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Monoclonal Antibody EIV-E12 Recognizes a Glycoprotein Antigen, Which Differs from the B-Cell-Specific chB6 (Bu-1) Antigen

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Abstract: The antigen (s) recognized by the EIV-E12 monoclonal antibody (mAb) is first detected in the embryonic bursal mesenchyme on the precursor to the bursal secretory dendritic cell. Later in bursal embryonic development the prebursal stem cells which enter the developing follicle express the antigen recognized by mAb EIV-E12. The goal of this project is to characterize the antigen recognized by mAb EIV-E12. In western blotting the deglycosylated bursal proteins showed reduced reactivity to mAb EIV-E12, indicating, that the EIV-E12 epitope of the antigen may consist of a carbohydrate group that is common to chicken B-cells. The EIV-E12 positive antigen is expressed by the plasma cells of terminal maturation of B-cells unlike the B-cell specific chB6 antigen. These differences in the expression patterns of EIV-E12 and chB6 positive antigens are confirmed by their different molecular weights of 200 and 65 kDa, respectively.

Key words: Bursal B-cell, monoclonal antibody EIV-E12, novel B-cell antigen

INTRODUCTION

In chickens, the bursa of Fabricius serves as the site of B-cell development (Glick *et al*., 1956), in which many non-lymphoid cells such as epithelial cells and bursal secretory dendritic cells (BSDC) may be important in the maturation of B-cells (Glick and Olah, 1993; Nagy *et al*., 2004; Olah and Vervelde, 2008). In a collaborative effort, Glick and Olah proposed that BSDC initiate the formation of the follicle and provide the appropriate microenvironment for B-cell maturation in the chicken (Olah *et al*., 1986; Glick and Olah, 1993; Felfoldi *et al*., 2005). Therefore, a series of monoclonal antibodies were raised against partially purified preparations of chicken splenic ellipsoid associated cells, that was proposed to be precursors of interdigitating and follicular dendritic cells (Olah and Glick, 1982; Igyarto *et al*., 2007) to provide reagents for studying the role of dendritic cells in B-cell development. One mAb, termed EIV-E12 was found to recognize a 200 kDa protein expressed on the cell surface of more than 80% of bursal cells (Pharr *et al*., 1995). The evaluation of EIV-E12 antigen expression during embryonic development revealed that the earliest cells in the bursal mesenchyme represented the precursor to the BSDC (Olah *et al*., 1990; Nagy *et al*., 2001, 2004). These EIV-E12$^+$ cells migrate through the basement membrane and enter bursal epithelium and initiate formation of the bursal follicle (Olah *et al*., 1986). The BSDC increase in number and exhibit a dendritic morphology (Olah *et al*., 1992; Nagy *et al*., 2004; Olah and Vervelde, 2008). The prebursal B-cells, characterized by the expression of surface IgM and surface glycoprotein (s) with a sialyl Lewis x (SLEX) moiety, start entering the developing bursal follicles by ED13 (Olah *et al*., 1986; Ratcliffe *et al*., 1986; Olah *et al*., 1990; Masteller *et al*., 1995a). Once in the follicles, the proliferating IgM$^+$ and SLEX$^+$ bursal B-cells begin to express the surface protein recognized by mAb EIV-E12 (Olah *et al*., 1990; Pharr *et al*., 1995). The immunocytochemical studies suggested that the EIV-E12$^+$ antigen (s) is shared between B-cells and BSDC in the bursal follicle (Olah *et al*., 1990; Pharr *et al*., 1995). By ED18, IgM$^+$ and EIV-E12$^+$ bursal B-cells differentiate and undergo a change in surface expression of the SLEX carbohydrate to a Lewis x (LEX) carbohydrate (Masteller *et al*., 1995a). The SLEX to LEX transition of bursal B-cells leads to diversification of the immunoglobulin heavy and light chain genes, which proceeds until sexual maturity (Thompson and Neiman, 1987; Reynaud *et al*., 1987; Masteller *et al*., 1995b). While the avian B-cell specific carbohydrate alloantigen (chB6) (Tregaskes *et al*., 1996; O'Laughlin, 2010) functions as a death receptor (Funk *et al*., 1997), because cross linking chB6 with an anti-chB6 antibody results in apoptosis in both bursal B-cells and DT40 cell line (Funk *et al*., 2003). The function of the EIV-E12$^+$ antigen is unknown. The objective of this manuscript is

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to characterize the EIV-E12$^+$ antigen that is shared between BSDC and B-cells and later functionally characterize this antigen.

