Purine nucleoside phosphorylase (PNP), the enzyme schematically next to adenosine deaminase in the purine salvage pathway, has been demonstrated cytochemically in peripheral blood lymphocytes of healthy subjects and chronic lymphocytic leukemia (CLL) patients. The enzyme activity is confined to the cytosol. In healthy subjects the majority of lymphocytes are strongly reactive for PNP, whereas the rest are devoid of cytochemically demonstrable activity. The percentage of PNP-positive cells largely corresponds to the number of E rosette-forming cells and is inversely proportional to the number of Ig-bearing cells. In six of seven CLL patients studied only a minor percentage of the lymphocytes showed strong PNP activity, whereas the large majority (88%-98%) possessed trace activity. Such patients have a high number of Ig-bearing cells and a low number of E rosette-forming cells. A different pattern of markers was found in the lymphocytes of the seventh CLL patient: 66% were strongly reactive for PNP, an important number formed E rosettes, and a minor percentage were Ig bearing. These data indicate that PNP can be useful as a "nonmembrane" marker in the differentiation of the B and T cell origin in CLL and deserves to be studied in other lymphoproliferative disorders.

PURINE NUCLEOSIDE PHOSPHORYLASE (PNP; EC 2.4.2.1), the enzyme schematically next to adenosine deaminase (ADA) in the purine salvage pathway, catalyzes the reversible reaction purine nucleoside + Pi = α-D-ribose-1-phosphate + purine base. Rubio et al. worked out a cytochemical method for the localization of PNP in which the reaction is driven towards the ribosyl transfer by using hypoxanthine as substrate in a high concentration.

In a recent study PNP was proposed as a possible marker of human circulating T lymphocytes. This assumption was based on the observations that (1) the number of PNP-reactive lymphocytes largely corresponds to the number of E rosette forming cells, (2) phytohemagglutinin-induced blasts are reactive for PNP, and (3) the majority of Ig-bearing lymphocytes are PNP negative and vice versa when these markers are simultaneously assessed.

Although the role of PNP, in conjunction with other enzymes of the purine salvage pathway, in lymphocyte maturation and/or transformation is far from clear, interest in this enzyme in cell-mediated immunity was raised after PNP deficiency was reported in four children whose T cell dependent immunologic functions were seriously compromised without affecting their B cell function and number.

The present study reports on the distribution of PNP in lymphocytes of chronic lymphocytic leukemia (CLL) patients and to evaluate its activity in...
conjunction with known membrane markers of T and B cells. Significant reduction of ADA and PNP levels in CLL has been reported.\textsuperscript{7,9}

The importance of immunologic classification of lymphoid malignancies into T and B cell types has been stressed repeatedly, since T cell CLL may differ in its course and prognosis from B cell CLL and may require different treatment.\textsuperscript{10} Although most cases of CLL are classified as being of B cell origin,\textsuperscript{11} the nature of lymphocytes in CLL has been disputed. Leukemic cells with variations in expression of membrane markers\textsuperscript{12} or with hybrid membrane phenotypes\textsuperscript{13} have been reported. It is thus probable that variable expression of surface markers may occur among neoplastic cells of T or B nature. An easily detectable histochemical enzyme marker that could help in the differentiation of subsets of lymphocytes in lymphoid malignancy would therefore be of considerable interest. PNP, which can be readily demonstrated at the light- as well as at the electron-microscopic level, requires a minimum of peripheral blood, and allows the differentiation of a large number of lymphocytes in a short time, may fulfill this need.

**MATERIALS AND METHODS**

Peripheral blood samples were obtained from five healthy subjects and from seven patients with CLL. Lymphocytes were purified from venous blood and anticoagulated with preservative-free heparin (20 U/ml) by Ficoll-Metrizoate gradient centrifugation.\textsuperscript{14} After centrifugation for 40 min at 400 g, the interface layer was collected and washed twice in Hanks' buffered salt solution. E rosette formation was performed according to the method of Jondal et al.\textsuperscript{15}

PNP activity at the light-microscopic level was assessed as follows: The cell pellet (2.5 x 10\textsuperscript{6} cells) was fixed in cold 2\% distilled glutaraldehyde buffered to pH 7.4 with 0.1 M sodium cacodylate for 5 min. Rinsing after fixation was done in the same buffer supplemented with 0.22 M sucrose for at least 1 hr. The cell pellet was frozen at -22°C for 15 min and thawed to allow a good penetration of constituents of the incubation medium.

