Effects of partial decerebration and hypophyseal allograft in the thymus of chicken embryos: thymostimulin localization and enzymatic activities

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Changes in chicken embryo thymus after partial decerebration (including the hypophysis) and hypophyseal allograft were investigated. Chicken embryos were partially decerebrated at 36-40 hr of incubation and on day 12 received a hypophyseal allograft from 18-day-old donor embryos. The embryonic thymuses were collected on day 18 and examined with histological methods, tested for the anti-thymostimulin-like immune-reaction, and for histoenzymatic activities and compared with normal and sham-operated embryos at the same age. After partial decerebration, the thymic cortical and medullary compartments diminished markedly in size. Anti-thymostimulin, succinic dehydrogenase and ATPase enzymatic activities tested, yielded negative reactions. In partially decerebrated hypophyseal allografted embryos, the same thymic compartments improved and anti-thymostimulin-like immune-reaction and enzymatic activities partially recovered.

These findings confirmed the key role of hypophysis in thymic ontogenic development and provided new information in metabolic enzymatic pathways and synthesis of a thymostimulin-like substance in the thymus

Key words: hypophysectomy, hypophyseal allograft, chicken thymus, thymostimulin, histoenzymology

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The primary lymphatic organs, in birds, the thymus and bursa of Fabricius, play an important role in producing adaptive immunological responses and their development starts during embryonal life (Le Douarin *et al.,* 1984; Cooper *et al.,* 1991; Glick, 1994). During chicken embryonic development the various reticular-epithelial cells and humoral factors, that make up the thymic microenvironment, process T-cell precursors (Aita, 1992; Boyd *et al.,* 1992; Aita *et al.,* 1995). On day 18 of embryonic life, each thymic lobe is divided into two distinct compartments: the cortex, where lymphocytes overlie the epithelial cells, and the medulla, where the few lymphocytes intermingle with isolated or clustered epithelial cells. In the cortex, Kendall (1980) described large pale and small dark epithelial cells, Sugimoto et al. (1977) described cells with electron lucent vacuoles containing dense bodies, probably released outside the vacuoles, and Romano et al. (1996) showed light, intermediate and dark epithelial cells whose vesicles varied in quality and quantity. In the medulla, pale cells may be associated with Hassall's corpuscles (Kendall 1980), other epithelial cells have numerous cytoplasmic vacuoles and vesicles suggesting secretory activity (Sugimoto *et al.,* 1977; Kendall, 1980), and intracellular or intercellular cysts with microvilli and cilia, whose walls contained other cells with dense granules and mucous producing cells (Sugimoto *et al.,*1977; Kendall 1980; Chan, 1991; Romano et al. 1996), persisting in post-hatching (Frazier,1973; Isler, 1976) and in adult chicken (Aita *et al.,* 1995). The chicken thymic stromal cells were studied for phenotypic characterization with monoclonal antibodies (Boyd *et al.,* 1992). Some of these epithelial cells are thought to produce hormonal factors deputed to intrathymic maturation and differentiation of pre-

T lymphocytes into mature thymocytes or T-cells.

In birds, only one specific hormonal factor, the avian thymic hormone (ATH), a parvalbumin, has been extracted and localized only in cortical reticular-epithelial cells (Brewer *et al.,* 1990; Barger *et al.,* 1991; Kiràly and Celio, 1993), where it promotes immune maturation of T-lymphocytes (Murthy and Ragland, 1992). On the contrary, in mammals, including humans, various thymic hormonal factors have been extracted and localized in the subcapsular, cortical and medullary epithelial cells (Greeps, 1981; Aita *et al.,* 1984; Fabien *et al.,* 1988; Monier *et al.,* 1988; Aita and Amantea, 1991; Aita, 1992).

Among them, a thymic factor, thymostimulin, extracted from calf thymus (Falchetti *et al.,*1977) was able to stimulate immunological functions in experimental (Falchetti *et al.,* 1981, 1982) and in clinical studies (Aiuti *et al.,* 1979; Lin *et al.,* 1987). Anti-thymostimulin immuno reactivity was detected in humans (Aita and Amantea, 1991) and others mammals (Aita *et al.,* 1984, 1989b). A thymostimulin-like immunoreactivity was also observed in post hatching and aging avian thymuses (Aita *et al.,* 1989a, 1995). Few medullary cells, in particular, vacuolar and cystic epithelial cells, located around an epithelial cluster or arranged in small groups, were immunoreactive to anti-thymostimulin serum. Up to 3 months of age, these medullary cells showed an evident anti-thymostimulin-like immune-reaction whereas in 6-month-old thymus, they were only weakly immune-reactive. Thymostimulin-like immune reactivity was also localized in embryonic and adult avian bursa of Fabricius, mostly in follicle associated epithelium (FAE) cells (Aita *et al.,* 1989a, 1992; Mazzone *et al.,* 2003).