MATERIALS AND METHODS

Whole bursal protein extracts: The bursas from ED20 and ED21 embryos were obtained from Ross X Ross 308 broiler hatching eggs obtained from Peco Farms (Gordo, AL, USA). The bursa tissue from 42 day old chickens was a generous gift from Dr. Alex Corzo (Department of Poultry Science, Mississippi State University). The bursa tissue from 4 week old chickens was from Hy-Line W-36 roosters, a generous gift from Dr. Scott Branton (USDA-ARS, Poultry Research Unit, Mississippi State University). Whole tissue extracts were prepared by homogenization of bursal folds in ice cold radioimmunoprecipitation buffer (1x Tris-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide and protease inhibitors; Santa Cruz Biotechnology, USA, No. sc-24948) as described (Felfoldi *et al*., 2008). The amount of protein in the extracts was determined by the bicinchoninic acid assay (Pierce, USA, No. 23227) and aliquots were stored at -80°C.

Antibodies: The EIV-E12 and BoA1 mAb were in the form of an equal mixture of hybridoma supernatants and sterile glycerol. The BoA1 mAb is an IgG1 heavy chain isotype and recognizes the chB6 (Bu-1) antigen (Igyarto *et al*., 2008). The isotype of mAb EIV-E12 was determined to be an IgG1 heavy chain with the Rapid Isotyping Kit (Pierce, USA, No. 26178).

Protein deglycosylation and western blot analysis: Proteins were deglycosylated with the enzymatic deglycosylation kit according to the manufacturer's protocol (Prozyme, USA, No. GK80110). Briefly, whole bursal protein extracts (200 µg) were incubated in reaction buffer (control) or with a mixture of N-glycanase, sialidase and O-glycanase at 37°C overnight. The reactions were terminated by the addition of an equal volume of 2x Laemmli sample buffer containing 2-mercaptoethanol (Laemmli, 1970). The reaction mixtures were then electrophoresed on 8% Tris-glycine SDS-PAGE gels (50 µg/well) for 2 h at 100 volts and electrophoretically transferred to nitrocellulose membranes for western blotting. The blots were washed and treated with monoclonal antibodies as described (Wan *et al*., 2004). The blots were blocked for 3 to 4 h and incubated overnight at 4°C in a 1:5 dilution of EIV-E12 or a 1:50 dilution of BoA1 hybridoma supernatants. After incubation with the primary antibodies, the blots were washed 5 times in Tris-buffered saline (TBS) containing 0.05% Tween-20 and one time with TBS. The blots were incubated with a 1:1250 dilution of goat anti-mouse immunoglobulin conjugated with alkaline phosphatase (Southern Biotechnology Associates, USA, No. 1010-04) for 1 h at room temperature. The blots were washed 5 times in TBS-tween and incubated for 5 min in the substrate Sigma Fast NBT/BCIP (Sigma, USA, No. B5655) to reveal protein bands.

Immunohistochemistry: Bursa, spleen and thymic tissues of 6 weeks old SPF chickens were embedded in liver and frozen in liquid nitrogen. The tissue samples were stored in a deep freezer (-80°C). The ten micron thick frozen sections were mounted on microscope slides pretreated with poly-L-lysine (Sigma, USA) and then fixed by incubation in cold acetone for 10 min. After rehydration in phosphate buffered saline (PBS) the mAb EIV-E12 was applied to the sections for a 45 min incubation period. The sections were washed in PBS and then incubated for 45 min with a secondary biotinylated anti-mouse IgG antibody (Vector Labs, USA). The sections were washed in PBS and then incubated with the avidin-biotinylated peroxidase complex (Vector Labs, USA). The substrate used for the peroxidase was 4-chloro-1-naphthol which results in a blue precipitate (Sigma, USA). The controls included incubation of sections with the primary antibody replaced by PBS and sections in which the secondary antibody was replaced with PBS.

RESULTS AND DISCUSSION

The antigen recognized by mAb EIV-E12 is first detected in the bursa on hematopoietic cells that migrate into the bursal mesenchyme and give rise to BSDC in the follicles (Olah *et al*., 1990; Nagy *et al*., 2001, 2004). Later in bursal embryonic development, the prebursal stem cells (B-cell progenitors) that enter the developing follicle express the EIV-E12 antigen (Olah *et al*., 1990; Pharr *et al*., 1995). Therefore it is important to identify and functionally characterize the 200 kDa antigen recognized by mAb EIV-E12.

