Research Note

Identification of the Avian B-Cell-Specific Bu-1 Alloantigen by a Novel Monoclonal Antibody

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ABSTRACT A panel of monoclonal antibodies was generated against the guinea fowl's bursal cells. One of the antibodies, designated BoA1, recognized both cortical and medullary B cells of bursal follicles and B cell dependent regions of peripheral lymphoid organs, like germinal centers and splenic periellipsoidal regions. The staining pattern of this monoclonal antibody is similar to other antibodies (L22, 11G2, AV20), which also identify the Bu-1 antigens. Under reducing conditions, the molecular weight of the BoA1 antigen is 70 to 73 kDa, and after immunoprecipitation it proved to be identical with the antigen recognized by the AV20 antibody. It is unique for this novel monoclonal antibody that it shows wide range cross-reactivity with different avian species, like chicken, quail, guinea fowl, and turkey. Therefore, this Bu-1-specific monoclonal antibody could be a versatile tool for studying the B cell development in different domesticated birds.

Key words: B lymphocyte, obese chicken, quail, turkey, guinea fowl

2008 Poultry Science 87:351–355
doi:10.3382/ps.2007-00365

INTRODUCTION

The Bu-1 (**chB6**) marker was originally recognized by Gilmour et al. (1977) using alloantisera a and b between inbred lines of chickens. The marker is expressed on early and mature B cells, except plasma cells, which are the terminal maturation forms of B cells. In splenic cell suspensions, 2 chB6-positive cell populations can be distinguished. The majority of the Bu-1-positive cells are small, dense, and strongly positive (Weber, 2000), and prone to apoptosis. This is confirmed by Funk and Palmer (2003), who observed rapid cell death after exposure to chB6 antisera. Immunoglobulin V-gene rearrangement in embryonic spleen cells occurs only in this cell population (Houssaint et al., 1991; Pickel et al., 1993). The minority of Bu-1-positive cells is larger, less dense, and weakly positive. It is suggested that the 2 chB6-positive cell populations represent B cells and monocyte/macrophage cell lines, respectively (Houssaint, 1987; Houssaint et al., 1986, 1989; Veromaa et al., 1988; Weber, 2000). Function of the chB6 cell surface molecule is unknown, but it has been reported to control cell survival and adhesion during B cell development (Funk et al., 1997). The polymorphism of chB6 molecule was discovered by antisera and subsequently by monoclonal antibodies produced against this Bu-1a and Bu-1b surface molecule (Pink and Rijnbeek, 1983; Veromaa et al., 1988). The chB6 molecule is a homodimer of a 70-kD transmembrane protein with conserved intracellular and a highly glycosylated extracellular domains (Tregaskes et al., 1996). The chB6 antibody is a useful marker for identifying B cells in sections and suspensions with immunocytochemistry and fluorescence activating cell sorter, respectively.

In this research report we add a novel Bu-1-specific monoclonal antibody to the recent repertoire of the Bu-1 antibody family: 11G2 (Veromaa et al., 1988), L22 (Pink and Rijnbeek, 1983), AV20 (Rothwell et al., 1996), and HIS-C1 (Jeurissen and Janse, 1998). This novel monoclonal antibody, designated BoA1, recognizes not only chicken Bu1a and Bu1b allotypes, but unlike the other members of Bu-1 antibodies, cross-reacts with other avian species like turkey, quail, and guinea fowl.

MATERIALS AND METHODS

Birds

Fertilized guinea fowl (*Numida meleagris*), New Hampshire chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*), and quail (*Coturnix coturnix japonica*) eggs and birds were purchased from commercial breeders. Fertilized obese chicken eggs (strain B-13) were obtained from Cornell University, Ithaca, NY. The eggs were incubated at 38°C