The thymus, as an endocrine gland is related to the hypophysis and to the other endocrine glands. These relationships are well described in mammals (Comsa *et al.,* 1982; Blalock, 1989,1994) but remain less clear in birds. After early partial decerebration (PD), including ablation of the hypophyseal anlage, the embryos decreased by 40% in total size, in comparison with normal, embryos. The thymus appears underdeveloped showing abnormally small cortex and medulla, scarce lymphocytes, fewer clusters with poorly differentiated reticular-epithelial cells than thymus from normal embryos (Jankovic *et al.,* 1981; Mastrolia *et al.,* 1986; Herradòn *et al.,* 1991). In an ultrastructure study, after partial decerebration Romano *et al.,* (1996) found evident variations in the cortical zone and the smaller medullary zone contains fewer clusters and fewer intracellular cysts than normal thymus. Decerebration also induces changes in T-cell differentiation. After a partial decapitation at 33-38 hrs of incubation, Moreno *et al.,* (1995) found that T-cell subsets, $CD4^+$ and $CD8^+$cells and TCRα,-expressing cells, declined.

After a hypophyseal allograft in partially decerebrated chick embryos, the total thymus increases in size; medullary epithelial cells increase in number (Mastrolia *et al.,*1986,1987) and cytologic features recover but do not altogether return to normal (Romano *et al.,*1996).

In this study we sought further information on thymic ontogenic development. We first investigated possible histological changes, and a thymostimulin-like immune-reaction in thymic medullary epithelial cells, from normal chick embryos, partial decerebrated embryo (PD), and hypophyseal allografted PD (PD+H). In the same three experimental groups we also investigated, with histoenzymatic methods, several enzymatic pathways and compared the results in thymus from normal chick embryos and adult aging chickens. .

Materials and Methods

Experiments

White Leghorn chicken embryos (*Gallus gallus domesticus*) were used for two series of experiments. In experiment 1, the prosencephalon, including the hypophyseal anlage, and the presumptive anlage of the Rathke pouch were removed from 36-40 hrs embryos using Fugo's technique (1940). In experiment 2, on day 12 of incubation, PD received a hypophyseal allograft onto the chorion-allantoic membrane from an 18-day-old donor embryo.

The experimental thymuses were compared with thymuses from normal and sham-operated chicken embryos of the same age. For the sham-operation a small window was opened in the embryonic shell, at 36 hrs and covered with a slide and sealed with paraffin wax at 40 hrs. On day 12, the sham-operated embryos were opened for the second time and then covered and sealed again. On day 18 of incubation, thymuses were collected from normal and

sham-operated embryos, PD, and PD+H. Five embryos per group were utilised. Each experimental chick was examined to ascertain hypophyseal removal. In allografted PD the grafted hypophysis was examined histologically. The embryonic stage was evaluated by the days of incubation and by Lillie's tables of development (Hamilton, 1952).

Thymic specimens

Two thymic lobes from right side and two from left side of the neck, of all groups, were fixed in Bouin's liquid, for 8 hrs at room temperature, dehydrated and embedded in paraffin wax. Serial sections 5 μm thick were stained by haematoxylin-eosin, for histological examination and other sections for anti-thymostimulin immune-reaction.

Anti-thymostimulin immune-reaction

The preparation and properties of rabbit anti-bovine thymostimulin (TP1 Serono-R) serum have been described in detail elsewhere (Aita *et al.,*1981). In brief, serum specimens were collected from hybrid rabbits for use as the control pre-immune serum. The same hybrid rabbits were then immunized with several intra-muscular injections using 1 mL of calf thymostimulin (10 μg/mL) emulsified in 1 mL of complete Freund's adjuvant. Antibody titres of rabbit anti-thymostimulin total serum or purified IgG were tested after extensive absorption with calf liver and spleen tissue powders or with liver, spleen and thymus cells, by immune-electrophoresis. Total serum or purified IgG were also tested against other thymic hormonal factors or sera of mammals and humans, using the double diffusion technique on agar-gel. To saturate the antibody, total serum was then adsorbed with thymostimulin powder (TP1 Serono-R). The 5-μm sections, dewaxed, dehydrated in absolute ethanol and incubated in 1.65% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase, were processed with the indirect peroxidase-anti-peroxidase (PAP) method (Sternberger *et al.,* 1970), overnight at 4° C, using rabbit anti-calf thymostimulin serum (dilution 1:100). For the ensuing steps an Ortho Diagnostic Kit was used as previously reported (Aita *et al.,* 1989b). The immune-reaction was revealed by 3-amino-9-ethyl-carbazole (EAC) in N-dimethyl formamide in sodium acetate buffer pH 5.0 (Ortho Diagnostic System). None of the sections were counter-stained. For negative immune-reaction controls, sections of thymus were treated with rabbit pre-immune or adsorbed serum instead of specific anti-serum. Calf, human or chicken thymus was used as a positive control (Aita *et al.,* 1984, 1995; Aita and Amantea, 1991).

