

Thymic development in surgically bursectomized embryonic chicken: expression of PCNA, CD3, CD4 and CD8 markers

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Little information is available on the functional relationship between bursa and thymus during chicken embryogenesis. We, therefore, investigated embryonic thymuses taken at 17 days *in ovo* from chickens bursectomized at 68-72 hours, with histological, histochemical (PAS, Alcian blue), and immunoreaction (anti-cytokeratin B, anti-PCNA/cyclin and anti-CD3, CD4 and CD8 antibodies) methods and compared these data with those from normal and sham-operated chickens of the same age. The bursectomized thymuses distinctly differed from normal and sham-operated thymuses: they were smaller, and the cortical zone was thinner and contained fewer epithelial cells and thymocytes. Only few cortical thymocytes were immunoreactive for PCNA, indicating low proliferative rate. More cortical thymocytes as compared with the normal, expressed CD3 on their cell membrane, whereas the thymocytes at the cortical-medullary border expressing anti-CD4 and anti-CD8 antibodies were less numerous than in normal thymus. The medullary zone contained few epithelial clusters made up of fewer cells than medullary clusters in normal chickens. Some cystic formations were enlarged and contained PAS- or Alcian-blue positive amorphous material. All these data suggest that early bursectomy affects both morphological and functional thymic development.

Key words: embryonal avian thymus, bursectomy, PCNA CD3 CD4 CD8 markers.

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The two primary lymphoid organs in birds are the thymus and the bursa of Fabricius; the thymus is responsible for cellular immunological responses and the bursa for humoral immunological responses (Glick *et al.*, 1956; Cooper *et al.*, 1991).

During chicken embryonic development, T-cell precursors are processed by the various reticulo-epithelial cells and humoral factors that make up the thymic microenvironment (Aita, 1992; Boyd *et al.*, 1992; Aita *et al.*, 1995); B-cell precursors are processed by the epithelial stroma and humoral factors that make up the bursal microenvironment (Mazzone *et al.*, 2003).

The importance of the bursa in antibody production has been investigated in hormonally or surgically bursectomized chickens during embryonal incubation or in post-hatching life.

Experiments using fertile eggs dipped in/or injected with testosterone propionate, at various ages of embryonal life, (Mueller *et al.*, 1960; Carey and Warner, 1964; Hirota *et al.*, 1976; Moriya and Ichikawa, 1990; Soos *et al.*, 1990), showed that this hormone prevented bursal differentiation and antibody production to various extents, depending on the doses used and when antigens were injected. Similar effects were obtained with hormone 19-nortestosterone (Papermaster and Good, 1962) and other hormones, including diethylstilbestrol (Glick and Sadler, 1961) progesterone, estradiol and cortisol (Norton and Wira, 1977), allylestriol (Soos *et al.*, 1990) and colchicines given during neonatal life (Romppanen *et al.*, 1983).

In experiments using surgical bursectomy performed at 52-64 hours of incubation, Jankovic *et al.*, (1975, 1976), Isakovic *et al.*, (1980) verified that the immune properties were low but not abolished nor did bursectomy prevents the specific bursa (Bu) antigen-bearing and IgM-containing lymphocytes from differentiating. When chickens were surgically bursectomized at 62 hours and the skin was

grafted within 24 hours from hatching skin allograft rejection was delayed (Medina and Pedernera, 1977). Whereas in surgically bursectomy made at 68-72 hours of incubation, Fitzsimmons *et al.*, (1973) noted the atrophy of the thymic cortex. Surgical bursectomy performed at the time of hatching or at the age of 6 months left antibody production unchanged and the thymus of bursectomized embryos was found to contain some plasma cells (Zaccheo *et al.*, 1969). When surgical bursectomy was done in a period varying from 2 to 20 weeks of age, antibody production peaked during the first few weeks after hatching and declined with increasing age (Chang *et al.*, 1957). Antibody responses were significantly lower in chickens surgically bursectomized at 1, 2, 5 and 10 weeks of age than in controls (Mueller *et al.*, 1960). None of these experimental studies in chicken provided detailed histological information on the bursectomized thymus.

Current knowledge assumes that the thymus and bursa are interrelated. For example, cells migrate from the bursa to the thymus (Woods and Linna, 1965) and bursectomy interferes with the T-suppressor cell population in the thymus and significantly reduces the number of thymocytes (Glick, 1983). In thymectomized embryos, Isakovic *et al.*, (1980), Jankovic *et al.*, (1981) noted a pronounced delay in the development of bursal follicles.

After the 90's no paper deals with the effect of bursectomy on thymus function. To extending previous research conducted by our laboratory (Aita *et al.*, 1999) to investigate primary lymphatic organs in birds and to take again this subject into account, in this study we sought morphological-functional changes in the thymus of chicken embryos after surgical bursectomy performed at 68-72 hours. We studied histological and histochemical aspects, using PAS, Alcian blue, anti-cytokeratin methods and we investigated the expression of anti-PCNA/cyclin and anti-CD3, CD4, CD8 markers.

