

## Immunoreactivity and morphological changes of bursal follicles in chickens infected with vaccine or wild-type strains of the infectious bursal disease virus

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**ABSTRACT.** Infectious bursal disease (IBD) is characterized by immunosuppression due to the depletion of lymphocytes in the atrophied bursa of Fabricius (BF). We have sometimes encountered contradictory findings: chickens infected with the vaccine IBD virus (IBDV) strain have sometimes exhibited a highly atrophied BF, but not immunosuppression. In this study, chickens administered vaccine or wild-type strains of IBDV were later vaccinated with the B1 strain of the Newcastle disease virus (NDV). Bursal changes were examined histologically with a focus on the bursal follicle. The immunoreactivity to NDV was also evaluated with the hemagglutination inhibition test. In gross examination, we observed a few chickens with a severely atrophied BF in vaccine strain-administered groups (vaccine groups), and the level of severity was the same as that in the wild-type strain-administered group (wild-type group). However, these chickens retained humoral antibody responses to NDV and were revealed to possess a higher number of bursal follicles than those of the wild-type group. These results indicated that macroscopic evaluation does not accurately reflect the immunoreactivity and degree of bursal damage in IBDV-administered chickens. We also found non-immunosuppressed chickens in the wild-type group. These non-immunosuppressed chickens retained a significantly higher number of normal follicles and total follicles according to our statistical analysis. Furthermore, a high correlation coefficient between the NDV-HI titer and the number of normal follicles was found in the wild-type group. These results implied that the retained number of normal follicles is important for the immunoreactivity of chickens infected with IBDV.

**KEY WORDS:** bursal follicle, infectious bursal disease, vaccine

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Infectious bursal disease (IBD), an acute and highly contagious viral infection in young chickens, is of worldwide importance for the poultry industry [26]. IBD sometimes results in death and mortality depending on the virulence of the IBD virus (IBDV), which is the causative agent of IBD, and the mortality rate is up to 60% in very virulent IBDV infection [4, 15]. In addition to its mortality, immunosuppression is the most serious sequela of IBD, since immunosuppression is an inescapable outcome regardless of IBDV virulence [9]. After the acute phase of infection, surviving chickens exhibit suppressed humoral antibody responses to other vaccines [7] and become more susceptible to secondary infection, such as inclusion body hepatitis [5], coccidiosis [1], Marek's disease [2, 25], hemorrhagic-aplastic anemia and gangrenous dermatitis [23], infectious laryngotracheitis [22], infectious bronchitis [18], chicken anemia agent [34], and salmonellosis and colibacillosis [33].

The immunosuppression of IBD is known to be caused by the depletion of lymphocytes in the bursa of Fabricius (BF) [10, 19, 26], which results in macroscopic BF atrophy, the

most characteristic lesion of IBD [12]. Live vaccine strains of IBDV also cause BF atrophy to various extents [17, 21]. Nevertheless, these live vaccine strains are confirmed to exhibit effective antigenicity and to be safe, and they do not induce immunosuppression, because it is widely accepted that the extent of atrophy caused by live vaccine strains is slightly less compared with that caused by the wild-type strain [4]. On the other hand, we sometimes find live IBDV vaccine strain-vaccinated chickens showing an unexpectedly high extent of BF atrophy without immunosuppression. In addition, recent studies have shown that some chickens that survived IBD were immunosuppressed, though their BFs were repopulated with B lymphocytes [31, 32]. These reports and our experience indicate that IBDV-induced immunosuppression may not be fully explained by atrophy of the BF and/or depletion of lymphocytes.

The bursal follicle is the smallest component of the BF parenchyma, which consists of the medulla, the cortex and the follicular structure [8, 14, 24]. The follicular structure of the BF is composed of the follicle-associated epithelium, the basement membrane (BM) and the BM-associated epithelium [8, 14, 24]. It provides a microenvironment for the development of B lymphocytes and is important for immunoreactivity in chickens [6, 8, 16, 24]. In spite of the important role of the follicular structure, it has not been fully evaluated in the study of immunosuppression by IBDV. The aim of the present study was to assess the BF pathologically with a focus on the bursal follicle and immunoreactivity in chickens administered a vaccine strain or wild-type strain of IBDV.

