Purine Nucleoside Phosphorylase (PNP) and Adenosine Deaminase (ADA) Activities Examined Cytochemically in Unfixed Lymphocytes of Patients With Lymphoproliferative Disorders

By Keizo Maeda, Kazuhiko Ito, and Nobuo Yamaguchi

New techniques have been devised for the cytochemical demonstration of purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA) activities in unfixed human lymphocytes. A suspension of living lymphocytes is mixed with agarose sol containing the reagents for the detection of PNP or ADA activity on a glass slide. The mixture solidifies, is incubated, and then dried for light-microscopic observation. Reactive cells are recognized by the diffusely deposited granules of formazan, the end-product of the cytochemical reaction, and are divided into three groups of the cell with the low, middle, and high enzyme activity by the number of the granule. In healthy adults, the mean percentages of PNP- and ADA-positive cells were more than 90% in unfractionated lymphocytes, T-cell fractions, and complement-receptor cell fractions and cells with middle PNP and ADA activities were predominant. The PNP and ADA staining was observed in lymphoid cells of patients with lymphoproliferative disorders. A decrease in the percentage of PNP-positive cells accompanied with a relative increase of cells with the low enzyme activity was observed in the lymphocytes of nine patients with chronic lymphocytic leukemia (CLL). Similar findings were obtained in the ADA staining of the lymphocytes of five patients with B-cell CLL.

MATERIALS AND METHODS

Patients

Seven patients with B-cell CLL, one with null cell CLL, one with T-cell CLL, two with adult T-cell leukemia, one with Sézary’s syndrome, one with null cell acute lymphocytic leukemia (ALL), two with leukemic lymphosarcoma, and two with hairy cell leukemia were studied. These patients were hospitalized at Research Institute for Nuclear Medicine and Biology of Hiroshima University, Kobe Municipal Central Hospital, the Center for Adult Diseases, Osaka, Kobe Shinko Hospital, Ekisaikai Hospital, and Kobe University Hospital. Twenty-one healthy adult volunteers served as normal controls.

Purification of Lymphocytes

Ten volumes of heparinized whole venous blood were mixed with one volume of a 5% suspension of silica gel (KAC-II, Japan Immunoresearch Laboratories Co., Takasaki, Japan) and incubated for 1 hr at 37°C with gentle agitation. Phagocytic cells with ingested silica were removed by Ficoll-Isopaque gradient centrifugation. The interface layer was aspirated and washed three times in phosphate-buffered saline (PBS) at pH 7.5, and is referred to as unfractionated lymphocytes (UFL). More than 96% of the cells were found to be

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viable by trypan blue dye exclusion. Less than 2% of the UFL were peroxidase-positive.

**T Cells**

T cells were identified and isolated from UFL by spontaneous rosette (E-rosette) formation with neuraminidase-treated sheep erythrocytes by a slight modification of the method of Sakane and Green. The rosetting-forming cells were washed 3 times in PBS, after which the erythrocytes were left to be lysed by incubation with distilled water for 20 sec. or by incubation for 6 min at 37°C with Tris buffer containing 0.83% ammonium chloride (pH 7.4). Rosette-forming cells were counted in a total of 500 lymphocytes stained with methylene blue. A rosette was defined as a lymphocyte with three or more sheep erythrocytes attached to its surface. The mean percentage of E-rosette-forming cells in UFL of 13 healthy adults was 70% (range 64%–77%).

**Complement-Receptor Cells**

Complement-receptor cells were identified and isolated from UFL by rosette formation with sheep or ox erythrocytes sensitized with rabbit IgM antibody to sheep or ox erythrocytes and human complements (EAC). EAC were prepared by a slight modification of the method of Galili and Schlesinger. Rabbit IgM antibody to sheep or ox erythrocytes was purchased from Cordis Laboratories, Miami, Fla., and that to ox erythrocytes from Japan Immunoresearch Laboratories. EAC and UFL were mixed in a ratio of 50:1 in PBS, centrifuged at 200 g for 5 min at 37°C, and incubated at 37°C for 1 hr. EAC-rosette-forming cells were enumerated and isolated by the procedure described for T cells. The mean percentage of E(OX)AC-rosette-forming cells in UFL of 10 healthy adults was 16% (range 12%–19%). Complement-receptor cell fractions of three healthy adults contained 39%–48% surface membrane immunoglobulin (SmIg) bearing cells. SmIg-positive cells were detected by the immunofluorescence method of Horwitz and Lobo, with the use of fluorescein-conjugated polyvalent goat anti-human immunoglobulins (IgG, IgM, IgA) obtained from Behring Institute, Marburg, West Germany.