The cyclin, an acidic polypeptide of MW 36 kDa, was detected in the nucleus of several cells. Its synthesis is followed closely by DNA synthesis and its level increased in S phase of the cell cycle (Bravo *et al.*, 1981; Bravo, 1984, 1986; Bravo and Graf, 1985; Bravo and Macdonald-Bravo, 1987).

PCNA (the proliferating cell nuclear antigen) was demonstrated to be identical with cyclin, in sub-cellular localization, peptide composition and biological properties (Mathews *et al.*, 1984).

Thereafter, an auxiliary protein specific for DNA polymerase δ , was extracted from foetal calf thymus (Tan *et al.*, 1986) or from calf thymus (Crute *et al.*, 1986). Bravo *et al.* (1987) reported that cyclin/PCNA is the auxiliary protein of DNA polymerase δ . The markers CD3, CD4 and CD8 are expressed in the cortical and medullary zone of the thymus, in the ontogenetic period, by T lymphocytes, during their differentiation till to become mature thymocytes (Le Douarin *et al.*, 1984; Chan *et al.* 1988; Strominger, 1989; Janossy *et al.*, 1989; Dunon and Imhof, 1996, 2000).

Materials and Methods

Thymic specimens

Thymuses of Golden Comet strain (*Gallus gallus domesticus*) were removed at 17 days *in ovo* from chickens bursectomized at 68-72 hours according to the method of Fitzsimmons *et al.*, (1973) (provided by Civinini and Gallo). The description of the bursectomy method is described elsewhere (Civinini *et al.*, 1993, 1994). Of 864 chicken embryos operated, the 14.2% survived until 17 days of incubation, when we removed the majority of them. Six embryos survived until 1 day after hatching. Thymic specimens were fixed in Bouin's liquid, for 8 hours at room temperature, dehydrated and embedded in paraffin wax. Serial sections of 5 μ m thickness were stained by haematoxylin-eosin, Alcian blue, PAS (Periodic Acid Schiff) (Pearse, 1972) for histological and histochemical examination. Other sections were processed with immunohistological methods (anti-cytokeratin B, anti-PCNA/cyclin and anti-CD3, CD4, CD8 antibodies), and compared with normal thymuses and thymuses from sham-operated chickens of the same age.

Immunoreactions

Anti-keratin B. Before dewaxing, the thymic sections were trypsinized, using 0.05% trypsin (Difco Lab.) plus 0.05% CaCl₂, pH 7.8 for 10 min at 37°C, then treated by the peroxidase-anti-peroxidase (PAP) method using a monoclonal anti-keratin B antibody (high molecular weight cytokeratins n.11, 10,5, and 1-56, 56.5, 58, and 68 kDa respectively) that normally reacts with all squamous and ductal epithelia (Kits and method supplied by Ortho Diagnostic immunostaining system). Anti-keratin immunoreactivity was revealed by 3-amino-9-ethyl-carbazole (AEC-Ortho Diagnostic);

no counterstaining was used. The slides were dehydrated and coverslipped with Tissue Adhesive PC-380k (Ortho Diagnostic). For the negative immunoreaction control, the specific anti-keratin B serum was replaced with a non-immune serum (Ortho Diagnostic).

Anti-PCNA/cyclin. The method for visualizing the antibody anti-PCNA/cyclin has been reported in detail elsewhere (Alunni *et al.*, 2001). Briefly, the thymic sections were incubated with the primary antibody (anti-PCNA, PC 10 mouse monoclonal antibody (moAb) IgG Sigma, n.P-8825) (Casasco *et al.*, 1993) at the dilution of 1:1000 in 1% normal horse serum in phosphate buffer, pH 7.2, overnight at 4°C. The immunoreaction was revealed by 0.05% 3-3' diaminobenzidine (DAB) tetrahydrochloride with 1% nickel-sulfate and 0.01% hydrogen peroxide in 0.05% M Tris-HCl buffer (Sigma-Aldrich). No counterstain was used. Slides were then dehydrated and coverslipped using Entellan (Merck-Germany). The specificity of the immunostaining was tested by replacing the primary antiserum with immune horse serum alone.

Anti-CD3. Thymic sections were treated with a PAP method using an anti-CD3 antibody (a pan-T marker, clone UCHT1 by DAKO). The immunoreaction was revealed by 3-amino-9-ethyl-carbazole (AEC-immunostaining DAKO PAP kit). No sections were counterstained. Slides were then dehydrated and coverslipped with Tissue Adhesive PC-380k (Ortho). A non-immune serum (DAKO) was used as a negative control.

