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Heterogeneity of 5′-Nucleotidase Activity in Lymphocytes in Chronic Lymphocytic Leukemia

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ABSTRACT The specific activity of 5′-nucleotidase was determined in lymphocyte plasma membranes from 14 normal subjects and 10 patients with chronic lymphocytic leukemia (CLL). Whereas the enzyme was present in the preparation from normal lymphocytes, in 7 out of 10 CLL patients the membranes had markedly decreased or no detectable 5′-nucleotidase activity. The lack of this activity from the lymphocytes of most patients with CLL constitutes an alteration in a plasma membrane enzyme from the normal cell. The presence of the enzyme in the lymphocytes of some patients with CLL and its decrease in others provide further evidence for biochemical heterogeneity among patients with this disorder.

INTRODUCTION

Despite the immunologic abnormalities described in chronic lymphocytic leukemia (CLL)[1] only a few differences have been documented between the lymphocytes found in normal subjects and patients with CLL. These include the decreased response to phytohemagglutinin stimulation (1), an altered hybridization pattern of the RNA (2), and the high percentage of cells bearing surface immunoglobulins in CLL (3). A membrane abnormality in these cells was suggested in the recent finding that CLL lymphocytes have fewer binding sites for mitogens than normal lymphocytes (4).

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[1] *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; PBS, phosphate-buffered saline; UDPG, uridine diphosphate glucose.

In an extension of studies on human lymphocyte membranes in which the activity of 5′-nucleotidase was used as a plasma membrane marker (5), a striking difference was observed between normal lymphocytes and the cells of most patients with CLL: While 5′-nucleotidase was present in plasma membranes purified from lymphocytes found in the blood of normal subjects, the activity was markedly decreased or absent in similar fractions prepared from the patients' lymphocytes.

METHODS

Sodium diatrizoate (Hypaque) was obtained from Winthrop Laboratories, New York, and Ficoll from Pharmacia Fine Chemicals, Piscataway, N. J. The reagents for the 5′-nucleotidase assay, 5′-AMP, and β-glycerophosphate were supplied by Sigma Chemical Co., St. Louis, Mo. Lymphocytes were isolated from the blood as follows: 1 vol of freshly drawn, heparinized blood was mixed with an equal volume of phosphate-buffered saline (PBS: 0.15 NaCl-0.01 M potassium phosphate buffer, pH 7.4) and layered on a separating mixture containing 6% Ficoll-10% Hypaque in 10 mM Tris-HCl, pH 7.4. After centrifugation at 600 *g* for 30 min at 4°C, the interphase containing lymphocytes as well as some platelets and monocytes was collected. The cells were washed once with PBS and resuspended in their own platelet-free plasma-PBS mixture to which ADP had been added at a final concentration of 10^{-5} M in order to agglutinate platelets. The suspension was incubated for 20 min at 37°C on a column (6) containing 1.5 g glass wool in a 50 ml glass syringe after which the column was washed with 4 vol of the plasma-PBS mixture. This procedure efficiently removed monocytes and platelets so that the final preparation contained over 99% lymphocytes with no red cells, a very rare monocyte or granulocyte, and less than 1 platelet per 5 lymphocytes. The lymphocytes were dis-

TABLE I

5′-Nucleotidase in Lymphocyte Membranes

Source of cells	Specific activity
	μmol/h/mg protein
Normal subjects (14)	6.5 (3.9–11.2)
Patients with chronic lymphocytic leukemia	
Sen.	9.6
Smo.	7.8
Tig.	3.8
Bro.	0.2*
Gd.	<0.2
S. G.	<0.2
Mar.	0.4‡
I. G.	0.6*
DeB.	<0.2
Fal.	<0.2

The 5′-nucleotidase assay mixture contained the following in a total volume of 0.1 ml: Tris-HCl pH 7.8, 5 μmol; NaK tartrate, 1 μmol; β-glycerophosphate, 2 μmol; $MgCl_2$, 1 μmol; membrane protein, 2–20 μg; and 5′-AMP, 0.1 μmol, which was omitted from the blank. The mixture was incubated for 30 min at 37°C after which 0.2 ml of 10% trichloroacetic acid was added. The samples were centrifuged for 10 min at 4,000 *g* and Pi was determined in the supernatant fluid by the method of Ames and Dubin (7).

* Average of two separate preparations.

‡ Average of three separate preparations.

rupted, plasma membranes prepared, and the activity of 5′-nucleotidase was assayed by previously described methods (5). Purification was also monitored by electron microscopy. All assays were done in duplicate. Specific activity is expressed in micromoles Pi liberated per hour per milligram protein. The techniques for fixation, sectioning, and electron microscopic examination of cells have been detailed elsewhere (5). 10 patients with CLL whose leukocyte count ranged from 40,000 to 100,000/mm^3 were studied. Eight of the patients had never received therapy, while the other two had not been treated for a period of at least 10 mo before the study. The lymphocytes of normal subjects and patients with CLL were subjected to identical purification procedures.

