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Enzyme analysis of thymic nurse cells

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Abstract. Thymic nurse cells (TNC) were characterized according to their enzyme content. The following enzymes: Lactate dehydrogenase (LDH) isoenzymes, adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) were evaluated. When the enzyme profile of the TNC were compared with the one found in the different thymocyte subpopulations, the ADA/PNP ratio was very low and similar to the early thymocytes, whereas the LDH isoenzymes approached the pattern of the cortical thymocytes. This enzyme profile suggests that the enzyme content of the TNC is dependent on the presence of at least two different thymocyte subpopulations, the early thymocytes and the cortical thymocytes.

Introduction

Subpopulations of thymocytes can be defined by a number of characteristics, such as reactivities with monoclonal antibodies [1–3], binding sites for peanut agglutinin (PNA) [4], sensitivity to steroids [5] or irradiation [6], sedimentation in density gradient [7], functional properties [8] and their enzyme content [9–18]. Enzymes that have been used for T lymphocyte differentiation in many species, including the mouse, are adenosine deaminase (ADA, E.C. 3.5.4.4), purine nucleoside phosphorylase (PNP, E.C. 2.4.2.1), terminal deoxynucleotidyl transferase (TdT, E.C. 2.7.7.31), alkaline phosphatase (AP, E.C. 3.1.3.1) and the lactate dehydrogenase (LDH, E.C. 1.1.1.27) [9–18].

The aim of the present investigation is to study the relationship between the thymic nurse cells and the thymic subpopulations in the mice according to their enzyme content.

Therefore we have examined the LDH isoenzyme distribution and the ADA/PNP ratio of different murine thymic subpopulations and of the

thymic nurse cells and evaluated these results with previous studies wherein the alkaline phosphatase and TdT [16–18] were evaluated.

The following thymic subsets were examined.

Cortical thymocytes

This major subpopulation of thymocytes (~85%) is located in the cortex. These cells are mostly small noncycling cells with the phenotype L3T4$^+$, Lyt2$^+$, Thyl$^+$. These cells are sensitive to steroids and irradiation and can be agglutinated by PNA. As these cells carry both the L3T4 and the Lyt2 antigen, they will be referred to as double positive cortical thymocytes.

In previous studies, these cells have been selected by agglutination with PNA and these cells exhibited a high ADA/PNP ratio [15] and a typical LDH isoenzyme pattern with predominant activity in the LDH-5 and 4 bands, but also with activity in the LDH-1, 2 and 3 bands, whereas peripheral T lymphocytes exhibit activity only in the LDH-3, 4 and 5 bands [12–13].

Medullary thymocytes

This subpopulation of thymocytes is located in the medulla. These cells are characterized by the expression of either Lyt2 or L3T4 and by their relative resistance to irradiation and cortisone. They are not agglutinated by PNA. As these cells carry either the L3T4 antigen or the Lyt-2 antigen, they will be referred to as single positive medullary thymocytes.

In previous studies these thymocytes have been selected by the above mentioned criteria and it was shown that these cells are characterized by a lower ADA/PNP ratio [15] and an LDH isoenzyme pattern with lower activity in the LDH-1, LDH-2 and LDH-3 bands as compared with the double positive cortical thymocytes [12–13].

Early thymocytes

This small subpopulation of thymocytes has the phenotype L3T4$^-$, Lyt2$^-$, Thy-1$^+$ and expresses the I1-2 receptor. This population is predominant in embryonic murine development as more than 80% of these cells with this phenotype are present in thymuses of embryo's before day 17. These cells will be referred to as early thymocytes.

In a previous study [14] these thymocytes have been selected by studying the thymocytes from 14 and 15 day old embryo's and we found that these cells are characterized by a LDH pattern with almost all activity in the

LDH-5 and LDH-4 and no activity in the LDH-1, LDH-2 and LDH-3 bands.

Thymocytes obtained from thymic nurse cells (TNC)

TNC are lymphoepithelial complexes which are thought to play a role in the early stages of the intrathymic differentiation pathway [19–22]. These cells will be referred to as TNC thymocytes.

The lymphoid blasts engulfed in TNCs contain membranous AP and TdT suggesting a relationship with very immature cortical blast cells [16–18].

In the present study the enzymes: LDH, ADA and PNP were studied in the TNC and compared with the enzyme content of the other thymic subpopulations.

Material and methods

Mice. Two month old C57 BL/Ka male mice were obtained from the laboratory of the Liege University.