Histo-enzymatic reactions

Other thymic lobes, from right and left side of the neck of all groups, were frozen by liquid nitrogen and sectioned by a Leitz microtome in a cryostat, 5 μm thick serial sections were submitted to the following enzymatic methods.

Succinate dehydrogenase (SDH-EC 1.3.99.1) was identified by the nitro blue tetrazolium (NBT) method of Nachlas *et al.,* (1957); lactate dehydrogenase (LDH-EC 1.1.1.27) was localized by the method of Verne *et al.,* (1961); NADH (EC 1.6.99.3) and NADPH (EC- 1.6.99.1) diaphorase were revealed by the procedure reported by Scarpelli *et al.,* (1958). Ca^{++} ATP-ase (EC 3.6.1.3) was identified at pH 8.5 by the method of Wegmann and Bankowski (1960). Mitochondrial α-glycero-phosphate dehydrogenase (α-GPDH-EC 1.1.1.8) activity was identified by the method reported by Pearse (1972).

Sodium glycerophosphate, sodium succinate, sodium lactate and nitro-blue tetrazolium were purchased from Sigma (Sigma Chemical Co., St. Louis MO); sodium NADH, sodium NADPH and sodium ATP were purchased from Boehringer Mannheim GmbH. Negative control sections, were incubated in media without specific substrates.

Results

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

Histological findings

In sections of thymus from 18- day old normal embryos, haematoxylin-eosin staining revealed a lobular structure (Figure 1A). The cortex was enriched with densely packed lymphocytes that masked the reticular-epithelial cells. The medulla was essentially located in the centre of the organ and typically consisted of large clusters made up of many epithelial cells, interspersed with evident

