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Research Article

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Pyridine Nucleotide Transhydrogenase in Normal Human and Leukemic Leukocytes *

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The rationale of the study of enzymes in leukemic cells is based on the supposition that malignant change causes an alteration in cell metabolism demonstrable by enzymological techniques. Since more sophisticated methods of cell separation have been reported in the last 20 years (1–4), quantitative analysis of enzymes present in a single cell type can be undertaken. Recent studies of leukemic cells have emphasized enzymes involved in purine and pyrimidine synthesis and the metabolism of folic acid (5–10) in the hope that their activity could be modified as a therapeutic measure. Little work has been carried out on the electron transport reactions of leukemic cells.

The TPNH-DPN or TD transhydrogenase enzyme catalyzes the following reaction: TPNH + DPN $\overset{\text{TD transhydrogenase}}{\rightleftharpoons}$ TPN + DPNH. A related reaction takes place between the oxidized and reduced forms of DPN. Thus, DPNH + *DPN $\overset{\text{DD transhydrogenase}}{\rightleftharpoons}$ DPN + *DPNH. Enzymes catalyzing the above reactions have been shown to be present in bacteria, animal tissues, and plants (11–15). In these tissues, TD transhydrogenase is present in the mitochondria and is apparently different from the soluble estrogen-dependent TD enzyme present in the endometrium, mammary, and pituitary glands (16). The function of the two transhydrogenase reactions *in vivo* is not yet clear, but they may well play an important part in regulating levels of reduced forms of the two pyridine nucleotide coenzymes.

In 1963, Silber, Huennekens, and Gabrio (17) reported the presence of both TD and DD transhydrogenase in normal and leukemic leukocytes. The leukemic cells exhibited more enzyme activity than the normal controls. These studies were carried out on the supernatant fraction of cell homogenates that had been dialyzed against 0.05 M potassium phosphate buffer.

Methods

Leukocytes for this study were obtained from 24 normal subjects and 47 patients with leukemia. The pathological specimens were restricted to blood from patients in which the differential leukocyte count was 70% or more of one cell type. For example, patients with chronic myeloid leukemia had 70% of cells of the myeloid series, though the maturity varied. More than half (12 of 18) had elevated leukocyte counts ($> 20{,}000$ per mm^3) with metamyelocytes and myelocytes present. Those with normal levels had polymorphonuclear cells and band forms. The patients with acute leukemia were all children and had 70% or more of blasts. The majority were considered to have lymphoblastic leukemia and six were distinguishable as myeloblastic. Most of the patients were on some form of antileukemic therapy. Twenty ml of blood was taken from normal subjects and 5 to 20 ml from patients with an elevated leukocyte count; the anticoagulant used was EDTA. An equal volume of 6% dextran and saline [1] was added, and the blood was allowed to sediment for 30 to 60 minutes at room temperature. During this sedimentation the supernatant fluid, containing leukocytes and some erythrocytes, was removed and centrifuged in an International refrigerated centrifuge at $200 \times g$ for 7 minutes. The cells that sedimented were washed with 5 ml of 5% dextrose and water and spun again for 7 minutes at $200 \times g$. The red cells were lysed with 0.2% hypotonic saline for 30 seconds, and the suspension was corrected to 0.9% before recentrifugation. Most of the hemoglobin was removed during this step. The final preparation was resuspended in normal saline, and a leukocyte differential count was carried out at this time. Centrifugation at $600 \times g$ for 10 minutes resulted

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in 0.1 to 0.2 ml of packed leukocytes which was stored at −10° C.

Before enzyme assay the frozen cells were resuspended in 0.01 M Tris (hydroxymethyl) aminomethane buffer, pH 7.5, and subjected to sonic oscillation for 2 minutes in a Raytheon 10-kc sonic oscillator. The solution was centrifuged in a model L Beckman Spinco centrifuge at 100,000 × *g* for 1 hour. The sediment was resuspended in 1 ml 0.01 M Tris buffer, pH 7.5, and homogenized for 1 minute in a Teflon hand homogenizer.

