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ABSTRACT

Infectious bursal disease virus (IBDV) is a pathogen that primarily infects B lymphocytes in domestic avian species. This viral infection has been associated with immunosuppression, clinical disease/mortality, and enteric malabsorption effects. The purpose of this experiment was to compare the effects of a classic (USDA-STC) and a new variant IBDV (RB-4, known to induce primarily the enteric disease) on immune cell populations in lymphoid organs. Seventeen-day-old specific-pathogen-free (SPF) White Leghorn chickens were either not infected (control) or inoculated with either USDA-STC or RB-4 IBD viral isolate. On days 3 and 5 post-inoculation (PI), lymphoid tissues were collected to prepare cell suspensions for immunofluorescent staining and cell population analysis by flow cytometry. Portions of the tissues were snap frozen for immunohistochemistry to localize various immune cells and IBD virus in the tissues. Tissue homogenates were prepared to test for IBDV by quantitative MTT assay. Both the USDA-STC and RB-4 viruses greatly altered lymphocyte populations in the spleen and bursa. At 5 d PI, bursal B cells were approximately 25% and 60% of lymphocytes in chicks infected with USDA-STC and RB-4, respectively, whereas in control birds, B cells constituted 99% of bursal lymphocytes. This reduction in the proportions of bursal B cells was associated with an infiltration of T cells. In the spleen, IBDV infection also reduced the percentage of B cells and increased the percentage of T cells. The differential effects of classic and variant IBDV infection on immune cell populations in lymphoid organs may explain the differences in clinical effects induced by these viruses.

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MEET THE STUDENT-AUTHORS

Christina L. Johnson, Primary Author

I am from Conway, Ark. I graduate from Conway High School in 2001 and I just completed my second year as a poultry science major. I heard about the Laboratory Rotations in Agricultural Research course during my first semester as a college student. I really liked the idea of learning about real research and actually conducting a research project, especially because I learn best by taking an active part in the learning process and I always liked the idea of working in a research laboratory. By taking the leadership role in preparing the manuscript for our team project, I learned about every aspect of research, including the background research, design of the project, actual hands-on experiments, team work, data analysis, and the many rewrites it takes to get a paper ready for submission. I learned a lot from this project and I am now conducting research in reproductive physiology with Dr. Keith Bramwell as part of my undergraduate program in poultry science. For the future I plan to go to graduate school.

Ashley K. Cox

I graduated in 2001 from Lincoln High School in Lincoln, Ark. A sophomore at the University in Arkansas majoring in poultry science with a minor in global agriculture, I plan to graduate with my B.S.A. degree in May 2005. During my time at the University of Arkansas I have been a part of many clubs including the Poultry Science Club and Sigma Alpha Professional Agriculture Sorority where I have served as the secretary for three semesters, and Arkansas Baptist Collegiate Ministry. During the spring or summer of 2004, I plan to study abroad in Scotland and further my knowledge of international agriculture. I have always been interested in science, and I believe that understanding and keeping abreast of current research is the key to being well informed in any area. With my experience in the research of the effects of the variant versus the classic IBDV, I gained some valuable skills, vocabulary, and insight that I will be able to refer back to as I further my education.

MEET THE STUDENT-AUTHORS, CONTINUED...

April D. Keeter

I am from Harrison, Ark. I graduated from Alpena High School in 2001 where I was valedictorian of my senior class. Throughout high school, I was an active member of Future Farmers of America and the BETA Honors Club. Based on my interest in animals and science, I enrolled in the Dale Bumpers College of Agricultural, Food and Life Sciences at the University of Arkansas. I recently completed my sophomore year in poultry science and pre-veterinary medicine. I enrolled in the Laboratory Rotations in Agricultural Research course during my freshman year because I wanted to know more about research and laboratory work. I liked this project because we could use the techniques we learned in the first part of the course and then apply them to a real disease problem in poultry. I know having this hands-on research experience will help me a lot with my science courses at the University of Arkansas and my studies in veterinary school.