Thymus dissociation and TNCs isolation. In each experiment, TNCs were isolated from 30 to 40 thymuses. Thymuses were minced with scissors and washed for 10 min in PBS. The fragments were dissociated by repeated incubations in the presence of dispase, Collagenase and DNase. TNCs were isolated from the resulting suspensions by successive runs of 1 g sedimentation by using a slight modification [23] of the method originally described by Weckerle and Ketelsen [20].

Cultures. To recover lymphoid cells from TNCs, the isolated TNC complexes were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, 2mM/ml L-glutamine, 1% non-essential amino-acids, 1mM/ml sodium pyruvate, 150 U/ml penicillin and 0.075 mg/ml streptomycin, and grown in 30 mm Petri dishes (Lux Scientific Corporation, Sanbio, The Netherlands). After a 20 h incubation, lymphoid cells were released and collected.

Preparation and separation of thymocyte suspensions. Suspensions of thymocytes were prepared by disrupting the thymuses mechanically by gentle pressure from a polysterene pestle in cold RPMI 1640 medium buffered with Hepes.

Thymocytes were separated into PNA positive and negative cells by flow cytometry as described [24–25].

Briefly, thymocytes were labeled with subagglutinating concentrations of PNA–FITC (Vector Laboratories, Burlingame, CA, USA). The cells were stained for 30 min at 4 C and subsequently diluted to 5×10^6 cells/ml without washing for sorting in a fluorescence-activated cell sorter (Ortho, diagnostic Systems, Westwood, MA, USA). The medium sized PNA negative cells (medullary thymocytes) and the small non dividing subset of PNA positive cells (cortical thymocytes) were collected separately. The mean proportion of these subsets were respectively 4–7%, and 75–85%. After sorting the cells were analysed with the flow cytometer to assess purity. For each cell fraction a purity of at least 98% was obtained. Early thymocytes were obtained by preparing thymocytes from thymuses of 14 day old embryos. Gestational age was determined by noting the appearance of vaginal plugs (day 0).

Determination of the LDH isoenzyme pattern. The separated cell suspensions were finally washed three times and adjusted to a concentration of 1×10^7 cells/ml in Tris buffer. These cells were freeze-thawed three times and were spun at 2000 × G. The LDH isoenzymes were determined by agar gel electrophoresis as decribed [11–14]. Because LDH is a tetrameric enzyme composed of two possible subunits, resulting in five isoenzymes – B4, B3A, B2A2, BA3, and A4 – respectively called LDH-1, LDH-2, LDH-3, LDH-4, and LDH-5, the B:A ratio was calculated by using the following formula:

$$\frac{\%\text{LDH-1} + \%\text{LDH-2} \times 0.75 + \%\text{LDH-3} \times 0.5 + \%\text{LDH-4} \times 0.25}{\%\text{LDH-5} + \%\text{LDH-4} \times 0.75 + \%\text{LDH-3} \times 0.5 + \text{LDH-2} \times 0.25}$$

Assay for PNP and ADA. The obtained cell fractions were adjusted to a concentration of 1×10^7/ml in 50 mM potassium phosphate buffer at pH 7.4. The suspensions were freeze-thawed three times and centrifuged in a microcentrifuge (Eppendorf, Hamburg, Germany).

ADA and PNP activities were determined by using a modification of the spectrophotometric method of uric acid production as described by Kalckar (26), adapted for an automated Trace III system (Beckman Instruments, Inc., Fullerton, CA). The change in optical density was read at 292 nm over a 10-min period at 37 C, with 1 min interval.

For measuring PNP activity, the reaction volume (0.5 ml) consisted of 0.3975 ml 50 mM potassium phosphate buffer, 0,0025 ml Xanthine oxidase

(Sigma X-4500), 0.05 ml inosine, 0.5 mM, and 0.050 ml cell extract. For measuring ADA activity, the reaction volume (0.5 ml) was the same as for PNP, but this time adenosine 0.5 mM was used instead of inosine and 0.0025 ml PNP (a 1/20 dilution) (Sigma 3003) was added. ADA and PNP activities were expressed as ADA/PNP ratio.

Results

Table 1 shows the LDH isoenzyme distribution, the B/A ratio and the ADA/PNP ratio of the different murine thymocyte cell populations. The zymograms of the different cell populations are shown in Fig. 1.

