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Research paper

Locally applied testosterone is a novel method to influence the development of the avian bursa of Fabricius☆,☆☆

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ABSTRACT

Bursa of Fabricius is a primary lymphoid organ in the birds and its microenvironment is responsible for the B cell maturation. The epithelial anlage develops through epithelial–mesenchymal interaction into which –as a third party – hemopoietic cells immigrate. Chemical bursectomy can be made by dipping or injecting eggs by testosterone solution. Both methods of treatments compromise the development of the whole embryo. Heparin beads of 70–150 μm in diameter were soaked in 5% testosterone solution and implanted into the tail bud of 4 day old chicken embryos before the bursal anlage began to develop at embryonic day 5. The locally applied testosterone inhibited the differentiation of the bursal secretory dendritic cell (BSDC) precursors, which were the inducer of the follicular epithelial bud formation. Testosterone treatment did not blocked the entering of B cells and other hemopoietic cells into the bursal mesenchyme, but in the absence of BSDC precursor cells the dendroepithelial microenvironment is not established, subsequently B cell precursors are lodged only in the bursal mesenchyme. This method is suitable for locally introducing other regulatory molecules, involved in bursal development.

1. Introduction

The epithelial–mesenchymal interaction is a fundamental process for the development of many organs, like tooth, intestine, lung, kidney and glands (Dassule and McMahon, 1998; Roberts et al., 1998; Ball and Risbridger, 2001; Nagy and Goldstein, 2006). The locally applied morphogens interfere with molecular cascade regulating the epithelial–mesenchymal interaction. During the last two-decade numerous signaling molecules and growth factors were discovered, which associated by epithelial–mesenchymal interactions. The effect of different signal molecules has been experimentally analyzed in *in vitro* tissue recombination studies or by *in vivo* microbead implantation experiments. Local application of growth factors by microbeads implanted into the embryo is a valuable tool for studying the mechanism of operation of the regulatory molecules. This method was originally developed by Eichele et al. (1984) to investigate the effects of retinoids on the chick limb development.

Bursa of Fabricius is a primary lymphoid organ, which is responsible for the B cell maturation (Glick, 1956). It is generally believed, that the epithelial anlage of the bursa develops from the hindgut around day 4.5 of incubation and grows into the mesenchyme of the tail bud. The bursal follicular medulla – which develops during the 11–13th days of embryogenesis – is a lymphoepithelial organ, like the thymus. The epithelial bud, as the bursal follicular rudiment grows into the mesenchyme of the bursal fold, and the hemopoietic B cell precursors invade in it (Glick, 1995). The formation of the epithelial bud is induced by invasion of precursors of bursal secretory dendritic cells (BSDC) into the surface epithelium of hindgut, which results in a transitory dendroepithelial tissue (Oláh et al., 1986; Nagy et al., 2004a; Oláh and Vervelde, 2008). Several hours later the microenvironment of the dendroepithelial tissue receives B cell precursors transforming the dendroepithelial tissue to lymphoepithelial one, establishing bursal follicular medulla (Nagy et al., 2004b).

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☆☆ Professor Bruce Glick who discovered the function of the bursa of Fabricius passed away on February 19, 2009. Imre Olah was working with him several years and we identified the bursal secretory dendritic cells (BSDC). We dedicate this paper for his memory.

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The androgens, such as the testosterone proprionate (TP), results in bursectomy by preventing the differentiation of precursors of BSDC and subsequently the normal development of the bursa of Fabricius (Glick and Oláh, 1984). The testosterone mediated chemical bursectomy has been made either by dipping the embryonated eggs into the testosterone solution or by intra-allantoic injection, therefore the testosterone influenced the development of the whole body of the embryo (Dixon and Fitzsimmons, 1980; Soós et al., 1990; Fennell and Scanes, 1992).

The microbead implantation allows to study the role of the regulatory molecules without significant effects to other organs. In this paper we report, that the implantation of testosterone soaked bead is a useful method for studying the follicle formation and avian B cell differentiation, even chemical bursectomy can be achieved.