I began my collegiate career at the University of Arkansas as a biology major without much of a focus on a specific niche to carve out for the future. This lack of specific focus continued until the final semester of my senior year. I always wanted to get involved in laboratory research but was not aware of the many opportunities available at the University of Arkansas. It was in my senior year that I, rather fortuitously, stumbled upon my first course in laboratory research and my first exposure to immunology research. I wished I had looked into opportunities to get involved in research earlier in my collegiate career. I learned a lot by conducting this multidisciplinary team project, including how much I love biomedical research, especially in immunology. I am now at the University of Pennsylvania pursuing a doctorate and further career in immunology.

William J. Quinn

INTRODUCTION

In chickens, as in mammalian species, the major players in protective immunity are T- and B-lymphocytes. In chickens, B cells develop in the bursa of Fabricius, which consists of numerous organized B-cell compartments (follicles) where pre-B cells differentiate into mature B cells. Mature B cells then leave the bursa to go to peripheral lymphoid organs such as the spleen where they can encounter foreign antigens and respond by producing antigen-specific antibodies. Antibodies to a particular antigen play a major role in the elimination of the antigen. T cells develop in the thymus gland where they differentiate into two major types of T-cell populations; helper T cells and cytotoxic T cells. Helper T cells, which can be identified based on their expression of CD4 molecules, play a major role in regulating the immune response providing activation factors to other T cells, B cells, and macrophages. Cytotoxic T cells, which can be identified by their expression of CD8 molecules, play an important role in killing virus-infected cells and tumor cells (cytotoxic T cells). Within these T cell populations there are further subsets that can be identified based on the type of antigen-receptor (T cell-receptor, TCR); the type of CD8 molecule ($\alpha\alpha$ or $\alpha\beta$); and the combination of CD4 and CD8 molecules.

Viruses that specifically infect T- or B- lymphocytes can have severe detrimental effects on the immune system and on the ability of an individual to defend against pathogenic microorganisms. The target cell for infectious bursal disease virus is the avian B cell. Infectious bursal disease virus (IBDV) is a double stranded RNA virus belonging to the family *Avibirnaviridae*. The virus particle is 60 nm in size and non-enveloped. There are three distinct age-associated disease syndromes that are linked to IBDV infection: immunosuppression, clinical/mortality, and malabsorption or enteric effects (Kim and Sharma, 2000). There are two recognized serotypes within this virus group as well as numerous variants of serotype I (Calnek et al., 1997). Within the classic

serotype I IBD virus group, clinical disease is associated with cytolysis, inflammation, edema, and heterophil infiltration, and viral infection is linked directly with immunosuppression (Allan et al., 1972). The variant serotype I IBD viruses induce similar but milder necrotic lesions, in the absence of acute inflammation. Newberry et al. (1997) isolated a variant IBDV (RB-4) from the proventriculus of a commercial broiler chicken; this variant induces mild tissue pathology, clinical signs, and associated enteric effects (Newberry et al., 1997). These differences in infection-associated symptoms may be due to the relative severity of B-cell loss and the type/effectiveness of the resulting IBDV-specific immune response.

At present, little is known with respect to how variant RB-4 IBDV differs from the classic USDA-STC IBDV during acute infection (e.g., one to 5 d PI). The objective of this study was to measure and compare the effects of the variant RB-4 (enteric origin) and classic USDA-STC IBD viruses on lymphocyte populations in spleen and bursa of specific-pathogen-free (SPF) White Leghorn chickens. Additionally, viral infection was confirmed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and by localization of virus within affected tissues.

