Expression of 5'-Nucleotidase (CD73) Related to Other Differentiation Antigens in Leukemias of B-Cell Lineage


Ecto-5'-nucleotidase (5'NT; CD73) expression was studied with a monoclonal antibody (7G2) and a radiochemical assay and compared with the expression of other antigens in B-cell-lineage leukemias on cells from 100 leukemic patients and two cell lines. A B-cell origin was confirmed by the expression of CD19 and HLA-DR. Four stages of B-cell leukemias were defined: stage I (pro-B) as CD10\(^+\), cytoplasmic \(\mu\) (\(\mu\)-); surface Ig (\(\text{slg}^+\)); stage II (cALL) as CD10\(^-\)/\(\mu^+\)/\(\text{slg}^+\); stage III (pre-B) as CD10\(^-\)/\(\mu^+\)/\(\text{slg}^-\); and stage IV (B) as CD10\(^-\)/\(\mu^+\)/\(\text{slg}^-\). A linear correlation was found between immunohistochemical and radiochemical determination of 5'NT (\(r = 0.86\)). 5'NT expression was low in T-cell leukemias and stage I, high in stages II and III, and low again in stage IV of B-cell leukemias. 5'NT expression was not related to \(\text{cp}^+\).

From several studies of acute lymphoblastic leukemia (ALL) it is known that CD10\(^+\) cells can have high 5'NT activities.\(^6,11\) The scarce data in these studies about 5'NT activity in the so-called non-T, non-B ALL and null-ALL stages have to be viewed with great care because these groups were poorly defined. The classification into these groups is out of date with the acquisition of new monoclonal antibodies (MoAbs) that define differentiation antigens. Also, MoAbs against 5'NT (CD73) have been developed recently.\(^12\) The aim of the present study was to define the expression of 5'NT in leukemias corresponding to the earliest stages of B-cell differentiation. Therefore, immunocytochemical and radiochemical detection of the enzyme was compared with the expression of other differentiation antigens.

MATERIALS AND METHODS

Reagents. The CD3, CD4, CD7, CD8, CD10, and CD34 MoAbs were purchased from Sanbio (Uden, The Netherlands); CD2, HLA-DR, \(\kappa\), and \(\lambda\) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands); CD19, CD22, and terminal deoxynucleotidyl transferase (TdT) from DAKO (DKT Diagnostics, de Kwakel, The Netherlands); CD20 from Becton Dickinson (Etten-Leur, The Netherlands); and CD21 from Coulter Clone (Hialeah, FL). The CD73 antibody used was 7G2.\(^12\) Fluorescein isothiocyanate (FITC)-conjugated antimouse IgG was from DAKO. [\(8-14\)C]-inosine monophosphate (IMP; 50 \(\mu\)Ci/ml, specific activity \(800 \text{Ci/mmol}\)) was derived from Amersham International (Buckinghamshire, UK); \(\alpha\)-methylene adenosine diphosphate (AOPCP), IMP, inosine, hypoxanthine, and adenosine from Sigma (St Louis, MO). Plastic sheets precoated with 0.1 mm polyethyleneimine (PEI)-cellulose were from Merck (Darmstadt, Germany).

Cells. Cells were obtained from bone marrow and peripheral blood samples of 100 leukemic patients, 97 patients with ALL, and three with chronic lymphocytic leukemia (CLL). The study was approved by the Institutional Review Board. Informed consent was obtained in each case according to the Declaration of Helsinki. In addition, two lymphoblastic cell lines, WI-L2 and 1254, were used.\(^13\) Mononuclear cells were isolated on a Ficoll Isopaque gradient and in most cases used after cryopreservation as described before.\(^14\)