2. Materials and methods

2.1. Animals

Fertilized White Leghorn chicken eggs were obtained from commercial breeders and incubated at 37.7 °C in humidified incubator. Embryos were staged according to the Hamburger and Hamilton table (1951) or the number of days of incubation (ED). The design and condition of the animal experiments were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary.

2.2. Antibodies

Monoclonal anti-chicken CD45 (clone: HIS-C7), CVI-ChNL-74.3 and CVI-ChNL-74.2 antibodies were purchased from CEDI-Diagnostics BV (Lelystad, Netherlands) and used as hemopoietic, dendritic cell and macrophage markers, respectively. Monoclonal anti-vimentin (AMF-17b) antibody was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa). M1 mAb identifies chicken IgM was purchased from Southern Biotech (Birmingham, Alabama). BoA1 monoclonal antibody was used to identify the Bu1 positive B lymphocytes (Igyártó et al., 2008).

2.3 Bead application and microsurgical procedures

The heparin-acrylic beads (70–150 μm; Sigma-Aldrich Co., St. Louis, MI) were incubated for 1 h in 5% testosterone propionate (Sigma), dissolved in 95% ethanol.

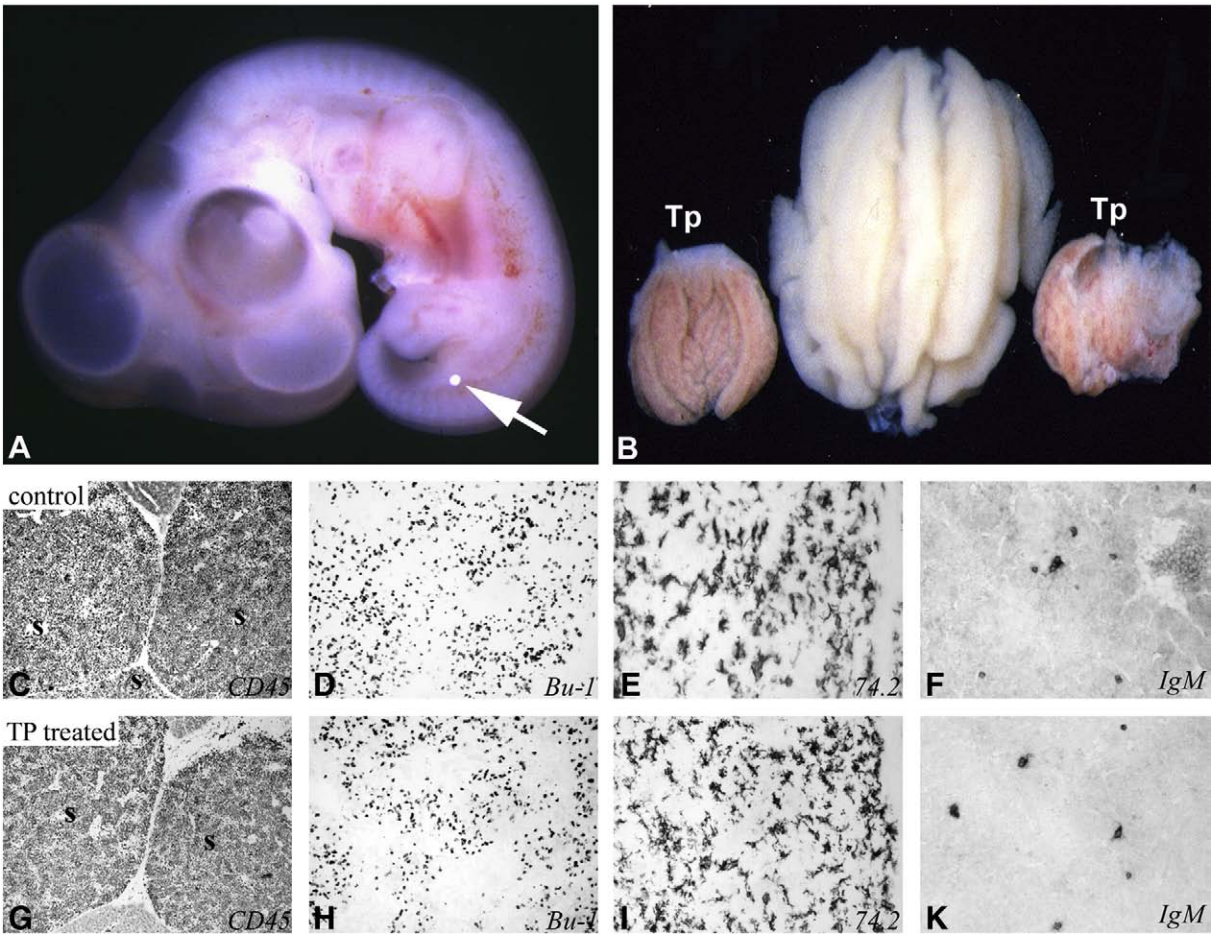

Fig. 1. A. Stereo micrograph shows the location of the implanted bead (arrow). B. Comparison of bursal size of control and TP treated bead implantation at ED 19. C to F and G to K figures taken from spleen (S) of 16 day old control and TP treated embryos, respectively. Hemopoietic (CD45; C and G); B cell (Bu-1; D and H); macrophage (74.2; E and I); immunoglobulin (IgM; F and K) markers have been used to demonstrate the different cell populations. No immunohistological differences can be seen between the control and TP bead treated embryos.

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For bead implantation, the tail bud of the 23–24 Hamburger–Hamilton stage (ED4) chick embryo was stabilized by means of humidified cotton thread. The right hind limb was drawn away with forceps, thus giving access to the cloacal region of the tail bud. The lateral ectoderm and mesenchyme of the tail bud were punctured with electrolitically sharpened tungsten needle to create a space for bead insertion. The embryos were further allowed to develop