Anti-CD4 and anti CD8. Thymic sections were treated with indirect immuno-fluorescence (IIF). They were incubated with mouse anti-CD4 or anti-CD8 monoclonal IgG antibody, respectively (Sigma-Aldrich, Dorset, UK, species reactivity: chicken), 1:20 diluted in phosphate buffer saline (PBS), pH 7.3, for 1 hour at room temperature. After three washes in PBS, the sections were incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (GAM-FITC, Sigma-Aldrich, Dorset, UK), or tetramethyl rhodamine isothiocyanate (GAM-TRITC, Sigma-Aldrich, Dorset, UK), to reveal the immune reaction with CD4 and CD8, respectively. Control slides were performed by replacing the primary antibody by mouse non-immune IgG. After washes in PBS, sections were mounted in glycerin and observed at fluorescence out fit microscope (Leitz, Germany).

Statistical analysis

In each specimen, multiple sets of consecutive sections were differentially immuno-stained with the anti-PCNA and anti-CD3 antibodies. Counts of immunoreactive cells (nucleated only, cell area ranging from 4 to 100 mm²) in 1 mm² areas of thymuses were performed with a computer-assisted image analysis system (Axioscope-KS300-Zeiss) by an observer unaware of treatments. The number of immunoreactive cells was then calculated by averaging the cell numbers from 5 specimens per treatments, and expressed as the mean \pm standard deviation (SD). Measurements of cell diameter (major axis) were performed in 5-8 specimens and pooled. Numerical results were analysed by means of analysis of two-tail-T Student test.

Results

Since no morphological or immune-histochemical differences were found between thymuses from normal and sham-operated embryos, we henceforward refer to both groups as normal embryos.

Histological and histochemical findings

Haematoxylin-eosin, Alcian-blue and PAS-stained slides of thymus removed from 17-day old embryos, from normal chickens showed a well-marked subdivision between the cortex and the central medulla in each lobe (Figure 1A). The abundant lymphocytes in the cortical zone made it difficult to visualize the cortical epithelial cells. The medullary zone contained fewer lymphocytes, than the cortex, and epithelial cell clusters were clearly visible. Some of them had vacuoles, while others had a cystic appearance of the intra- or intercellular type (Figure 2A). The cytoplasm of a few intracellular cysts stained with Alcian blue, whereas the membrane of some intercellular cysts stained with PAS.

Conversely, the thymuses removed from bursectomized chickens were always smaller than those from controls. The cortex and medulla were also smaller but well separated and proportional in size (Figure 1B). The thymic cortex was thinner than normal cortex and contained fewer lymphocytes. The medulla contained few epithelial cell clusters made up of fewer cells, filled with amorphous materials (Figure 2B). Some Alcian blue positive cystic formations were dilated and irregularly shaped (Figure 2C). Other cystic cells reacted only to PAS.

Immunohistochemical reactions

Anti-cytokeratin B. In every normal thymus examined anti-cytokeratin B induced an intense immunoreaction seen in the subcapsular zone as a continuous line. The reaction was also visible in the cortex as thin filaments. In the medulla, anti-cytokeratin B immunoreactivity was concentrated in epithelial cell clusters and in reticular processes forming a large, compact and continuous mesh (inset Figure 1A). In bursectomized thymuses, as in normal thymuses, anti-cytokeratin B induced an intense immunoreaction in the subcapsular zone. In the cortex, the thin filaments were less numerous than those in the control thymuses. The medulla contained some small clusters, made up of few, immune-reactive epithelial cells; the reticular processes formed a loose mesh (inset Figure 1B) .

The results concerning anti-PCNA, -CD3, -CD4 and -CD8 are summarized in Table 1.

Anti-PCNA/cyclin. In normal thymuses the cortical lymphocytes exhibited an intense immune reaction indicating efficient proliferative activity. None of the medullary lymphocytes were immune reactive (Figure 3A). In bursectomized thymuses there were a significantly decreased number of strongly PCNA-immune reactive elements in the external cortex. The few other cortical lymphocytes visible were only weakly immune-reactive. None of the medullary lymphocytes were immune reactive. (Figure 3B, Figure 4).

Anti-CD3. In normal thymuses the cortical lymphocytes showed strong membrane immune staining forming the characteristic ring. The medullary lymphocytes were also positive (Figure 5A).

In bursectomized thymuses the statistical analysis showed a significantly increased number of positive cells in the cortex, whereas in the medulla there were a significant decrease of positive cells, located only in some zone of the medulla (Figures 5B, Figure 6).

Anti-CD4. In normal thymuses the thymocytes were intensely immune reactive at the border between cortex and medulla and in the medullary zone (Figure 7A). In bursectomized chickens the thymocytes, less numerous than in normal thymus, were strongly immune reactive in the medullary zone (Figure 7B).

Anti-CD8. In normal thymuses, the thymocytes of the medullary zone showed strong immune reactivity (Figure 7C). In bursectomized chickens, the thymocytes, less numerous than in normal thymus,

revealed an intense immune reaction (Figure 7D).

Thymic sections from normal, sham-operated and bursectomized chicken embryos treated with pre-immune sera were always negative.

Table 1. Immunological findings.