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## MATERIALS AND METHODS

**Chickens:** Four-day-old specific pathogen-free White Leghorn chickens (Nisseiken Co., Ltd., Tokyo, Japan) were used. Chickens were bred in groups in a state of mixing males and females under *ad libitum* conditions. All procedures were in accordance with the guidelines of the Animal Research Committee of the National Veterinary Assay Laboratory and were approved by the committee (approval number O-034).

**Experimental design and virus:** Chickens (n=55) were divided into the following 5 groups: the Vac-Ch (n=9), Vac-IM (n=9), Vac-LC (n=10), IBDV wild-type strain-administered (n=15) and control groups (n=12). Chickens in the Vac-Ch group were administered the live IBDV vaccine strain for chicks (strain S706). Chickens in the Vac-IM group were administered the live IBDV vaccine strain for chicks (intermediate virulence type; strain 228E). Chickens in the Vac-LC group were administered the live IBDV vaccine strain for large chicks (strain MB-1). Chickens in the IBDV wild-type strain-administered group (wild-type group) were administered the IBDV wild-type strain (strain K-1). Chickens in the control group were not administered any viruses. The 3 vaccine strains are used in commercial vaccines in Japan. The IBDV wild-type strain was originally isolated from laying hens in 1992 in Niigata Prefecture, Japan. The titers of the administered viruses in the Vac-Ch, Vac-IM, Vac-LC and wild-type groups were  $10^{5.4}$ ,  $10^{4.5}$ ,  $10^{5.5}$  and  $10^{4.7}$  EID<sub>50</sub>/ml [50% embryo infectious dose (EID<sub>50</sub>)], respectively. Each group was kept in a separate isolator.

On day 1, all 4-day-old chickens, except for controls, were orally administered 0.2 ml of viral specimens using feeding needles. The control group was administered 0.2 ml of phosphate-buffered saline using feeding needles. At 7 days post infection (DPI), all chickens were vaccinated oculonasally with one dose of the commercial live vaccine of the Newcastle disease virus (NDV) containing the B1 strain according to the manufacturer's instructions. Blood was collected at 28 DPI for the hemagglutination inhibition (HI) test of the antibody titers to NDV, as described below. At 35 DPI, the chickens' body weights were measured, and they were humanely euthanized. Subsequently, their BFs were extracted and macroscopically examined and weighed. The BF weight to body weight ratio (F/B ratio) was calculated with the following formula: F/B ratio=BF weight (g)/body weight (g) × 100. All BFs were collected and fixed in 10% neutral-buffered formalin for further histological examination. The chickens that died during the experiment were excluded from the analysis.

**HI test:** The collected sera were subjected to the NDV-HI test to evaluate immunoreactivity. The serum antibody titers to the hemagglutination antigen of NDV were measured with the HI test with the commercial hemagglutination antigen of NDV (Kaketsuken, Kumamoto, Japan) according to the manufacturer's instructions.

**Histopathological examination:** Fixed BFs were transected at the point of maximum cross section to evaluate each BF under the same conditions. The specimens were em-

bedded in paraffin, sectioned (4 μm thick) and stained with hematoxylin and eosin and the periodic acid–Schiff reaction.

**Immunofluorescence:** For antigen retrieval, the deparaffinized sections were heated at 98°C in an immunosaver (Nisshin EM Corp., Tokyo, Japan) for 45 min. The sections were then incubated with an anti-keratin AE1/AE3 antibody (1 in 200 dilution; Dako Denmark A/S, Glostrup, Denmark) or anti-chicken Bu-1 antibody (clone AV20; 1 in 100 dilution; SouthernBiotech, Birmingham, AL, U.S.A.) at 4°C overnight and visualized with an Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1 in 1,000 dilution; Life Technologies Corp., Gaithersburg, MD, U.S.A.). The fluorescent signals in the sections were observed with a fluorescence microscope (FSX100, Olympus Corp., Tokyo, Japan). The follicle-associated epithelium and B lymphocytes in the BF of a control chicken were used as a positive control for the anti-keratin AE1/AE3 antibody and anti-chicken Bu-1 antibody, respectively. Negative controls were obtained by omitting the primary antibody.