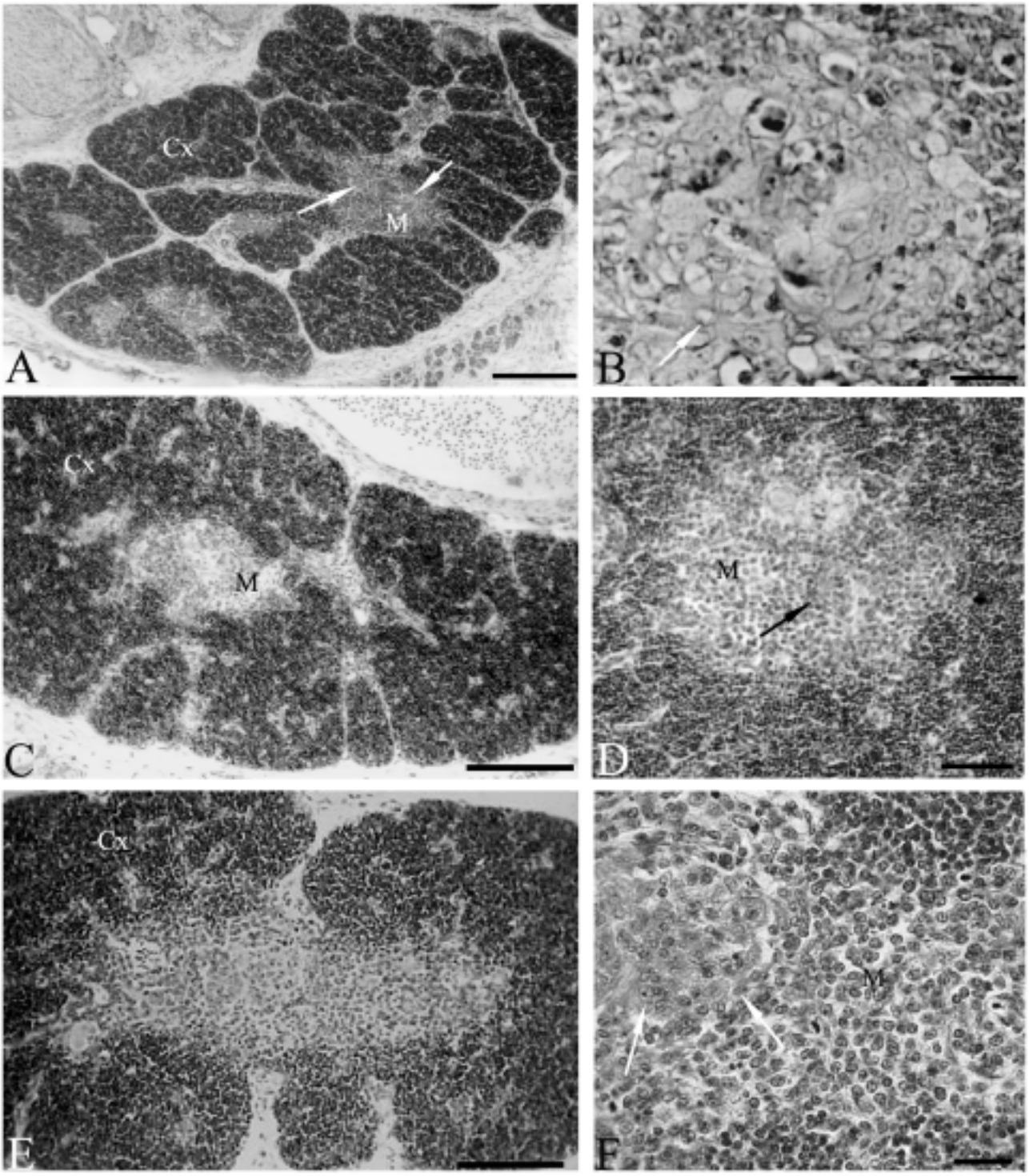

Figure 1. Staining with haematoxylin-eosin (Cx=cortex; M=medulla): (A) Normal embryo thymus. (B) Partially decerebrated embryo (PD) thymus. Note that the medulla is very small. (C) PD with a hypophyseal allograft (PD+H) thymus, showing the evident recovery of the total size and of the medulla. (D) Normal embryo thymus, in the medulla an epithelial cluster(arrow). (E) PD thymus, in the medulla isolated epithelial cells and a very small cluster (arrow). (F) PD+H thymus, showing a partial recovery of a medullary epithelial cluster (arrow). Scale bar (A,B,C),40 µm; (D,E), 22 µm; (F), 15 µm.