The evaluation of mAb EIV-E12 with immunohistochemistry is shown in Fig. 1. The developing B-cells in the posthatch bursal follicles express the antigen recognized by mAb EIV-E12 (Fig. 1a). Therefore, developing bursal B-cells continue expression of the EIV-E12 antigen into the posthatch period. Peripheral B-cells expressing the EIV-E12 antigen were observed in the peri-ellipsoid white pulp or B-cell zone of the spleen (Fig. 1b). In the medullary region of the thymus (Fig. 1c), EIV-E12$^+$ round and stellate-shaped cells are found, while the thymic cortex reveals mainly stellate-shaped EIV-E12$^+$ cells. The observation of peripheral B-cells in the thymus is not unusual, as in mammals peripheral B-cells colonize the thymic medulla and play an important role in negative selection (Yamano *et al*., 2015).

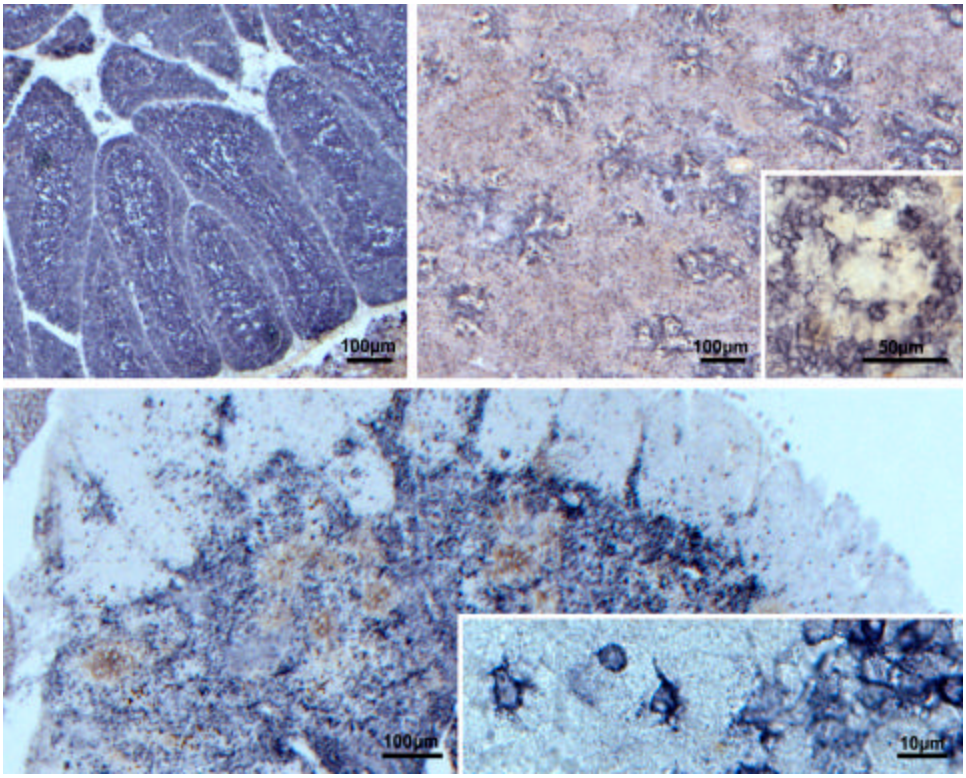

Fig. 1: Immunohistochemistry analysis of mAb EIV-E12 reactivity in primary and secondary lymphoid tissues. (a) Bursa of Fabricius. The EIV-E12 mAb recognizes both cortical and medullary B-cells, (b) Spleen. The EIV-E12 positive cells outline the ellipsoid. Inset: higher magnification of an ellipsoid (unstained area), which is surrounded by ellipsoid associated cells and B cells. Inside the ellipsoid few positive cells occur, possibly representing migrating cells from the circulation, (c) Thymus. The thymic medulla contains EIV-E12 positive cells, but their localization is inhomogeneous. High and low cell density areas are intermittently recognized. Inset: in higher magnification the cortex reveals scattered positive cells with round and stellate-shaped appearance

