5' Nucleotidase Activity in Mitogen-Stimulated Adult and Cord Blood Lymphocyte Cultures

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Summary

Ecto 5'Nucleotidase (5'N) activity was determined in adult and cord blood lymphocytes before and after stimulation by phytohemagglutinin (PHA) and pokeweed mitogen (PWM). After 7 days of culture, the mean 5'N level $\pm$ S.E. in unstimulated adult cells was 15.9 ± 3.21 nm/h/ 10^6 cells. Cells stimulated by PHA and PWM for 7 days had 5'N levels of 44.65 ± 6.69 nm/h/ 10^6 cells and 33.24 ± 7.67 nm/h/ 10^6 cells, respectively. Unstimulated cord blood cells cultured for 7 days had a mean 5'N level of 3.22 ± 0.94 nm/h/ 10^6 cells whereas the level in PHA-stimulated cultures was 39.50 ± 10.52 nm/h/ 10^6 cells and in PWM stimulated cultures 46.01 ± 8.93 nm/h/ 10^6 cells. T-cell enriched and T-cell depleted subpopulations stimulated with mitogen generally did not show as great a degree of enhancement of 5'N activity as did unfractionated cells.

This study indicates that 5'N activity is enhanced in cells that have been activated by mitogen stimulation. Although unstimulated cord blood cells have lower levels of 5'N activity than adult cells they have the capability upon stimulation to express levels equal to those found in adults.

Abbreviations

AET, 2-aminoethylisothiuronium bromide
AMC, adult mononuclear cells
CBMC, cord blood mononuclear cells
CLL, chronic lymphocytic leukemia
Con A, concanavalin A
5'N, 5'nucleotidase
PBM, peripheral blood mononuclear cells
PHA, phytohemagglutinin
PWM, pokeweed mitogen
TCD, T cell depleted
TCE, T cell enriched

5'Nucleotidase (E.C. 3.1.3.5) is an enzyme in the purine catabolic pathway that catalyzes the hydrolytic dephosphorylation of nucleoside 5'monophosphates. Deficiencies in two other enzymes in this pathway, adenosine deaminase and nucleoside phosphorylase, are associated with distinct immunodeficiency syndromes (3). Diminished levels of lymphocyte ecto-5'N are not associated with a single clinical entity but rather with multiple clinical conditions (4, 12, 22, 24, 27, 30). Most of these conditions are associated with impaired differentiation of lymphocyte function; therefore, it has been postulated that 5'N activity may be a marker for lymphocyte differentiation (4, 8).

It has been shown previously that CBMC have diminished 5'N activity when compared to the enzyme levels of AMC (20, 24). The present study was designed to evaluate the effect of mitogen stimulation on 5'N activity in both AMC and CBMC. Our results indicate that such stimulation enhances 5'N activity of CBMC as well as AMC.

MATERIALS AND METHODS

Isolation of mononuclear cells. Mononuclear cells were separated from fresh heparinized venous blood from normal adult subjects aged 25-35 years or from cord blood specimen obtained at the time of delivery by a modification of the Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Inc., Piscataway, NJ, Hypaque, Winthrop Laboratories, Sterling Drug Co., New York) buoyant density gradient method of Boyum (6, 25). Isolated PBM were fractionated into TCE or TCD populations. There was no attempt to deplete monocytes from the PBM. For fractionation purposes equal volumes of PBM ($2-4 \times 10^6$ /ml) and a 1% suspension of sheep erythrocytes, which had been treated with AET (19), were mixed in 50-ml centrifuge tubes; centrifuged for 8 min at $800 \times g$ at room temperature; and incubated for 1 h at 4°C. The cell mixture was then resuspended, underlayered with Ficoll-Hypaque, and centrifuged for 30 min at $1300 \times g$ at room temperature. The nonrosetted cells were removed from the interface and the sheep erythrocytes in the pellet were lysed with Tris-NH₄Cl. TCE and TCD populations were washed three times in phosphate buffered saline plus 2% fetal bovine serum. Cell populations were characterized with immunofluorescent techniques using the presence of surface immunoglobulin as a marker for B-cells, the monoclonal antibody OKT3 to detect T cells and the monoclonal antibody OKM-1 to determine monocytes and null cells.