Immunohistochemistry. Immunoperoxidase staining was performed using cytospin cells to detect \(\text{cp}^+\). Aliquots of 50 \(\mu\)l of 0.5 × 10\(^8\) cells/mL Earle’s balanced salt solution with 5% human serum albumin were cytotoxicelugrifuged and dried on silica-gel. Cells were...
fixed in buffered formaldehyde acetone (4°C) for 30 seconds. After rinsing with water and phosphate-buffered saline (PBS), cells were incubated with 50 μL of MoAbs at room temperature for 60 minutes. Next, the slides were rinsed with PBS three times. Rabbit antimouse peroxidase with 2% pooled human serum was added for 60 minutes. Peroxidase staining was performed using diaminobenzidine with 0.05 mol/L imidazole in 0.05 mol/L Tris buffer (pH 7.6) for 10 minutes, and with copper sulphate 0.5% for 60 minutes. Peroxidase staining was performed using diamino benzidine with 0.05 mol/L imidazole in 0.05 mol/L Tris buffer (pH 7.6) for 10 minutes, and with copper sulphate 0.5% for 60 minutes.

After rinsing twice, FITC-conjugated rabbit antimouse IgG with 2% pooled human serum and 10% rabbit serum was added for 30 minutes. Cells were rinsed twice and resuspended in 0.3 mL PBS/BSA/sodium azide. Fluorescence was measured with a FACStar (Becton Dickinson). Flow-cytometric detection of intracellular TdT was performed after fixation of the cells with buffered formaldehyde acetone as described by Slaper-Cortenbach et al.

For detection of surface antigens, cells were fixed with 1% paraformaldehyde diluted in PBS for 5 minutes followed by rinsing with PBS containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide. Immunofluorescence staining was performed with MoAbs CD2, CD3, CD4, CD7, CD8 (as T-cell markers), CD10, CD19, CD20, CD21, CD22, CD34, TdT, HLA-DR, k, λ, and CD73. After rinsing twice, FITC-conjugated rabbit antimouse IgG with 2% pooled human serum and 10% rabbit serum was added for 30 minutes. Cells were rinsed twice and resuspended in 0.3 mL PBS/BSA/sodium azide. Fluorescence was measured with a FACStar (Becton Dickinson). Flow-cytometric detection of intracellular TdT was performed after fixation of the cells with buffered formaldehyde acetone as described by Slaper-Cortenbach et al.

**Definition**: Samples were considered antigen positive if the percent of cells expressing the antigen was greater than 20% after substracting of control. The samples of the B-cell-lineage leukemias were classified into four stages. The B-cell origin of these samples was confirmed by virtue of the expression of CD19 and HLA-DR. The most mature, stage IV B-cell leukemias were CD10+, CD4+, and sIg+. Less mature, early pre-B cells that did not express CD10 were classified into two stages based on the expression of CD10 according to the model of Nadler et al.

**Radiochemical assay.** 5'NT was determined radiochemically as follows. Cells were suspended in 50 mmol/L Tris buffer, pH 7.4, and 1 mmol/L EDTA at a density of 5 x 10⁶ cells/mL. Cells were disrupted by sonification on ice with four bursts of 5 seconds with 10-second intervals. Twenty microliters of this lysate was spotted onto PEI-thin layer chromatography sheets. IMP, inosine, and hypoxanthine were separated on distilled water; the spots were identified under ultraviolet excitation at 254 nm, cut out, and quantitated by liquid scintillation counting. Enzyme activities were expressed as nanomoles of IMP converted to inosine and hypoxanthine per hour per 10⁶ cells. 5'NT was defined as the AOPCP-inhibited fraction. The results shown are the means of duplicate determinations. The median coefficient of variation of the individual determinations is 4.7%.

**Statistics.** Statistical testing was performed with the χ²-test and the Mann-Whitney U test at a two-sided significance level of 0.05.

**RESULTS**

There were 19 T-ALL samples and 83 samples of B-cell lineage including the two cell lines. Four samples were pro-B-ALL (stage I), 43 cALL (stage II), 28 pre-B- (stage III), and 8 B-cell (stage IV) leukemias.