RESULTS

When membrane preparations obtained from normal lymphocytes were assayed for 5′-nucleotidase, the reaction was linear with time over a 1 h period using between 2 and 10 μg of membrane protein. A pH response optimum of about 7.5 was noted. The specificity of the enzyme for the 5′-mononucleotide was supported by the finding that no Pi was released when 2′,3′-AMP was substituted for 5′-AMP. The level of 5′-nucleotidase in the membranes prepared from lymphocytes of normal subjects is shown in Table I. Whereas a relatively narrow range of values was obtained with these lymphocyte membranes, 5′-nucleotidase activity was absent or barely detectable in membrane preparations from 7 out of the 10 patients with CLL studied. Increasing the amount of protein in the assay to four times the level used with lymphocyte membranes from normals and lengthening the incubation time of the reaction mixture from the standard 20 min to 2 h was needed to reveal any activity in these preparations. The assays were repeated on fresh lymphocyte membranes prepared from three patients on several different occasions at intervals of 1–8 wk; the patients deficient in lymphocyte membrane 5′ nucleotidase on one occasion always lacked activity when tested on fresh lymphocytes membranes prepared from subsequent blood samples. There was no relationship between the white blood cell count and 5′-nucleotidase level in the CLL patients studied.

In order to determine whether the failure to detect enzyme activity could be attributed to the presence of an inhibitor in the CLL membrane, a "mixing" experiment was performed. When membranes from a normal and a CLL lymphocyte preparation lacking 5′-nucleotidase

TABLE II

5′-Nucleotidase Activity in Crude Lymphocyte Homogenates

Source of cells	Specific activity
	μmol/h/mg
Normal subjects (8)	0.8 (0.3–1.2)
Patients with chronic lymphocytic leukemia	
Smo.	1.0
Mar.	<0.2
I. G.	<0.2
DeB.	<0.2
Fal.	<0.2*

* Homogenates were prepared from lymphocytes as described above. Lymphocytes from patient Fal. were also obtained directly from the buffy coat, omitting the gradient or column steps. This shortening of the purification procedure had no effect on 5′ nucleotidase activity. The purified lymphocytes suspended in 0.01 M Tris-HCl, pH 7.5-0.002 M dithiothreitol were homogenized with five strokes of a tight-fitting Dounce homogenizer (5). The homogenate was centrifuged at 4,000 *g* for 10 min. The assay for 5′-nucleotidase activity in the supernatant fluid was performed as described in Table I except for lengthening the incubation period to 2 h, and increasing the protein to 80 μg. In some experiments, portions were dialyzed for 16 h against 10 mM Tris-HCl, pH 7.5-1 mM β-mercaptoethanol without affecting the results.