**Quantitative analysis of microscopic findings:** For the quantitative evaluations, histological scoring evaluations of follicular lesion were performed according to previous reports [12, 13, 20, 21, 26, 27, 29]. In brief, the scoring evaluations were based on the percentage of affected follicles, i.e. those showing lymphocyte depletion, in all the follicles: 0 ≤ 1%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 ≥ 75%.

In addition to the scoring evaluation based on previous reports, we classified and counted the number of bursal follicles. The BM of the bursal follicle was stained with the periodic acid–Schiff reaction, and the associated epithelium was positive for cytokeratin. According to the population of lymphocytes and state of the follicular BM-associated structures, namely the BM and BM-associated epithelium, we classified the bursal follicle of the present chickens as follows: a follicle that retained lymphocytes and the BM-associated structures was classified as a normal follicle, and that exhibiting depletion of lymphocytes and a lack of a discernible cortex and medulla but retained the BM-associated structures was classified as a small follicle. We counted the numbers of normal follicles and small follicles and calculated the number of total follicles by summation of the numbers of normal and small follicles. In the case of inappropriate specimens in which the mucosal folds on the luminal surface of the BF were not transected vertically, the specimens and chickens were excluded from the following statistical analyses (Nos. 8 and 9 in the Vac-Ch group and No. 15 in the Vac-IM group).

**Statistical analysis:** The data are expressed as the arithmetic mean ± standard deviation (SD), except for the HI titers, which were assessed by the geometric mean and expressed as the geometric mean ± geometric SD. For the HI titer, undetectable values were calculated as 1. The HI titer was expressed on a base-2 logarithmic scale for the following statistical procedures. Statistical significance was determined with the Student's *t*-tests or one-way analysis of variance (ANOVA), which was followed by Tukey–Kramer post hoc tests for multiple comparisons. Spearman's rank correlation coefficients (*r*) between the HI titer and other

parameters were calculated. The analyses were conducted with GraphPad Prism ver. 5 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). In addition to the group statistical comparisons, chickens in the wild-type group were subdivided into 2 subgroups according to the presence or absence of an anti-NDV HI antibody response and subjected to the same statistical analysis as described above: the chickens with detectable NDV-HI antibody production ( $n=6$ , partly immunoreactive subgroup) and the chickens without antibody production ( $n=7$ , immunosuppressed subgroup).

## RESULTS

**Clinical manifestations:** No clinical signs were observed in the vaccine strain-administered groups (vaccine groups). In the wild-type group, 2 chickens died at 3 DPI, and the others showed depression and ruffled feathers at 3–7 DPI.

**Macroscopic findings of the BF and HI titers:** At necropsy, we observed macroscopically mild to severe atrophy in the vaccine groups (Fig. 1A–1C) and severe BF atrophy in the wild-type group (Fig. 1D). In the Vac-IM and Vac-LC groups, unexpectedly severe BF atrophy was sometimes observed (Fig. 1B). The mean F/B ratios were significantly decreased in the vaccine groups compared with the control group (Table 1). The mean F/B ratios were significantly decreased in the wild-type group compared with the other groups. The F/B ratios of the severely BF-atrophied chickens in the vaccine groups were less than 0.10 (0.05, Nos. 7 and 13 in Vac-IM, 0.07, No. 15 in Vac-LC; 0.08, No. 25 in Vac-LC) and fell within the mean F/B values  $\pm$  2SD of the wild-type group.

HI antibody responses ranged from 1:5 to 1:160 in the control group and vaccine groups. The mean HI titers of the vaccine groups did not significantly differ compared with the control group or among vaccine groups (Table 1). Notably, the chickens with very low F/B ratios in the vaccine groups also showed antibody responses (HI titers of 1:40, 1:5, 1:20 and 1:40 for Nos. 11, 13, 15 and 25, respectively). The mean HI titer of the wild-type group was significantly lower than those of the other groups. However, 6 chickens showed HI antibody responses.