Lymphocyte cultures. Unfractionated PBM, TCE or TCD populations were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate, 2 mM L-glutamine, 2.5 mM Hepes buffer (Gibco, Grand Island, NY) and 5% heat inactivated fetal bovine serum (M.A. Bioproducts, Walkersville, MD). For 5'N studies the cells were cultured at a concentration of 10^6 cells/ml in a volume of 5 ml in 12×75 mm polystyrene tubes (No. 2003, Falcon Labware, Cockeysville, MD). Cultures were left unstimulated (controls) or were stimulated with either 5 μ l/ml of PWM (Gibco, Grand Island, NY) or 10 μ l/ml of PHA (Difco, Detroit, MI). Cultures were harvested at 1, 3, or 7 days. Before the 5'N assay, the cells were washed three times in PBS + 2% fetal bovine serum and counted. For studies measuring tritiated thymidine incorporation, the cells were cultured as described previously (25).

5'Nucleotidase assay. The procedure of Chatterjee *et al.* (7), with minor modifications, was used in determining the 5'N activity of the sample cell suspensions. The substrate was prepared by adding 87 mg of 5'AMP and 200 μ l of 0.01 μ Ci of [¹⁴C]-5'AMP (New England Nuclear, Boston, MA) to 100 ml of MgCl₂ (12.5 mM)-Tris-HCl (63 mM), pH 7.5 buffer. The 100 ml of prepared substrate was divided into two 50-ml aliquots. To one 50-ml aliquot, 200 mg of Con A, a specific inhibitor of 5'N, was added (32). Each 50-ml portion was divided further into 1-ml aliquots and stored at -20°C for later use. One hundred μ l of the 5'AMP substrate solution either with or without Con A was added to 10^6 cells suspended in 50 μ l of MgCl₂ Tris-HCl buffer, pH 7.5. The samples were placed in a Dubnoff shaking incubator for 1 h at 37°C. The reaction was terminated by the addition of 0.2 M ethylene-diamino-tetracetate acid disodium.

The reaction mixture was then applied to columns consisting of 0.6 g neutral alumina (Biorad, Laboratories, Richmond, CA) and packed into disposable polyethylene columns. Immediately before use, the columns were equilibrated with Tris-HCl buffer, pH 7.5. All samples were done in duplicate. The hydrolytic product [^{14}C]-adenosine was eluted from the column with 7 ml of Tris-HCl buffer, pH 7.4. The eluate was collected in scintillation vials to which 10 ml of Aquasol-2 (New England Nuclear, Boston, MA) was added. The radioactivity in each sample was determined using a Packard Tri-Carb liquid scintillation spectrophotometer. 5'N activity was expressed as that part of the total phosphatase activity inhibitable by Con A.

RESULTS

5'N activity in AMC and CBMC. The mean value of 5'N activity in fresh AMC from 22 individuals was 11.72 ± 2.07 (S.E.) nm/h/ 10^6 cells. Most of the subjects were studied on more than one occasion and similar values were obtained on each determination. The 5'N activity of 26 different CBMC specimens had a mean value of 5.67 ± 0.83 nm/h/ 10^6 cells. The difference in enzyme activity between AMC and CBMC was significant at a P value <0.01 . Figure 1 depicts the range in enzyme activity seen in both CBMC and AMC. The adult values displayed a very broad range. If the highest adult value and lowest two adult values are removed from this data the mean value for AMC is not significantly changed (11.72 versus 12.36).