**L Cells**

E-rosette-forming cells and then EAC-rosette-forming cells were removed from UFL by rosette formation as described above. The remaining cells were defined as L cells.

**Cytotoxic Procedures for Demonstration of PNP and ADA Activities**

About 6 × 10⁶ lymphocytes suspended in 1 ml of PBS were mixed with 50 μl of agarose sol containing the reagents for the detection of PNP or ADA activity in the circle with a diameter of 14 mm on a glass slide at 37°C within 20 sec. Immediately after the agarose sol solidified at room temperature, the mixture was incubated in moist air at 37°C for 1 hr, shielded from the light. The mixture was then air-dried at room temperature. The cells in the filmy agarose layer were counterstained with 0.005% neutral red and then observed with a light microscope through an oil-immersion lens. The percentage of PNP- or ADA-positive cells was calculated by counting at least 1000 lymphocytes in each sample. The agarose sol used for the detection of PNP activity was prepared by dissolving 20 mg of inosine (Kohjin Co., Kyoto, Japan), 10 mg of phenazine methosulfate (Nakarai Chemicals, Kyoto, Japan), 30 mg of MTI tetrazolium (Sigma Chemical Co., St. Louis, Mo.), and 1.1 U of xanthine oxidase (Sigma) in an agarose sol kept at 37°C, the sol containing 550 mg of agarose (Agarose A-37, Nakarai) in 100 ml of 25 mM sodium potassium phosphate buffer (pH 7.5) containing 116 mM NaCl. For the preparation of the sol for the detection of ADA activity, 40 mg of adenosine (Nutritional Biochemicals Corp., Cleveland, Ohio) replaced inosine, and 1.6 U of PNP (Boehringer Mannheim Corp., New York, N.Y.) were added.

For investigation of the sensitivity of the method, UFL of healthy adults were heat-treated at 56°C for 30 and 37 min in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Nakarai), pH 7.4, and used as samples for determination of PNP and ADA activities, respectively. More than 84% and 65% of the UFL heat-treated for 30 and 37 min, respectively, were found to be viable by trypan blue dye exclusion. The number of cells was not decreased during the treatment.

In the cytochemical reaction of PNP or ADA, each substrate was finally converted to uric acid if PNP or ADA was present in the cells. The two-stage oxidative reaction of hypoxanthine to xanthine and then to uric acid is coupled with the reduction of MTT tetrazolium into insoluble blue formazan in the presence of phenazine methosulfate.

**Negative Controls**

Negative control experiments were carried out with an agarose sol from which inosine, adenosine, inorganic phosphate, or xanthine oxidase was omitted. For the preparation of an agarose sol without inorganic phosphate, 25 mM Tris-maleate buffer (pH 7.5) was substituted for phosphate buffer. Potent competitive inhibitors of PNP and ADA—formycin B and cofomycin, respectively—were introduced into the agarose sol at a concentration of 1 mM. Both reagents are gifts from Dr. Aoyagi of the Microbial Chemistry Research Foundation, Institute of Microbial Chemistry, Tokyo, Japan.

**PNP and ADA Spectrophotometric Assays**

PNP- and ADA-specific activities of lymphocytes were assayed spectrophotometrically by a modification of the method of Hopkinson et al. After contaminating erythrocytes were removed by the procedure described above, lymphocytes were resuspended in 50 mM sodium potassium phosphate buffer, pH 7.5, and sonically disrupted by four 15-sec bursts with cooling. The cell debris was pelleted at 10,000 g for 3 min with cooling in a Fisher Model 59 microcentrifuge (Fisher Scientific Co., Pittsburgh, Pa.). The resulting clear supernatant was used for enzyme assays. For PNP assay, 50 μl of the supernatant was added to 1.0 ml of the phosphate buffer containing 0.2 μmole inosine and 0.05 U xanthine oxidase. For ADA assay, 50 μl of the supernatant was added to 1.02 ml of the phosphate buffer containing 0.2 μmole adenosine, 0.05 U xanthine oxidase, and 0.05 U PNP. The PNP and ADA reactions were carried out at 37°C against a blank solution from which adenosine and inosine had been omitted, respectively. The rate of production of uric acid was measured at 293 nm. Protein concentration was determined by the method of Lowry et al., using bovine serum albumin (Sigma) as a standard. The specific activity is expressed as nanomoles per hour per milligram of protein.