In agreement with our previous studies [12–14] unseparated thymocytes, double positive cortical thymocytes and single positive medullary thymocytes showed an LDH pattern with activity in the five bands. The single positive medullary thymocytes have a significantly higher activity in the LDH-5 band. This results in a lower B/A ratio than for the double positive cortical thymocytes. The early thymocytes showed, however, a different LDH pattern with no activity in LDH-1 and higher activity in LDH-5, resulting in a very low B/A ratio.

When examined for the ADA/PNP ratio, the unseparated thymocytes, double positive cortical thymocytes and single positive medullary thymocytes had a high ADA/PNP ratio. This ratio was the highest for the double positive cortical thymocytes. The early thymocytes showed, however, a different ADA/PNP ratio with a much lower ADA/PNP ratio of (on average) 1.63.

When the enzyme profile of the freshly isolated thymic nurse cells is studied an interesting dissociation was seen. Although the ADA/PNP ratio was very low (similar to the early thymocytes), the LDH isoenzymes approached the pattern of the unseparated or double positive cortical thymocytes. When TNC preparations are freed from the thymic stromal cells by overnight incubation, the released TNC thymocytes have the same low ADA/PNP ratio.

Discussion

In this study we have analysed the LDH isoenzyme pattern of different T cells subsets. The data confirm in C57BL mice our previous observations obtained in the outbred NMRI strain [12–14]. The data clearly show the

Table 1. Enzyme analysis of different T cell subsets in thymus.

Source	n	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	B/A	A/P
C57Bl 5–8 wks. unseparated thymocytes	7	4.8 ± 0.6	10.6 ± 1.0	16.6 ± 0.9	27.3 ± 1.7	40.6 ± 2.1	0.386 ± 0.024	5.94 ± 0.55
C57Bl embryo "prothymocytes"	4	0	1.0 ± 0.7	14.5 ± 15.4	15.6 ± 14.3	79.6 ± 18.0	0.072 ± 0.062	1.63 ± 0.36
C57Bl 5–8 wks. "thymic nurse cells"	16	6.1 ± 0.7	11.5 ± 1.4	14.2 ± 1.4	20.4 ± 1.5	47.8 ± 3.1	0.365 ± 0.033	1.93 ± 0.25
C57Bl 5–8 wks. "TNC" after incubation	10				not done			2.14 ± 0.56
C57Bl 5–8 wks. PNA pos. thymocytes	5	7.0 ± 0.6	13.6 ± 1.3	18.4 ± 1.1	23.8 ± 1.2	36.9 ± 1.6	0.477 ± 0.020	8.69 ± 0.39
C57Bl 5–8 wks. PNA neg. thymocytes	5	0.8 ± 0.5	3.1 ± 0.9	12.5 ± 1.7	34.0 ± 1.3	49.6 ± 3.4	0.217 ± 0.027	3.11 ± 1.92

Each value represents the mean (± SD) obtained from n experiments. A = adenosine deaminase; P = purine nucleophosphorylase.

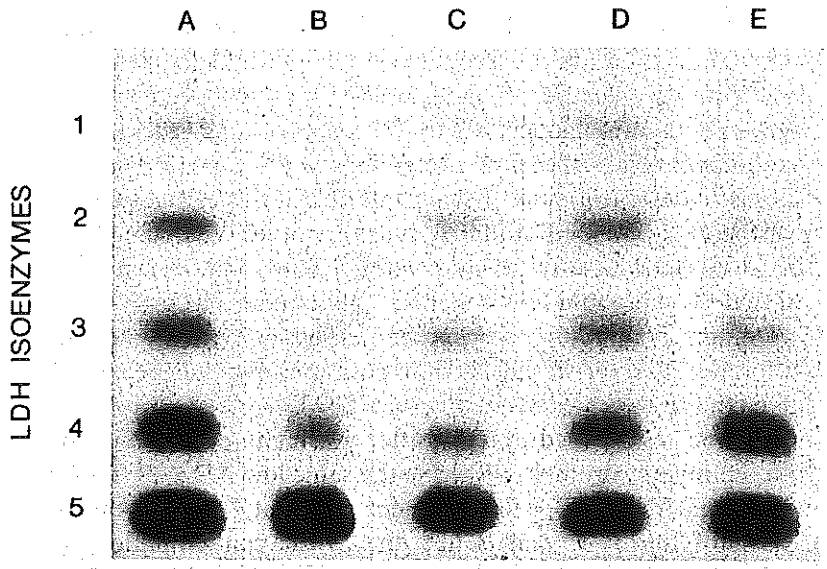

Fig. 1. Typical zymograms of the different thymocyte subpopulations. A. Unseparated thymocytes. B. Prothymocytes (thymocytes from 14 days old embryo). C. Thymic nurse cells. D. cortical thymocytes (PNA positive thymocytes). E. Medullary thymocytes (PNA negative thymocytes).

drastic changes during T cell development and that the enzymes, LDH, ADA and PNP can be used as a parameter of T cell differentiation in the mice.

