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Thymidine Phosphorylase Activity in Plasma: A Cancer Marker or an Artifact of Ultrafiltration? (40640)[1]

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Thymidine phosphorylase (EC 2.4.2.4, thymidine:orthophosphate deoxyribosyltransferase) catalyzes the reversible reaction: thymidine + orthophosphate ⇋ thymine + 2-deoxy-α-D-ribofuranose-1-phosphate (1). The enzyme is present in many normal and neoplastic eukaryotic cells (1–3) and is found also in prokaryotes (1, 4).

In human subjects, thymidine phosphorylase activity is present in a variety of tissues (1–3), including the leukocytic fraction of blood obtained from both normal subjects and patients with leukemia (5). Recently, however, it was reported that thymidine phosphorylase activity is present also in a cell-free plasma fraction obtained from normal subjects (6), as well as from patients hospitalized with neoplastic diseases, some of whom had received chemotherapy (7–9). In the latter group, the reported mean level of thymidine phosphorylase activity in 40 μl of plasma (52.7 ± 17.6%, as percentage degradation of thymidine ± SD) was approximately twofold higher than that determined for the healthy donors (23.9 ± 13.7%). It was proposed, therefore, that an elevated level of the enzyme activity could be used as a diagnostic marker in the detection of certain forms of cancer (7).

Recent studies on the distribution and substrate specificity of various pyrimidine nucleoside phosphorylase activities (P. W. Woodman, unpublished data) have demonstrated that the thymidine phosphorylase activity of normal human blood is located primarily in the granulocyte fraction, a finding that is consistent with earlier observations by Gallo and Perry (10), using less purified preparations of granulocytes and lymphocytes.

The absence of thymidine phosphorylase activity from the plasma (2.9 ± 0.2%, percentage degradation of thymidine (80 pmole) per 25 μl of plasma ± SD) was demonstrated by the use of procedures that prevented either lysis of cells or contamination of the plasma with granulocytes, or both. Accordingly, the method used by Pauly and co-workers (6–9) to obtain their cell-free plasma fractions was examined, to determine whether or not it was responsible for an artifactual localization of thymidine phosphorylase activity in the plasma.

Evidence is presented to show that thymidine phosphorylase activity is not usually present in plasma from healthy subjects, but only appears there when the technique used to fractionate the blood causes either cell lysis or contamination of the plasma fraction with granulocytes or both.

Materials and methods. Fractionation of plasma. All chemicals used were of analytical grade. Twenty milliliters of human blood were collected in heparinized syringes from each of nine healthy subjects. The blood was centrifuged at 500*g* for 12 min; the plasma supernatant fraction (A) was removed, divided in half, and stored on ice until processed. One-half of the plasma fraction (A) was centrifuged at 10,000*g* for 30 min at 4°; the supernatant fraction (B) was harvested and the cell pellet (C) obtained was resuspended in a 20 m*M* sodium phosphate/10 m*M* β-mercaptoethanol buffer, pH 8.0 (Buffer 1),[2] and the cells were lysed by freeze/thawing. An aliquot of the supernatant material (B) was saturated to the level of 55% with ammonium sulfate (Schwarz–Mann, Orangeburg, N.Y.) at 4°. The precipitate was harvested by centrifugation at 16,000*g* for 20 min at 4° and the clear supernatant fraction was discarded, while the pellet (D) was resuspended in Buffer 1 and dialyzed against the

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[2] Abbreviation used in this paper: Buffer 1:20 m*M* sodium phosphate/10 m*M* β-mercaptoethanol, pH 8.0.

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same buffer for 4 hr with four changes of buffer.

The remaining half of the plasma (A) was processed according to the method used by Pauly and co-workers (6–9); thus, fraction (A) was filtered through a 0.22-μm membrane (Millipore Corp., Bedford, Mass.) to remove cells and debris, which yielded a filtrate free of cells (but not the products of their earlier rupture). Because of the number of cells still present in fraction A, the 0.22-μm membrane rapidly became clogged; thus, it was not possible to filter more than 150 μl of plasma with one filter. To obtain a larger volume of cell-free plasma (E), it was necessary to recentrifuge the plasma supernatant material (A) at 800*g* for 10 min before filtering it through the 0.22-μm membrane. An aliquot of E was saturated to the 55% level with ammonium sulfate at 4°, and the precipitate (F) was harvested and treated as described for D.

Samples were taken at each stage of the fractionation and differential blood cell counts made on smears stained with Wright's solution. Fractions A to F were stored at −22° until assayed. Protein levels were determined by the method of Lowry *et al.* (11).