**Statistical Analysis**

Results were compared by using the two-tailed Student's t test.

**RESULTS**

**Cytotoxic Demonstration of PNP and ADA Activities in Lymphocytes**

PNP and ADA activities in circulating lymphocytes of healthy adults were demonstrated cytochemically
by our method. Either PNP- or ADA-positive cells were easily recognized by many large blue granules of formazan, the end-product of the cytochemical reaction, which are deposited diffusely as shown in Fig. 1. The normal shape of the cells and nuclei was clearly retained in more than 90% of the cells observed. In negative control reactions without each of the reagents, as described by Edwards et al.26 and Spencer et al.;27 cells with no granules or minute granules were observed. Formazan production was markedly decreased by the addition of formycin B, a potent competitive inhibitor of PNP, or by coformycin, an inhibitor of ADA, to the agarose sol (Fig. 2). These negative controls provided evidence that PNP and ADA are responsible for the production of large formazan granules. A PNP- or ADA-positive cell was defined as a lymphocyte that has one or more large granules of formazan.

The sensitivity of our cytochemical method was examined with heat-treated UFL of healthy adults. The heat-treated UFL had only 8% PNP and 30% ADA activities of the initial values measured biochemically, but 89% PNP- and ADA-positive cells.

In addition, as seen in Table 1, UFL of patient 13 had 18% PNP activity of the mean value of normal controls, but 90% PNP-positive cells. UFL of patient 2 had 42% ADA activity of the mean value of normal controls, but 88% ADA-positive cells. These results indicate that our method can detect at least 10% PNP and 30% ADA activities of those of normal UFL.

Distribution of PNP and ADA Activities in Normal T and Complement-Receptor Cells

The percentages of PNP- and ADA-positive cells were examined in T-cell and complement-receptor cell fractions of healthy adults (Table 1). The mean percentages of PNP-positive cells were 97% in UFL, 93% in T-cell fractions, and 93% in complement-receptor cell fractions. The very high value in complement-receptor cell fractions indicates that the majority of B cells are PNP-positive, because most SmIg-bearing lymphocytes have complement receptors.26

The mean percentages of ADA-positive cells were 91% in UFL, 95% in T-cell fractions, and 95% in complement-receptor cell fractions. The distribution of ADA in T and B cells was similar to that of PNP.
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<th>Surface Markers (%)</th>
<th>Lymphocyte Fraction</th>
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<th>Percent PNP-Specific Activity of Cells (%)</th>
<th>ADA Activity</th>
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**Table 1.** PNP and ADA Activities in Lymphocyte Fractions of Patients With Lymphoproliferative Disorders

- PNP- and ADA-positive cells are divided into three groups: low, middle, and high enzyme activity, which contains from 1 to 4, from 5 to 9, and 10 or more large formazan granules, respectively.
- Specific activities are measured spectrophotometrically as described under Materials and Methods. Percent specific activities of UFlL from patients with B-cell, T-cell, and null cell malignancies are the percentage of the mean specific activities of normal complement-receptor cell fractions, T-cell fractions, and UFlL, as controls, respectively.
- Expressed as n mole/hr/mg of protein. Mean ± standard error.
- Patient 1 was reexamined 1 yr later.
- Patient 17 was reexamined after receiving the treatment for skin lesions with externally applied prednisone for 6 wk.
- The figures in normal controls represent mean and range.

SmIg, surface membrane immunoglobulin; CR cell, complement-receptor cell; ND, not determined; n, the number of subjects tested.

