediately air-dried, fixed in acetone at 4°C for 10 min, and rehydrated in phosphate-buffered saline (PBS, pH 7.2). Tissue sections were first covered with PBS containing 10% heat inactivated horse serum for 30 min to block nonspecific binding. The sections were then incubated for 30 min with the primary monoclonal antibodies (mAbs). Subsequently, the sections were incubated with biotinylated anti-mouse IgG (Vector Labor-

atories, Inc., U.S.A.) for 30 min. After blocking endogenous peroxidase with 0.3% H₂O₂ in methanol, the sections were then incubated with the avidin-biotin peroxidase complex (Vector Laboratories, Inc., U.S.A.). Finally, the sections were colored with the 3,3'-diaminobenzidine (0.2 mg/ml) and 0.01% H₂O₂. Between each incubation the slides were washed three times with PBS. The sections were then counterstained with Mayer's hematoxylin (Merck, Germany), dehydrated in graded alcohols, cleared in xylene, and mounted for microscopic observation. The following mouse monoclonal antibodies were used as primary antibodies: CT3 (CD3 specific), L22 (Bu-1a specific), 11G2 (Bu-1b specific), 3E8 (IgG Fc specific), B-4D-4 (IgA α chain specific) and A-13 (IgM specific). To detect Bu-1 antigen, a mixture of L22 and 11G2 was used.

The lymphoid follicles in the cecal tonsils were more poorly developed in the GF chickens than in the CV chickens. Germinal centers were observed in the lymphoid follicles of the CV chickens, and the lymphoid follicles contained CD3, IgG, IgM and IgA positive cells (Fig. 1A). On the other hand, no germinal centers were observed in the lymphoid follicles of the GF chickens, and neither IgA nor IgG positive cells were observed in the lymphoid follicles (Fig. 1B), though CD3 and IgM positive cells were detected.

In the villous region of cecal tonsils, the number of CD3 positive cells in the GF chickens was less than that in the CV chickens (Fig. 2). Moreover there were many IgA positive cells in the CV chickens, but very few in the GF chickens (Fig. 3). In the CV chickens, the number of IgM and IgG positive cells was much less than that of IgA, and was negligible in the GF chickens.

In the spleen of neither the GF nor the CV chickens, any germinal centers were observed. The tissue distribution of B cells was similar in both the GF and the CV chickens, though that of T cells was a little different. CD3 positive cells distributed more in the CV chickens than in the GF chickens (Fig. 4).

In the bursa of Fabricius, the development of lymphoid follicles was similar in both the GF and the CV chickens. A similar density of lymphocytes in the bursal follicles was observed. Most of the bursal lymphoid cells were IgM

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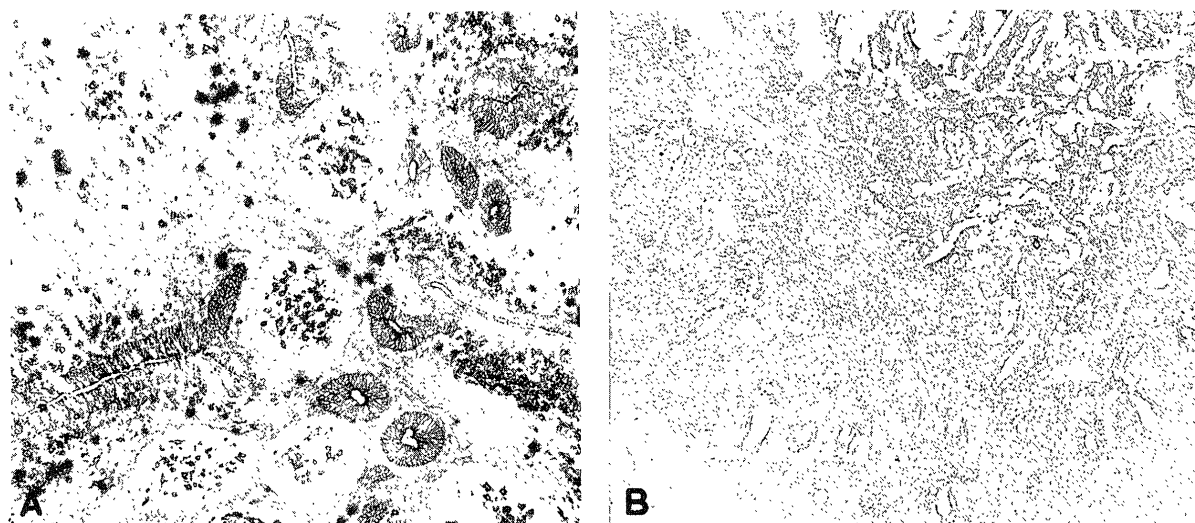


Fig. 1. (A) IgA positive cells in the lymphoid follicle of the cecal tonsils in the 4-week-old CV chickens. The lymphoid follicle contains IgA positive cells. Many germinal centers also contain IgA positive cells. Epithelial cells are positive for IgA. $\times 100$. (B) The lymphoid follicle of the cecal tonsils in the 4-week-old GF chickens. The lymphoid follicle is poorly developed, and no germinal centers or few IgA positive cells are detected. $\times 100$.