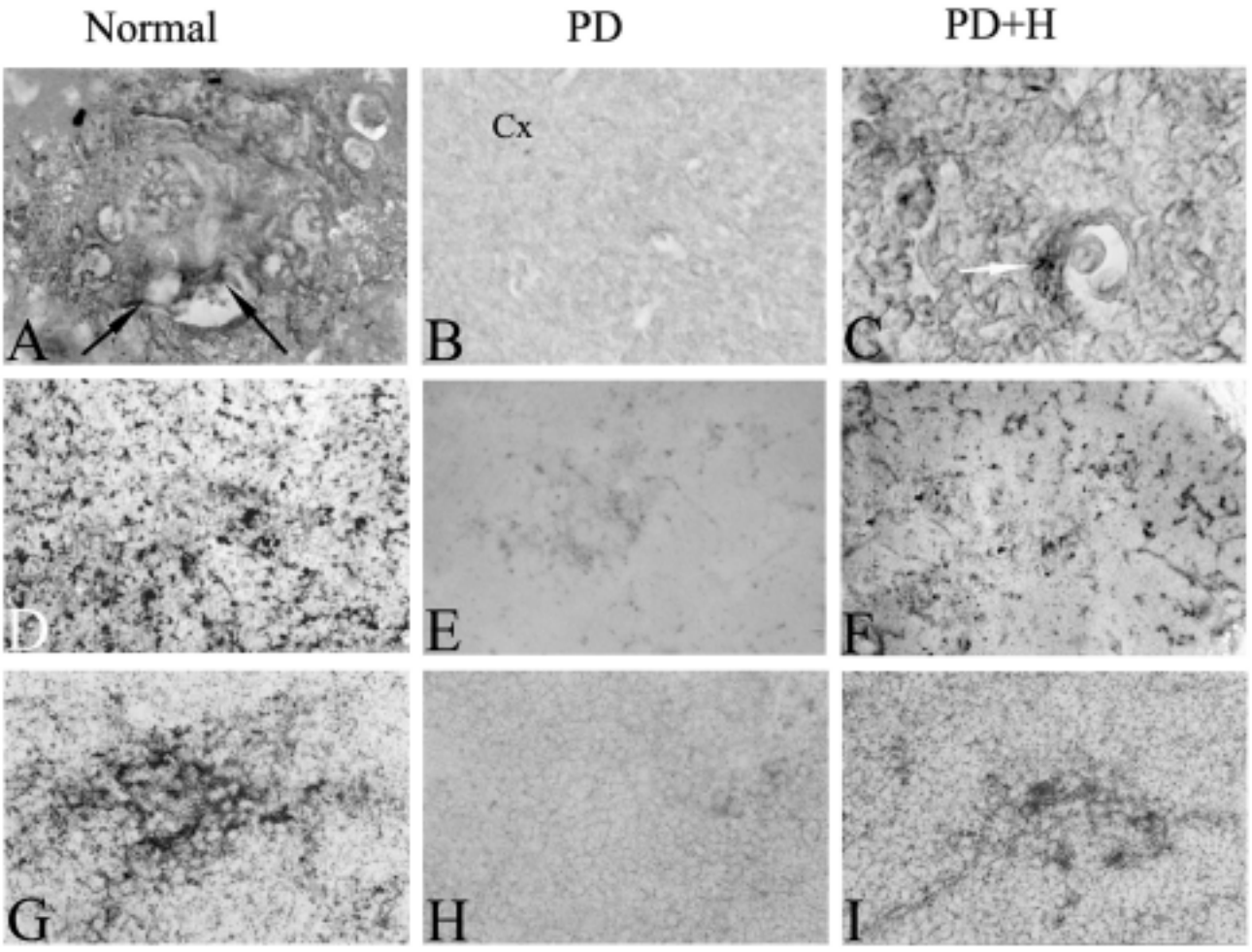

Figure 2. (A, B, C) Anti-thymostimulin reaction. (A) The reaction is located in some external epithelial cells of the cluster (arrows). (B) The reaction is negative, Cx=cortex, M=medulla. (C) Some medullary epithelial cells of the cluster express a faint reaction (arrow). (D, E, F) ATPase pH 8.5 reaction: (D) The enzymatic reaction is localized in the cortical reticular-epithelial cells with their thin processes, in the connective septa, and in the medullary macrophages. (E) The enzymatic reaction is negative. (F) The reaction is positive, less intense than in NE, at the level of the connective septa, in the few cortical reticular-epithelial cells and in the few medullary macrophages. (G, H, I) SDH reaction. (G) The epithelial cells of the cluster are positive. (H) The reaction is negative. (I) The reaction has recovered but is fainter than in normal embryos. Scale bar, 12 μm.

intracellular or intercellular cysts (Figure 1 B). No myoid cells or Hassall's corpuscles were observed.

The cortex from PD was abnormally narrow (Figure 1C) and the connective septa contained eosinophilic leukocytes. The epithelial cell clusters in the small medullary zone in the centre of the organ were drastically reduced in number and cell density; cystic cells were hard to identify (Figure 1D).

The cortex from PD+H contained a rich lymphocyte population (Figure 1 E) and fewer eosinophilic leukocytes than the cortex from PD. The medullary epithelial clusters and cysts increased in number but always remained less numerous than they were in normal embryos (Figure 1 F). The epithelial clusters were interspersed with numerous lymphocytes.

The histological analysis of the grafted hypophysis stained with haematoxylin-eosin showed a well-preserved cordonal-cellular organization. The graft contained mainly adenohypophyseal tissue, with scarce neurohypophyseal fibres (*data not shown*).

Anti-thymostimulin reaction

In normal embryos, the only elements that expressed strong anti-thymostimulin-like immunereactivity were the reticular-epithelial cells of the medullary clusters, especially the intercellular and intracellular cysts (Figure 2 A). The cortical and