A good linear correlation was found between the percentage of 5'NT⁺ cells determined by the FACS and the 5'NT activity determined by the radiochemical assay (r = 0.86; r = .84 if only B cells were evaluated; Fig 1). The percentage of 5'NT⁺ cells is related to the immunologic subtypes, as shown in Table 1 and Fig 2. The radiochemical 5'NT activity of the different immunologic subtypes is shown in Table 1 and Fig 3. It is clear that in the least mature stage I the 5'NT expression is low. It is high in the CD10⁺ stages II and III and low again in stage IV. 5'NT was low in T-ALL, including one CD10⁻ T-ALL.

We evaluated whether there was a correlation between the expression of 5'NT and other differentiation antigens of the B-cell lineage. No significant correlation could be detected between the percent of cells positive for 5'NT and the percent of cells positive for CD20, CD21, CD22, CD34, or TdT. For CD19 and HLA-DR, such a correlation was not performed because all B-lineage samples were positive for these two antigens.

There was a significant correlation between the percent of CD10⁺ and 5'NT⁺ cells (P < .05; Table 2). The mean (±SD) 5'NT activity of CD10⁺ cells was 6.1 ± 12.2

**Table 1. Percentages of 5'NT⁺ Cells Determined by Immunofluorescence Staining and 5'NT Activity Determined Radiochemically in T-ALL and the Four Stages of B-Cell-Lineage Leukemias**

<table>
<thead>
<tr>
<th>Immunophenotype</th>
<th>Median % 5'NT⁺</th>
<th>Range % 5'NT⁺</th>
<th>Median 5'NT (nmol/h/10⁶ cells)</th>
<th>Range 5'NT (nmol/h/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-ALL</td>
<td>2.0</td>
<td>0.0-9.1</td>
<td>0.8</td>
<td>0.0-2.6</td>
</tr>
</tbody>
</table>

**B-lineage:**

- **Stage I, pro-B**: 1.5 (0.6-9.4) 3.0 (1.9-6.0)
- **Stage II, cALL**: 36.3 (0.4-91.0) 30.0 (0.0-109.9)
- **Stage III, pre-B**: 48.4 (6.9-93.2) 23.2 (1.6-82.5)
- **Stage IV, B**: 5.3 (0.8-31.5) 0.6 (0.0-11.7)
nmol/h/10^6 cells (median 1.8) compared with 36.9 ± 28.8
nmol/h/10^6 cells (median 41.4) of CD10^+ samples. The
range of 5'NT values in the CD10^- stages II and III is very
wide, as is illustrated in Table 1 and Figs 2 and 3; several
CD10^- cases were even 5'NT-. High 5'NT activities were
not found in stages I and IV, but were always associated
with CD10 expression. Two-color fluorescence studies have
been performed in a few cases (Fig 4). These confirm the
relationship between 5'NT and CD10 expression. Cases
that were CD10^- and cases that were CD10^+ did not show significant differences in 5'NT expression. Among
stage III samples, the number of CD10^- samples was too
low (n = 3) to compare their 5'NT activity with that of
CD10^+ cases.

Within the CD10^- group, there was no significant correlation
between the percent of cells positive for 5'NT and the percent of cells positive for some CD10, CD20, CD21, CD22, CD34,
and TdT.

A significant inverse correlation between the percent of cells positive for κ or λ and the percent of cells positive for 5'NT was found (P < .01; Table 2). The mean (±SD) 5'NT activity of κ^- samples was 35.7 ± 28.5 nmol/h/10^6 cells (median 36.0) compared with 2.2 ± 3.9 nmol/h/10^6 cells
(median 0.6) of κ^- samples. The three CLL samples were
κ^- or λ^- and were therefore classified as stage IV B-cell
leukemias. The percentages of 5'NT^- cells of these samples
were 31.5%, 0.8%, and 1.4%. In the latter two cases, 5'NT
activity was determined radiochemically: 0.9 and 0.1 nmol/
h/10^6 cells. These data are also shown in Figs 2 and 3.

Cell line 1254 is a spontaneous mutant of WI-L2. 1254
shows a more mature phenotype with κ expression and is
5'NT-, opposite to WI-L2 cells, which are κ^- and 5'NT^-.

