DEVELOPMENTAL CHANGES IN TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE OF THE CHICKEN THYMUS*

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Received December 15,1976

SUMMARY : Terminal deoxynucleotidyl transferase activity begins to be detectable in the thymus of 14-day old chicken embryos. It reaches a maximum 3 weeks after hatching, and persits at a fairly high level in 21-weeks old chickens. Multiple chromatographic forms of TdT are detected, and the relative proportions of these forms change during the development of the chicken.

INTRODUCTION

Among the eukaryotic deoxynucleotide-polymerizing enzymes, terminal deoxynucleotidyl transferase (TdT)has two unique properties : its ability to catalyze the synthesis of polydeoxynucleotides in the absence of template (1-3), and its tissue distribution which is restricted to the thymus (4) and, at a low level, to the bone marrow (5,6) of all the animal species examined. It is also present in certain types of leukemic cells (5-10). In the thymus of humans (6), mouse (11), and rat (12), TdT is unequally distributed among the different types of cells, and seems to be a marker of "pre-T cells" ; these latter cells might also be responsible of the low activity found in the normal bone marrow. The physiological role of TdT is unknown but its remarkable tissue distribution and its catalytical properties *in vitro* have led Baltimore (13) to propose that it might act as a somatic mutator, implicated in the generation of antibody diversity.

The chicken thymus also contain high levels of TdT, whose properties are very similar to those of the mammalian enzyme (our data, not shown here). Because the differentiation organs of the T-cells and B-cells are separate in the chicken (14), the avian system is of particular interest for immunological studies, and the easy availability of embryos is very useful for developmental studies.

* Supported by grants obtained from INSERM (A.T.P. n° 14 75 37) and from NATO (n° 1134).

In this communication we present experiments showing the important changes of TdT activity that occur during the development of the chicken embryo and of the chicken from the time of hatching up to five months.

MATERIALS AND METHODS

Animals : Leghorn chicken embryos (S.O.R.G.A., Paris) and virus-free Leghorn chickens (Institut Gustave Roussy, Villejuif) were used in this study.
Tissue preparation : Thymuses were dissected after killing of the animals by ether (chicken) or decapitation (embryos). In the case of the smallest embryos (8 days), the whole neck was taken. The isolated thymuses were washed in phosphate-buffered saline (PBS), frozen in liquid nitrogen and kept at -80°C until enzyme extraction. For microscopic examination, the thymuses were minced over a stainless steel mesh, washed in PBS enriched with normal calf serum, and stained with Giemsa.
Enzyme extraction and phosphocellulose chromatography : Thymuses (1 to 2 g) were suspended in 10 ml of TEM buffer (50 mM Tris-HCL pH 7.9,1 mM EDTA, 1.4 mM β-mercaptoethanol)containing 0.4 mM phenyl methyl sulfonyl fluoride (PMSF) and 0.5 % Nodinet. The suspension was homogenized 1 minute in the Sorvall Omni-Mixer. 10 ml of TEM containing 3 M KCl were added, and another homogenization (30 sec.) was carried out to reduce the viscosity. The "PMSF-detergent-high salt extract" was centrifuged for 60 minutes at 140,000 xg. The pellet was discarded and the supernatant dialyzed either for 16 hours against 2 changes of TEM buffer containing 20 % glycerol (TEMG) or for only 3 hours against the same buffer and diluted until molarity of the KCl (checked by conductivity measurement) was below 0.1 M. The turbid dialysate was centrifuged for 20 minutes at 12,000 xg. The extract was adsorbed onto a phosphocellulose (P II, Whatmann) column (0.5 x 8 cm) previously equilibrated in TEMG buffer containing 50 mM KCl, at a flow-rate of 10 ml/hr. The column was washed with 5 to 10 volumes of 0.1 M KCl-TEMG buffer containing 0.5 mg/ml of bovine serum albumin (TEMG-BSA). The enzyme was eluted either stepwise by 0.6 M KCl-TEMG-BSA buffer or by a linear gradient from 0.1 to 0.6 M KCl in TEMG-BSA (30 ml in each chamber). In some cases, the KCl concentration of the final buffer was 0.8 M. Fractions of 1 ml were collected and assayed for TdT activity.
Terminal deoxynucleotidyl transferase assay : The assay mixture (100 µl) contained 50 mM Tris-HCl (pH 7.9), 2 mM DTT, 0.4 mM $MnCl_2$, 0.5 µg $d(pA)_{10}$ (Collaborative Research), 0.02 mM dGTP, 2 µCi of ^3H dGTP (Amersham) and 10 to 25 µl of the enzyme fraction. In all case, the concentration of EDTA