MATERIALS AND METHODS

Experimental Animals

In this experiment, 60 SPF White Leghorn chickens (Sunrise Farms, Catskill, N.Y.) were hatched at the University of Arkansas Poultry Health Laboratory. The chickens were randomly placed in five Horsfal isolation units with 15 chicks per isolation unit. Throughout the study, feed (prepared to meet or exceed National Research Council standards) and water were provided ad libitum. The environmental temperature was maintained at 35°C for the first week, followed by decreases of 2.8°C per week for the rest of the experiment. Group I was designated as the negative control, Groups II and IV were challenged with USDA-STC IBDV, and Groups III and V were challenged with variant RB-4 IBDV. The viral challenge inocula for both virus strains were standardized to a concentration of ~10^3 EID_{50}/mL and administered bilaterally to the eye (30 µL per eye). Chicks in Groups III and V, and chicks in Groups II and IV were inoculated with virus on days 17 and 19 post-hatch, respectively. On day 22 post-hatch (day 3 and 5 post-challenge), six chicks from each group (Group I-V) were randomly selected, weighed, humanely killed, and subjected to necropsy. Organ weights were recorded and tissue samples were collected for further analysis.

Immunofluorescent Staining of Spleen and Bursa Lymphocyte Suspensions and Cell Population Analysis by Flow Cytometry

For each chicken, individual single cell suspensions were prepared from spleen and bursa by gently pushing tissues through a 60 µm nylon mesh in ice-cold Dulbecco's phosphate buffered saline (PBS) with 10% calf serum. The bursal lymphocyte suspensions were placed in 15 mL Falcon tubes and centrifuged at 250 x g for 10 min. The supernatant fluid was removed, and the cell pellets were resuspended in PBS and washed again by centrifugation. After the final wash, pellets were resuspended in PBS+ (PBS with 1% bovine serum albumin and 0.1 % sodium azide) and the concentration was adjusted to 2 x 10^7 lymphocytes/mL. For spleen cell suspensions, lymphocytes were separated from red blood cells by slow-speed centrifugation following the procedure for whole blood described in Erf and Smyth (1996). Following isolation, splenic lymphocytes were then processed in the same manner as described for the bursal lymphocyte suspensions. To identify various types of lymphocyte populations, cells (1 x 10^6) were then stained with a panel of fluorescence- or biotin-labeled mouse monoclonal antibodies specific for chicken lymphocyte markers (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Included were fluorescein isothiocyanate (FITC)-conjugated Bu-1 specific antibodies (Bu-1-FITC), CD4-FITC, CD8α-phycoerythrin, CD8β-biotin, TCR1-biotin, TCR2-biotin, and TCR3-biotin to identify B cells, CD4+ T cells (T helper cells), CD8+ T cells (cytotoxic T cells), T cells with γδ T cell receptors (TCRs), T cells with αβ1 TCRs, and T cells with αβ2 TCRs, respectively. The binding of biotinylated antibodies was detected using Quantum red-labeled streptavidin (Sigma Chemicals, St. Louis, Mo.). Additionally, to determine the proportions of lymphocytes in each cell suspension, lymphocytes were identified using unlabelled K55 mouse monoclonal antibody (a gift from Dr. H. S. Lillehoj, USDA-ARS, Beltsville, Md.) and goat-anti-mouse IgG-FITC antibody (Sigma Chemical Company, St. Louis, Mo.) in an indirect staining method. Immunofluorescent cell population analyses were then carried out using a FACSCalibur flow cytometer and the Cell Quest cell population analysis program (Becton Dickinson Flow Cytometry Systems, Mountain View, Calif.) as described by Erf et al. (1998). Data were expressed as the percentage of lymphocytes (K55+ cells) in each sample. The effect of treatment on the percentage of each cell population in a tissue was determined by analysis of variance (ANOVA). Differences between means were detected using Fisher's LSD test using the Systat 8.0® Statistical Analysis Software (SPSS Inc., Chicago, Ill.).