in ovo for 12 or 15 days. The 16 and 19 day old treated and control (ethanol soaked bead implantation) embryos were killed and if bursal rudiment was found, it has been removed together with the thymus and spleen for histological procedures. This study included a total of 27 experimental and 15 control embryos resulting from 3 separate series. During the first pilot experiment, the body weight of the TP treated and control embryos was measured at day 19 of incubation before tissue samples were taken.

2.4. Immunocytochemistry

For immunohistochemical studies bursal rudiments were fixed in 4% paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for 1 h, rinsed with PBS and infiltrated with 15% sucrose/PBS overnight at 4 °C. The medium was changed for 7.5% gelatine containing 15% sucrose at 37 °C for 1–2 h, and the tissue samples were rapidly frozen at −60 °C in isopenthane (Fluka). 10 µm thick frozen sections were made and collected on poly-L-lisine coated slides (Sigma) and air dried.

From each of group of birds the spleens and thymuses were collected in PBS. Generally, 3 spleens and 3 thymuses were put together and embedded into liver, which were placed on a 15×15 mm size cardboard. The prepared tissue blocks were frozen in liquid nitrogen (the liver protects the tissue samples from the drastic and sudden cooling effect). The 10 µm thick frozen sections were fixed for 10 min in ice cold acetone and air dried. Immunocytochemistry was made by standard techniques. Briefly, after rehydration in PBS the sections were incubated at room temperature with primary antibodies for 45 min, followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) and avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen-peroxide (Sigma) in PBS for 10 min before ABC. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphtol (Sigma).

2.5. Histological procedures

For histological examination the tissue samples were fixed in 4% buffered glutaraldehyde solution for overnight and postfixed in 1% osmium tetroxide for 2 h. After dehydration in graded ethanol, the tissue samples were embedded in Polybed/Araldite 6500 mixture (Polyscience, Inc., Warrington, PA). The 1 µm thick semithin sections were stained by toluidine blue.