Thymidine phosphorylase assays. Thymidine phosphorylase activity was assayed in the catabolic direction, using two types of conditions. Assay 1 employed essentially the conditions of the microassay developed by Pauly *et al.* (6–9), while Assay 2 utilized saturating substrate conditions. In Assay 1, 40 μl of the plasma fraction were added to 60 μl of RPMI medium 1640 (pH 7.2) containing 1.6 μCi [*methyl*-^3H]thymidine (Amersham/Searle, Arlington Heights, Ill.: sp. act., 24 Ci/mmole).

For Assay 2, the 100-μl incubation mixture contained 0.1 *M* sodium phosphate buffer at pH 6.4, β-mercaptoethanol (2.5 m*M*), and 2.0 μCi [*methyl*-^3H]thymidine (Amersham/Searle, sp. act. 24 Ci/mmole), 25 μl of the plasma fraction, and 1.6 m*M* unlabeled thymidine (Sigma Chemical Co., St. Louis, Mo.). The mixtures were incubated at 37° for 3 hr and the reaction was terminated by boiling for 2 min. [^3H]Thymine and [^3H]thymidine were separated from each other on silica gel uv_{254} polygram plates (Brinkman, Westbury, N.J.), using chloroform:methanol (9:1). Radioactivity was measured in a liquid scintillation counter (Amersham/Searle MK III). The results obtained are expressed as either picomoles of thymidine cleaved per milligram of protein, or as the percentage degradation of thymidine (6–9). Student's *t* tests were performed, using a formula modified for unequal variances (12).

Results. As shown in Table I, the highest levels of thymidine phosphorylase activity were detected in the lysates of the pellets (C) (composed predominantly of white blood cells, derived from the centrifuged crude plasma of each of the nine subjects studied) by use of both Assays 1 and 2. In contrast, the two cell-free plasma fractions, B and E, both contained relatively low levels of thymidine phosphorylase activity (Table I).

High-speed centrifugation of the initial "plasma" fraction A significantly decreased the level of enzyme activity present; thus, the plasma supernatant fraction obtained at

TABLE I. DISTRIBUTION OF THYMIDINE PHOSPHORYLASE ACTIVITY DURING THE FRACTIONATION OF CELL-FREE PLASMA FROM NORMAL HUMAN BLOOD[a]

Fraction	Assay 1	Assay 2
(A) Plasma supernatant fraction from whole blood (centrifuged at 500*g* for 12 min)	4.6 (±1.8)[b]	6.9 (±3.2)
(B) Cell-free plasma supernatant fraction from (A) (centrifugation at 10,000*g* for 30 min, 4°)	0.6 (±0.5)	1.6 (±0.8)
(C) Lysate of the pellet formed from (A) by (B)	490.0 (±399.0)	476.7 (±384.7)
(D) 0–55% ammonium sulfate "cut" of (B)	3.0 (±3.3)	1.8 (±0.4)
(E) Ultrafiltrate of plasma supernatant fraction from (A) (800*g* for 10 min)	1.4 (±0.6)	2.7 (±1.2)
(F) 0–55% ammonium sulfate "cut" of (E)	6.3 (±3.2)	5.7 (±3.3)

[a] Twenty milliliters of blood were collected from each of nine normal subjects and fractionated as described under Materials and Methods. Fractions were assayed in duplicate for thymidine phosphorylase activity, using both Assays 1 and 2. Values are the mean of those determined for each of the nine subjects ± the standard deviation (SD).

[b] pmole of thymidine cleaved/mg protein (±SD).

TABLE II. DIFFERENTIAL CELL COUNT OF LEUKOCYTE CONTAMINANTS PRESENT IN THE PLASMA SUPERNATANT FRACTION A[a]

Subject No.	% Granulocytes	% Lymphocytes/ Monocytes
1	49	51
2	51	49
3	60	40
4	64	36
5	52	48
6	72	28
7	67	33
8	52	48
9	75	25

[a] Fraction A was obtained by centrifuging 20 ml of normal human blood for 12 min at 500*g*. Smears were made from the supernatant fractions and stained for differential cell counts.

10,000*g* (B), which was completely free of cells, contained levels of thymidine phosphorylase activity that were about one-eighth ($P < 0.001$) and about one-fourth ($P < 0.002$) of those originally present in the plasma prepared at 500*g* (A), as determined by Assays 1 and 2, respectively. As shown in Table I, removal of the cellular contaminants from A by low-speed centrifugation, coupled with ultrafiltration, in the manner described by Pauly *et al.* (6–9), only lowered the level of thymidine phosphorylase activity of crude plasma (A) to about one-third (E), as measured by Assays 1 and 2 ($P < 0.001$ and $P < 0.01$, respectively); consequently, the level of enzyme activity in the latter type of plasma filtrate (E), was about twice that ($P < 0.02$) found in the cell-free plasma fraction (B) produced by high-speed centrifugation.