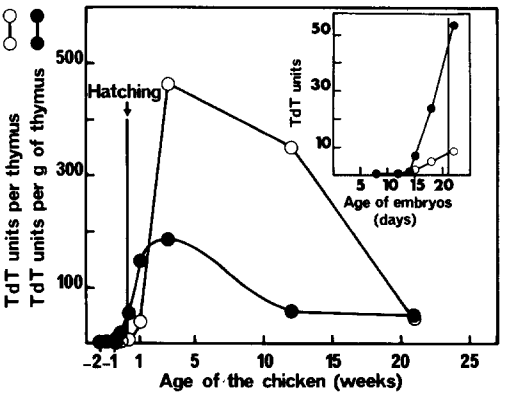

Figure 1 : Changes in the total TdT activity in the thymus of developping chicken. The values indicated were obtained by totalling the activity found in the active fractions recovered after phosphocellulose chromatography. Three separate determinations gave similar results.

in the assay mixture was maintained at 0.25 mM which is the molarity of EDTA when the higher amount of enzyme fraction is added. The reaction mixture was incubated for 30 minutes at 37°C, and 75 µl were adsorbed on Whatmann 3 MM paper disks presoaked in 10 % trichloracetic acid (TCA). The disks were washed 3 times in 5 % TCA and twice in absolute ethanol, dried by ether and counted in a toluene-PPO-POPOP scintillation mixture.

To determine very low activities or to verify negative results, we occasionally replaced the paper disk technique by filtration over GF/C disks, which gives lower backgrounds.

One unit of TdT is defined as the amount of enzyme incorporating 1 nanomole of dGMP into an acid-insoluble product in 30 minutes in the conditions described.

RESULTS

Evolution of TdT activity : Figure 1 shows the levels of activity found in the fractions obtained after chromatography of thymus extracts on phosphocellulose. Stepwise chromatography was used for the youngest embryos (8 and 12 days), and we controlled with older embryos and chickens that this technique gives similar results to linear gradient elution for the quantitation of total activity. When detected by our method, TdT begins to appear in the thymus of 14-day old embryos. The activity per gram of thymus increases very rapidly from 1 week before hatching (0.5 unit per g) to 3 weeks after hatching, when it