Immunoreaction	Normal embryos thymocytes in thymus	Bursectomized embryos thymocytes in thymus
anti-PCNA	++ total cortex -- medulla	++ external cortex -- medulla
anti-CD3	++ total cortex ++ medulla	+++ total cortex ± medulla
anti-CD4	++ border cortex-medulla ++ numerous in medulla	-- border cortex medulla ++ few in medulla
anti-CD8	++ numerous in medulla	++ few in medulla

-- = negativity ; ± = weak reactivity ; ++ = positivity ; +++ = strong positivity.

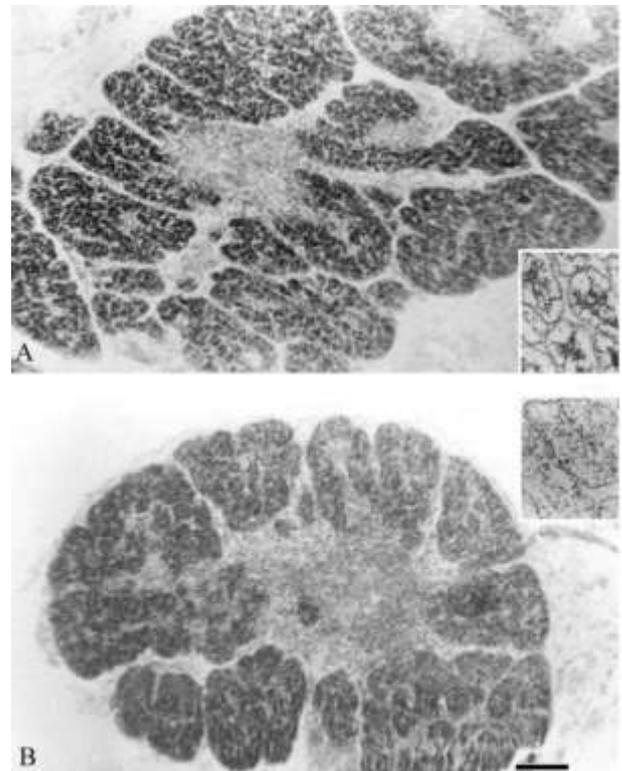


Figure 1. Haematoxylin-Eosin- A) Normal thymus. Inset: anti-cytokeratin B- The immunoreactivity is visible in the subcapsular zone, and in the cortex as thin filaments. Medullary clusters are strongly positive. B) Bursectomized thymus: note the size reduction and the rarefaction of cortical lymphocytes. Inset: anti-cytokeratin B- The subcapsular zone and the thin filaments are immunoreactive, but the medullary zone contains few immunoreactive epithelial cells. Scale bar (A), 80 µm; (B), 48 µm; (inset), 50 µm.

Discussion

Our findings provide new and detailed information on distinct morphological-functional changes in the thymus taken from bursectomized chick embryos, clearly showing that bursectomy interferes with thymic development.

These changes in hormonally or surgically bursectomized chickens have not been previously described in detail, because other investigators