**Histological findings of the BF:** Histologically, the bursal tunica intimae in the vaccine groups were occupied by normal and small follicles (Fig. 2A–2C). In the wild-type group, a few small follicles and a few normal follicles were interspersed with slight to moderate fibrosis in the lamina propria

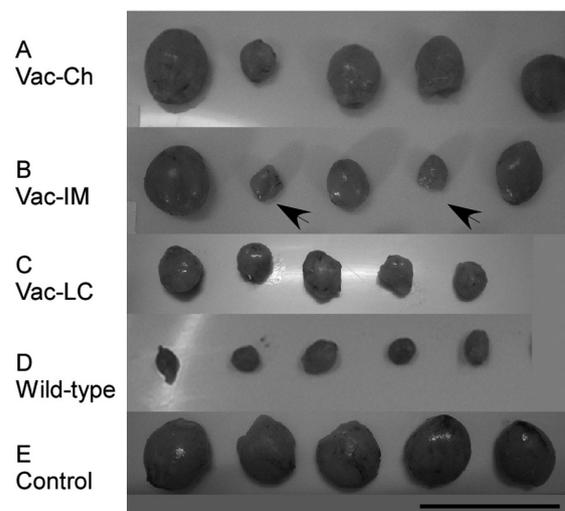


Fig. 1. Macroscopic appearances of the BF. Severely atrophied BFs were observed in the Vac-IM and Vac-LC groups (arrows). (A) The Vac-Ch group (Nos. 1–5), (B) Vac-IM group (Nos. 10–14), (C) Vac-LC group (Nos. 19–23), (D) wild-type group (Nos. 29–33) and (E) control group (Nos. 44–48). The bar in Fig. 1A–1E represents 5 cm.

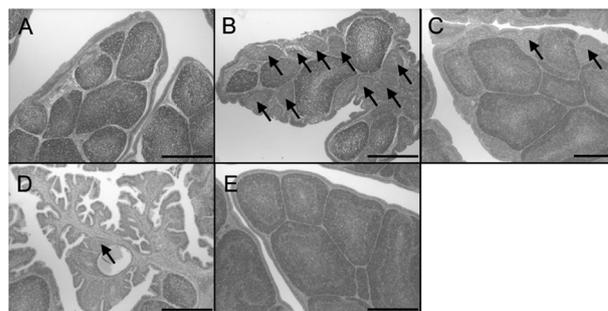


Fig. 2. Histological appearance of the BF. In the vaccine and wild-type groups (A–D), small follicles (arrow) were interspersed among the normal follicles. (A) The Vac-Ch group (No. 4), (B) Vac-IM group (No. 15), (C) Vac-LC group (No. 21), (D) wild-type group (No. 33) and (E) control group (No. 45). Hematoxylin and eosin stain. The bars in Fig. 2A–2E represent 400  $\mu$ m.

Table 1. Summed results of statistical analysis for each group

| Group     | F/B ratio (%)                  | HI titer                     | Number of follicles        |                            |                              | Lesion score                   |
|-----------|--------------------------------|------------------------------|----------------------------|----------------------------|------------------------------|--------------------------------|
|           |                                |                              | Normal                     | Small                      | Total                        |                                |
| Vac-Ch    | 0.35 $\pm$ 0.14 <sup>a</sup>   | 27.2 $\pm$ 2.2               | 510 $\pm$ 175 <sup>a</sup> | 51 $\pm$ 60                | 561 $\pm$ 146 <sup>a</sup>   | 1.00 $\pm$ 0.58 <sup>a</sup>   |
| Vac-IM    | 0.24 $\pm$ 0.19 <sup>a</sup>   | 40.0 $\pm$ 3.2               | 361 $\pm$ 204 <sup>a</sup> | 190 $\pm$ 215 <sup>a</sup> | 551 $\pm$ 58 <sup>a</sup>    | 2.00 $\pm$ 1.31 <sup>a,c</sup> |
| Vac-LC    | 0.22 $\pm$ 0.11 <sup>a</sup>   | 37.3 $\pm$ 2.3               | 343 $\pm$ 123 <sup>a</sup> | 170 $\pm$ 75 <sup>a</sup>  | 513 $\pm$ 92 <sup>a</sup>    | 1.80 $\pm$ 0.63 <sup>a</sup>   |
| Wild-type | 0.06 $\pm$ 0.02 <sup>a,b</sup> | 3.2 $\pm$ 4.3 <sup>a,b</sup> | 13 $\pm$ 19 <sup>a,b</sup> | 122 $\pm$ 89               | 136 $\pm$ 100 <sup>a,b</sup> | 4.00 $\pm$ 0.00 <sup>a,b</sup> |
| Control   | 0.52 $\pm$ 0.12                | 30.0 $\pm$ 2.2               | 706 $\pm$ 76               | 2 $\pm$ 2                  | 709 $\pm$ 75                 | 0.08 $\pm$ 0.29                |