The levels of thymidine phosphorylase activity in the fractions obtained by ammonium sulfate precipitation, expressed as the percentage degradation of thymidine (± SD), as determined by Assays 1 and 2, respectively, were 2.3 (±2.1)% and 3.1 (±0.9)% for fraction D and 6.2 (±2.7)% and 6.0 (±3.0)% for fraction F. These values represent an approximate doubling ($P < 0.002$ and $P < 0.001$, respectively) of the thymidine phosphorylase activity in cell-free plasma filtrate E, as compared to that present in fraction B.

The results in Table II clearly show the presence of leukocytes, especially granulocytes, in the initial plasma fraction A. Similar results were obtained with fraction C (the cell pellet). A small number of erythrocytes and platelets also were present in fractions A and C, although the data are not tabulated. Previous work, however, has shown that normal red blood cells (1, 5) and platelets (P. W. Woodman, unpublished data) do not contain detectable levels of thymidine phosphorylase activity. There were no cells or debris present in either the cell-free plasma fractions B and E or their respective ammonium sulfate fractions D and F.

Discussion. The data in Tables I and II are consistent with findings that the thymidine phosphorylase activity of blood of healthy donors is located primarily in the granulocytes ((10) and P. W. Woodman, unpublished data) and ordinarily is not detected in the plasma in significant amounts, irrespective of either the assay conditions used (i.e., either Assays 1 or 2) or the way in which enzyme activity is expressed (either percentage degradation of thymidine or picomoles of thymidine cleaved per milligram of protein).

The plasma B, prepared from A by high-speed centrifugation, contained no cellular contaminants; hence, the extremely low levels of thymidine phosphorylase activity present may have come from lysis of the granulocytes in fraction A, produced by low-speed centrifugation (500*g* for 12 min) of the whole blood. In addition, there is the possibility that the granulocytes had released some of their thymidine phosphorylase activity into the blood prior to fractionation (5, 13). On the other hand, the cell-free plasma fraction produced by low-speed centrifugation and ultrafiltration, E, contained a twofold higher level ($P < 0.02$) of thymidine phosphorylase activity than that of fraction B (produced by high-speed centrifugation (10,000*g*) for 30 min). Treatment of the two cell-free plasma fractions with ammonium sulfate also increased the thymidine phosphorylase activity to a more significant level with fraction E than was the case with fraction B. It is suggested, therefore, that the granulocytes present in the original plasma (A) were ruptured during the procedures employed by Pauly *et al.* (6–9), i.e., ultrafiltration through a 0.22-μm membrane, resulting in the release of their thymidine phosphorylase activity into the "plasma filtrate" (E).

Studies by others have shown that the levels of thymidine phosphorylase activity are

higher in normal human leukocytes than in those obtained from patients with chronic myelogenous leukemia (5, 10) or with either acute or chronic lymphocytic leukemia (5). In view of these findings (5, 10), as compared to those of Pauly *et al.* (9), who reported an elevated level of thymidine phosphorylase activity in the "plasma" they obtained from a patient with chronic myelogenous leukemia, it will be interesting to reevaluate the level of enzyme activity in a cell-free plasma fraction prepared without the use of ultrafiltration.

Because polymorphonuclear leukocytes can selectively release their lysosomal enzymes into the plasma (14), it is conceivable that intact leukocytes could also release traces of thymidine phosphorylase activity (5, 13). The present evidence strongly suggests, however, that the reported presence of thymidine phosphorylase activity in the plasma (6–9) may be an artifact of the method of its preparation, i.e., the rupture of granulocytes during the process of ultrafiltration.

Summary. The possibility was investigated that thymidine phosphorylase activity is released from leukocytes during ultrafiltration of crude "plasma," thus accounting for reports of this enzyme in "cell-free" plasma. Blood samples from nine healthy donors, when centrifuged at only 500*g* for 12 min, yielded plasma fractions that contained cells identified as leukocytes, erythrocytes and platelets, as well as significant levels of thymidine phosphorylase activity. Centrifugation of the crude plasma at 10,000*g* for 30 min produced a cell-free plasma that contained negligible levels of thymidine phosphorylase activity. By contrast, the sediment, which contained identifiable granulocytes, possessed very significant levels of enzyme activity. Centrifugation of the crude plasma at 800*g* for 10 min, when followed by ultrafiltration, yielded a cell-free plasma that contained a twofold higher level of thymidine phosphorylase activity ($P < 0.02$) than did the truly cell-free plasma produced by high-speed centrifugation.

Our findings substantiate earlier reports, which had indicated that thymidine phosphorylase activity is located primarily in the granulocytes, and suggest that significant levels of the enzyme ordinarily do not occur in true plasma obtained from normal blood, unless the granulocytes are ruptured. Consequently, the proposal that elevated levels of thymidine phosphorylase activity in plasma can be used as diagnostic markers for certain neoplastic states requires reevaluation.

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