The cells were incubated in the medium prepared as described previously\textsuperscript{16} and containing in final concentrations 48 mM Tris-maleate buffer pH 7.4, 3 mM lead nitrate, 4 mM α-D-ribose-1-phosphate, 10 mM hypoxanthine (solubilized by adding concentrated NaOH before addition to the medium; the final pH of the medium was adjusted to pH 7.2 with 1 N HCl), and 220 mM sucrose. Incubation proceeded with very slow shaking at 37°C for 30 min. After centrifugation, the pellet was washed twice in 7.5\% aqueous sucrose solution at 4°C and treated with 0.2\% aqueous ammonium sulfide to visualize the lead precipitate. After washing twice again, 2-3 drops of 4\% bovine serum albumin were added to the pellet; the cells were suspended therein and smeared on ordinary microscope slides. After drying, the smear was counterstained as follows: distilled water, 1 min; 0.005\% methylene blue in 0.005\% borax pH 8.5, 7 sec; distilled water, 5 sec; series of ethanol (70\%, 90\%, twice at 100\%), each 5 sec; xylol, two times 5 sec; mounted in Canada balsam (some synthetic media solubilize either the counterstain or the lead sulfide after a while; Canada balsam preserves the stains for at least 2 yr).

For electron microscopy, the procedure was the same including the incubation step. After rinsing, the cells were postfixed in 2\% osmium tetroxide, buffered to pH 7.2 with 0.05 M veronal acetate (containing 0.12 M sucrose) for 30 min at 4°C, dehydrated in graded ethanols, and routinely embedded in epon. Ultrathin sections were examined either unstained or briefly counterstained with uranium acetate and lead citrate.

Surface immunoglobulins were demonstrated as follows: After fixing and rinsing the cells as described above, these were incubated according to the method of Sternberger\textsuperscript{17} in the following successive antibody solutions, diluted in Tris-buffered saline pH 7.6 (TBS): normal goat serum (1/30) (Nordick Pharmaceuticals & Diagnostics, Antwerp, Belgium) for 10 min; rabbit antiserum against human immunoglobulins (1/100) (Nordick), supplemented with 1\% normal goat serum, for 30 min; goat antiserum against rabbit immunoglobulins (1/20) (Nordick) for 30 min; peroxidase-antiperoxidase (PAP) complex (1/300) supplemented with 1\% normal goat serum. After
each incubation, the cells were collected at 1400 rpm for 5 min and washed in TBS for 30 min. The reaction with 3,3’-diaminobenzidine to visualize the peroxidase was carried out at pH 7.6 for 10 min at room temperature according to the method of Graham and Karnovsky. After a wash in TBS, the cell pellet was postfixed in osmium tetroxide and further processed for electron microscopy as described above.

Control tests for specificity of the reactions. For PNP, either ribose-1-phosphate or hypoxanthine was omitted from the incubation medium or ribose-5-phosphate was substituted for ribose-1-phosphate. For surface immunoglobulins, the incubation step in the rabbit antisera against human immunoglobulins was omitted or replaced with an incubation step in normal rabbit serum.

RESULTS

Localization of PNP in lymphocytes of healthy subjects. Confirming previously reported results on a large number of subjects, strong PNP activity was found in 65%-80% of the peripheral blood lymphocytes (Table 1). The enzyme activity in the cytosol was easily detected on lymphocyte smears after a 30-min incubation by the presence of a black-brownish precipitate of lead sulfide, the insoluble end product of the cytochemical reaction (unreactive cells stain blue as a result of the counterstaining). Electron microscopy confirmed the light-microscopic counts and showed that the PNP reaction product confined to the cytosol and excluded from the outer plasma membrane, nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, and almost all single-membrane-limited bodies. Centrioles and some inclusion bodies, possibly secondary lysosomes containing auto- or heterophagous cytosol material, stained intensively. Other leukocytes contaminating the enriched lymphocyte population such as neutrophils and monocytes were more lightly stained than reactive lymphocytes.

Prolongation of the incubation time up to 2 hr resulted in intensification of the stain, however, without shifting the percentage of positive cells.

Localization of PNP in lymphocytes of CLL patients. As shown in Table 1, the distribution of PNP in six of seven patients differed markedly from that in healthy people. Under standard conditions of incubation (30 min at 37°C of a suspension containing 2–5 x 10⁶ cells), the large majority of lymphocytes (up to 98%) showed trace activity only. Owing to the combination of weak enzymatic