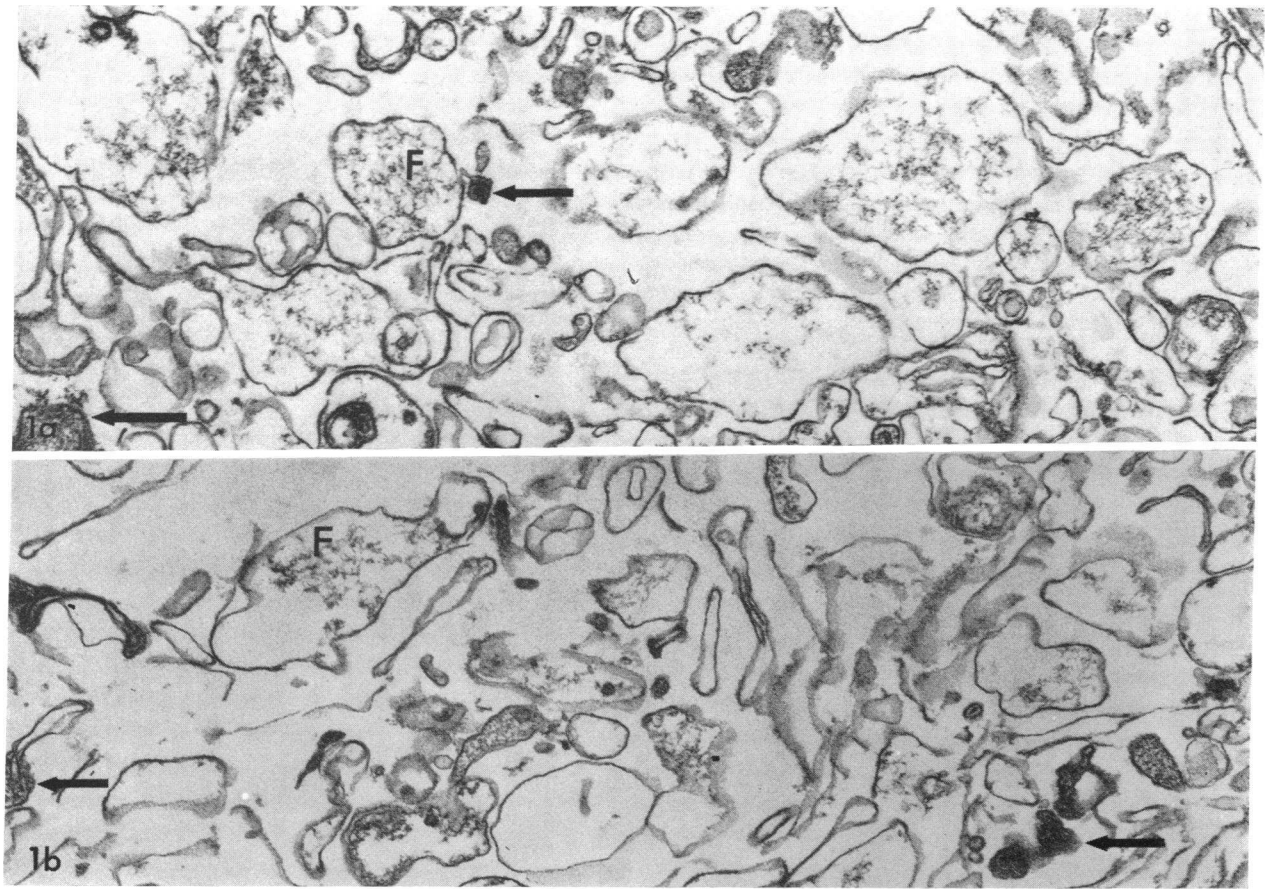

FIGURE 1 (*a*) Representative survey of lymphocyte membranes obtained from a normal subject. (*b*) Lymphocyte membranes from a patient with chronic lymphocytic leukemia. Both preparations show "clean" sheets as well as some membrane-associated fibrillar material (F). Occasional dense bodies (arrows) are also seen in both preparations. Magnification × 36,000.

were incubated separately and in a series of mixtures of varying proportions, the 5′-nucleotidase specific activity corresponded with the values predicted from the ratio of the two membrane preparations in the mixture. This finding rules out the presence of an inhibitor in the CLL or of an activator in the normal membrane.

Conceivably, the lack of 5′-nucleotidase on the membrane of CLL lymphocytes could reflect a difference in subcellular fractionation behavior from normal lymphocytes leading to contamination of membrane preparations in the CLL membranes, with nonmembrane protein, thereby resulting in lower specific activities in these cells. The following three experimental observations provided strong evidence against this possibility: (*a*) There was no difference in the morphology or the purity of the membranes prepared from lymphocytes of normal subjects and those from patients with CLL when examined with the electron microscope (Fig. 1*a* and *b*). (*b*) The specific activity of NADH diaphorase, another plasma membrane marker (8), was not diminished in CLL lymphocytes (9). (*c*) When the 5′ nucleotidase activity was determined in crude homogenates of lymphocytes from four patients whose membrane preparations were deficient in enzyme, the activity was also lacking in the crude preparation from which the membranes were derived (Table II). The enzyme was readily detected in the crude homogenate of lymphocytes from patient Smo. whose membrane activity is shown in Table I. A "mixing" experiment performed with crude homogenates from normal and CLL lymphocytes did not detect an inhibitor or activator of 5′-nucleotidase activity.

Since it has been reported that an increased proportion of the lymphocytes in the blood of patients with CLL are cells of bone marrow origin (B lymphocytes), the possibility was considered that the lack of 5′-nucleotidase reflected a shift in the type of lymphocyte rather than the neoplastic process. Although studies in patient Sen. who had a normal and DeB. with decreased activity

failed to establish a correlation between the enzyme level and percentage of B cells, a definitive answer to this question awaits the development of methods to separate B and T cells in man (3, 10).

DISCUSSION

The data presented above suggest that lymphocytes from patients with CLL fall into at least two groups based on the presence of the membrane marker 5′-nucleotidase. This view is consistent with the kinetic and immunologic evidence of heterogeneity. It is also supported by the finding of a decreased level of the lysosomal enzymes β-glucuronidase and acid phosphatase in the cells of some patients with CLL (11). Further studies are required to determine the possible effect of cell age, bone marrow vs. thymic origin, or the clinical course of the disease on this enzyme. Studies on 5′-nucleotidase in lymphocytes from patients with other diseases are also warranted to determine the effect of other clinical conditions on its activity. Purification of the enzyme and preparation of an antibody will be needed to determine if the enzyme protein is actually lacking or whether instead, the decreased activity reflects instability or functional impairment.

Since the in vivo function of 5′-nucleotidase in eukaryotic cells (or even of the highly purified *Escherichia coli* enzyme [12]) is not certain, the significance of the decrease in activity reported above must remain conjectural. Based on the in vitro specificity, at least two roles for membrane 5′-nucleotidase can be envisaged. The enzyme catalyzes the degradation of 5′-mononucleotides to nucleosides and therefore may be of importance in determining the level of precursors available for the polymerization reactions leading to nucleic acid synthesis. In addition, 5′-nucleotidase in *E. coli* (12) has been shown to hydrolyze uridine diphosphate glucose (UDPG) and therefore may play a role in the catabolism of this compound which is a major cofactor in the biosynthesis of the sugar moiety of membrane glycoproteins. An impaired function of 5′-nucleotidase, either by increasing the pools of 5′-nucleotides for macromolecular synthesis or by influencing the level of UDPG might be of advantage to a neoplastic cell. This concept is supported by the recent finding of a marked decrease in 5′-nucleotidase activity during liver regeneration (13).

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