*NPN- and ADA-positive cells are divided into three groups of the cell with the low, middle, and high enzyme activity, which contains from 1 to 4, from 5 to 9, and 10 or more large formazan granules, respectively.*

†Specific activities are measured spectrophotometrically as described under Materials and Methods. Percent specific activities of UFlL from patients with B-cell, T-cell, and null cell malignancies are the percentage of the mean specific activities of normal complement-receptor cell fractions, T-cell fractions, and UFlL, as controls, respectively.

‡Expressed as n mole/hr/mg of protein. Mean ± standard error.

§Patient 1 was reexamined 1 yr later.

¶Patient 17 was reexamined after receiving the treatment for skin lesions with externally applied prednisone for 6 wk.

The figures in normal controls represent mean and range.

SmIg, surface membrane immunoglobulin; CR cell, complement-receptor cell; ND, not determined; n, the number of subjects tested.
When PNP- and ADA-positive cells of UFL, T-cell, and complement-receptor cell fractions are divided into three groups of the cell with the low, middle, and high enzyme activity, which contains from 1 to 4, from 5 to 9, and 10 or more large formazan granules, respectively, cells with middle PNP activity and middle ADA activity were predominant, as shown in Table I.

**Distribution of PNP and ADA Activities in Lymphocytes of Patients With Various Lymphoproliferative Disorders**

Table I shows the percentages of cytochemically determined PNP- and ADA-positive cells and the specific activities of the two enzymes measured spectrophotometrically in circulating lymphocytes of patients with various lymphoproliferative disorders.

CLL in patients 1–7 was classified as being of B-cell origin, since 59% of the UFL from patient 1 were SmIg-positive and 50% or more of the UFL from the others have complement receptors. The percentages of PNP-positive cells among UFL of patients 1, 2, and 5 and complement-receptor cell fractions of patients 3, 4, 6, and 7 were significantly decreased to various degrees, as compared with those in normal controls (p < 0.001). The percentage of PNP-positive cells was also decreased in the UFL from patients with null cell CLL and T-cell CLL (p < 0.001). The specific activities of PNP in the lymphocytes of patients 1, 2, 3, 6, and 9 were significantly decreased to various degrees, as compared with those of normal controls (p < 0.05). A strong decrease in the number of PNP-positive cells is mainly responsible for the low PNP activity of lymphoid cells in CLL patient 1 (Fig. 3). In the other patients with CLL, cells with low PNP activity containing from 1 to 4 large formazan granules were predominant among PNP-positive cells, except for the UFL of patient 7. A depression of the enzyme activity in each PNP-positive cell, as well as a decrease in the number of PNP-positive cells, was observed in these patients.

In contrast to the findings in CLL patients, the percentage of PNP-positive cells was over 80% in the lymphoid cells of patients with the other lymphoproliferative disorders, as shown in Table I. Cells with low PNP activity were predominant in patients 10 and 11 with adult T-cell leukemia and patient 13 with null cell ALL (Fig. 4) and were 45% of the PNP-positive cells in patient 12 with Ségary’s syndrome. The specific activity of PNP was significantly decreased in the UFL of patients 10, 12, and 13 (p < 0.05). These results indicate that a depression of the enzyme activity in each PNP-positive cell is mainly responsible for the low PNP activities of lymphoid cells in these patients. In lymphoid cells of patients with leukemic lymphosarcoma and hairy cell leukemia, cells with middle PNP activity were predominant together with normal or higher percentages of cells with high PNP activity.

The percentage of ADA-positive cells in lymphocytes was also significantly decreased to various degrees in B-cell CLL patients 1, 3, 5, and 6 (p < 0.001), but there was not a close correlation with that of PNP-positive cells. In patients with B-cell CLL and B-cell lymphosarcoma, cells with low ADA activity were predominant among ADA-positive cells. The specific activity of ADA was also significantly decreased in patients 1, 2, 3, and 14 (p < 0.05). In contrast, in patients with the other diseases, cells with middle ADA activity were predominant.

The UFL of three patients 9, 10, and 12 with T-cell malignancies, including T-cell CLL, adult T-cell leukemia, and Ségary’s syndrome, mostly composed of

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**Fig. 3.** Light microscopic appearance of PNP activity in UFL of CLL patient 1. The picture shows nonreactive cells with no large granules of formazan. Black substances recognized in the cells are not formazan deposits but parts of a nucleus stained with neutral red. They could be easily differentiated in a color print.