Each value is expressed as the mean value  $\pm$  SD, a) Versus control group ( $P < 0.05$ ), b) Versus other 4 groups ( $P < 0.05$ ), c) Versus Vac-Ch group ( $P < 0.05$ ).

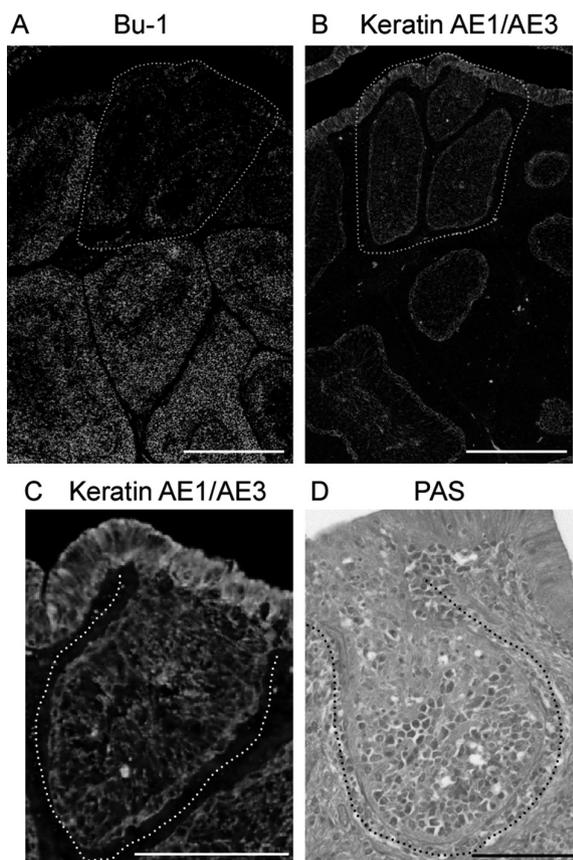


Fig. 3. Histological and immunofluorescent appearance of the small follicle observed in a chicken of the Vac-IM group (No. 13). Almost all lymphocytes were depleted in the small follicle area (A), but the BM-associated epithelium was retained (B). The small follicle area is shown as dotted lines in Fig. 3A and 3B. The small follicle lacked a cortical area, and the BM and BM-associated epithelium lined the outer boundary (C and D). The outer boundary of the small follicle is shown as dotted lines in Fig. 3C and 3D. The bars in Fig. 3A and 3B represent 200  $\mu\text{m}$ , and the bars in Fig. 3C and 3D represent 80  $\mu\text{m}$ . (A–C) Immunofluorescence and (D) periodic acid–Schiff reaction.

mucosae (Fig. 2D). In the control group, no significant lesions were observed, and normal follicles were retained (Fig. 2E). In the vaccine groups, lymphocytes and normal follicles were decreased to various extents, and the lesion scores were higher than that of the control group (Table 1). The chickens with very low F/B ratios in the vaccine groups showed more severe reductions in their numbers of normal follicles (101, 30 and 184 normal follicles for Nos. 11, 13 and 25, respectively) and worse follicular lesion scores (4, 4 and 3 for Nos. 11, 13 and 25, respectively) than the other chickens in the same groups. All chickens in the IBDV wild-type group had severe bursal damage and lesion scores of 4, regardless of their NDV-HI antibody titers.