©2008 Poultry Science Association Inc.
Received August 30, 2007.
Accepted November 7, 2007.
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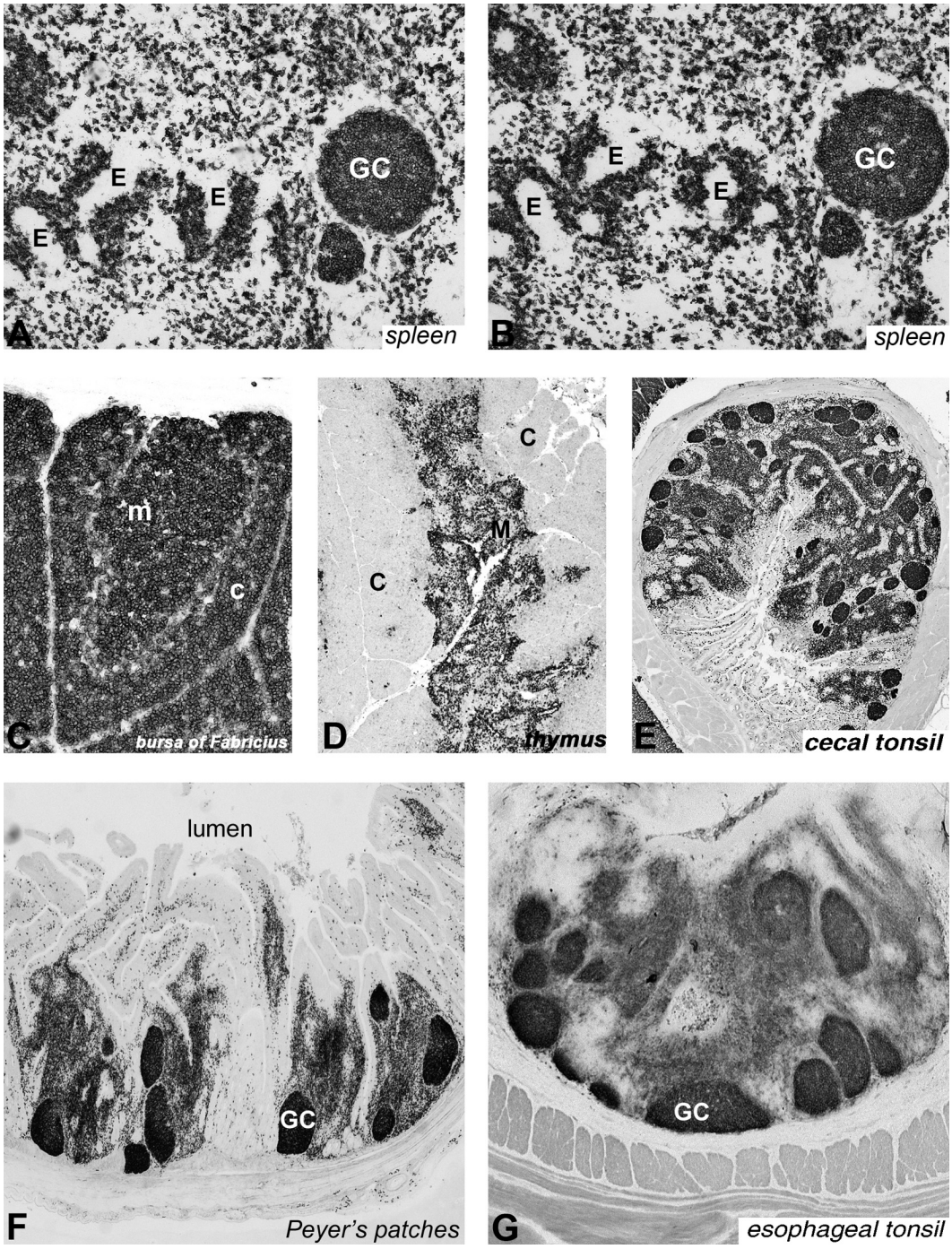

Figure 1. A. Bu-1b (11G2) monoclonal antibody (mAb) stained chicken spleen. The white pulp regions; germinal center (GC) and the periellipsoidal white pulp around the ellipsoid (E) are positive. Over the red pulp many Bu-1b-positive cells are scattered. Magnification: 50×. B. Parallel section with the Figure 1a stained with BoA1 mAb. The staining pattern is identical with the Figure 1a. Magnification: 50×. C. Bursal follicle stained with BoA1. Both cortical and medullary lymphocytes are positive. The BoA1 negative "line" separates the cortex (C) from the medulla (M). Magnification: 70×. D. Medullary region of the thymus is loaded with BoA1-positive cells. Magnification: 15×. E. Cecal tonsil. Strongly positive cells locate in the germinal centers, but many cells are scattered over the interfollicular region. Magnification: 15×. F and G. Peyer's patches and esophageal tonsil: both gut-associated lymphoid organs show similar staining pattern to cecal tonsil. Magnification: 30×.

in a humidified incubator. For monoclonal antibody (**mAb**) production BALB/c mice were purchased from the National Institute of Oncology, Budapest, Hungary. The design and conditions of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

Antibodies

A panel of mAb was generated against guinea fowl lymphoid cells following the protocol used by Magyar et al. (1994). Briefly, the Balb/c mice were immunized intraperitoneally with a bursal cell suspension of guinea