**Fig. 4.** Light microscopic appearance of PNP activity in UFL of ALL patient 13. The picture shows less reactive cells, with only a few large granules of formazan (×790).
E-rosette-forming cells, had lower PNP and higher or normal ADA-specific activities than the levels of the two enzymes in normal T-cell controls, which are not significantly different from each other (p > 0.05).

DISCUSSION

PNP activity was demonstrated in at least 90% of complement-receptor cells and about 97% of unfracti
tioned lymphocytes of healthy adults in our cyto-
chemical studies. This finding shows that PNP is
present in normal circulating B cells, because it has
been reported that about half of complement-receptor
cells have SmIg and correspond to about 80%
of SmIg-positive cells.25 In contrast, Borgers et al.3
reported in recent years that PNP activity is absent
from normal human circulating B cells, because they
failed to detect PNP activity in about 65% of SmIg-
bearing lymphocytes, which correspond to about 25% of
peripheral blood lymphocytes, in their cytochemical
studies of fixed cells. The discrepancy between the two
findings may be ascribed to a decrease in the enzyme
activity in the course of cell fixation with glutaralde-
hyde performed by the method of Borgers et al.3 Our
method has been devised to be carried out on unfixed
cells. The reagents for demonstrating enzyme activity
penetrate lymphocytes through cell membranes dam-
age when agarose sol solidifies. When PNP activity
of normal complement-receptor cell fractions was
measured spectrophotometrically, it was not signifi-
cantly different from that in normal T-cell fractions
(p > 0.05) (Table 1). B-cell lines of normal or
leukemic cell origin have been reported to have rather
higher PNP activity than T-cell lines when assayed
spectrophotometrically.5,6 These findings support our
results obtained cytochemically.

Recently, unusually high levels of deoxyguanosine
triphasphosphate were found in erythrocytes of patients
with a hereditary PNP deficiency. Deoxyguanosine
triphasphosphate has been proposed as a possible toxic
metabolite to lymphocytes.27-29 However, the definite
mechanism causing selective T-cell immune dysfunc-
tion in this disorder remains unclear. The thesis that
PNP is selectively distributed in T cells is not accept-
able, as stated above.

It is well known that various immune defects are
often present in patients with lymphoproliferative
disorders, including CLL, Hodgkin’s disease, and non-
Hodgkin’s disease. Very low PNP and ADA activities
biochemically measured have been reported in CLL
lymphocytes.9-13 The present paper has clarified by the
cytochemical analysis that the decrease in PNP activ-
ity in CLL cells is mainly due to a decrease in the
number of the enzyme-positive cells in one of nine
CLL patients studied, and a depression of the enzyme
activity in each PNP-positive cell as well as a decrease
in the number of the enzyme-positive cells occurs in
the other CLL patients. PNP may be utilized for
assessing the maturation of B cells and for distinguis-
ching biochemical subtypes of B-cell CLL. Borgers et al.
recently demonstrated trace activity of the enzyme in
88%-98% of malignant B CLL cells and presumed
that PNP might be present in small amounts of
immature B cells, contrary to the absence of the
enzyme activity in normal circulating B cells.10 Their
presumption is incompatible with ours. Elevated PNP
and normal ADA contents in circulating lymphocytes
in Hodgkin’s disease and variable ADA activities in
those in untreated non-Hodgkin's disease have been
reported.10,13 The PNP staining was in the normal
range in two cases with non-Hodgkin’s disease (Table
1).

Barton et al. recently have reported that PNP and
ADA activities vary inversely during rat T-cell differ-
entiation, because cortical thymocytes have high ADA
and low PNP levels, whereas low ADA and high PNP
levels are found in medullary thymocytes.31 In UFL of
three patients with T-cell malignancies, including T-
cell CLL, adult T-cell leukemia, and Sézary’s
syndrome, we have noted lower PNP and higher or
normal ADA levels than the levels in normal T-cell
controls. These enzyme activities would also change
during human T-cell maturation.

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Purine nucleoside phosphorylase (PNP) and adenosine deaminase (ADA) activities examined cytochemically in unfixed lymphocytes of patients with lymphoproliferative disorders

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