5′-NUCLEOTIDASE ACTIVITY OF TWO POPULATIONS OF MOUSE THYMOCYTES SEPARATED BY PEANUT AGGLUTININ AGGLUTINATION

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1. Introduction

The adenosine-generating enzyme 5′-nucleotidase is localized on the external surface of the plasma membrane of many cells [1], including lymphocytes [2,3]. This enzyme is a concanavalin A (con A) receptor [4] and is inhibited by the lectin binding [3,5]. Although little is known about its function, 5′-nucleotidase plays a critical role in lymphocyte adenosine metabolism; it is involved in adenosine uptake from 5′-AMP [6,7] and regulates intracellular cyclic AMP level [8] through membrane adenosine receptor sites [9]. As adenosine appears to exert a negative control on lymphocyte proliferation [10,11] the distribution of 5′-nucleotidase activity among lymphocyte subpopulations is a very important problem. Non-uniform distribution of 5′-nucleotidase among lymphoid tissues has been described [12–17] but these results are sometimes contradictory; moreover completely different distributions are reported for different animal species.

However low 5′-nucleotidase levels are unambiguously found in lymphocytes from patients with chronic lymphocytic leukemia (CLL) ([17–20] and ourselves) or hypogammaglobulinemia [21,22] and in cord blood lymphocytes [22,23]. As CLL cells are thought to be arrested at an early stage of development [24] and as cord blood cells might also be immature [25], it was hypothesized that the 5′-nucleotidase deficiency in hypogammaglobulinemic patients may reflect a stage of maturation arrest in T and B lymphocytes [23].

As others [13,14,16] we found that 5′-nucleotidase activity in thymocytes is 6–10-fold lower than in splenocytes. We checked the hypothesis that this low activity may reflect the fact that the major thymocyte subpopulation (85–95%) which is hydrocortisone-sensitive and immature displays very low 5′-nucleotidase activity.

2. Materials and methods

Thymocytes were obtained from 4–7-week-old male mice (C57/BL6, DBA2, C3H/eb, CB-20 or Swiss) killed by cervical luxation. Thymuses were removed, minced in Hank's salt and passed through a fine stainless steel mesh to give a single cell suspension ($1–1.5 \times 10^8$ cells/thymus); when necessary erythrocytes were selectively killed by 0.14 M ammonium chloride. Cell viability was 95–98%. Hydrocortisone-resistant thymocytes were obtained by the same procedure 48 h after intraperitoneal injection of 2.5 mg hydrocortisone acetate. Splenocytes were prepared as in [7].

Mature and immature thymocyte populations were isolated by agglutination with peanut agglutinin (PNA) as in [26]. Thymocytes, 2×10^8 in 0.25 ml PBS, were incubated with 0.25 mg PNA in 0.25 ml PBS for 10 min at 25°C; the mixture was then layered on 8 ml FCS 30% in PBS. After 30 min sedimentation ($1 \times g$) agglutinated (immature) cells (bottom layer) were removed from non-agglutinated (mature) cells (top layer), dissociated into single cells by 10 min incubation at 37°C with 5 ml 0.2 M D-galactose in PBS, washed twice with 5 ml 0.2 M D-galactose and twice with PBS. Unagglutinated cells (5–10% of total thymocytes) received the same treatment. Viability of both populations was 95%.

5′-Nucleotidase activity was determined using 5′-[^{32}P]AMP as substrate. Cells ($2.5–5 \times 10^6$) were incubated 30 min at 37°C in 250 μl 199 Hepes

medium with 0.2 mM 5′-[^{32}P]AMP. The reaction was stopped with 150 μl 0.1 N HCl and $^{32}P_i$ was determined as in [3]. The contribution of non specific phosphatases to 5′-AMP hydrolysis was negligible [3] and increases in P_i up to 0.1 M had no effect on the enzyme activity [1,8]. 5′-Nucleotidase activity was expressed as nmol P_i . h^{-1} . mg $protein^{-1}$. The amount of proteins corresponding to 2.5–5 X 10^6 cells was determined by Lowry's method.