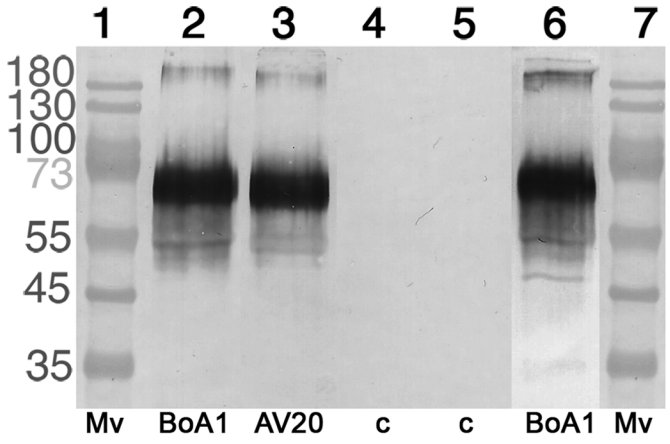

Figure 2. Immunoprecipitation of the chB6 antigen with AV-20 and BoA1 monoclonal antibody (mAb) and analyzed on SDS-PAGE gel under reducing condition. Line 1 and 7, molecular weight marker. Line 2, biotinylated B-cell extract precipitated with BoA1 mAb coated beads. Line 3, biotinylated B-cell extract precipitated with AV20 coated beads. Line 4, biotinylated B-cell extract precipitated with beads coated with isotype control antibody. Line 5, unlabeled B-cell extract stained with ABC. Line 6, unlabeled B-cell extract precipitated with AV20-coated beads, and after eluting the antigen was probed with BoA1 mAb. The protein precipitated with AV20 mAb is recognized by our novel mAb.

fowl. Booster injections were given at 2-wk intervals. The spleen cells of immunized mice were fused with Sp2/0-Ag14 mouse myeloma cells. The hybridomas were screened by immunocytochemistry and the selected ones were recloned at least twice. The isotype of the mAb was determined by ELISA using an immunotyping kit (Sigma-Aldrich, Budapest, Hungary).

Monoclonal antibody clone 5-11G2 against chicken Bu-1b antigen was purchased from Southern-Biotech (Southern Biotechnology Associates, Birmingham, AL), whereas AV20 (antiBu-1a/b), L-22 (antiBu-1a), and EIVE12 antibodies, which recognize avian B lymphocytes and plasma cells, were a generous gift from Pete Kaiser (Compton, UK), Olli Vainio (Oulu, Finland), and Todd Pharr (Mississippi State University, Mississippi State), respectively.

Immunohistochemistry and Immunofluorescence

The embryonic and adult lymphoid tissues were embedded in liver and snap-frozen in liquid nitrogen. Eight-micrometer-thick cryostat sections were air-dried and fixed with cold acetone. The endogenous peroxidase activity was quenched by 3% hydrogen peroxide (Sigma-Aldrich) in PBS. After rehydration in PBS the sections were incubated with primary antibodies for 45 min, followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and avidin-biotinylated peroxidase complex (ABC, Vector Laboratories). The peroxidase activity was visualized with 4-chloro-1-naphtol (Sigma-Aldrich).

Immunoprecipitation, SDS-PAGE, and Western Blot

Bursal tissue was placed on lens paper and minced in PBS. The minced bursal tissue was wrapped and gently pressed to collect the bursal cells. Then, 3×10^6 freshly isolated cells were mixed with CFA and injected intraperitoneally. The remaining cells were kept in liquid nitrogen in frozen media until the next injection. For booster injections, these cells were mixed with IFA and PBS for the last injection.

The surface molecules of 2×10^7 living bursal B-cells were biotinylated using water soluble biotin (EZ-Link Sulfo-NHS-LC-Biotin, Pierce, Rockford, IL) according to the protocol. After the labeling the cells were lysed for 1 h on ice with 1 mL of RIPA buffer (Sigma-Aldrich) containing protease inhibitor tablet (Roche, Budaörs, Hungary). The lysate was centrifuged at 21,000 $\times$ *g* at 4°C for 15 min. Five hundred microliters of cleared lysates was incubated with 100 μL (5 to 10 μg/mL) of our novel mAb, designated BoA1, AV20, and with isotype-matched control at 4°C. After 45 min of incubation 50 μL of anti-mouse Ig conjugated Dynal magnetic beads (10 mg/mL, Invitrogen) were added to each tube and incubated for further 45 min. The precipitated protein was washed extensively using RIPA buffer and magnetic separator. The beads were boiled in 50 μL of reducing sample buffer for 4 min, and the eluted protein (20 μL/well) was separated on SDS-PAGE (10%). Following electroblotting, the nitrocellulose membrane (0.45 μm; Amersham Bioscience) was blocked with fat-free milk and the precipitated and biotinylated protein was incubated with ABC for 30 min. The binding of ABC was visualized using NovaRed peroxidase substrate kit (Vector Laboratories).