<table>
<thead>
<tr>
<th>Subjects</th>
<th>WBC (x 10⁶)</th>
<th>Lymphocytes (%)</th>
<th>E Rosettes (%)</th>
<th>Ig-bearing Lymphocytes (%)</th>
<th>PNP Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy (n = 5)</td>
<td>ND</td>
<td>ND</td>
<td>57-75</td>
<td>10-25</td>
<td>68-80</td>
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<tr>
<td>A.R.</td>
<td>81.6</td>
<td>99</td>
<td>3</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>D.H.</td>
<td>79.0</td>
<td>94</td>
<td>6</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>E.Y.</td>
<td>30.2</td>
<td>93</td>
<td>10</td>
<td>95</td>
<td>8</td>
</tr>
<tr>
<td>S.A.</td>
<td>108.0</td>
<td>96</td>
<td>3</td>
<td>85</td>
<td>3</td>
</tr>
<tr>
<td>V.R.</td>
<td>45.0</td>
<td>85</td>
<td>10</td>
<td>86</td>
<td>8</td>
</tr>
<tr>
<td>M.A.</td>
<td>56.0</td>
<td>83</td>
<td>6</td>
<td>92</td>
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<tr>
<td>S.C. 1*</td>
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<td>95</td>
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<tr>
<td>S.C. 2*</td>
<td>38.2</td>
<td>97</td>
<td>19</td>
<td>18</td>
<td>66</td>
</tr>
</tbody>
</table>

ND, not determined.
*Two blood samples, the second obtained 14 days after the first.
staining and the blue counterstain, such cells appeared as light green stained cells (Fig. 1), quite distinct from normally reactive (black-brown) and unreactive (blue) cells. The latter possibly represent nonmalignant cells as judged by their different morphology at the light- and electron-microscopic levels. The few contaminating monocytes and neutrophils reacted as in healthy individuals and stained much more intensively than the neoplastic lymphocytes.

Electron microscopy confirmed the presence of trace activity in the cytosol of the malignant lymphocytes (Figs. 3 and 5). As in lymphocytes from healthy subjects, more precipitate deposits occurred after a 2-hr incubation, although the amount remained weak when compared with positive lymphocytes of healthy people, which are strongly overstained by that time.
Fig. 3. Electron-microscopic counterparts of Fig. 1. Patient M.A. With exception of one strongly stained (arrow) and one unstained (arrowhead) cell, all lymphocytes show trace precipitate of lead phosphate in cytosol. x3750.

Fig. 4. Electron-microscopic counterpart of Fig. 2. Patient S.C. Majority of cells were strongly labeled with precipitate, while remainder (arrows) were completely devoid of stain. Reactive red blood cell indicated by arrowhead. x3750.
Fig. 5 and 6. Larger magnifications of lymphocytes showing trace activity for patient E.Y. (Fig. 5) and strong activity for patient S.C. (Fig. 6). Note that precipitate is confined to the cytosol. x10,000.

Fig. 7. Patient M.A. Demonstration of surface Ig. All lymphocytes but one (arrow) bore low amounts of peroxidase reaction product. x10,500.
A total different pattern of PNP activity was demonstrated in the lymphocytes of patient S.C. Blood samples taken 14 days apart showed cells that reacted strongly to PNP activity (Fig. 2). Respectively, 77% and 66% of the lymphocyte populations from these samples were PNP positive, and no "trace activity" cells were seen. This patient's total lymphocyte count, however, was within the range found in the other patients (Table 1). Both by light and electron microscopy (Figs. 4 and 6) the amount of precipitate in the PNP-positive lymphocytes of this patient was estimated to be at least as high as that in healthy subjects.

**Surface markers.** In healthy subjects the number of PNP-positive cells largely corresponds to the number of E rosette-forming cells and is inversely correlated with the number of surface Ig-bearing cells (Table 1).

In the six patients with a high number of lymphocytes with PNP trace activity, a high number of Ig-bearing cells and a low number of E rosette-forming cells were obtained (Table 1). The amount of visualized peroxidase-reaction product, reflecting the density of surface Ig, was much lower in the lymphocytes of these patients than in those of healthy people (Fig. 7).

In contrast to the cases described above, the lymphocytes of patients S.C. showed a low number of Ig-bearing cells and a considerable number of E rosette-forming cells. The surface Ig per reactive cell amounted to the level usually found in healthy subjects.

Since neutrophils and monocytes were labeled for endogenous peroxidase in the visualization procedure of surface Ig, we were able to distinguish lymphocytes from the former cell types, thereby establishing that the counts obtained and partition of lymphocytes are not influenced by contamination of other cell types.

**Control reactions.** Omitting ribose-1-phosphate from the medium or substituting ribose-5-phosphate for ribose-1-phosphate resulted in the complete absence of the reaction product. When hypoxanthine was excluded from the medium a very weak precipitate remained in some cells.

For surface Ig demonstration, deletion of the incubation step in rabbit anti-human antiserum or its substitution by normal rabbit serum resulted in a total loss of surface membrane staining.