3. Results

Fig. 1A shows the place of the implanted bead ventral to the somites in the tail bud. By the 19th ED the bursal growth is remarkably retarded in the birds with implanted beads (Fig. 1B) while the body weights do not show any difference between control and TP treated embryos (Table 1). Similar results were obtained on spleen by immunocytochemistry using hemopoietic (Fig. 1C,G), B lymphocyte (Fig. 1D,H) and macrophage specific markers (Fig. 1E,I). The IgM production of B cells indicates their functional activity (Fig. 1F,K), which can be suppressed by injection of heterologous antibody to IgM (Kincade and Cooper, 1973).

In control bursa the folds fill up the bursal lumen by day 16 of incubation, and rudiments of the follicles are lined up along the surface epithelium (Fig. 2A). The cell density in the mesenchyme of the fold is much higher close to the epithelium, than in the center of the folds (Fig. 2A,B). In close proximity and inside the epithelial bud of the follicles heavily stained cells are remarkable (Fig. 2B), which are the BSDC precursors and epithelial bud inducers. In the bead implanted birds the bursal folds are developed, but lower in size, the lumen is large, and no sign of the follicle formation (Fig. 2C). The surface epithelium of the fold reveals vacuolization, the subepithelial fibrocytes are oriented perpendicularly to the surface, and the cell density in the center of the mesenchyme is high, because it is packed with round shaped cells, (Fig. 2D). The latters are proved to be hemopoietic cells expressing CD45 antigen (Fig. 3A).

At day 16 of incubation significant number of blood-borne hemopoietic cells expresses Bu-1 antigen indicating, that some B cells are differentiated before follicle formation, but they do not come together in the epithelial buds of the follicle (Fig. 3B), unlike the control one (Fig. 3C). During the development of the follicle the BSDC differentiate and produce vimentin type intermediate filament (Fig. 3D), and begins to express 74.3 dendritic cell antigen (Fig. 3E), while in bead implanted embryos no sign of presence of BSDC using either anti-vimentin or 74.3 monoclonal antibodies (Fig. 3F,G), respectively. By ED19 the scattered B cells over the mesenchyme of the fold assemble and occasionally follicle formation occurs, but the histological difference is still remarkable between the control and bead implanted birds (Fig. 3H,I).

4. Discussion

It is well-known, that the testosterone treatment of early chicken embryos results in chemical bursectomy with severe B cell depletion (Wilson and Boyd, 1990). The introduction of testosterone takes place either by dipping the whole embryonated eggs into the testosterone solution or intra-allantoic injection (Dixon and Fitzsimmons, 1980; Glick and Oláh, 1984; Fennell and Scanes, 1992). Both methods compromise the development of the whole embryo, because

Table 1
Comparison of body weight (in grams) of TP treated and control embryos at day 19th of embryogenesis

Embryos	TP treated	Control
1	23	26
2	25	24.3
3	28	24
4	22	24
5	23	22.2
6	25	23
Average	24.3	23.9