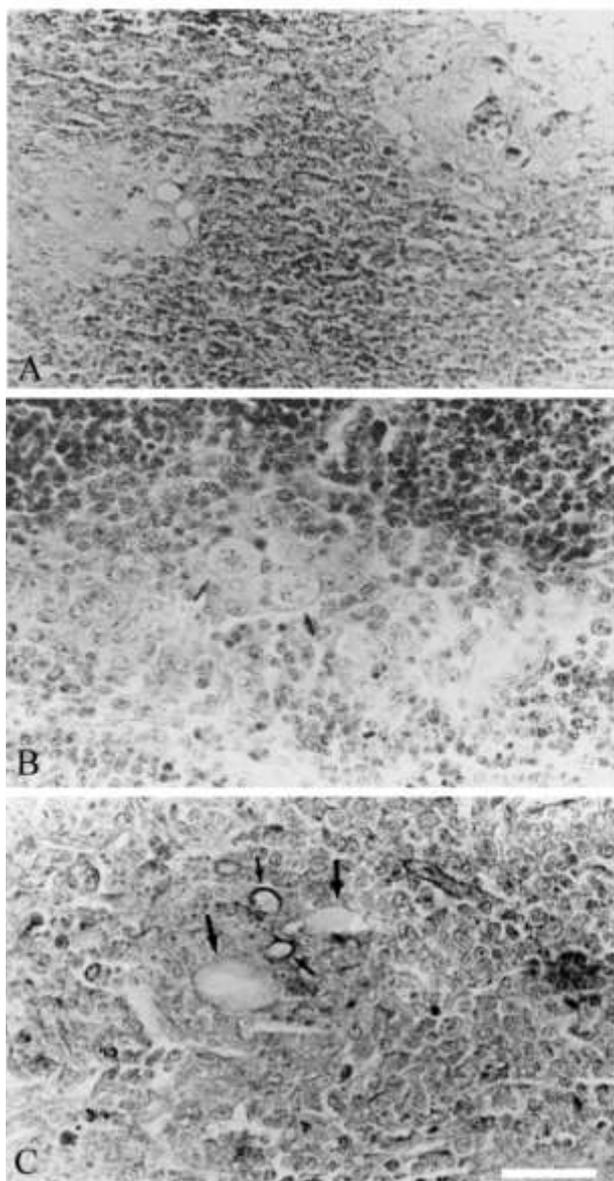


Figure 2. Haematoxylin-Eosin- A) Normal thymus: the medullary clusters are made up of epithelial cells, some have cystic appearance of the intra- or intercellular type. B) Bursectomized thymus: note that the cluster contains few cells, which are enlarged and contain granular material (arrows). C) Alcian blue- Bursectomized thymus: the cluster contains few cells, two are Alcian blue positive at the external line (arrows) and two are enlarged (headarrows). Scale bar (A,B,C,) 48 μ m.

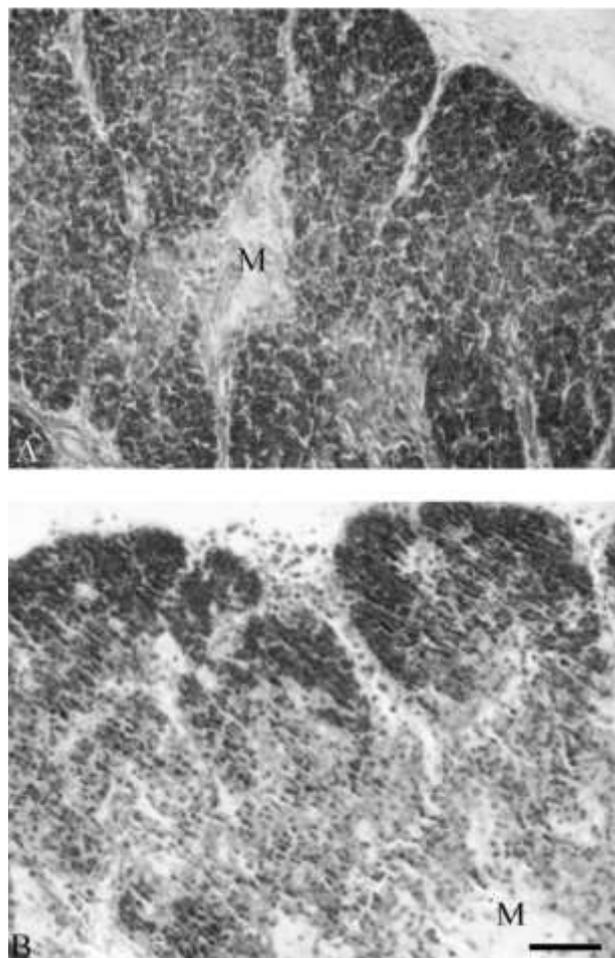


Figure 3. Anti-PCNA- A) Normal thymus: thymocytes throughout the cortex are strongly immunoreactive. B) Bursectomized thymus: only the thymocytes in the external zone of the cortex and other scattered inside the cortex show strong immunoreactivity. Scale bar (A, B), 60 μ m.

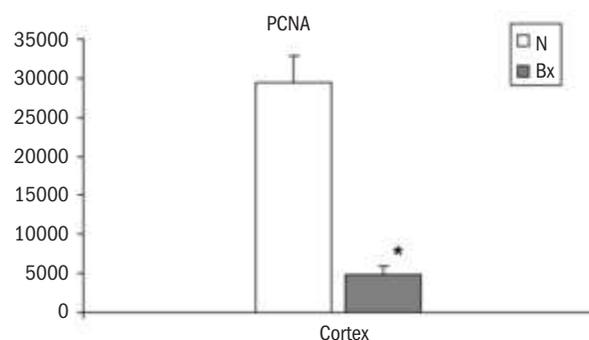


Figure 4. Anti-PCNA- Density (number of cells/mm²) of PCNA+ thymocytes (detected by immunohistochemistry) in thymus of normal (N) and bursectomized (Bx) chicken embryos. Data refer to quantitative analysis on tissue sections and are expressed as the mean \pm SD. The two tail Student t test for unpaired data shows a significantly decreased number of positive cells in cortex ($*p < 0,001$) of bursectomized as compared with normal embryos.