The goal of the experiment shown in Fig. 2 was to determine if the protein recognized by mAb EIV-E12 is glycosylated and if identical with the B-cell specific antigen chB6 recognized by mAb BoA1 (Igyarto *et al*., 2008). A reduction in the molecular mass of the 200 kDa protein recognized by EIV-E12 would indicate that this protein is glycosylated. Therefore, bursal protein extracts were incubated with reaction buffer (control) or with reaction buffer containing a mixture of deglycosylases and then samples were evaluated with western blotting. The blots were cut in half and incubated with mAb EIV-E12 or mAb BoA1. The deglycosylation experiment was conducted with both post-hatch (Fig. 2a) and embryonic bursal extracts (Fig. 2b). The protein extracts incubated with the deglycosylase mixture and reacted with mAb BoA1 showed a reduction in molecular mass to the 50 kDa range (lane 4), whereas bursal extract samples incubated in a reaction mixture without the enzymes (lane 3) or untreated (lane 2) gave signals in the expected 70 kDa range for the chB6 glycoprotein (Igyarto *et al*., 2008). In the case of the EIV-E12 mAb, the deglycosylation treatment resulted in a significant decrease in antigen detection from the protein lysates (lane 4). It is possible/likely that the epitope recognized by EIV-E12 mAb is partially or completely located on the carbohydrate side chains of the glycoprotein, therefore deglycosylation treatment reduced reactivity to the antibody. The results of the experiment with bursal proteins from both the embryonic and post-hatch periods suggest that part of the epitope recognized by mAb EIV-E12 may consist of a carbohydrate group, while the epitope recognized by mAb BoA1 is not related to those carbohydrate side chains.

To further characterize the EIV-E12$^+$ epitope, bursal protein extracts (from ED20 and 4 week old chickens) were incubated separately or with a mixture of the deglycosylases and then evaluated with western blotting to determine if the epitope consists of an N-linked

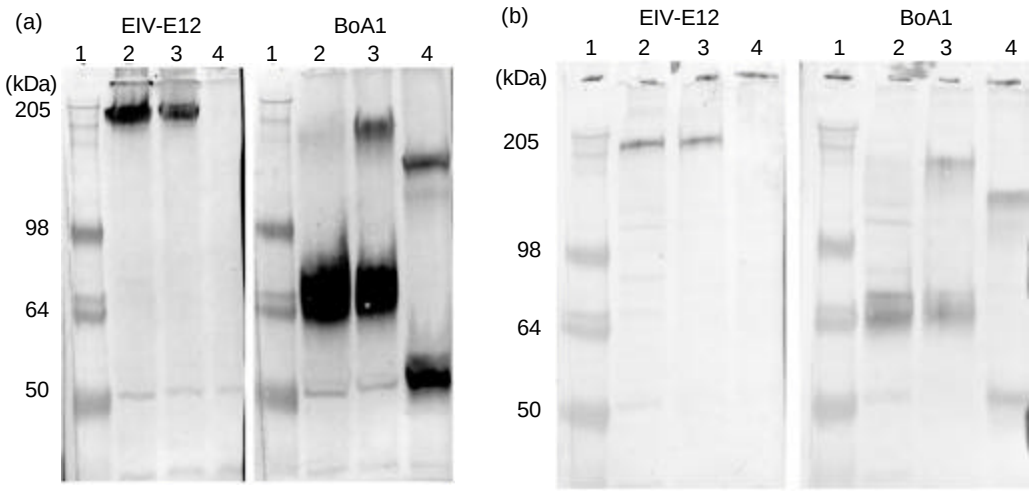

Fig. 2: Western blotting analysis of deglycosylated bursal extracts. Western blot analysis shows that the EIV-E12 and the B-cell specific chB6 antigens are different. (a) Western blotting analysis of day 42 post-hatch bursal extracts with mAb EIV-E12 and BoA1. Lane 1: Molecular mass standard. Lane 2: Day 42 bursal extract. Lane 3: Bursal extracts incubated in enzyme reaction buffer only (control). Lane 4: Bursal extracts incubated with an equal concentration of N-glycanase, Sialidase A and O-glycanase. (b) Western blotting analysis of ED21 bursal extracts with mAb EIV-E12 and BoA1. Lane 1: Molecular mass standard. Lane 2: Embryonic day 21 bursal extract. Lane 3: Bursal extracts incubated in enzyme reaction buffer only (control). Lane 4: Bursal extracts incubated with an equal concentration of N-glycanase, Sialidase A and O-glycanase