Viral Titration Assay

A viral titration assay was utilized to determine levels of viral infectivity in the tissues harvested at 3 and 5 d PI. A crude tissue homogenate was prepared from the spleen and bursa (1:5 w/v) in physiological saline (0.85%) containing antibiotics. Serial two-fold dilutions of each homogenate were then prepared in Eagle's Minimum Essential medium in sterile 96-well tissue-culture microtiter plates. Following sample dilution, each well of the microtiter plate was seeded with 100 µL of a BGM-70 cell suspension (1 x 10^6 cells/mL) (Jackwood et al., 1987). The plates were then incubated in a humidified incubator at 37°C in 5% CO_2 for 4 d. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Company, St. Louis, Mo.) assay was performed based on the method of Mosmann (1983). Briefly, MTT was prepared at 5 µg/mL in 0.85% physiological saline, sterile-filtered, and dispensed 20 µL per well. Microtiter plates were then returned to the 37°C incubator for 2 h. Following incubation, BGM 70 cells were lysed using an acid/ethanol mixture and the MTT dye conversion levels (levels are directly related to relative amounts of living, metabolically active cells) were determined at an absorbency of 570 nm using a spectrophotometer (96-well Universal Microplate Reader, Bio-Tek Instruments, Winooski, Vt.).

Immunohistochemical Staining

To identify and localize virus and various lymphocyte populations in spleen and bursa, frozen tissue sections were prepared and stained as described by Erf et al. (1995). Briefly, the tissue samples of spleen and bursa were placed in labeled aluminum-foil cups with freezing medium (O.C.T. Compound, Tissue-Tek, Sacura Finetek, USA Inc., Torrance, Calif.) and snap frozen in liquid nitrogen. Tissues were stored at -80°C until use. For immunohistochemical staining, the frozen tissue samples were cut into 6 µm sections using a Microm Microtome Cryostat (MICROM Laborgeräte GmbH, Walldorf, Germany) and placed on poly-L lysine-coated microscope slides. To block non-specific binding sites on the tissues, the tissue sections were incubated over night in PBS 10% horse serum at room temperature in a humidified chamber. To identify and localize various lymphocyte populations in the tissues, sections were immunochemically stained using the same panel of lymphocyte-specific antibodies as described for immunofluorescent staining, except lymphocyte specific antibodies were not directly conjugated to a label. The presence and location of IBDV virus were detected using an IBDV-specific mouse monoclonal antibody (University of Maryland, College Park, Md.; Intervet, Inc., Millsboro, Dela.). The binding of unlabeled detection antibodies, all of which were mouse IgG antibodies, was revealed using horse-anti-mouse IgG antibodies conjugated with biotin. The binding of biotinylated antibodies was detected using peroxidase-labeled biotin-avidin reagent (ABC reagent, VECTASTAIN Elite ABC" staining kit, Vector Laboratories, Inc. Burlingame, Calif.). The binding of ABC reagent to the complex formed by detection antibody and biotinylated antibody was detected by adding DAB substrate, which was converted by the biotin-conjugated peroxidase enzyme into a brownish-red product that precipitated at the site formation. Tissue sections were then counterstained with Methyl Green stain, covered with glass cover-slips, and examined by bright field microscopy.

RESULTS AND DISCUSSION

This study was conducted to address how the immune system was affected by classic compared to variant IBDV during acute infection by focusing on the effects of IBDV infection on immune-cell population profiles in spleen and bursa.

Based on results obtained from quantitative MTT assay and immunohistochemistry (IHC), IBDV was present in experimental birds challenged with USDA-STC or RB-4 IBD viruses but not in uninfected controls (MTT data not shown, IHC see Fig. 1 d and h). Comparison of viral activity in tissue samples obtained at 3- versus 5-d PI, revealed higher and more consistent levels of viral activity at 5 d PI than at 3 d PI. Additionally, at 5 d PI, the level of viral infection in spleen and bursa was similar in chicks infected with classic and variant IBDV. Hence, results reported and discussed here will focus on results obtained at 5 d PI.