The emergence of small follicles was observed in the vaccine groups (Fig. 2B and 2C) and wild-type group (Fig. 2D). As defined above, a small follicle exhibited depletion of lymphocytes in both the cortex and medulla (Fig. 3A and 3D)

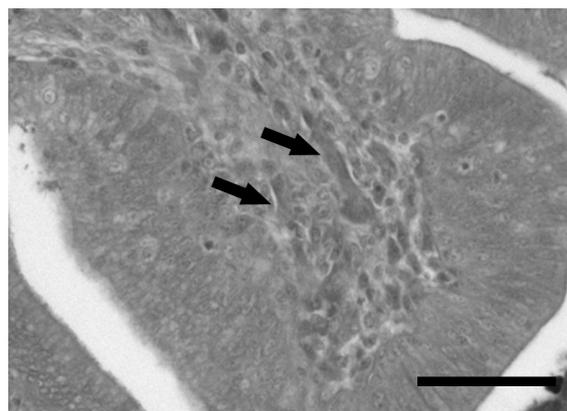


Fig. 4. Aggregated BM lacking an associated epithelium (arrows) in a chicken of the wild-type group (No. 29). The bar represents 50  $\mu\text{m}$ . Periodic acid–Schiff reaction.

Table 2. Correlation coefficients between HI titer and each parameter

|                            | Total chickens      | Vaccine and control groups | Wild-type group    |
|----------------------------|---------------------|----------------------------|--------------------|
| F/B ratio (%)              | 0.39 <sup>a)</sup>  | –                          | –                  |
| Number of normal follicles | 0.53 <sup>b)</sup>  | –                          | 0.71 <sup>a)</sup> |
| Number of small follicles  | –                   | –                          | –                  |
| Total number of follicles  | 0.49 <sup>b)</sup>  | –                          | –                  |
| Lesion score               | –0.44 <sup>b)</sup> | –                          | –                  |

–)  $P > 0.05$ , a)  $P < 0.05$ , b)  $P < 0.0005$ .

and lacked a discernible cortex and medulla (Fig. 3D), but retained an intact BM (Fig. 3D) and BM-associated epithelium (Fig. 3C). In addition, aggregated PAS-positive membranous structures were sometimes observed in the lamina propria mucosae, but only in the wild-type groups (Fig. 4).

**Quantitative analysis:** Chickens in the vaccine groups had a similar number of total follicles regardless of the F/B ratios, number of normal follicles or lesion scores (Table 1). The chickens with very low F/B ratios in the vaccine groups had average numbers of total follicles, and the numbers were higher compared with that in the wild-type group (total number of follicles, 591, 590 and 437 for Nos. 11, 13 and 25, respectively). The chickens in the wild-type group had a significantly smaller number of follicles compared with the control and vaccine groups.

**Correlation coefficients between NDV-HI titers and other parameters:** To identify the factors that correlated with immunoreactivity, the correlation coefficients between the NDV-HI titer and the following observational data were calculated: F/B ratio, number of normal follicles, total number of follicles and lesion scores (Table 2). In all the chickens, correlations were found for NDV-HI titer with the F/B ratio ( $r = 0.39$ ,  $P < 0.05$ ), the number of normal follicles ( $r = 0.53$ ,  $P < 0.0005$ ) and the total number of follicles ( $r = 0.49$ ,  $P < 0.0005$ ). No correlation was found between the NDV-HI titer and the number of normal follicles in chickens in the vaccine strain and control groups ( $r = 0.04$ ,  $P > 0.05$ ; Table 2). On the other hand, within the wild-type group, a high cor-

Table 3. Summed results of statistical analysis for the wild-type group

| Subgroup                       | n | HI titer                 | F/B ratio (%) | Number of follicles   |           |                         |
|--------------------------------|---|--------------------------|---------------|-----------------------|-----------|-------------------------|
|                                |   |                          |               | Normal                | Small     | Total                   |
| Partly immunoreactive subgroup | 6 | 12.6 ± 2.6 <sup>a)</sup> | 0.06 ± 0.02   | 25 ± 23 <sup>a)</sup> | 170 ± 113 | 194 ± 123 <sup>a)</sup> |
| Immunosuppressed subgroup      | 7 | 1 ± 1*                   | 0.05 ± 0.02   | 3 ± 5                 | 83 ± 32   | 86 ± 33                 |

Each value is expressed as the mean value ± SD, a) Significantly large compared with the immunosuppressed subgroup ( $P < 0.05$ ), \*Values below the detection limit were considered to be 1.

relation was found with the number of normal follicles ( $r = 0.71$ ,  $P < 0.05$ ; Table 2).

