Mitochondrial Electron Transport Enzymes in Normal and Leukemic Human Leukocytes

By AUDREY E. EVANS AND GODFREY S. GETZ

THE DISCOVERY OF CHANGES in the metabolism of malignant cells, as demonstrated by enzymologic technics, will lead, hopefully, to the better understanding of the nature of the neoplastic process, which is the ultimate necessity for satisfactory therapy. In 1963, Silber, Huennekens, and Gabrio reported the presence of increased amounts of two pyridine nucleotide transhydrogenase enzymes in human leukemic leukocytes. The TD transhydrogenase enzyme catalyses the transfer of hydrogen between reduced triphosphopyridine nucleotide (TPNH) and diphosphopyridine nucleotide (DPN) as shown in the following reaction:

\[ \text{TPNH} + \text{DPN} \xrightarrow{\text{TD transhydrogenase}} \text{TP} + \text{DPNH} \]

A related reaction takes place between the oxidized and reduced forms of DPN. Thus,

\[ \text{DPNH} + \text{DPN}^* \xrightarrow{\text{DD transhydrogenase}} \text{DPN} + \text{DPNH}^* \]

In 1966, Evans and Kaplan showed that TD transhydrogenase activity was increased in the particulate fraction obtained from leukocytes of patients with chronic lymphocytic and acute leukemia compared to normal controls. At that time there was insufficient data to demonstrate that the enzyme was situated in the mitochondria. Preparations of pure lymphocytes were not assayed to provide a more satisfactory comparison for the lymphocytic types of leukemia. The present study was undertaken to clarify these points and to investigate other mitochondrial electron transport enzymes.

METHODS

Leukocytes were obtained from five normal subjects, five patients with polycythemia vera, and twenty-two patients with leukemia. Several pints of normal blood were used to yield five preparations of normal lymphocytes. The cells were prepared by means of dextran sedimentation of whole blood, and red cells were lysed with hypotonic saline. White cells obtained from simple dextran sedimentation of normal blood consisted mainly of polymorphonuclear leukocytes (PMN), as a larger percentage of lymphocytes sediment with the red cells and are lost. Pure lymphocytes were prepared from leukocyte-rich plasma, following incubation for one hour on a glass bead column after the method of Rabinowitz.

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The final sample contained greater than 90 per cent lymphocytes. The blood from patients with acute leukemia contained 80 per cent or more blasts; no attempt was made to separate the different types of acute leukemia. However, the majority of these specimens came from children with acute undifferentiated or lymphoblastic leukemia. The packed white cells were stored at −10 C. until used for investigation.

Prior to enzyme assay the thawed cells were suspended in 0.3 M sucrose to make a 10 per cent solution, and homogenized by means of a Teflon homogenizer. Nuclei and debris were sedimented at 600 × g for 10 minutes and mitochondria were recovered from the 600 × g. supernatant fraction by centrifugation at 8,000 × g. for 10 min. The resultant 8,000 × g. sediment was suspended in 1 ml. 0.3 M sucrose and layered on a continuous sucrose density gradient ranging from 1 to 2 M. After additional centrifugation for one hour at 50,000 × g. in a No. 50 swinging bucket rotor, five 1 ml. fractions were obtained from the gradient.

Enzyme assays were carried out on the original homogenate and the five gradient fractions. The TD transhydrogenase reaction was measured by the use of the acetyl pyridine analog of DPN (AcPyDPN) as in the previous paper.2 A millimolar extinction coefficient of 5.1 was used to calculate the extent of the reaction. Succinate cytochrome C reductase activity was measured by the increase in absorption at 550 μm of cytochrome C for 90 sec. following incubation with a solution of enzyme.4 A millimolar extinction coefficient of 19.1 for reduced cytochrome C was used to calculate the concentration changes in the reaction. Beta-glucuronidase activity was measured by the amount of free phenolphthalein liberated after 30 min. incubation of phenolphthalein glucuronide with enzyme.5 Protein determinations by the Lowry method6 were made on the homogenate and fractions.

Preparations of the whole homogenate, the sediment following 8,000 × g. centrifugation, and the fractions from the gradient were examined by the electron microscope. The preparations were fixed between four and eighteen hours in 1 per cent phosphate buffered osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon 812.7

The significance of the results of the enzyme assays was calculated by the Scheffé test for simultaneous multiple comparisons using 95 per cent confidence limits.8

RESULTS

The specific activity of TD transhydrogenase and succinate cytochrome C reductase was expressed as μmole of substrate reduced per minute per milligram protein. For the sake of brevity, these units are omitted throughout the text. In the cells investigated, all three enzymes were present in the whole homogenate and some of the fractions from the gradient. The peak specific activity of both TD transhydrogenase and the mitochondrial enzyme succinate cytochrome C reductase occurred in the 1.5 M or third fraction from the gradient (Fig. 1). Despite the concentration of transhydrogenase in the third fraction, the enzyme was present to some extent throughout the gradient. In contrast, there was little succinate cytochrome C reductase activity in any gradient fraction other than the third.