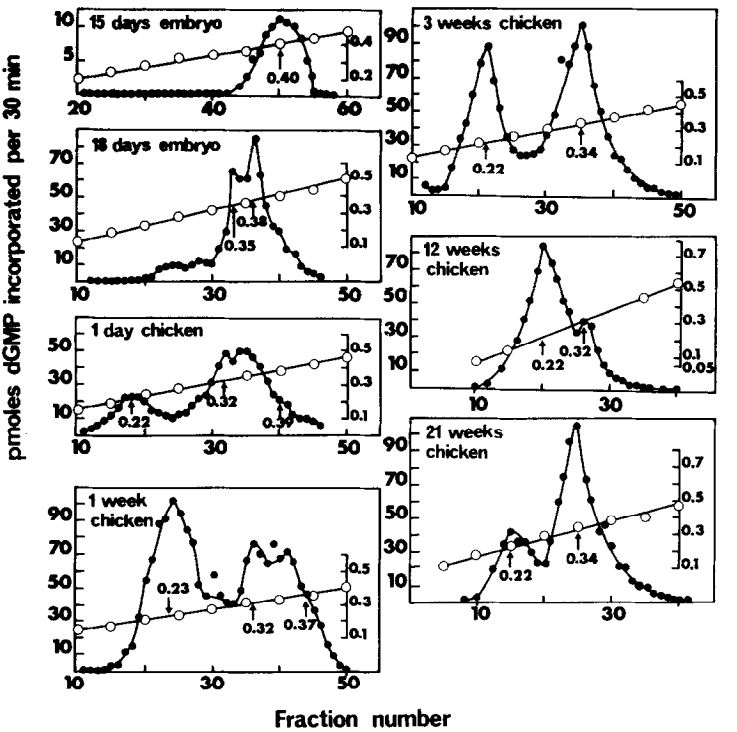

Figure 2 : Phosphocellulose chromatography of TdT. The quantities of thymus were : 15-day old embryos : 2g ; 18-day embryos : 3g ; 1-day chicken : 2g ; 1 week-chicken : 1.5g ; 3-week chicken : 1.2g ; 12-week chicken : 1g ; 21-week chicken : 1.8 g. In the two last cases, the gradient was from 0.1 to 0.8 M KCl. For technical details, see materials and methods. TdT activity (●——●) was measured with 10 µl of each enzyme fraction. The KCl molarity (o——o) was estimated by conductivity measurement.

reaches a maximum (184 units per gram). After that age, TdT activity decreases to a level which remains nearly constant from 12 to 21 weeks (50-60 units per gram). During the same period, the weight of the thymus undergoes very important changes, so that the evolution of the TdT activity per animal is even more important than that of the activity per gram of thymus. Possible interpretations of these results are presented in the discussion.

Evolution of chromatographic forms of TdT : Gratient elution of TdT from the phosphocellulose column never yielded a unique activity peak. In our study, and depending on the age of the animals, three different forms of TdT can be defined, depending on the molarity of KCl at which they are eluted from the phosphocellulose : approximately 0.22, 0.34, and 0.38 M. As shown in Figure 2 the 0.38 - 0.40 M peak is clearly present in the embryos, but can only hardly be detected as a shoulder in the young chicken. From the chromatogra-

phic profiles, it is not possible to determine whether there is a progressive shift (from 0.38 to 0.34 M) of the molarity of elution of the form of TdT found in the embryos or whether there is replacement of the first form by the second. The 0.34 M peak is present at every age with the exception of the 15-day embryos. The 0.22 M peak appears only after hatching, and increases progressively : it is nearly equal to the 0.34 M peak at 3 weeks and higher at 12 weeks. The inversion of the profile between 12 and 21 weeks was reproductibly found.

DISCUSSION

Although the chicken thymus has been less studied than the mammalian thymus, the availability of chicken embryos has permitted important developmental studies. Using interspecific chimeras, Le Douarin et al (15) described the different stages of the colonization of the thymus rudiment by stem cells and lymphocytes. The first lymphocytes appear in the thymus of 9 1/2-day old embryos, after the large basophilic cells. At 12 days, The lymphocytes represent 86 % of the embryonic thymus cells (16). As shown in our study, TdT is not detectable before the 14th day of embryo life. Two interpretations could be proposed for this late appearance of TdT : either the thymus is colonized only at that age by TdT - containing cells (which could be different from the first colonizing lymphocytes), or TdT is induced at 14 days in cells preexisting in the thymus. When comparing our results with those reported in the literature we were unable to find a simple correlation between the TdT activity and the evolution of the physical properties of thymocytes (17, 18), nor with the expression of thymic or bursal antigens (19). However, it is clear that maximum TdT activity is found during the period of the formation of the immune system of the chick, in contact with antigens in the external medium (the first month after hatching). We also note that the TdT activity per gram of thymus remains nearly constant from 3 to 5 months, the weight of the thymus decreasing nearly 6 times during the same period. This means that the loss of cells by the thymus affects TdT-active as well as TdT-inactive cells.

The occurence of several chromatographic forms of TdT has already been reported for mice and humans (6,11). Since no differences in catalytic properties could be found between these forms (6, 11, our results not shown), the interpretation of this observation is not clear. The multiplicity of enzyme forms might be due to a partial (proteolytic ?) degradation of the enzyme during the preparation, but it persists in the presence of PMSF (used throu-

ghout this study) ; it was not reduced when the time of preparation was shortened from 24 to 6 hours. However, the possibility of a degradation cannot be completely ruled out ; it could take place inside the cell, occuring differently at different ages of the chicken. It might possibly play a specific role in a selection process of thymocytes. Another hypothesis is that each form corresponds to a certain type of cells, as was suggested by Kung et al. for mice (10). In that case, the evolution of the chromatographic profile should be related to changes in the cellular composition of the thymus.

Cell separation experiments, and intracellular detection of the enzyme, using specific antibodies (20,21) are necessary to check these hypotheses. Experiments along this line are in progress.

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