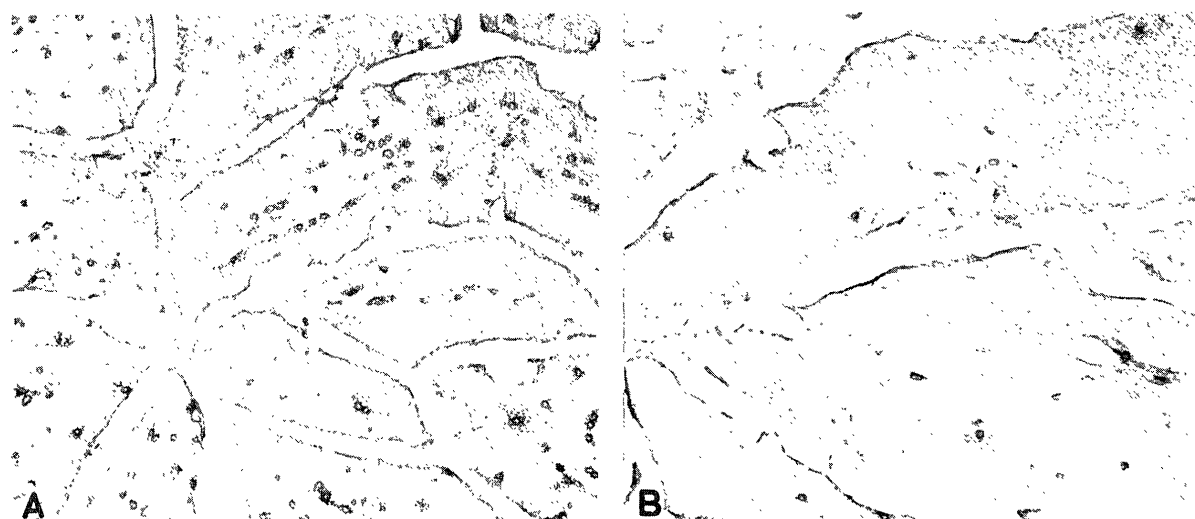


Fig. 2. CD3 positive cells in the villous region of the cecal tonsils. (A) Many CD3 positive cells are seen in the villous region in the 4-week-old CV chickens. $\times 200$. (B) There are fewer CD3 positive cells in the 4-week-old GF chickens than in the CV chickens (A). $\times 200$.

positive. A small population of bursal cells was IgG positive, and a few cells were IgA positive. No CD3 positive cells were detected in the bursal follicles of either type of chicken.

In the thymus, there was no significant difference between the GF and the CV chickens in the tissue distribution of lymphoid cells.

One of the immunohistochemical features of the lymphoid organs of the GF chickens was a decrease in the relative number of B and T cells in the cecal tonsils despite the lack of a difference in the immunohistochemical distribution of both primary organs of the bursa of Fabricius and thymus in the 4-week-old GF and CV

chickens. In addition, germinal centers of the lymphoid follicles in cecal tonsils well developed in the CV chickens, though no germinal center was detected in the GF chickens. This suggests that the development of intestinal immunity may be enhanced by intestinal flora. In particular, IgA positive cells were fewer in the GF chickens, although there were IgM positive cells in the lymphoid follicles even in the GF chickens. Class switch mechanisms may also be affected by intestinal flora. It has not yet been determined whether this tendency in tissue distribution continues in adult chickens or not, but at least, it is assumed from the data for GF mice and rats [1] that the lymphoid system may be more poorly developed in GF