Thymidine uptakes induced by PHA Difco (7 μl/ml) or con A Pharmacia (5 μg/ml) were determined as in [11]. Cells 250 μl (3 X 10/ml) were cultured in RPMI medium supplemented with 15% fetal calf serum for 60 h at 37°C under an air–CO_2 (95:5) atmosphere. For the last 5 h the cells were pulsed with [^{3}H]thymidine and harvested as in [11].

3. Results

The separation of mouse thymocytes into two subpopulations (mature and immature) was done by the method in [26] for mouse thymocytes and recently applied to human thymocytes [27]. This method is based on the observation that immature thymocytes bind PNA while mature cells do not. Agglutinated cells (80–90% of total thymocytes) were dissociated into single cells by incubation with D-galactose which bind specifically PNA; this population was unambiguously characterized as immature and hydrocortisone-sensitive, while non-agglutinated cells were found identical to mature hydrocortisone-resistant thymocytes [26,27]. Table 1 confirms these characterizations; it reports the stimulation of unseparated thymocytes and thymocyte subpopulations by PHA or con A. PNA^+ (agglutinated) lymphocytes were not stimulated by PHA, while PNA^- lymphocytes and thymocytes from hydrocortisone-treated mice were highly stimulated.

Table 2 shows the 5′-nucleotidase activity of unseparated, PNA^+ and PNA^- thymocytes. Ten experiments were performed with Swiss mouse thymocytes and one with each of the other mouse strains. The percentage of PNA^- thymocytes was 5–15%; the 5′-nucleotidase activity of this subpopulation was 3–5-fold higher than that of unseparated thymocytes. PNA^+ thymocytes accounted for 80–90% of total thymocytes and displayed a 5′-nucleotidase activity 1.5–2-fold lower than unseparated cells. 5′-Nucleotidase activity of PNA^- thymocytes was thus 5–11-fold higher than that of PNA^+ cells. PNA had no effect on pure 5′-nucleotidase from pig lymph node lymphocytes [5] (not shown). Thymocyte 5′-nucleotidase was neither affected by incubation with 0.2 M D-galactose for 10–60 min, nor by preincubation with PNA (≤50 μg/

Table 1
Mitogenic response of mouse thymocytes to Con A and PHA

Mitogen	Unseparated thymocytes	PNA^+ thymocytes	PNA^- thymocytes	Hydrocortisone-resistant thymocytes
None	600 ± 200	520 ± 50	900 ± 300	650 ± 60
Con A	93 000 ± 7000	23 000 ± 3000	210 000 ± 15 000	80 000 ± 10 000
PHA	1500 ± 300	600 ± 200	80 000 ± 6000	63 000 ± 4000

[^{3}H]Thymidine incorporation (cpm) in mouse thymocyte populations, 60 h after stimulation by PHA or con A

Table 2
5′-Nucleotidase activities (nmol P_i . h^{-1} . mg $protein^{-1}$) of thymocytes (unseparated, PNA^+, PNA^-) from different mouse strains

	C3H/eb	CB20	C57BL6	DBA2	Swiss
Unseparated thymocytes	30	40		30	15–35
PNA^+ thymocytes	18	25	11.2	20	8.3–25
PNA^- thymocytes	130	130	125	150	90–210

Table 3
Effect of hydrocortisone in vivo on the 5′-nucleotidase activity of mouse thymocytes

	Expt 1	Expt 2	Expt 3
Thymocytes from hydrocortisone-treated mice	194	144	162
Unseparated thymocytes from untreated mice	30	35	30
PNA^+ thymocytes from untreated mice	20	17.5	18
PNA^- thymocytes from untreated mice	110	133	130

5′-Nucleotidase activities (nmol P_i . h^{-1} . mg $protein^{-1}$) of thymocytes from hydrocortisone-treated Swiss mice and of control thymocytes (unseparated, PNA^+, PNA^-) from untreated Swiss mice

2.5×10^6 cells), which ruled out the possibility that the low 5′-nucleotidase level of PNA^+ thymocytes was due to D-galactose treatment or to inhibition by PNA uncompletely removed from the cell membrane.