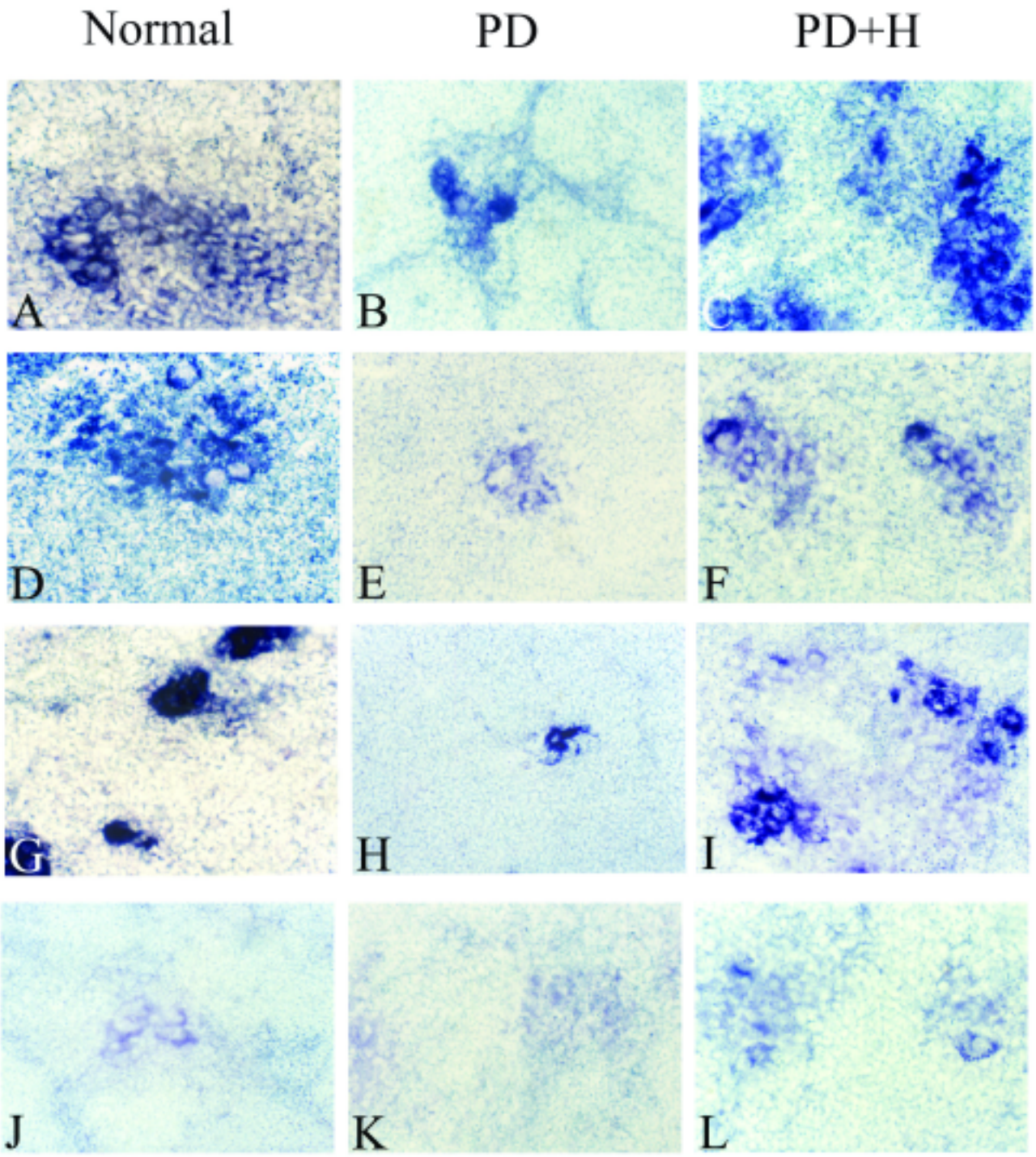

Figure 3. (A, B, C) LDH reaction. (A) Strong reactivity in the epithelial cluster. (B) Strong reactivity in the small cluster. (C) Strong reactivity in the recovered clusters. (D, E, F) NADH reaction. (D) Strong reactivity in the epithelial cluster. (E) Faint reaction in the small cluster. (F) Strong reactivity in the recovered clusters. (G, H, I) NADPH reaction. (G) Very strong reactivity in the epithelial clusters. (H) Strong reactivity in a very small cluster. (I) Strong reactivity in the recovered clusters. (J, K, L) a-GPDH reaction. (J) Faint reaction in some epithelial cells of the cluster. (K) Very faint reaction in some epithelial cluster. (K) Recovered reaction in the clusters. Scale bar, 12μm.

medullary lymphocytes lacked anti-thymostimulin-like immune-reactivity. In PD, no elements were immune-reactive for anti-thymostimulin (Figure 2B). In PD+H, some medullary epithelial cells were immune-reactive for anti-thymostimulin, but they never stained as intensely as they did in normal embryos (Figure 2C).

Negative control thymuses, treated with pre-immune or adsorbed serum, showed no immune-reactivity.

Histoenzymatic reactions

ATP-ase. In normal embryos, the capsule, connective septa and the cortical-reticular epithelial cells showed a marked reaction whereas lymphocytes did not. In the medulla, macrophages were also positive (Figure 2D). In PD, neither the cortex nor the medulla were reactive (Figure 2E). In PD+H, the connective septa and a few cells in the cortex and in the medulla were reactive; this reactivity was less intense than in normal embryos (Figure 2F).