Effect of mitogen stimulation on 5'N activity. Both CBMC and AMC were cultured in the presence of either PHA or PWM and the effect on 5'N activity determined. The 5'N levels of unstimulated and stimulated AMC cultures were determined on days 1, 3, and 7 of culture (Table 1). Unstimulated cultures of AMC showed only a minimal change in enzyme activity on each day studied. By contrast, 7-day cultures stimulated by PHA or PWM showed a 2–3-fold increase in activity over unstimulated cultures (Fig. 2). PHA stimulation enhanced enzyme activity slightly more than PWM. A rise in 5'N activity was seen in all AMC cultures stimulated by PHA and in 9/12 cultures stimulated with PWM.

Because stimulated AMC failed to show any enhancement of 5'N activity after 24 h, no attempt was made to determine enzyme activity in CBMC stimulated for a single day. 5'N levels of

Fig. 1. Range of 5'N activity in adult mononuclear cells and in cord blood mononuclear cells.

Table 1. 5' Nucleotidase activity in mitogen-stimulated adult mononuclear cells (AMC) and cord blood mononuclear cells (CBMC) (mean $\pm$ S.E.)

Day of culture	Specimen	Unstimulated	Phytohemagglutinin stimulated	Pokeweed mitogen stimulated
1	AMC (7) ¹	18.06 ± 2.27^2	27.10 ± 4.56	18.49 ± 2.15
	CBMC	ND ³	ND	ND
3	AMC (6)	26.04 ± 4.85	35.85 ± 4.68	21.665 ± 4.01
	CBMC (4)	10.93 ± 3.26	14.21 ± 5.26	8.60 ± 2.38
7	AMC (12)	15.91 ± 3.21	44.65 ± 6.69	33.24 ± 7.67
	CBMC (6)	3.22 ± 0.94	39.50 ± 10.52	46.01 ± 8.93

¹ () represents number of subjects studied.

² Expressed in nm/h/ 10^6 cells.

³ ND, not done.

Fig. 2. Ratio of 5'N activity in stimulated cultures versus nonstimulated cultures at different days in culture.

stimulated and unstimulated CBMC were evaluated on days 3 and 7. PHA and PWM stimulation for 7 days resulted in an increase in 5'N activity to levels comparable to those seen with adult cells stimulated for 7 days (Table 1). But the ratio of enzyme activity in stimulated cells to activity in unstimulated cells was much higher in CBMC than in AMC (Fig. 2).

5'N activity in lymphocyte subpopulations. CBMC and AMC were fractionated into TCE or TCD populations before being put into culture. Phenotypic characterization of AMC cell populations is depicted in Table 2. Preliminary studies indicate CBMC populations are similar. (Ault, B. and Herrod, H., unpublished observations). Cultures were harvested at day 7 and 5'N activity determined (Table 3). Both the absolute and relative increase in 5'N activity after mitogen stimulation of TCE populations was not as great as that seen in unfractionated populations. Both AMC and CBMC TCD populations stimulated by PWM had levels of 5'N activity similar to that of unfractionated cells; however, the relative increase over unstimulated population was always less than 3:1 (Fig. 3).

AMC and CBMC transformation in the presence of PHA and PWM. Table 4 shows the results of stimulation of CBMC and AMC as measured by incorporation of tritiated thymidine. Peak responses to PHA occurred on day 3 or 4 whereas the peak response to PWM was on day 5 or 6. Overall CBMC had higher

Table 2. Characterization of mononuclear cell subpopulations¹

Cell population	sIg + (%)	OKT3 + (%)	OKM - 1 + (%)
Unfractionated (range)	7.5 ± 3.3 ² (6-11%)	66.7 ± 6.7 ² (60-73%)	19.8 ± 4.2 ² (13-24%)
T cell enriched	1.8 ± 1.3 (0-3%)	79.2 ± 5.1 (72-84%)	8.0 ± 7.0 (1-15%)
T cell depleted	28.8 ± 10.2 (15-43%)	6.0 ± 2.6 (2-9%)	55.4 ± 13.8 (36-74%)

¹ Values represent means from five different individuals.² Results expressed as % positive ± S.D.