The TD transhydrogenase reaction was measured by the use of the acetyl pyridine analogue of DPN (AcPyDPN) according to the following equation (14): TPNH + AcPyDPN ⇌ TPN + AcPyDPNH. The reaction was measured by following the increase in absorption at 375 mμ for 10 minutes. The reaction mixture contained 100 μmoles of phosphate buffer, pH 6.5, 1 μmole KCN, 0.3 μmole TPNH, and 0.6 μmole AcPyDPN in 1 ml. The assays were carried out in duplicate at room temperature. Two blanks containing enzyme were run simultaneously; in one of them the AcPyDPN was omitted and in the other, TPNH. Readings were made every minute and standardized against the blank containing TPNH only. The results were corrected by the amount of increased absorption of the blank containing AcPyDPN only. A millimolar extinction coefficient of 5.1 was used to calculate the concentration changes in the reaction. Protein concentration was determined by the method described by Lowry, Rosebrough, Farr, and Randall (18).

Results

The results were calculated in millimicromoles of AcPyDPN reduced per hour per milligram of protein in the homogenate. The significance was calculated by the Scheffé test for simultaneous multiple comparisons using 95% confidence limits (19).

TD transhydrogenase activity was found in both the supernatant fraction and the sediment (Table I). The amount of enzyme in the supernatant fraction was between 55 and 92 mμmoles per hour per mg protein and did not vary significantly among the various cell types. Considerable substrate reduction of AcPyDPN took place in the supernatant fraction making a large correction of the figures necessary.

TD transhydrogenase activity was present in the particulate fraction at a five- to tenfold higher level than in the supernatant fraction. In these assays there was practically no endogenous reduction of the AcPyDPN. The normal cells had a TD activity of 241 mμmoles AcPyDPN reduced per hour per mg protein. The result of 366 mμmoles obtained from the cells of 18 patients with chronic myeloid leukemia was higher than the normal preparations, but the difference was not significant. The 584 mμmoles found in cells from patients with chronic lymphocytic leukemia was significantly higher than the activities of normal cells and those in chronic myeloid leukemia. Cells from acute myeloid and acute lymphocytic leukemia had activities of 558 mμmoles and 1,079 mμmoles, respectively. Both were significantly higher than normal. The results of acute myeloid leukemia were not significantly different from chronic myeloid leukemia, but the value from acute lymphocytic leukemia was significantly higher than chronic lymphocytic leukemia.

TABLE I

*TD transhydrogenase in normal and leukemic leukocytes**

Cell type	No. of subjects	Transhydrogenase activity: Supernatant fraction	Transhydrogenase activity: Particulate fraction
		mμmoles/hour/mg protein	
Normal human	24	55 ± 8 (12–168)	241 ±16† (138–390)
Chronic myeloid leukemia	18	55 ±10 (0–134)	366 ±19 (222–558)
Chronic lymphocytic leukemia	13	68 ±12 (14–180)	584 ±34 (402–756)
Acute myeloid leukemia	6	88 ±26 (24–183)	558 ±76 (492–858)
Acute lymphocytic leukemia	10	92 ±21 (24–261)	1,079 ±118 (552–1,860)

* TD transhydrogenase = TPNH-DPN transhydrogenase.
† 1 standard error.

Discussion

The results reported by Silber and co-workers (17) showed that there was more enzyme activity in leukemic than in normal cells. However, the amount of TD activity was considerably less than that found in the present study. This is probably accounted for by their method of preparation, which included dialysis against 0.05 M potassium phosphate buffer and discarding of the resultant sediment. In the present study, assay of the DD transhydrogenase was done on a small number of preparations. More DD than TD activity was found in the supernatant fraction, and results were approximately the same as those of Silber and his colleagues for the DD enzyme.

If TD transhydrogenase is a mitochondrial en-

zyme in human leukocytes as it is in most animal tissues, its presence in the soluble fraction requires explanation. The degree of substrate reduction of the AcPyDPN that occurred in the assays of the supernatant fractions makes it difficult to evaluate the existence of the enzyme in this fraction at all. In the final calculations the degree of nucleotide reduction that resulted from transhydrogenation was frequently less than that derived from the substrate. It is possible that after sonic oscillation a small amount of the enzyme was eluted from the mitochondria and did not sediment at 100,000 $\times g$. The TD enzyme was assayed using the pyridine nucleotide analogues thionicotinamide DPN and pyridine-3-aldehyde DPN; the ratio of activity found in the supernatant and particulate fractions was similar to that of the TD enzyme prepared from beef heart mitochondria (13).