SDH. In normal embryos, the cortical and medullary lymphocytes showed mitochondrial enzymatic reactivity. In the medulla a positive reaction was visible in the epithelial clusters (Figure 2G). In PD, SDH reactivity was less intense in lymphocytes than in normal embryos, and epithelial cell clusters gave a negative reaction (Figure 2H). Although the enzymatic reactivity was more intense in PD+H, it never reached the intensity seen in normal embryos (Figure 2I).

LDH. In normal embryos, the cortical (*not shown*) and medullary lymphocytes and the medullary reticular epithelial and cysts showed cytoplasmic enzymatic reactivity (Figure 3 A). In PD, the cortical (*not shown*).and medullary lymphocytes showed weaker enzymatic reactivity than they did in normal embryos. The few epithelial cells of the central medulla were clearly reactive (Figure 3 B).

In PD+H, the cortical lymphocytes (not shown), revealed a positive reaction and the medullary lymphocytes stained less intensely than they did in normal embryos. Whereas the improved medullary clusters were strongly reactive (Figure 3 C).

NADH. In normal embryos, the cortical (not shown) and medullary lymphocytes were intensely stained. Strong reactivity was also visible in medullary epithelial clusters (Figure 3 D). Also in PD, the cortical and medullary lymphocytes were reactive. Small clusters made up of fewer epithelial cells than in normal thymus were weakly reactive (Figure 3 E). In PD+H, both the lymphocytes and the reticular epithelial cells of the improved medullary clusters were NADH positive (Figure 3 F).

NADPH. In normal embryos, lymphocytes showed an evident reaction and medullary reticular-epithelial clusters displayed a very intense reaction (Figure 3G). In PD, the reaction was particularly evident in a few epithelial cells of the medulla (Figure 3 H). In PD+H, the reticular-epithelial cells of the clusters showed enzymatic reactivity but it was invariably weaker than that seen in normal embryos (Figure 3 I).

α-GPDH. In normal embryos, the cortical (not shown) and medullary lymphocytes were positive. The medullary epithelial cells of the cluster showed a faint positive reaction (Figure 3J). In PD, the few medullary epithelial cells showed a fainter reaction than in normal embryos (Figure 3K). In PD+H, lymphocytes and medullary epithelial cells were more reactive than in PD (Figure 3L).

No enzymatic reactions were detected in the control sections incubated without the specific substrates.

Discussion

The distinct morphological-functional changes we observed by comparing histological, enzymatic and immunological features in normal chicken embryos, PD and PD+H provide new information on thymic ontogenic development. As well as confirming previous histological observations, this study provides new data showing differences in the thymustimulin-like immunoreaction in thymic medullary cystic epithelial cells from normal, PD, PD+H chick embryos. Overall, these findings suggest a possible role of cysts in the thymostimulin production: the presence of thymostimulin-like immunoreaction in the medulla of birds could has some immunomodulatory function, additional or complementary to those of ATH. In PD, our observations about TS-like immunoreactivity was clearly negative. On view of this data it could be suggest that PD might has lacked the thymostimulin-like reactivity because hypophysectomy either blocked thymostimulin synthesis or prevented epithelial cysts from differentiating earlier. In previous study

(Herradòn *et al.,*1991) of 17 day-old thymuses after hypophysectomy (at 33-38 hrs) it was observed a delayed of epithelial cysts formation. Using ultramicroscopy, others have described normal cysts in embryonal life (Chan, 1991,1994; Romano *et al.,* 1996). Although the ultrastructural evidence indicated secretory or absorptive activities, or both (Chan, 1991) the punctual function of these activities remains unclear.

Our histoenzymatic study, investigating several enzymatic metabolic pathways, identified important differences between thymus from normal, PD and PD+H embryos. For example, we observed less LDH and SDH reactivity in the cortical and medullary lymphocytes in PD than in normal embryos, suggesting an interference of hypophysis also in aerobic and anaerobic glycolytic pathways. Moreover, the medullary epithelial clusters, even if drastically reduced in number and made up of fewer epithelial cells, showed LDH and NADPH reactivity, but only a weak reaction for NADH, α-GPDH, intervening in the formation of gkycerolipids, and a negative reaction for SDH and ATP-ase, compromising the aerobic glycolitic pathway and the storage of ATP. Others also found negative ATP-ase and α-esterase reactions in PD (Herradòn *et al.,* 1991). Overall, these data support the idea that partial decerebration, interferes with normal thymic development, with lymphocyte differentiation and most important, with the maturation of epithelial clusters, epithelial cysts and the synthesis of thymostimulin, and therefore intervenes also in some metabolic pathways.