The body weights of all IBDV-challenged SPF chickens were lower than those of their age-matched control chickens (Table 1), which may be attributed to the infection- and/or immune response-associated loss in appetite, heightened metabolic activity, and potential enteric effects. Both absolute and body weight-adjusted spleen weights were higher in IBDV-infected chicks compared to non-infected controls. Spleen weights were not different in chicks infected with classic or variant IBDV (Table 1). This increase in spleen weight is likely due to the anti-IBDV immune responses initiated in the spleen. Antigen-specific immune responses in secondary lymphoid organs such as the spleen are generally associated with recruitment of lymphocytes from the circulation into the spleen and proliferation of antigen-specific lymphocytes. As shown by cell-population analysis, the proportions of T cells, particularly the proportions of the more sophisticated αβ T cells (compared to γδ T cells), increased in both groups of IBDV-infected chicks (Table 2). These T lymphocytes consisted of both helper T cells (CD4+CD8) and cytotoxic T cells (CD8αβ+). It

Table 1. Body weights (BW), lymphoid organ weights, and proventriculus weights in specific-pathogen-free White Leghorn chickens infected with classic (USDA-STC) or variant (RB4) infectious bursal disease virus (IBDV) at 17 d of age and examined 5 d post-infection.[z]

Variable	Control	USDA-STC IBDV	RB4 IBDV
Body weight (g)	130 ± 4.17 [a, y]	113 ± 4.77[b]	112 ± 4.52[b]
Bursa weight (g)	0.750 ± 0.053[a]	0.403 ± 0.026[b]	0.404 ± 0.035[b]
Bursa (%BW)	0.571 ± 0.028[a]	0.371 ± 0.036[a]	0.364 ± 0.032[b]
Spleen weight (g)	0.215 ± 0.018[b]	0.323 ± 0.023[a]	0.303 ±0.032[a]
Spleen (%BW)	0.165 ± 0.013[b]	0.289 ± 0.019[a]	0.267 ± 0.025[a]

[z] IBDV was administered by eye-drop, 30 µL of 10^3 EID_{50}/mL.

[y] Mean ± SEM based on six birds per treatment; for each weight, treatment means that do not share a common letter are different ($P \leq 0.05$).

is interesting to note that the viral infection-associated changes in the proportions among splenic lymphocytes differed between infection with USDA-STC and RB-4. The percentage of ab T cells in spleens from chicks infected with RB-4 was higher than in spleens from chicks infected with USDA-STC. Additionally, while infection with classic IBDV did not affect the proportions of CD4+CD8^{low} and CD8αα subpopulations, infection with variant IBDV resulted in increased proportions of these CD4- and/or CD8-defined lymphocyte subsets. Little is known about lymphocytes with either the CD4+CD8^{low} and CD8αα phenotypes in peripheral tissues such as the spleen. However, the recruitment of these unique cell types to the site of IBDV infection (IBDV localization data not shown) may in part be responsible for the less severe symptoms observed following infection with variant compared to classic IBDV. Similarly, the presence of these cell types may be an indication of a more effective immune response to IBDV infection. The notion that the presence of these lymphocyte subsets may be an indication of a more effective immune response is based on observations by Ward (2000), who reported the presence of these cell types in Rous sarcoma virus-induced tumors that were actively regressing.

Although the organization of the lymphoid areas in the spleen was affected by IBDV infection, including substantial reduction in the size of B-cell follicles and infiltration of T cells into B-cell areas (data not shown), the bursa was more severely affected by IBDV infection. IBDV infection with either the classic or variant strain decreased both the absolute and body weight-adjusted weight of bursae compared to non-infected controls. This effect on bursa weights was similar in both groups of IBDV-infected chicks and was associated with greatly reduced proportions of B cells (Table 3), less densely populated B-cell areas (follicles), and infiltration of T cells into follicles (Fig. 1 f and g). In uninfected controls, the bursa of Fabricius, which is the site of B-cell development, consists primarily of lymphocytes and more than 99% of these lymphocytes are B cells (Table 1). The few T cells isolated from bursae of uninfected chicks were primarily located in the spaces between the B-cell follicles and likely represent T cells present in bursal blood vessels (Fig. 1 a-c). Infection with either the classic or variant IBDV strain resulted in similar qualitative changes in bursal lymphocyte populations. However, the magnitude of these alterations differed between virus strains. Compared to infection with variant IBDV, infection with classic IBDV resulted in a greater loss of B cells.