As shown in detail in Table 1, the specific activity of transhydrogenase and succinate cytochrome C reductase was two to five times higher in fraction 3 than in the original homogenate except in the cells of chronic lymphocytic and acute leukemia, when the difference of activity between homogenate and fraction 3 was ten to twelvefold. Beta-glucuronidase activity was detected in fractions 2, 3, and 4 from the gradient and no sharp peak occurred. There was little difference in the glucuronidase specific activity and gradient fractions in the myeloid cells, but in the case of the leukemic lymphocytes, the most active gradient fraction was four times as active as the homogenate.
Activity of Fractions from 2M - 1M Continuous Sucrose Density Gradient

Fig. 1.—Specific enzyme activity of cells from acute myeloid leukemia measured in five fractions from a sucrose density gradient. Peak activity of both enzymes occurs in fraction 3.

The results of the enzyme activity of the homogenate and fraction 3 are given in Table 1. The types of diseases and the numbers of specimens assayed are listed. In chronic lymphocytic and acute leukemic cells, the TD transhydrogenase and succinate cytochrome C reductase activities were significantly elevated over the other cell types including the normal lymphocytes. In acute leukemia, a higher succinate cytochrome C reductase activity was found in fraction 3 than in the corresponding fraction of chronic lymphocytic leukemia. The statistical significance of the results of the various types of leukemia are given in Table 2.

Beta-glucuronidase activity in the homogenate ranged from 0.07 μg, free phenolphthalein liberated per milligram protein in the cells of chronic lymphocytic leukemia, to 0.27 μg, in normal PMN. This difference between leukemic lymphocytes and PMN was significant. The range of activity of the gradient fractions extended from 0.21 μg, free phenolphthalein liberated per...
Table 1.—TD Transhydrogenase and Succinate Cytochrome C Reductase Activity in Normal and Leukemic Human Leukocytes

<table>
<thead>
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<tbody>
<tr>
<td>Normal polymorphous</td>
<td>5</td>
<td>2.2 ± 0.44†</td>
<td>2.9 ± 0.09</td>
<td>7</td>
<td>11.1 ± 1.3</td>
<td>9.1 ± 0.26</td>
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<td></td>
<td>(0.6-3.3)</td>
<td>(1.6-5.7)</td>
<td>(6.9-16.2)</td>
<td>(3.8-14.0)</td>
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<td>Polycythemia vera</td>
<td>5</td>
<td>3.9 ± 0.63</td>
<td>3.1 ± 0.10</td>
<td>5</td>
<td>13.2 ± 1.2</td>
<td>9.8 ± 0.38</td>
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<tr>
<td></td>
<td>(1.8-4.9)</td>
<td>(1.9-5.2)</td>
<td>(12.5-15.4)</td>
<td>(3.1-17.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>5</td>
<td>3.1 ± 1.01</td>
<td>5.0 ± 0.06</td>
<td>5</td>
<td>12.8 ± 1.7</td>
<td>10.9 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>(1.7-7.1)</td>
<td>(4.0-5.8)</td>
<td>(7.9-16.1)</td>
<td>(6.4-16.5)</td>
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<td>Normal lymphocytes</td>
<td>4</td>
<td>4.6 ± 0.92</td>
<td>5.2 ± 0.27</td>
<td>4</td>
<td>21.2 ± 6.3</td>
<td>36.0 ± 1.72</td>
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<tr>
<td></td>
<td>(5.1-6.2)</td>
<td>(4.1-9.5)</td>
<td>(13.3-39.0)</td>
<td>(30.0-64.0)</td>
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<tr>
<td>Chronic lymphocytic leukemia</td>
<td>9</td>
<td>5.3 ± 0.71</td>
<td>10.4 ± 0.16</td>
<td>9</td>
<td>73.2 ± 12.5</td>
<td>103.6 ± 2.25</td>
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<tr>
<td></td>
<td>(3.5-9.8)</td>
<td>(6.8-14.0)</td>
<td>(23.1-128.3)</td>
<td>(50.0-164.0)</td>
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<td></td>
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<tr>
<td>Acute leukemia</td>
<td>8</td>
<td>5.8 ± 0.77</td>
<td>12.0 ± 0.43</td>
<td>9</td>
<td>69.0 ± 6.4</td>
<td>161.0 ± 2.36</td>
</tr>
<tr>
<td></td>
<td>(3.9-8.7)</td>
<td>(5.2-18.5)</td>
<td>(56.2-101.9)</td>
<td>(100.0-231.0)</td>
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</table>

TD Transhydrogenase = TPNH + DPN TD Transhydrogenase = TPN + DPNH.