Work is now in progress using crude fractionation of leukocyte homogenates centrifuged at 600 and 8,000 $\times$ g. The specific activity of the TD transhydrogenase increases in the 600-g supernatant fraction and is greatest in the 8,000-g precipitate. Electron microscopic examination of this precipitate demonstrated the presence of mitochondria.

In examining the findings obtained from assay of the particulate fraction two facts emerge; more enzyme activity was present in lymphocytes than in myeloid cells, and the greatest activity was present in the immature cells of acute leukemia. The blood used for the normal controls contained a mixture of polymorphonuclear leukocytes and lymphocytes. However, this type of dextran sedimentation allows a larger number of lymphocytes to be lost with the red cells resulting in a final preparation of 70 to 90% polymorphs. This provides a good control of normal granulocytes for comparison with the cells of myeloid leukemia but is not a completely satisfactory control for abnormal lymphocytes.

The activity of 366 mμmoles per hour per mg protein in chronic myeloid leukemia was higher than the 241 mμmoles found in normal cells, but the difference is not significant at the 5% level. The high normal or increased transhydrogenase activity contrasts with many other enzymes in chronic myeloid leukemia, which are reduced, such as alkaline phosphatase (20). In contrast, four patients with myeloid metaplasia were studied, and the mean was 140 mμmoles, which is in the low normal range. In this myeloid disease, the alkaline phosphatase is usually increased in contrast to the relatively low transhydrogenase activity.

Both the chronic and acute lymphocytic forms of leukemia had significantly higher activity in the particulate fraction than the normal controls did. There was a significant difference between 1,079 mμmoles of enzyme activity in the acute lymphocytic cells and 584 mμmoles in the cells of chronic lymphocytic leukemia. The chronic lymphocytic results were significantly higher than the 366 mμmoles found in chronic myeloid leukemia. Although there was not a good control of normal lymphocytes, it would appear that lymphocytes have more transhydrogenase activity than the cells of the myeloid series. The more immature cells had increased TD enzyme activity in contrast to leukocyte alkaline phosphatase, which is absent in myeloblasts and undifferentiated stem cells (21).

Mitochondria are present in both lymphocytes and polymorphonuclear cells but more numerous in lymphocytes (22, 23). They are also larger and more numerous in the earlier stages of development (24). Increased numbers and sometimes structural alterations of mitochondria have been noted in leukemic myeloblasts and mouse leukemic lymphocytes (25, 26). It is possible that the increase in size and number of mitochondria in lymphocytes and leukemic cells accounts for the varied TD activity found in this study. Another possible explanation is that leukemic cells are more sensitive to sonic oscillation, and the mitochondria are more easily broken, releasing a large number of small fragments. However, Morrow, Bierman, and Jenkins (27) showed that leukemic cells were more resistant to sonic oscillation and required two to four times longer exposure than normal cells to produce similar enzyme activation. In this study, increasing the time of sonic oscillation from 2 to 5 minutes did not increase the amount of available enzyme in the particulate fraction obtained from normal cells.

We made no attempt to evaluate the effect antileukemic therapy had on the leukemic leukocytes, since insufficient specimens were obtained from patients who were not receiving treatment. Sil-

ber and associates felt that in their results there was no difference between the patients who were and were not on chemotherapy (17).

Summary

TPNH-DPN (TD) transhydrogenase activity has been assayed in the supernatant and particulate fractions of the leukocytes from 24 normal subjects and 47 patients with leukemia. The results were calculated as millimicromoles of AcPyDPN reduced per hour per milligram of protein. Little TD activity was found in the supernatant fraction, and no difference was noted among the types of cells. The enzyme activity in the sediment of cells from patients with chronic lymphocytic, acute myeloid, and acute lymphocytic leukemia was significantly higher than that in cells from normal controls. The enzyme activity of the cells in acute and chronic lymphocytic leukemia was significantly higher than that in acute and chronic myeloid leukemia.

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