Table 2. Proportions among lymphocyte populations in the spleen of specific-pathogen-free White Leghorn chickens infected with classic (USDA-STC) or variant (RB4) infectious bursal disease virus (IBDV) at 17 d of age and examined 5 d post-infection.[z]

Cell population (% total lymphocytes)	Control	USDA-STC IBDV	RB4 IBDV
B cells	40.2 ± 2.27[a, y]	10.6 ± 3.40[b]	9.82 ± 4.71[b]
γδ T cells	12.2 ± 0.93[b]	17.7 ± 1.46[a]	15.7 ± 1.51[ab]
αβ T cells	45.2 ± 1.62[c]	62.1 ± 2.02[b]	71.0 ± 6.06[a]
$CD4^+CD8^-$ cells	15.0 ± 1.09[b]	24.7 ± 3.00[a]	27.3 ± 1.85[a]
$CD4^+CD8^{low}$ cells	7.27 ± 1.45[b]	3.75 ± 1.84[b]	14.6 ± 5.27[a]
$CD8\alpha\beta^+$ cells	24.3 ± 2.48[b]	33.6 ± 4.00[a]	32.8 ± 2.41[a]
$CD8\alpha\alpha^+$ cells	13.7 ± 2.12[b]	10.4 ± 2.08[b]	21.2 ± 4.52[a]

[z] IBDV was administered by eye-drop, 30 µL of 10^3 EID_{50}/mL.

[y] Mean ± SEM based on six birds per treatment; for each cell population, treatment means that do not share a common letter are different ($P \leq 0.05$).

The loss of B cells due to IBDV infection was associated with extensive infiltration of T cells, particularly cytotoxic T cells. As viral presence was similar in bursae from both IBDV-infected groups, it is possible that heightened anti-viral cytotoxic activity mediated by infiltrating cytotoxic T cells may in part be responsible for the enhanced loss of B cells observed during infection with classic IBDV compared to variant IBDV. An important role of T cells in IBDV infection was recently demonstrated by Kim et al. (2000) and Rautenschlein et al. (2002). The exact contribution of T cells to the resolution and pathogenesis of IBDV infection, however, is not fully understood. Rautenschlein et al. (2002) support the role of the avian T cell as an IBD viral modulator by first limiting early-phase viral replication and secondarily increasing the level of tissue damage through the release of cytokines. Additionally, the study by Kim et al. (2000) supports a role of cytotoxic T cells in viral clearance.

In conclusion, both the classic USDA-STC and variant RB-4 IBDV strains had profound effects on the proportions among lymphocyte populations in the spleen and bursa at 5 d PI. The differences in the extent of alterations in immune-cell profiles of spleen and bursa observed with the classic versus the variant strain may be responsible for the differences in clinical symptoms induced by these viruses (i.e., immunosuppression and enteric effects versus primarily enteric effects, respectively). Further studies will be needed to characterize the role of the various T lymphocyte populations altered during acute IBDV infection.

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Table 3. Proportions among lymphocyte populations in the bursa of specific-pathogen-free White Leghorn chickens infected with classic (USDA-STC) or variant (RB4) infectious bursal disease virus (IBDV) at 17 d of age and examined 5 d post-infection.[z]