*Fraction 3 from 2M.–1M. sucrose gradient.
†Mean and 1 standard error.

Specific enzyme activity of a crude homogenate of leukocytes and the 1.5 M. fraction of a continuous sucrose density gradient TPNH-DPN or TD transhydrogenase was measured by the use of the acetyl pyridine analogue of DPN. The reaction mixture contained 100 µmole phosphate buffer, pH 6.5, 1 µmole KCN 0.3, µmole TPNH 0.6, µmole AcPy-DPN in 1 ml. The reaction was measured by following the increase in absorption at 375 m. for 5 minutes.

Succinate cytochrome C reductase activity was measured by observing the optical density change at 550 m. following 3 minute incubation of enzyme and sodium succinate; the reaction was initiated by the addition of excess cytochrome C. The reaction mixture of 3 ml. contained 1 x 10⁻³ M. sodium succinate, 3 x 10⁻³ M. oxidized cytochrome C, 4 x 10⁻³ M. potassium phosphate buffer of pH 7.4, 5 x 10⁻⁴ M. KCN of pH 7.4, and 1 per cent serum albumin.

Consideration was given to the possibility that myeloid cells might contain a latent inhibitor of mitochondrial respiratory enzymes which was activated during preparation, thus causing the lower values found in these cells. This possibility was investigated in the following manner. A crude homogenate of cells from chronic myeloid leukemia was assayed at time of preparation of the homogenate and after standing for two hours at 4 C. and 22 C. There was little change in the activity of TD transhydrogenase at either of these temperatures,
Table 2.—Statistical Significance of Enzyme Activity of F<sub>3</sub> Comparing Cell Types

<table>
<thead>
<tr>
<th></th>
<th>PMN</th>
<th>P. Vera</th>
<th>CML</th>
<th>NL</th>
<th>CLL</th>
<th>ALL</th>
<th>PMN</th>
<th>P. Vera</th>
<th>CML</th>
<th>NL</th>
<th>CLL</th>
<th>ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TD Transhydrogenase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Succinate Cytochrome C Reductase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

*+ denotes that the difference in enzyme activity of these two cells was statistically significant within 95 per cent confidence limits.

while succinate cytochrome C reductase decreased by one third in the sample incubated at 22 C.

In most of the assays of the gradient fractions, the hypertonic sucrose was not removed or diluted to isotonic levels when the enzyme activity was measured. Using acute leukemic cells, the effect of hypertonic sucrose was studied by repeat centrifugation of fraction 3 from the gradient and resuspension in 0.3 M sucrose. The succinate cytochrome C reductase activity remained unaltered and the TD transhydrogenase activity increased by about a third.

The effect of storage of cells on the enzyme activity was measured by assaying some preparations fresh and after varying lengths of time stored, up to two months. There was no significant deterioration of enzyme activity.

**DISCUSSION**

In the previous study, using the sediment from a crude homogenate of white cells, it was noted that leukemic lymphocytes had more TD transhydrogenase activity than normal PMN. Owing to the method of preparation, the following questions were not answered in that study: (1) whether TD transhydrogenase is a mitochondrial enzyme in human leukocytes, (2) whether the lower activity of myeloid cells could be due to the fact that fractions analyzed from these cells were less pure than the fractions analyzed from lymphocytes, and (3) whether the increased activity was due to a larger number of mitochondria present in lymphocytes and leukemic cells.

The technics employed in this study were aimed at producing a pure mitochondrial preparation. Assay of the enzyme throughout the various stages of preparations showed the greatest specific activity to be in the third or 1.5 M fraction from the sucrose gradient. A known mitochondrial enzyme, succinate cytochrome C reductase, was assayed on the same preparations and its activity was found to parallel that of transhydrogenase, thus providing strong evidence for the mitochondrial location of this latter enzyme.

Four preparations of normal lymphocytes were studied. In fraction 3, the activity of both enzymes was two to three times greater than that of normal PMN, but the wide range of results was such that this difference was not significant. Further assays of normal lymphocytes were not carried out because of the large amount of normal blood required for each preparation. The specific
activity of both enzymes in the lymphocytes of chronic and acute leukemia was significantly greater than that found in normal lymphocytes.