Using the chick-embryo hypophyseal allograft, in this histo-immunological and histo-enzymatic study, we confirm that these morphological-functional changes arose not from the nervous system but from the hypophysis gland itself. We are in agreement with Betz (1967) who made a partial decerebration, using a modification of Fugo's method (1940), at 33-45 hrs of incubation and chorio-allantois grafted after nine days, with pars distalis from ten day-old donors. In grafted embryos, the weight and the histological structure of analysed organs, thyroid, adrenal glands, testes and left ovaries were recovered. The conclusion of the author was that the defects observed in hypophysectomized embryos could be correlated with the lack of pars distalis rather than the hypothalamus or other parts of the brain, and that the ectopic embryonic pars distalis may secrete normal amounts of somatotropic, thyrotropic, gonadotrophic and adrenocorticotrophic hormones. Woods and Thommes (1984), Woods (1987), Méndez *et al.,* (2005) reach to the same conclusions, studying left ovaries and testis of chicken embryos hypophysectomized at 44-46 hrs of incubation and grafted after nine days, with an adenohypophysis from ten days-old donors.

To our knowledge, our laboratories were the first to graft a hypophysis onto PD (Mastrolia *et al.,* 1987; Romano *et al.,* 1996) to verify the influence of hypophysis in the development of the morpho-functional functions of the thymus. The reason why we chose to graft a hypophysis taken from an 18 day-old embryo in a 12 day-old decerebrated embryo is that the hypophysis is completely active at 18 days. In fact, Tixer-Vidal (1973) reported that, using tinctorial or cytochemical methods, the first signs of cellular differentiation in the embryonal adenohypophysis appear, in the cephalic lobe, on day 6 or days 10-11, whereas in the caudal lobe, much later, on day 15 or days 18-19. By day 12 the normal thymus should be sufficiently developed because its development begins at day 5 of incubation (Romanoff, 1960). Thus, the six days of incubation from day 12 to 18 allowed thymic morphological, enzymatic and endocrine functions to improve even if they do not return to normal. These morphological changes matched the marked improvements described in an ultrastructural study (Romano *et al.,*1996). Hence our data provide definitive evidence of the necessity of hypophysis for efficient and complete development of the thymus. The data strongly support the existence of an active endocrine-immune circuit but leave open the question of the hypophyseal hormone or hormones involved. We may assume that growth and thyrotropic hormones are necessary for a correct development, as ascertained by Betz (1967) for other embryonic organs. In an early study on birds Scanes *et al.,* (1986) also performed a hypophysectomy in chickens at 20-22 days post-hatching and treated them either with growth hormone, or with the thyroid hormones T3 and T4 between 25 and 49 days of age. After hypophysectomy the thymus decreased by 31.3% in weight and after T3 injection increased by 93.8%. After injection of growth hormone it increased by 56.1%. Unfortunately no histological study was done. Which hormones of the adenohypophysis may

intervene on the regulation of the production of thymostimulin in birds thymuses is not known. Blalock (1989) and Fabris *et al.,* (1997) reported some immune-hormonal interactions between thymus and endocrine glands only in mammals.

As concerns the comparison of the histo-enzymatic reactions we observed in normal embryonal thymus in these experiments with those in the adult aging thymus in a previous study (Aita *et al.,* 1995) showed that the cortical and medullary lymphocytes exhibited similar enzymatic reactivity at both periods. Whereas the embryonal cortical epithelial cells reacted only weakly to these enzymes, except to ATP-ase and were therefore harder to identify, the medullary epithelial clusters and cysts exhibited the same enzymatic reactivity shown in the adult, except for α-GPDH, which in the adult thymus was reactive only in the myoid cells, cells that are also reactive to ATP-ase. These diverse findings may reflect the need for an ontogenetic period of differentiation before the thymus reaches complete maturation after hatching. Even in 6 month-old chickens, an age when the thymus is already regressing, the scarce medullary clusters display intense enzymatic activity, possibly expressing continued, albeit reduced, thymic vitality and functionality.

In conclusion, our findings in chick embryos confirm the role of the hypophysis in lymphocyte-differentiation and epithelial cell growth during thymic ontogenic development. They also provide new information on the role of the hypophysis in modulating some metabolic enzymatic pathways and synthesis of the thymostimulin in the thymus.

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