Cell population (% of total lymphocytes)	Control	USDA-STC IBDV	RB4 IBDV
B cells	99.9 ± 0.38[a, y]	23.9 ± 7.17[c]	58.1 ± 22.5[b]
γδ T cells	0.45 ± 0.05[b]	9.75 ± 1.06[a]	8.91 ± 2.52[a]
αβ T cells	0.56 ± 0.12[b]	54.1 ± 8.11[a]	40.9 ± 14.8[a]
$CD4^+CD8^-$ cells	0.33 ± 0.06[b]	18.5 ± 3.59[a]	16.5 ± 5.21[a]
$CD4^+CD8^{low}$ cells	0.11 ± 1.45[b]	2.74 ± 0.60[a]	4.23 ± 1.61[a]
$CD8\alpha\beta^+$ cells	0.21 ± 0.05[c]	25.3 ± 4.43[a]	13.1 ± 4.33[b]
$CD8\alpha\alpha^+$ cells	0.43 ± 0.07[b]	13.6 ± 1.12[a]	10.2 ± 3.69[a]

[z] IBDV was administered by eye-drop, 30 µL of 10^3 EID_{50}/mL.

[y] Mean ± SEM based on six birds per treatment; for each cell population, treatment means that do not share a common letter are different ($P \leq 0.05$).

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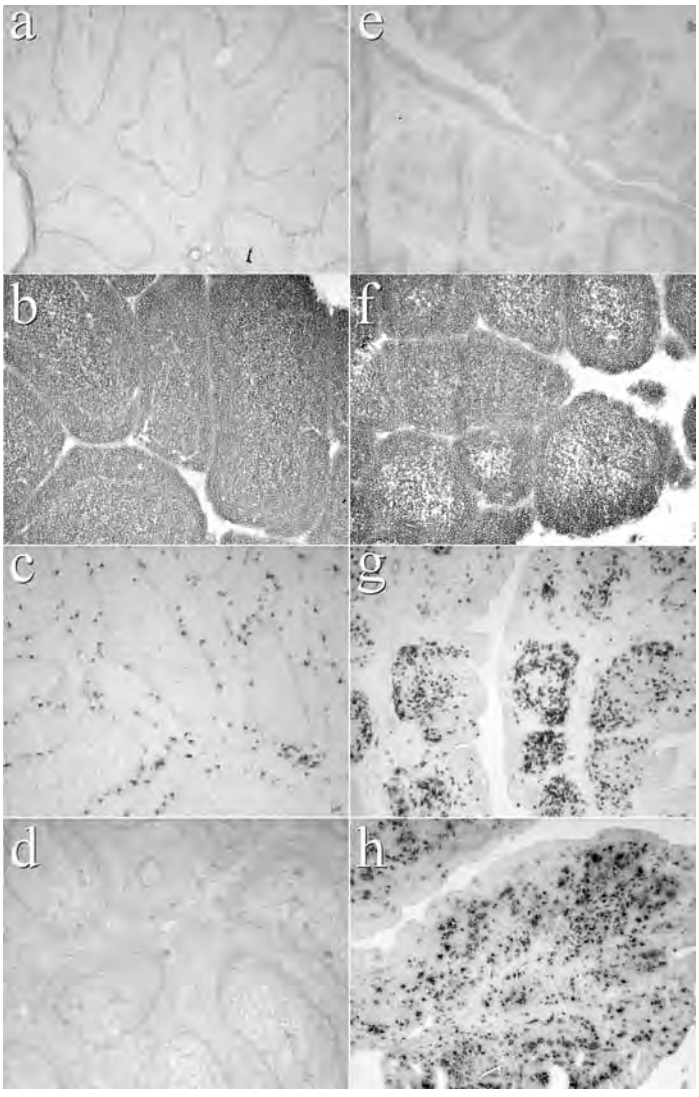

Fig. 1. Localization of B cells, T cells, and infectious bursal disease (IBD) virus in bursas from 22-day-old specific-pathogen-free White Leghorn chickens that were not infected or infected with variant RB-4 IBD virus 5 d prior to tissue collection. Immunochemically stained cells appear as dark cells on this black-and-white photograph. Pictures a-d and e-h represent bursal sections from uninfected and RB-4-infected chicks, respectively. Pictures (a) & (d) non-specific staining control (isotype control); (b) & (f) B cells; (c) & (g) T cells; (d) & (h) IBDV. The tissues were viewed by light bright-field microscope at 100x magnification.