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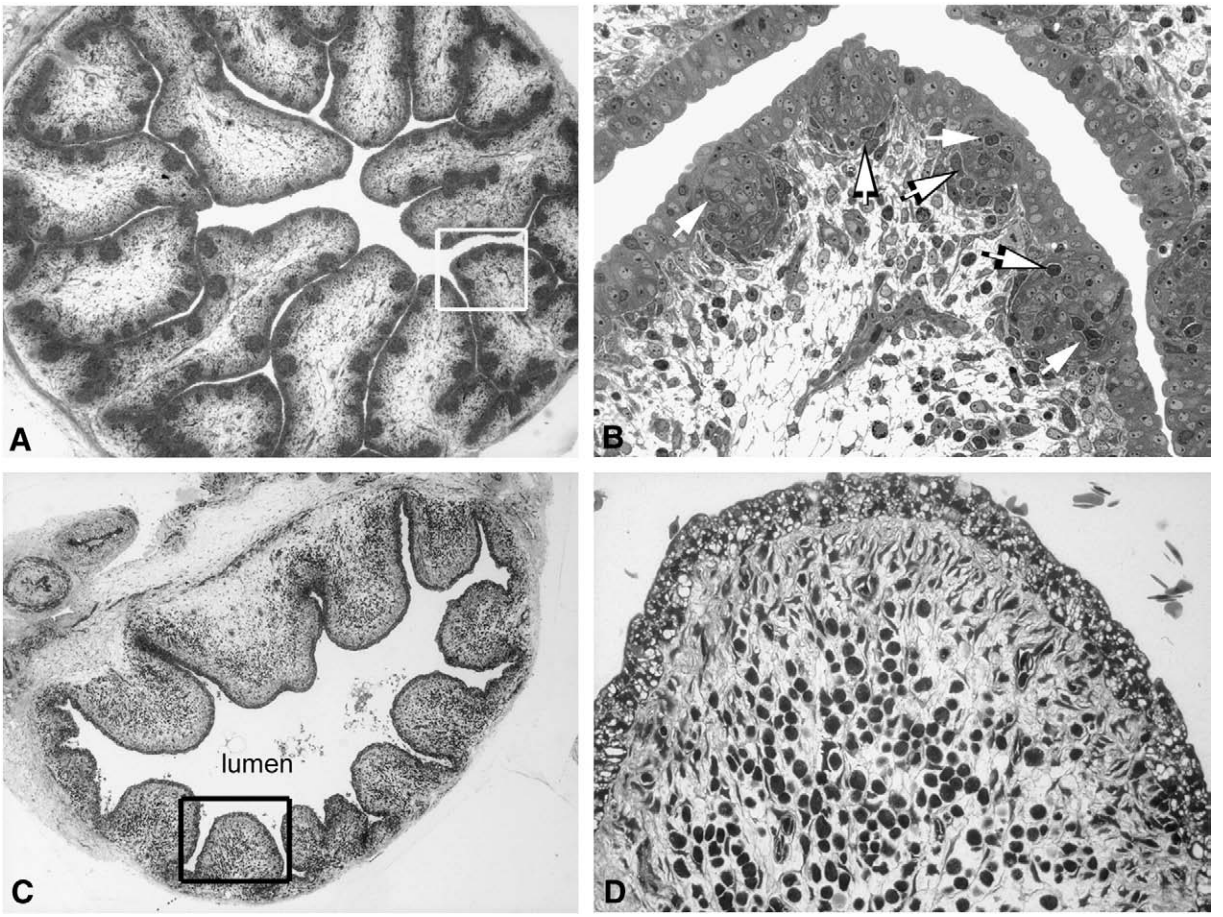

Fig. 2. A. Cross-section of a control bursa at ED16. The folds fill up the bursal lumen and the follicular rudiments as dark dots are attached to the surface epithelium. The outlined area is shown on B. B. Heavily stained BSDC precursors found inside and around the follicular rudiments (arrows). C. Bead implanted bursa at ED 16. The plicae are developed, but follicular rudiments are absent. The outlined area is shown in D. D. The surface epithelium is vacuolized, the subepithelial mesenchymal cells look like fibrocytes and show highly polarized appearance. The center of the fold is loaded with hemopoietic cells, proved by immunocytochemistry.

the testosterone as an antiproliferative drug interferes with the mitotic activity of the embryo. The method, which is shown here, introduces the testosterone locally; heparin-acrylic bead is implanted into the tail bud, close to the cloacal membrane. The testosterone is released from the bead, shows up in high, effective concentration only around the cloacal region. Different concentration of testosterone can be used or introducing more than one bead (Amthor et al., 2006) to regulate the effect of testosterone. The effectiveness of the method has been proved by histological and immunocytochemical methods. During the seventies and early eighties, when the chemical and surgical bursectomy flourished mainly classical histological and some immunological methods (antibody measurement in young adult birds) were available for studying the effect of bursectomy and the avian B cell differentiation and maturation. In this paper we have used few antibodies to identify more precisely the cell types and cell migration, which were presumably touched by testosterone treatment.