To study mature thymocytes, we used hydrocortisone treatment to deplete the immature cell population [26]. Table 3 represents the 5′-nucleotidase activity of Swiss mouse thymocytes, two days after an intraperitoneal injection of 2.5 mg hydrocortisone acetate. Hydrocortisone-resistant cells were pooled from 10 mice ($\sim 15 \times 10^6$ cells/thymus); they did not agglutinate with PNA. Their 5′-nucleotidase activity was compared with that of thymocytes from untreated mice (unseparated, PNA^+, PNA^-). 5′-Nucleotidase activity of hydrocortisone-resistant cells was 5- and 8–10-fold higher than that of unseparated and PNA^- control thymocytes, respectively. It appeared that PNA^- control thymocytes and hydrocortisone-resistant thymocytes, which have been claimed to be identical (mature) population [26] had similar 5′-nucleotidase activities.

5′-Nucleotidase activity of splenic lymphocytes from Swiss mice was determined in 10 different expt; its mean value was 320 ± 70 nmol P_i . h^{-1} . mg^{-1}. As no difference between T and B splenocyte 5′-nucleotidase activity was evidenced [14], it appeared that PNA^- or hydrocortisone-resistant thymocytes had lower 5′-nucleotidase activity than T splenocytes.

4. Discussion

Using the general technique in [28] we separated mouse thymocytes into PNA^- thymocytes identified as mature cells (medulary) and PNA^+ thymocytes which are immature (cortical). As it was shown [26,27] that PNA agglutination and subsequent dispersion with galactose yielded fully functional cells, and as these treatments had no effect on 5′-nucleotidase activity, we can state that the low 5′-nucleotidase activity of mouse thymocytes results from the presence of 90–95% immature cells. In mouse thymus immature cells can be selectively eliminated by hydrocortisone treatment, the remaining mature cells had the same high 5′-nucleotidase activity than PNA^- control thymocytes. These repeatedly obtained results confirmed that mature thymocyte 5′-nucleotidase activity is 10-fold higher than that of immature thymocytes. No difference for 5′-nucleotidase activity were found in thymocytes from hydrocortisone-treated rats or untreated control rats (410 ± 105 and 370 ± 110 nmol P_i . h^{-1} . mg^{-1}, respectively) [15]; however it must be emphasized that the 5′-nucleotidase value reported for control thymocytes was abnormally high as compared to rat splenocyte 5′-nucleotidase (890 nmol P_i . h^{-1} . mg^{-1}), as generally the thymocyte activity is 6–10-fold lower ([13,14] and ourselves) or even more in rabbit thymocytes [16].

5′-Nucleotidase activity in PNA^- mature thymocytes appeared lower than in T splenocytes, if we assume that T and B splenocytes display the same mean activity [14]. This result can be explained if thymus maturation generates several subclasses of T-cells [29] with different 5′-nucleotidase activity and if PNA^- thymocyte subpopulation is heterogeneous [30]. On the other hand a small subpopulation of mature lymphocytes bearing PNA receptors is mostly found in the thymus [29] and could account for the 5′-nucleotidase activity of PNA^+ thymocytes.

5′-Nucleotidase activity appears to be restricted to hydrocortisone-resistant thymocytes. It was recently shown that the steroid-resistant intrathymic pool is a resident subpopulation which is not exported to the periphery [31]. We do not know if 5′-nucleotidase increase in peripheral T cells occurs after migration from the thymus to secondary lymphoid tissues or before.

Recent reports on the low 5′-nucleotidase activity in lymphocytes from patients with CLL [17–20] or hypogammaglobulinemia [21,22] increase the interest of studying the function and distribution of this enzyme among various lymphocyte populations.

T and B cells from cord blood have a lower activity than adult lymphocytes [22,23]. Malignant transformed cells display also very low activity [32]. Most of these cells are thought to be arrested at an early stage of maturation [24,25]. As we found a low 5′-nucleotidase activity in immature thymocytes, the absence of 5′-nucleotidase activity might be considered as a marker of a selective maturation arrest. This was confirmed by the absence of detectable 5′-nucleotidase in new-born cat liver cells and in cells from regenerating liver [33].

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