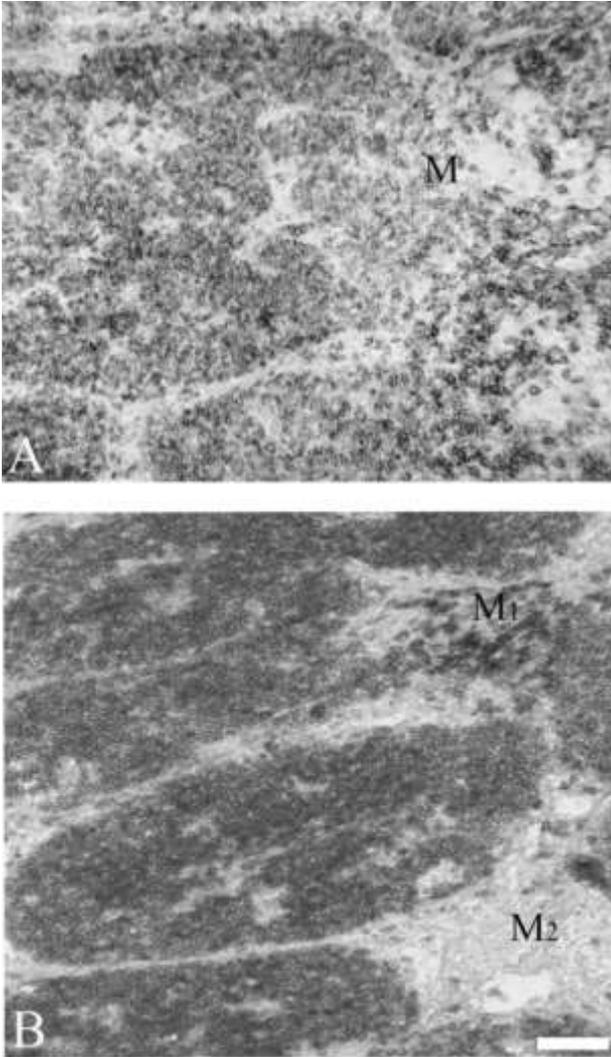


Figure 5. Anti-CD3- A) Normal thymus: every thymocyte in the cortex (C) and in the medulla (M) exhibits immunoreactivity. B) Bursectomized thymus: numerous cortical thymocytes are immunoreactive. Medullary thymocytes are immunoreactive in some zones (M 1) or completely lacking in other zones (M2). Scale bar (A,B), 55 μ m.

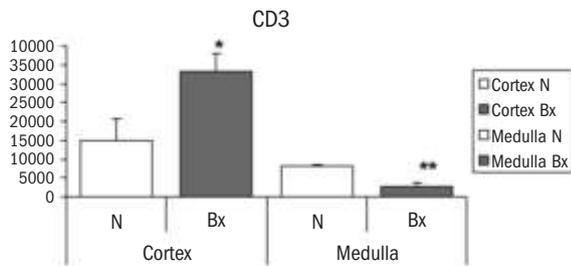


Figure 6. Anti-CD3- Density (number of cells/mm²) of CD3⁺ thymocytes (detected by immunohistochemistry) in thymus of normal (N) and bursectomized (Bx) chicken embryos. Data refer to quantitative analysis on tissue sections and are expressed as the mean \pm SD. The two-tail Student t test for unpaired data shows a significantly increased number of positive cells in cortex (* p <0,01) and a decrease of positivity in medulla (** p <0,001) in bursectomized as compared with normal embryos.