Three histological components: a) surface epithelium b) mesenchyme of the fold and c) hemopoietic cells contribute to the bursal follicle formation. The epithelial anlage of the bursa seems to develop well and the plication also takes place. It seems, that up to the follicle formation stage, the bursal development is not blocked by TP but the epithelium becomes vacuolated before epithelial bud formation. The subepithelial mesenchyme also reveals remarkable alteration: the mesenchymal cells became elongated, reminding to the fibrocytes, and oriented perpendicularly to the surface epithelium. We do not know, if the alteration in the epithelium or the subepithelial mesenchyme comes first, but during the organogenesis the mesenchyme determines the phenotypic manifestation of epithelium. It has been shown, that testosterone treatment inhibits the capability of mesenchyme to support epithelial differentiation of mammary gland (Kratochwil and Schwartz, 1976). Thus, we believe, that the epithelial vacuolization is the consequence of the histological and functional changes of the mesenchyme. It is possible, that the survival and further differentiation of the epithelium requires some stimulation from the mesenchyme and/or blood-borne BSDC precursors. However, the latter cells do not differentiate and migrate to induce epithelial bud formation, thus subsequently the epithelium becomes vacuolated, getting to degenerate. It is unfortunate,

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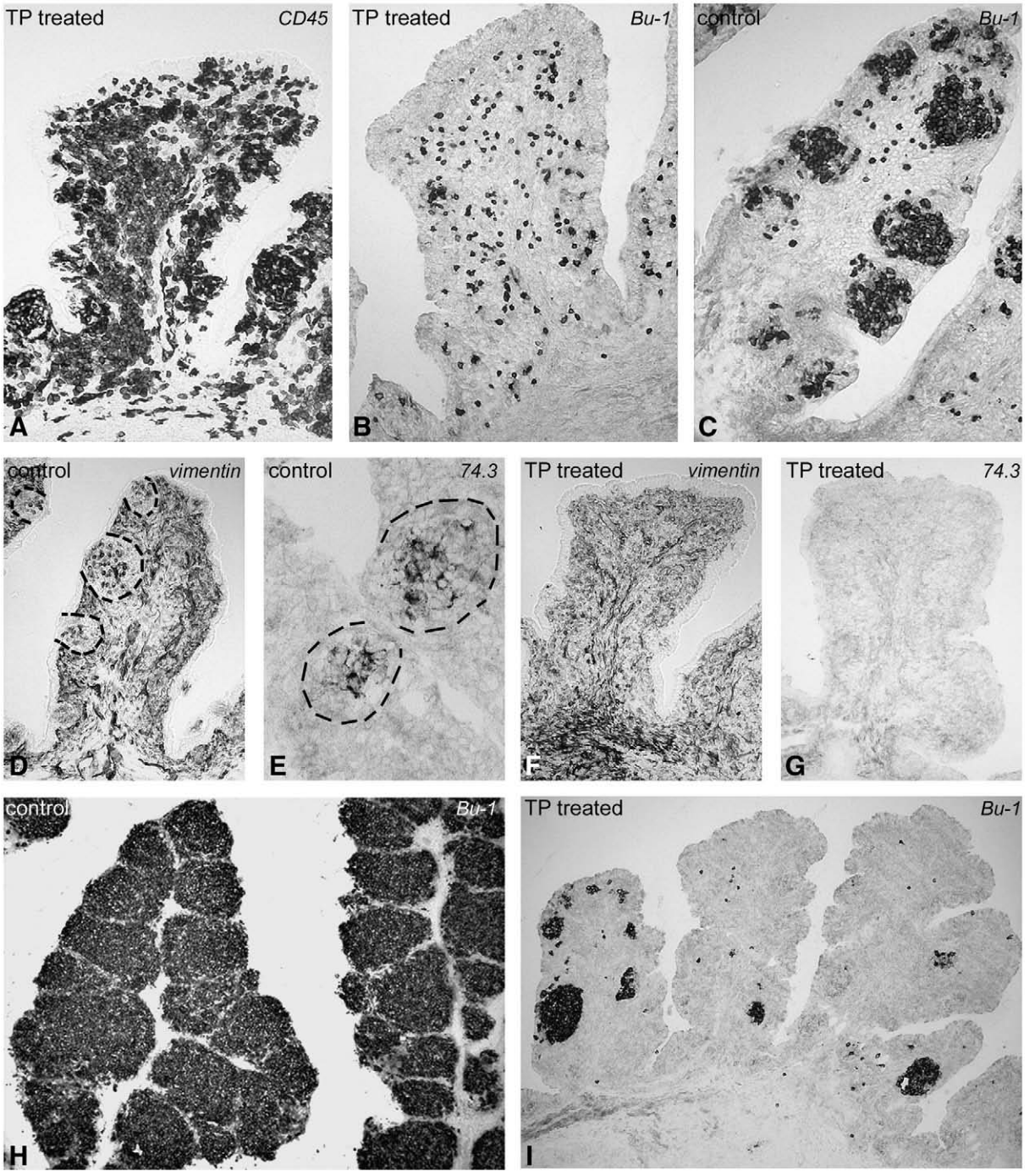