Table 3. 5' Nucleotidase activity in mononuclear cell cultures stimulated by mitogen for 7 days

Cell population	Unstimulated	Phytohemagglutinin stimulation	Pokeweed mitogen stimulation
Unfractionated AMC (4) ¹	5.61 ± 2.74 ²	47.93 ± 6.86	20.13 ± 5.10
CBMC (8)	0.87 ± 2.82	24.58 ± 5.43	22.3 ± 4.70
T cell enriched AMC (4)	9.37 ± 2.90	14.72 ± 1.97	8.88 ± 1.55
CBMC (3)	2.68 ± 2.0	3.47 ± 2.65	6.75 ± 3.41
T cell depleted AMC (4)	11.67 ± 5.39	17.09 ± 7.42	26.71 ± 5.10
CBMC (4)	7.44 ± 2.83	13.76 ± 5.86	19.23 ± 9.3

¹ () represents the number of subjects studied. AMC, adult mononuclear cells and CBMC, cord blood mononuclear cells.² Expressed in nm/h/10⁶ cells.Table 4. In vitro incorporation of tritiated thymidine by unfractionated AMC and CBMC stimulated by PHA and PWM¹

Cell source	No. subjects	PHA	SI	PWM	SI
AMC	5	85,711 ² 188	456	32,529 291	112
CBMC	15	149,926 819	183	41,640 1097	38

¹ Definitions: AMC, adult mononuclear cells; CBMC, cord blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; and SI, stimulation index.² Values represent counts per min of stimulated cells over counts per min from unstimulated cultures.

cells was decreased by 96% on both days (5821 cpm versus 145,760 cpm, day 4). 5'N activity after 7 days of culture was 22.1 nm/h/10⁶ cells in irradiated cells versus 67.3 nm/h/10⁶ cells in nonirradiated cells.

DISCUSSION

The observation that CBMC have diminished levels of 5'N compared to adult cells has previously been made (20, 24). This does not appear to be due to significant differences in the relative % of cells with different phenotypes in these two populations (1, 16). Previous studies have suggested that this difference is a reflection of the degree of maturation of newborn cells (20, 24). Despite this initial difference, stimulation of CBMC by either PHA or PWM results in expression of 5'N levels comparable to stimulated adult cells.

Previous studies in which 5'N activity has been evaluated in mitogen-stimulated cells have yielded conflicting results. In studies reported by Quagliata *et al.* (22), PHA stimulation failed to affect 5'N activity in cultured cells from normal individuals or patients with CLL. But the maximum culture period in these studies was only 5 days. In another study, stimulation of tonsillar-derived human lymphocytes with four different mitogens induced a decline in 5'N activity during a 4-day culture period (23). Studies in mouse spleen cells demonstrated a 1.5-3-fold increase in 5'N activity on day 2 when cells were 62-67% blast forms as determined by the fluorescent-activated cell sorter. Subsequently, there was a decline in 5'N activity in all cultures except those stimulated by lipopolysaccharide (18).

The results of our study raise the question of whether the mitogen-induced enhanced expression of 5'N is a result of differentiation of stimulated cells or whether it reflects some other phenomena. There are several lines of evidence to support the concept that 5'N activity may represent a differentiation marker in lymphocytes. First, lymphocytes from patients with conditions that have been associated with diminished ecto-5'N activity such as X-linked agammaglobulinemia (4, 10, 12, 24, 27, 30), common variable hypogammaglobulinemia (4, 10, 12, 24, 30) CLL (21, 22) and cord blood lymphocytes (20, 24) are thought to have altered maturation of lymphoid cells. Second, this concept is further supported by investigations of the distribution of 5'N activity in different murine, human, and rabbit lymphoid tissues. These studies have demonstrated that 5'N activity was lowest in intrathymic T lymphocytes and highest in the supposedly more differentiated T cells found in the peripheral blood and lymph nodes (2, 9, 11, 13, 29). Third, factors that promote T-cell differentiation, such as thymosin and human thymic epithelium conditioned medium, have been shown to enhance 5'N activity (9). Finally, both *in vitro* studies of lymphoblastoid cultures over time, as well as studies in aging human subjects show changes in 5'N activity with age (5, 28).