**DISCUSSION**

Up to now the relationship of 5'NT to B-cell maturation,
in particular in the very early stages of B-cell development,
has not been clearly defined. In this study we established
the place of 5'NT in the maturation scheme by comparing
its expression, determined by immunocytochemical and
radiochemical assays, in relation to a panel of other
differentiation antigens. Leukemic B-cell–lineage samples
were categorized into four stages depending on their
expression of sIg, κ, and CD10. Stage I (pro-B) samples
did not express any of these three markers. The B-cell
origin of these samples was confirmed by the expression of
CD19 and HLA-DR. According to the model of Nadler et
al., pro-B cells are of a less mature type than the cells that
already express CD10^+ and were called stage II in the
present study. Normal counterparts for leukemic pro-B cells have been recognized among fetal and adult bone marrow cells.16

Our results clearly show that the median 5'NT is low or absent in the stage I pro-B cells, increases to very high activities in the CD10+ stages II and III, and decreases again in stage IV. 5'NT is only expressed on the cell surface together with CD10, but is clearly not present on all CD10+ leukemias. This finding is reflected in the wide ranges of 5'NT activities in stages II and III. In conclusion, 5'NT is expressed in CD10+ stages and its activity decreases before \( \kappa \) or \( \lambda \) appears on the cell surface.

Our findings are based on a study of malignant cells and it can be questioned whether the results can be extrapolated to normal B cells. A study to determine the 5'NT activity of non-malignant CD10+ cells has to show whether the high 5'NT activity on leukemic CD10+ cells is a malignant phenomenon or a normal maturation phenomenon. In a previous report we have used a histochemical lead staining method that is less sensitive than the methods used in the present report to study the prognostic significance of 5'NT in a group of 33 children with CD10+. It appeared that a high 5'NT activity was related to a poorer prognosis among children with CD10+ ALL. This prognostic factor was found to be independent from sex, age, and white blood cell count. Very recently, other investigators confirmed the prognostic significance of 5'NT activity among adults with CD10+ ALL.

Several hypotheses have been postulated to explain the prognostic value of 5'NT by its possible role in resistance to the thiopurines 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) or to methotrexate.20,21 If 5'NT plays an important role in drug resistance, one could expect an increased sensitivity of T-cell leukemias to methotrexate or thiopurines. The sensitivity to these drugs depends, however, on many other factors that might differ between T-ALL and B-lineage ALL. For example, the activity of hypoxanthine-guanine phosphoribosyl transferase (HPRT), the enzyme that converts the inactive 6-MP and 6-TG to their cytotoxic nucleotides, is lower in T-ALL than in B-lineage ALL.22

Recent findings suggest that 5'NT plays a role in signal transduction pathways and lymphocyte proliferation. CD73 antibodies stimulated DNA synthesis specifically in 5'NT+ lymphocytes. Also, CD73 antibodies induced a fraction of normal B cells to leave G0 phase and enter the G1 phase of the cell cycle. The same antibodies stimulated H-thymidine uptake in malignant B cells from patients with CLL.23,24 Thus, 5'NT+ CD10+ ALL cells might have different control mechanisms of cell proliferation than 5'NT+ CD10+ ALL cells. The present study did not show differences in expression of other B-cell antigens between 5'NT+ CD10+ and 5'NT+ CD10- ALL.

We conclude that the place of 5'NT (CD73) in leukemias corresponding to stages of early B-cell development has been characterized. Future studies should make clear whether a high expression of this enzyme in CD10+ leukemias is a normal maturation phenomenon or a malignant phenomenon. The role of 5'NT in drug resistance and cell proliferation has to be studied.

REFERENCES


Expression of 5'-nucleotidase (CD73) related to other differentiation antigens in leukemias of B-cell lineage

R Pieters, LF Thompson, GJ Broekema, DR Huismans, GJ Peters, ST Pals, E Horst, K Hahlen and AJ Veerman

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