Fig. 3. A–G and H, I were taken from 16 and 19 days old embryos, respectively. A. CD45 immunostaining shows that large number of hemopoietic cells invades in the bursa B. Bu-1 immunostaining reveals scattered B lymphocytes over the mesenchyme of the fold. C. In control embryo the B lymphocytes already settle down in the bursal follicle. D. Anti-vimentin immunostaining show BSDC precursors in the bursal follicles (outlined with dashed line). E. The differentiating BSDC getting to express 74.3 antigen. F. and G. After implantation of TP treated bead no sign of follicle formation using anti-vimentin and 74.3 monoclonal antibodies, respectively. H. Bursal follicles are loaded with B cells. I. After TP treated bead implantation few bursal follicles have been formed.

that we still do not have specific marker(s) for BSDC precursors, therefore we cannot recognize them in the mesenchyme, unlike the epithelial bud. The first circumstantial histological evidence for the TP treatment is the absence of the heavily stained cells, which are the BSDC precursors namely, epithelial bud inducers (Oláh et al., 1986). The absence of the epithelial bud inducer cells has been confirmed by anti-vimentin and 74.3 monoclonal antibody staining (see Fig. 3F,G). The hemopoietic cells, as the third component, enter the bursal mesenchyme, but remain around the vessels.

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Interesting, that during the late embryonic development few follicles have been formed. We speculate, that the testosterone concentration, by time, gradually decreases and under a critical concentration small number of BSDC precursors (inducer cells) has been formed and induced epithelial bud and subsequently follicle formation (see Fig. 3i). This type of bud and follicle formation may be similar to the small-sized follicle formation which occurs in tardy embryogenesis and during regeneration described by Withers et al. (2006). Development of the bursa of Fabricius shows two morphogenetic periods: 1. formation of epithelial anlage between 4,5 and 7 ED, and 2. epithelial bud and follicle formation between 11 and 13 ED. The TP influences the second morphogenetic period by eliminating or hold up the differentiation of BSDC.

Bu-1 positive B cells emerge in the bursal mesenchyme before follicle formation providing evidence for extrabursal B cell differentiation (Glick, 1995; Rattclife, 2006). After surgical or chemical bursectomy the birds are capable for high level antigen specific IgM, but not IgG, antibody production (Glick, 1956). In mammals 5–10% of B cells express CD5 antigen. This B cell population is produced in the peritoneum, expresses a limited V gene repertoire and spontaneously secretes IgM antibodies. In chicken, the B cells which emerge before bursal follicle formation and appear in the bursal mesenchyme and spleen, but do not go through the bursal follicle, might be identical with the mammalian CD5 B cell population. Anti-IgM staining indicates their immunoglobulin production providing functional evidence for extrabursal B cell population.

In conclusion the paper introduces a classical developmental biology method into the avian immunology research to study B cell differentiation. The immunocytochemistry completes our earlier histological knowledge; namely there is B cell formation without bursa of Fabricius, and the testosterone inhibits the differentiation of BSDC precursors, resulting in absence of follicle formation. The bead implantation is a suitable method for local administration of different signal molecule either to study immunological and developmental biology aspects of bursa of Fabricius or to achieve bursectomy depending on the testosterone concentration.

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