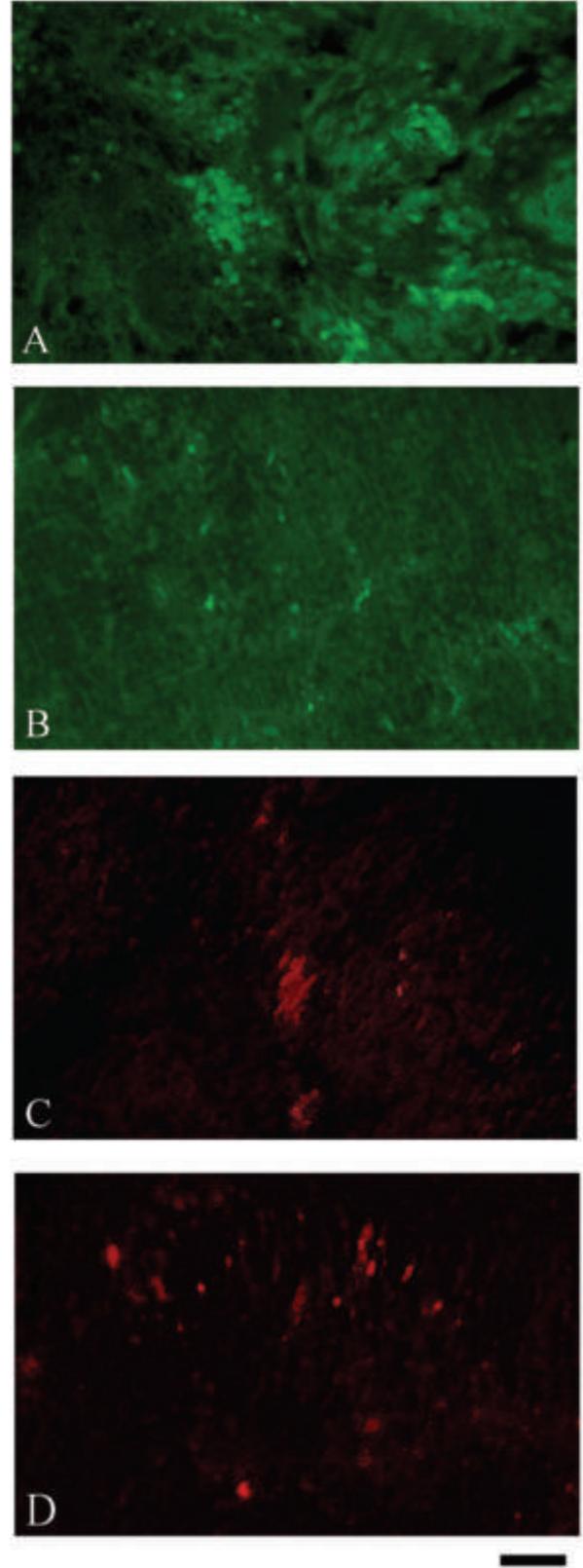


Figure 7. Anti-CD4- A). Normal thymus: thymocytes located at the cortical-medullary border and in medulla are immunoreactive. B) Bursectomized thymus: few thymocytes are immunoreactive in medulla.- Anti-CD8- C) Normal thymus: thymocytes are immunoreactive in the medulla. D) Bursectomized thymus: few thymocytes are positive in the medulla. Scale bar (A), 30 μ m, (B,C,D), 15 μ m.

focused their attention on antibody production rather than on the thymus morphology. Only in a study conducted in hormonally bursectomized chickens (Szenberg and Warner, 1962) the thymus was found normal in 60% of cases, had moderate cortical atrophy in 30% and complete cortical atrophy and severe degenerative changes in the medulla in 10%.

Conversely, in the present study, distinct differences in the thymus from surgically bursectomized chicken embryos and control thymus were found. The lack of bursa markedly affected epithelial cell development, well detected using the anti-B-cytokeratin serum immune-reaction, that normally reacts with all squamous and ductal epithelia of vertebrates and of normal and pathological human thymuses (Aita and Amantea, 1991). In the thymic cortical zone a reduction of the epithelial cells was evidenced; in these cells normally there is the production of the avian thymic hormone (ATH), a parvalbumin (Brewer *et al.*, 1990; Barger *et al.*, 1991; Kirali and Celio, 1993), that promotes immune maturation of T-lymphocytes (Murthy and Ragland, 1992). In the medullary zone there is the reduction in size and in number of clusters with some cystic formations abnormally large, and irregularly shaped with amorphous material stained weakly for PAS and Alcian blue, or strongly only to PAS. Other authors have described normal cysts in embryonic life, by electron microscopy (Chan, 1991, 1994; Romano *et al.*, 1996), but their function remains unclear. Previous ultrastructural evidence indicated secretory or absorptive activities, or both (Chan, 1991). Aita *et al.*, (1989a, 1995), Aita and Romano (2006) found that the external epithelial cells of some cysts in the cluster were positive to anti-thymostimulin-like immunoreaction. Thymostimulin, a calf thymic factor, is able to stimulate immunological functions and was detected in humans (Aita and Amantea, 1991) and in mammals (Aita *et al.*, 1984, 1989b).

We may suppose that the reduction of the thymic epithelial compartment is followed by an altered production of the hormonal factors, necessary for the maturation of T-lymphocytes.

As concern PCNA immune-reactivity we noted a markedly difference in control and bursectomized thymuses. The strong PCNA thymocytes labelling in control thymus indicated an efficient proliferative rate in the G1/S phase and is in contrast with the bursectomized thymus. The strong PCNA labelling

was present only in the external cortex of bursectomized thymus, whereas in few, scattered thymocytes of the remaining cortex there was a faint PCNA reaction. These differences are, in some way, difficult to explain in view of previous finding in human. In fact, it was reported that unstimulated human peripheral blood T-lymphocytes are PCNA negative, and its expression is evident only after stimulation and is regulated by a signal after IL2 binding and that PCNA is an indicator for T-lymphocytes committed to DNA synthesis and occurs later in G1 phase of the cell cycle (Kurki *et al.*, 1987). PCNA functions as a cofactor of DNA polymerase δ and is necessary for DNA replication in proliferating eukaryotic cells (Casasco *et al.*, 1993). In human Turka *et al.*, (1993) found that a PCNA protein higher level is present in immature double positive thymocytes ($CD4^+ CD8^+$) than both in single positive thymocytes ($CD4^+$ or $CD8^+$) and in peripheral blood T cells. The PCNA double positive thymocytes displayed low RNA content, characteristic of the resting cells in G0 phase. The authors (Turka *et al.*, 1993) indicated that the high levels of PCNA in these resting cells might mean a differential regulation during lymphoid development and contribute to the process of thymic selection. Even if another marker, the proto-oncogene *c-myc*, was found to be highly expressed in quiescent avian thymic lymphocytes (Thompson *et al.*, 1986). In contrast with the histo-immune localization of PCNA in different tissue (Yu *et al.*, 1992), few papers deal with thymocytes and thymus. At our knowledge we are the first to describe PCNA localization in normal embryonic avian thymus and much more in bursectomized thymus.