**DISCUSSION**

The results of this study indicate that peripheral blood lymphocytes of CLL patients that have been characterized by surface markers as belonging to the non-T lineage(s) have a distribution of PNP activity differing markedly from that in healthy subjects. Contrary to the presence of high PNP activity in a large part of the lymphocytes of healthy people, lymphocytes of B cell CLL have trace activity only. One patient, on the other hand, had a high number of cells that stained strongly for PNP. Since this patient showed a concomitantly low number of Ig-bearing cells and a considerable amount of E rosette-forming cells, his malignant lymphocytes were probably of non-B cell origin, illustrating that PNP may be a useful enzyme marker for the differentiation of malignant cells of B and non-B origin. The main difference from other subset markers is that PNP is a nonmembrane marker but is evenly distributed over the cell cytosol. The determination of PNP at the light-microscopic level is relatively
simple to perform, requiring a small number of cells. Its evaluation in CLL is very reproducible, since contamination with other cell types such as monocytes is very limited. When a lymphocyte population of high purity can be obtained, such as is the case in CLL, biochemical quantitation of PNP activity may be a suitable diagnostic means.

Although we recognize that these results bear on only a small number of CLL patients, we feel that the uniformity of response as far as intensity of the reaction and the number of cells involved is striking. We may not preclude, however, the possibility that upon study of a larger number of cases, intermediately reactive cell populations may be found.

The sole use of surface markers in the determination of the origin of malignant proliferating lymphocytes is not always reliable and represents a serious drawback in the immunologic analysis of neoplastic lymphocytes. The evaluation of subsets with surface markers is complicated by the fact that individual cells can bear both T and B surface markers and by the variable expression of surface markers that may occur among cells belonging to these lineages. Obvious variations of the membrane-staining intensity for the demonstration of Ig have been found in CLL. Moreover, lymphocytes of B cell CLL apparently possess receptors for *Helix pomatia* A hemagglutinin, a protein that in healthy people binds to T lymphocytes only. Although CLL of T cell origin appears to be rare in Western countries, Uchiyama et al. recently reported 16 cases of adult T cell leukemia in Japan.

A non-membrane marker of enzymatic origin such as PNP could therefore be an aid to a more accurate diagnosis in CLL and possibly also in acute lymphocytic leukemia (ALL). The origin of malignant cells in the latter disease was described as T cell in 25% of cases, null cell in the majority, and rarely B cell. The evaluation of PNP would therefore be warranted in ALL. Such an approach would not only be of possible diagnostic value but might also be of help in therapeutic monitoring of the patients, since T, B, and null cell ALL have distinctive clinical features, have different prognoses, and respond to different therapy.

If in healthy subjects PNP were absent from peripheral blood B lymphocytes as hypothesized earlier, the fact that trace activity can be found in malignant B CLL cells suggests that PNP may be present in small amounts in immature B cells, since B CLL cells are affected basically by a block in their maturation process. On the other hand, the appearance of trace activity of PNP in malignant B cells might be the result of an uncontrolled gene expression.

The implication of PNP in immune phenomena is highlighted by the observation of severely impaired T cell function with normal B cell function in four children with PNP deficiency. The role of PNP in the maturation and proliferation of lymphocytes is far from clear. Normal function of lymphocytes may be impaired by relative deficiencies of ribose-1-phosphate and/or hypoxanthine and of pyrophosphoryl ribosyl phosphate. Cell proliferation can be prevented by the accumulation of purine nucleosides.

Strongly decreased PNP and ADA activities have been reported in CLL lymphocytes when compared with those of healthy people. The same authors found that lymphocytes in Hodgkin disease contained elevated levels of PNP but had unchanged ADA contents. On the other hand, the elevated PNP...
levels in such patients, despite the fact that they possess lower numbers of E rosette-forming cells, can be explained by a high PNP content per reactive cell. Unfortunately, we have not yet had the opportunity to evaluate PNP in Hodgkin disease. A decrease in ADA has been seen in CLL lymphocytes versus normal cells.8 Interestingly, the low ADA activity in CLL lymphocytes becomes elevated in the cells of those patients who responded well to chemotherapy.9 Moreover, Trich and Minowada have shown that lymphoid T cell lines have a 25-fold higher ADA content than lymphoid cells of non-T origin and proposed the elevated ADA activity as a biochemical means of characterizing T cell leukemic blasts.26

Another cytochemically detectable enzyme that shows decreased activity in CLL is 5'-nucleotidase, which is localized at the plasma membrane.27,28 5'-Nucleotidase precedes ADA and PNP in the metabolic pathway of purines. This makes three enzymes consecutive in the pathway that show a decrease of activity in CLL lymphocytes of presumed B cell origin.

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large population of lymphocytes forming non-
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