For immunoprecipitation, unlabeled bursal B-cell extracts were prepared as written above. Then 500-μL B-cell extracts were incubated with 50 μL of AV20 conjugated Dynal magnetic beads. Fifty microliters of anti-mouse Ig conjugated Dynal beads was incubated overnight with 3 mL of AV20 supernatant. After washing with RIPA buffer, the beads were used for precipitation. After 1 h the beads were washed and the precipitated proteins eluted by boiling in 50 μL of reducing sample buffer. The eluted proteins were separated by SDS-PAGE and electroblotted. The nitrocellulose strips containing the protein eluted from AV20 beads were incubated with BoA1 antibody. The primary antibody was followed by peroxidase labeled anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed with NovaRed peroxidase substrate kit.

RESULTS AND DISCUSSION

A novel monoclonal antibody was produced against the guinea fowl's bursal B cells, and characterized. Immunohistochemical characterization was compared with other members of the B-cell-specific Bu-1 antibody family, like 11G2, L22, and AV-20. The staining patterns

Table 1. Monoclonal antibodies recognizing chB6 transmembrane molecules in avian species[1]

Item	Chicken a allele	Chicken b allele	Obese chicken	Turkey	Guinea fowl	Quail
11G2	–	+	–	–	–	–
L22	+	–	+	–	–	+
AV20	+	+	+	–	–	–
HIS-C1[2]	+ (no information for alleles)		?	+	?	–
BoA1	+	+	+	+	+	+

[1]The symbols + and – indicate whether or not the avian species reacted with the listed monoclonal antibody.

[2]Jeurissen and Janse (1998). HIS-C1 mAb has not been tested in our lab; thus, we have not had information concerning the allelic background of HIS-C1.

of different antibodies on lymphoid organs are identical with that of our mAb BoA1 (isotype IgG_1); therefore, we show only 2 parallel sections from chicken spleen stained with 11G2 (Figure 1a) and BoA1 (Figure 1b) to justify the above statement. The B cell regions of the spleen, that is, the germinal centers and the periellipsoidal white pulp, histologically consist of small, dense and larger, less dense cell populations, respectively (Olah and Glick, 1982).

Both cortical and medullary B cells of the bursa of Fabricius are recognized by BoA1 mAb, leaving an unstained line at the cortico-medullary epithelium, suggesting that this distinct area contains BoA1-negative cells (Figure 1c). The antibody labels many B cells in the thymic medulla where they are scattered in a different density (Figure 1d). In the gut-associated lymphoid tissue, cecal tonsil (Figure 1e), Peyer's patches (Figure 1f), and esophageal tonsil (Figure 1g), the germinal centers are sharply demarcated from the environment and strongly BoA1-positive, whereas many positive cells are scattered in the interfollicular region.

From B-cell extracts, the BoA1 mAb under reducing conditions precipitated a 70- to 73-kDa molecule, which is closely identical to those of L22 and AV20 reported by Pink and Rijnbeek (1983) and Rothwell et al. (1996), respectively. This finding was confirmed also by precipitating the bursal B cell extract with AV20-coated beads. The beads sorted out the chB6 antigen, which was eluted from the beads and reacted with BoA1. The result indicated that the AV20 precipitated antigen also was detected with BoA1 antibody (Figure 2).

The L22 and HIS-C1 mAb cross-react with quail (Nagy et al., 2004) and turkey, respectively (Jeurissen and Janse, 1998). The BoAl mAb is less species-restricted than the other members of Bu-1 mAb family because it cross-reacts with all domesticated birds (chicken, turkey, guinea fowl, and quail; Table 1). Each of the Bu-1-specific mAb was raised against chicken cells, unlike the BoA1, which was produced against guinea fowl cells. This may confirm that the polymorphism of the chB6 is really an avian B-cell-specific feature (Tregaskes et al., 1996).

Pharr et al. (1995) reported a mAb, designated EIV-E12, that shows an identical staining pattern with the BoAl mAb. The expression of chB6 antigen is shut down during terminal maturation of B cells, namely the plasma cells do not express this antigen. However, the expression of EIV-E12-positive antigen goes on and is expressed by the plasma cells. In chicken embryonic bursa, before follicle formation, the EIV-E12- and Bu-1b-specific 11G2 mAb identify dendritic cell precursors and B cells, respectively (Olah et al., 1993). After follicle formation bursal B cells also express the EIV-E12 antigen. These observations suggest that the avian B cells have another B cell surface molecule, which emerges in a later developmental stage than the chB6 motif and its expression continues after their terminal maturation.

In conclusion, the BoA1 mAb is a novel member of the Bu-1-alloantigen-specific mAb family, which was produced against guinea fowl cells and reacts with chicken, turkey, guinea fowl, and quail B cells.

ACKNOWLEDGMENTS

The authors thank Jutka Fügedi and Vidra Zsuzsa for laboratory assistance. Grant sponsor: OTKA; grant numbers: T-042558 and K-69061.

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