*Immunoreactivity and bursal follicles in the wild-type group:* We divided the wild-type group into 2 subgroups: the partly immunoreactive subgroup ( $n = 6$ ) and the immunosuppressed subgroup ( $n = 7$ ) (Table 3). Although both subgroups had severe lymphocyte reductions and severe follicular lesion scores, the partly immunoreactive subgroup had a larger number of normal follicles and total number of follicles compared with the immunosuppressed subgroup ( $t$ -test,  $P < 0.05$ ).

## DISCUSSION

In gross examination, we observed severe atrophy of the BF in the chickens of the vaccine groups, and the level of severity was the same as in the wild-type group. However, these chickens retained immunoreactivity to exogenous antigens and were found to possess a higher number of total follicles than the wild-type group histologically and statistically. These results indicated that macroscopic evaluation does not accurately reflect the immunoreactivity and the degree of bursal damage in IBDV-administered chickens.

In the histological examination, we observed the emergence of small follicles, which lost almost all lymphocytes and lacked the cortical area, but retained their BM-associated structures. Similar small follicles had been reported in previous reports of IBDV-infected chickens and had been thought to not be fully functional [31, 32]. In the present study, almost equal numbers of small follicles were observed in the wild-type group, regardless of the immunoreactivity of the chickens. This result also demonstrated that the small follicles were not functional and not associated with immunoreactivity of IBDV-infected chickens. On the other hand, the emergence of the small follicles was a pathological change due to IBDV administration, because these findings were not detected in the control chickens but significant increases were detected in the vaccine-administered chickens. B lymphocytes in the BF are the main target of IBDV, but the bursal stromal components also exhibit susceptibility to IBDV [11]. Taking into account the observation that the small follicles retained BM-associated structures, the small follicles might represent mild lesions in IBDV infection that were enough to damage lymphocytes in bursal follicles but not too severe to damage BM-associated structures. Small follicle-like structures have also been reported in cyclophosphamide-treated chickens [3, 28]. Since cyclophosphamide is a selective toxicant to avian and mammalian B lymphocytes [30], the resemblance of the small follicles also implied

the small follicles were mild pathological lesions of IBDV that were enough to damage lymphocytes without damaging stromal cells. All the chickens in the vaccine groups, including the chickens with macroscopically severe BF atrophy, retained larger total numbers of follicles, which were calculated by summation of the numbers of normal and small follicles, than that in the wild-type group. In other words, they retained larger numbers of BM-associated structures. Although it is necessary to investigate using larger numbers of wild-type IBDV and long-term experiments for a proper evaluation, preservations of BM-associated structures may be related with the virulence of IBDV and an important key to analysis of the effect of IBDV on chickens.

We also found non-immunosuppressed chickens in the wild-type group, despite the fact that all the chickens in the wild-type group showed severe BF atrophy in gross examination, a decreased F/B ratio and the worst follicular lesion score. These non-immunosuppressed chickens retained a significantly higher number of normal follicles and total number of follicles. Furthermore, a high correlation coefficient between the NDV-HI titer and the number of normal follicles was found in the wild-type group. These results suggested that the retained number of normal follicles is important for immunoreactivity in chickens infected with IBDV, in addition to a previously reported factor, namely the extent of depletion of lymphocytes in the BF [10, 19, 26].

In addition to the small follicles, we observed small nests of PAS-positive membranous structures only in the wild-type groups. It is reported that highly virulent IBDV infects stromal cells more frequently than a moderately virulent IBDV wild-type strain [27]. It is suspected that the PAS-positive membranous structures might be due to the destruction of BM-associated structures resulting from infection of stromal cells of bursal follicles after administration of IBDV, but the detailed mechanisms remain unclear. It is necessary to analyze histopathological effects in chickens infected with a larger number of immunosuppressive strains of IBDV to determine the relationship between state of immunoreactivity and histopathological findings.

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