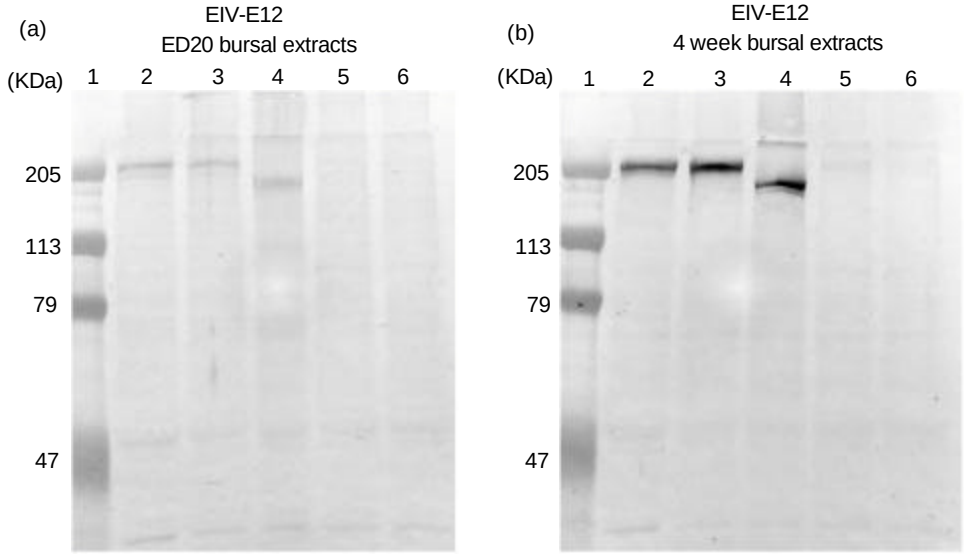

Fig. 3: Western blotting analysis of bursal extracts treated with N-glycanase, sialidase A or a mixture of sialidase A and O-glycanase. The EIV-E12^{+} glycoprotein antigen is sensitive to sialidase A. (a) Western blot with mAb EIV-E12 and ED20 bursal extracts. Lane 1: Molecular mass standard. Lane 2: Embryonic day 20 bursal extract. Lane 3: Bursal extracts incubated in enzyme reaction buffer only (control). Lane 4: Bursal extracts incubated with N-glycanase. Lane 5: Bursal extracts incubated with sialidase A. Lane 6: Bursal extracts incubated with an equal concentration of sialidase A and O-glycanase. (b) Western blot with mAb EIV-E12 and 4 week old bursal extracts. Lane 1: Molecular mass standard. Lane 2: Four week old chicken bursal extract. Lane 3: Bursal extracts incubated in enzyme reaction buffer only (control). Lane 4: Bursal extracts incubated with N-glycanase. Lane 5: Bursal extracts incubated with sialidase A. Lane 6: Bursal extracts incubated with an equal concentration of sialidase A and O-glycanase

carbohydrate, O-linked carbohydrate or sialic acid. The mixture of sialidase and O-glycanase is required to digest some O-linked carbohydrates, as sialic acid groups on O-linked carbohydrates can interfere with the activity of the O-glycanase enzyme (Liu *et al*., 2007). The results are shown in Fig. 3. When bursal extracts were incubated with sialidase (lane 5) or a mixture of sialidase and O-glycanase (lane 6) the reactivity of EIV-E12 with the 200 kDa glycoprotein was drastically reduced. The results shown in Fig. 3 suggest that part of the epitope may consist of sialic acid or possibly a sialylated O-linked carbohydrate (Watanabe *et al*., 1995). Alternatively, it is also possible that removal of carbohydrates recognized by sialidase A and O-glycanase could result in an overall structural change in the protein, therefore changing the conformation of the epitope recognized by mAb EIV-E12 (Loirat *et al*., 1999). Additional studies will be required to address these possibilities.

Conclusions: The EIV-E12$^+$glycoprotein may be involved in carbohydrate lectin interactions in the bursa (Pohlmeyer *et al*., 2005). Our hypothesis is that the developing B-cells (bursal stem cell stage) may receive a differentiation signal from the follicular microenvironment to which the secreted product of BSDC has significant contribution- resulting in upregulation of the 200 kDa glycoprotein expression in B cells.

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