Obviously we cannot deduce if the strong reaction in every cortical thymocytes of the normal thymus may represent PCNA content only in resting cells or in S phase cells or in both. Anyway, also in the study of Turka *et al.*, (1993) thymocytes, more or less, were positive to PCNA reaction. On the contrary, in the bursectomized thymocytes, the faint reaction may indicate that the thymocytes are in a different period of the cell cycle, as indicated in an immune fluorescence study also in 3T3 cells, by Bravo and Macdonald-Bravo (1987). The authors described the existence of two populations of PCNA/cyclin expressed in resting cells and during the S phase from G1 to G2. Thereafter Morris and Mathews (1989) found in HeLa cells a fluctuating synthesis of PCNA during S phase and that these

cells maintain PCNA in excess of the amount involved directly in DNA replication.

In the medulla we do not find immune-reaction to PCNA in the thymocytes both of the normal and of the bursectomized thymus. This finding, in our opinion, reveals that thymocytes have ceased to proliferate, or that the reaction is very weak and not detectable.

Like PCNA, the expression of the CD3 marker also differed, to some extent, in normal and bursectomized thymus. In all cortical thymocytes of control and bursectomized thymuses, CD3 was well expressed. This finding fits in with evidence that at the time when pro-thymocytes proliferate and differentiate in the thymus, the cellular expression of both the T cell receptor (TCR) and the non-polymorphic parts of the TCR complex, are referred to as CD3 antigens (Janossy *et al.*, 1989). At different times in ontogeny various types of TCR-expressing cells have been evidenced: CD4⁻ CD8⁻ (double negative) TCR- $\gamma\delta$; CD4⁺ CD8⁺ (double positive), CD4⁺ CD8⁻, or CD4⁻ CD8⁺ TCR- $\alpha\beta$ (Strominger, 1989). The TCR associated with the molecular complex CD3, is necessary for the membrane expression of the $\alpha\beta$ heterodimer and serves to transmit the signal generated at the cell surface to the interior and thereby to induce the appropriate effectual function (Blackman *et al.*, 1990). The kinetics of T cell differentiation and maturation in the thymus were studied in mammals and in human (Campana *et al.*, 1989; Janossy *et al.*, 1989; Strominger, 1989). The immune system of the chicken functions in a similar way (Vainio and Imhof, 1995). Chan *et al.*, (1988) identified the avian homologues of the antigens CD4 and CD8 concluding that their expression is highly conserved in birds and mammals.

In chick embryo there are three waves of stem cells seeding in the thymus, recruited by chemotactic peptides produced by epithelial thymic cells (Champion *et al.*, 1986). The first wave occurs at 6-7 days, the second at 11-12 days and the third at 17 days (Le Douarin *et al.*, 1984; Penit *et al.*, 1985; Dunon and Imhof, 1996, 2000). After 5 days of initial influx a subpopulation of thymocytes begins to express the TCR- $\gamma\delta$ /CD3 complex and after 8 days TCR- $\alpha\beta$ /CD3 complex (Dunon and Imhof, 2000). In our experimental study, we may suppose that the thymus has received at least two waves of prothymocytes, one at 6-7 days and the second at 11-12 days. In fact in all cortical thymo-

cytes of bursectomized thymuses, CD3 was well expressed but it is noticeable that the number of these thymocytes is higher than that found in the control thymus as revealed by statistical analysis, and moreover, an interesting finding is that normal thymocytes expressed the CD3 marker in the total area of the medulla, whereas bursectomized positive thymocytes are less numerous and located only in restricted zones of the medulla. As concerns CD4⁺ and CD8⁺ markers, they are well expressed at cortico-medullary border and in medullary normal thymocytes, whereas in bursectomized thymus the thymocytes expressing these markers are less numerous. These findings prove, once again, that bursectomy prevents a normal, consequential development of thymocytes, blocking partially the differentiation and the expression from CD3 to CD4 and CD8.

We might put forward the hypothesis that in the condition of bursectomy there is the lack of its endocrine influence on thymus development as it happens for the adrenal glands or testis (Pedernera *et al.*, 1980) and in ovaries (Civinini *et al.*, 1993, 1994) where there is an alteration of steroidogenic activity. A partial restoration of the adrenal function was made by the administration of the hormone bursin isolated from the bursa (Audhya *et al.*, 1986; Ramade *et al.*, 1988; Youbicier-Simo *et al.*, 1993). In our study it is evident the decrease of the population of cortical and medullary epithelial cells and consequently this might mean a possible reduction of the synthesis of the hormones ATH and thymostimulin, necessary for the maturation of T-lymphocytes.

In conclusion, the morphological and functional methods we used provide well documented evidence that bursectomy interferes with thymic development, at least during embryonic life and that there is a functional interrelation between bursa and thymus.

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