The enzyme profile of the TNC preparations indicates that this cell population is composed of at least two different populations. The high activities in the LDH-1 and LDH-2 bands found in the TNC preparations are similar to the high activities that were found in the small non dividing subset of PNA positive cells, which are generally accepted to be highly enriched for double positive Lyt-2, L3T4 cortical thymocytes. The high activities in LDH-4 and LDH-5 found in the TNC preparations are similar to the high activities that were found in early thymocytes located in thymus rudiments of 14 day old embryo's. Although early thymocytes in embryonic thymuses differ from the double negative thymocytes in adult thymuses in some properties, both populations share many characteristics and it is tempting to relate these enzyme levels in the TNC preparations to the presence of early thymocytes. Therefore we assume that the double positive cortical thymocytes contribute to the high activities in the LDH-1 and LDH-2 bands, whereas the early thymocytes contribute to the high activities in the LDH-4 and LDH-5 bands. This results in the mixed pattern that is found in the TNC preparations.

The ADA/PNP ratio of the TNC preparations is also intermediate between the low ratio found in the early thymocytes and the high ratio found in the double positive cortical thymocytes.

The method of thymus dissociation used in the experiments described here allow to obtain a cell suspension containing very few macrophages and dendritic cells. After 4 to 5 runs of sedimentation of this cell suspension, 80 to 85% of the cells obtained are nurse cells, the other cells being lymphoblasts. Because each nurse cell contains about ten lymphocytes, it can be estimated that the lymphoid cells engulfed within nurse cells represent more than 97% of the lymphoid cell suspension. The enzyme profile and the ADA/PNP ratio observed in the TNC preparations can thus be considered to be due to the thymocytes located within TNC.

As the thymocytes released by TNC after overnight incubation and thus freed from the thymic stromal cells, have the same low ADA/PNP ratio we can conclude that this ratio reflects the enzyme content of the thymocyte compartment of the TNC and is not due to the contamination by the stromal cells.

The high number of cells required for the LDH analysis did not allow us to study the isoenzyme pattern of the TNC thymocytes after culture. However, as these stromal cells have a LDH isoenzyme pattern composed of only the LDH-5 band (unpublished observation), we conclude that the high B/A ratio must be ascribed to the high LDH-1, LDH-2 and LDH-3 content of the TNC thymocytes.

Conclusion

Our conclusion is that the enzyme content of the thymic nurse cells is dependent on the presence of two different thymocyte subpopulations. Previous phenotypic studies had shown that most intra TNCs lymphocytes are Thy-1, Lyt-2 and L3T4 positive and thus display the classical cortical phenotype [16, 27]; however, a minority of cells share several features with the most immature thymocytes since they express alkaline phosphatase [17] and nuclear TdT [16]. Some cells do express Il–2 receptors (Wekerle: personnal communication, Defresne et al.: unpublished results). Therefore we may assume that a part (less than 20%) of intra TNC lymphocytes are similar to, or close to, early thymocytes, whereas the remaining part is composed of cortical double positive lymphocytes. The enzyme profile indicates that the absolute activities of ADA and PNP are more important in the early thymocytes therefore resulting in an ADA/PNP ratio of the TNC similar to the one found in the early thymocytes. On the contrary the

activities in the LDH-1, LDH-2 and LDH-3 bands that are present in the double positive cortical thymocytes remain in the TNC, although the activity in LDH-5 is increased due to the presence of the early thymocytes. When early thymocytes are mixed with unseparated thymocytes in a proportion of 1 to 4, an enzyme profile is obtained that is similar to the one found in TNC (data not shown). Additional phenotypic and enzyme analysis with particular attention to the absolute activities of the different enzymes of the TNC, should allow a more accurate assessment of the relative proportion of the early thymocytes and double positive cortical thymocytes in the TNC. However, the variable number of thymocytes per TNC and the high number of cells that are required for an accurate measurement of the absolute enzyme activities per number of cells hinders this study.

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