It also is clear that mitogen stimulation may induce some changes associated with differentiation. For example, PWM stimulation induces some B cells to differentiate into immunoglobulin secreting cells (25). Stimulation with Con A or PHA may in some circumstances induce T suppressor cell activity (14, 26). Stimula-

Fig. 3. Comparison of ratios of 5'N activity in stimulated cultures to unstimulated cultures on day 7 of culture. Stimulation ratios in fractionated cells were always less than in the unfractionated cells. UF, unfractionated; T, T cell enriched; and B, T cell depleted.

peak responses to PHA than did AMC. Thymidine incorporation by unstimulated cells was also higher in CBMC. Similar findings were observed after PWM stimulation.

Effect of irradiation on 5'N activity in stimulated cells. Efforts to evaluate 5'N activity after 7 days of culture in mitomycin-treated, mitogen-stimulated cells were unsuccessful because of poor cell yields at the end of the culture period. AMC given 2000R and cultured in the presence of PHA, were evaluated for incorporation of tritiated thymidine at day 4 and 5 and for 5'N activity after 7 days of culture. Incorporation of tritiated thymidine by irradiated

tion of responsive cells with mitogen results in other changes not necessarily associated with differentiation such as increased DNA synthesis and changes in the cell membrane. These latter changes include enhanced glucose transport across the membrane, changes in surface receptors, activation of cellular proteases bound to the plasma membrane, activation of membrane ATPases, and enhanced phospholipid turnover (17).

It is possible that these membrane-associated changes result in enhanced expression of the ecto enzyme 5'N independent of the state of differentiation of the cell. It is evident that induction of one mitogen-induced effect is not always associated with other mitogen dependent effects. For example, it has been observed that despite a proliferative response to PWM that is as good as that seen in adult cells (Table 4), PWM stimulation generally fails to induce differentiation of CBMC to immunoglobulin synthesis (15, HGH, unpublished observations). The observation that 5'N activity in PHA-stimulated cultures is greater at day 7 than at day 3 or 4, when PHA-induced DNA synthesis is at its peak, suggests that 5'N activity increases are not solely a reflection of the degree of activation. This is further supported by our study showing that incorporation of tritiated thymidine by irradiated cells was far more decreased than was expression of 5'N activity by similarly treated cells.

We also found that 5'N levels were generally higher in stimulated unfractionated cells than in TCE populations. This was not due to an intrinsic difference in 5'N levels in TCE and TCD populations as they were similar in fresh, unstimulated cells (Table 3). Previous studies also have indicated that TCE populations respond well in transformation assays to both PHA and PWM (31). This finding could indicate that optimal expression of the enzyme in stimulated cultures may be dependent upon cellular interactions.

PWM stimulation of the TCD population resulted in 5'N activity similar to that seen in unfractionated cells. This mitogen stimulates both T and B cells as opposed to PHA, which stimulates T cells only. Our TCD populations had a high % of OKM-1 positive cells as well as apparently adequate numbers of T cells to induce both proliferation and enhanced 5'N activity (31).

The effect of monocytes in these studies is not clear. Based on our method of detecting these cells (OKM-1 monoclonal antibody) it appears that both TCE and TCD populations should have enough monocytes for ready activation by mitogen. Perhaps the relative deficiency of OKM-1 positive cells in TCE populations is sufficient for activation and proliferation but does not provide adequate help for expression of 5'N. If this is the case, it would indicate that the expression of 5'N is more monocyte-dependent than is lymphocyte transformation.

How mitogen stimulation enhances 5'N activity expression by mononuclear cells is not clear. Our bias is that this enhanced activity is a reflection of membrane events that are not necessarily associated with differentiation. Further studies using this model may provide insights into the relationship of this enzyme with lymphocyte function.

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