Beta-glucuronidase, a lysosomal enzyme was measured in an attempt to assess the amount of nonmitochondrial protein present in the samples. This assessment was based upon the expectation that the specific leukocyte granules or lysosomes would be the most likely contaminants of mitochondrial preparations of these cells. The low level noted in the homogenate of lymphocytes and the higher level in PMN is similar to the results reported by Anlyan and Follette, although their greatest activity was found in cells of CML. The amount present in any of the gradient fractions was small, and no significant difference between the cell types was noted. Presumably this was due to the method of preparation and storage of the cells, which is quite likely to result in rupture of the lysosomes and dispersion of their contents during differential centrifugation.
The electronmicrographs of the fractions from the gradient were unsatisfactory in spite of several washings of the fractions with isotonic sucrose. The mitochondria were damaged by the exposure to hypertonic sucrose, and the preparations contained some granules and sucrose crystals. However, two preparations from cells of chronic and acute myeloid leukemia showed that most of the particles were mitochondria. This, taken together with the lack of difference between the beta-glucuronidase concentration in the gradient fraction 3 of myeloid and lymphoid cells, suggested that the fraction 3 of myeloid cells was a relatively pure preparation of mitochondria. Thus, there is no reason to believe that the lower enzyme activity of these cells was the result of contamination by nonmitochondrial protein.

From the specific-activity measurements of the two respiratory enzymes examined in relatively pure mitochondrial fractions, it appears that the mitochondria of cells from chronic lymphocytic and acute leukemia are more active than those of the other cell types investigated. Mitochondria are larger and more numerous in leukemic mouse lymphocytes than in normal cells, but Wolf found fewer mitochondria in the cells of humans with chronic lymphocytic leukemia than in normal human lymphocytes. Aisenberg suggests that tumor mitochondria are qualitatively similar to the mitochondria of normal tissue, but fewer in number or smaller in size. The very small difference in respiratory enzyme activity found between the homogenates of PMN, normal lymphocytes and lymphoblasts, and the marked difference found following purification of mitochondria from these homogenates indeed suggests that there may be fewer mitochondria in the leukemic blast cells but that they have greater than normal activity.

It is possible that the differences in enzyme activity observed were due to differences in accessibility of the respective substrates to the mitochondria of normal and leukemic leukocytes. Intact mitochondria are relatively impermeable to TPNH and are not freely permeable to succinate, but thawing and homogenization of the frozen leukocytes during preparation resulted in a degree of mitochondrial damage as seen by electron microscopy. Succinate cytochrome C reductase assays were done following a 3 min. preincubation at 37 C. in hypotonic 0.1 M. phosphate buffer in the presence of succinate. It seems unlikely that the concentration of substrate was limiting the measured enzyme activities in these studies on partly damaged mitochondria. Indeed, brief sonication of some mitochondrial preparations did not result in any increase of measured activity of either respiratory enzyme.

The age of circulating leukemic cells is uncertain and they may be older than their primitive histologic appearance suggests. Lin and Bouroncle suggested that the generation time for the leukemic pool of cells was thirty-six days compared to a normal of thirty-two hours, and Dameshek suggests that the cells circulating in patients with chronic lymphocytic leukemia are quite mature and are the result of accumulation rather than rapid proliferation. It is not possible to say from this study whether the increase in enzyme activity found in leukemic lymphocytes is a function of cell immaturity or of neoplasia. If it is a function of neoplasia, the effect is seen only in the cells of the lymphocytic series as no increase was noted in the myeloid cells of CML.
Assays of TD transhydrogenase and succinate cytochrome C reductase carried out on the homogenate of normal and leukemic human leukocytes and on the 1.5 M fraction (fraction 3) of a continuous sucrose density gradient analysis of a crude mitochondrial fraction, provided strong evidence for the mitochondrial location of TD transhydrogenase. A slightly higher activity of these enzymes was found in the homogenate of cells from chronic lymphocytic and acute leukemia compared to those of normal polymorphonuclear leukocytes, normal lymphocytes, and cells from chronic myeloid leukemia. These differences were very much more marked when purified mitochondrial fractions from these cells were examined. The significance of these findings is discussed.

SUMMARIO IN INTERLINGUA

Essayage pro transhydrogenase de TD e pro reductase de succinatic cytochroma C effectuate in homogenato de leucocytos human tanto normal como etiam leiscemic e in le fraction 1,5 M (i.e., le fraction 3) de un continue analyse a gradiente de densitate de sucrosa de un crude fraction mitochondrial provideva forte evidentia pro le these de un location mitochondrial de transhydrogenase de TD. Un levemente plus alte activitate de iste enzimas esseva trovate in le homogenato de cellulas ab chronic leucemia lymphocytic e leucemia acute in comparation con illos de normal leucocytos polymorphonucleari, lymphocytos normal, e cellulas ab chronic leucemia myeloide. Iste differentias esseva multo plus marcate quando purificate fractiones mitochondrial ab iste cellulas esseva examine. Le signification de iste constatationes es commentate.

ACKNOWLEDGMENTS

The authors acknowledge with gratitude the technical assistance of Cecilia Lin, whose meticulous work formed the basis of this report.

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REFERENCES


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