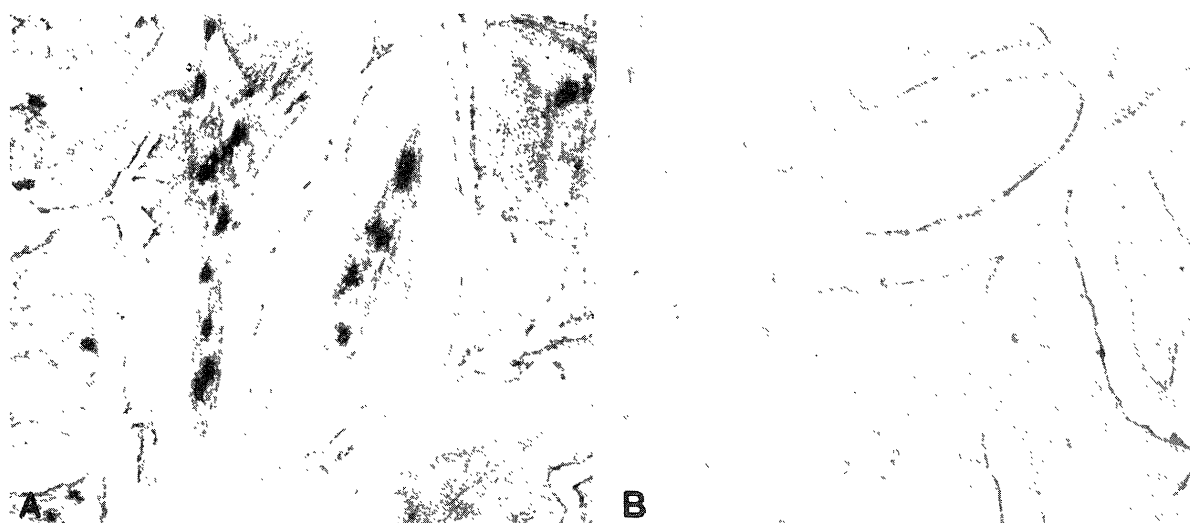


Fig. 3. IgA positive cells in the villous region of the cecal tonsils. (A) IgA positive cells are seen in the lamina propria of the villous region of the 4-week-old CV chickens. $\times 200$. (B) IgA positive cells are not detected in the villous region of the 4-week-old GF chickens. $\times 200$.

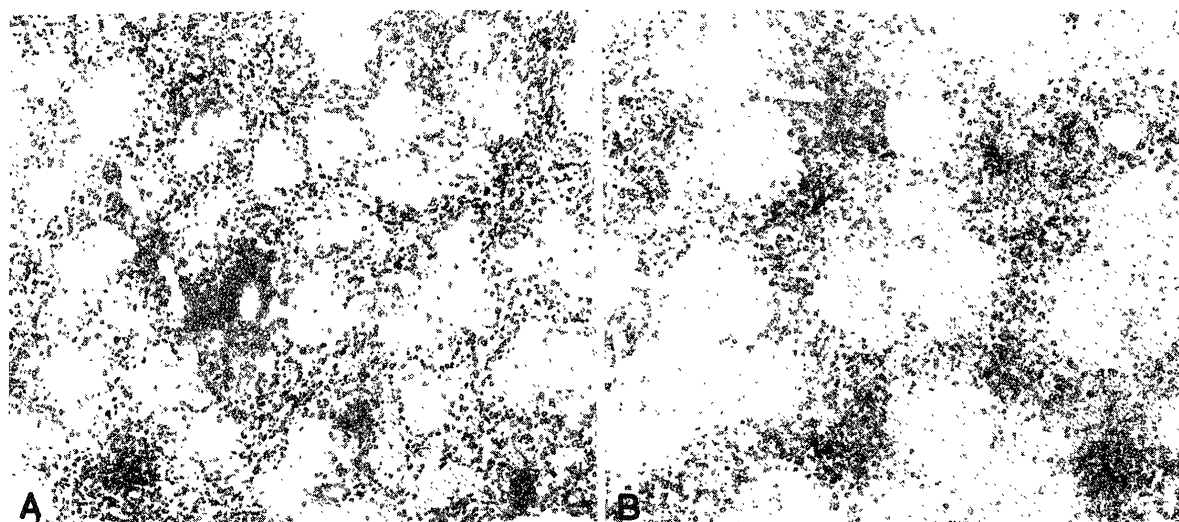


Fig. 4. CD3 positive cells in spleen. (A) CD3 positive cells more widely distributed in the 4-week-old CV chickens. $\times 100$. (B) CD3 negative area in a GF chicken is wider than that in the CV chickens. $\times 100$.

chickens than in CV.

To the best of our knowledge, almost no effect has been observed due to the structural difference between the bursa of Fabricius in 4- and 8-week-old GF and CV chickens, suggesting that the development of bursal lymphocytes is not dependent on exogenous antigen stimulation [6]. On the other hand, follicles of the bursa of Fabricius isolated from environmental stimuli by ligation of the bursal duct developed poorly [3]. In our study, there was no clear difference in the tissue distribution of lymphocytes in the primary organs, thymus and the bursa of Fabricius, of the CV and GF chickens. This suggests that the development of these organs is not affected by bacterial flora. More detailed studies, especially based on the functional aspects of lymphocytes, are needed.

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