ABSTRACT

Methods have been developed to isolate mitochondria from small amounts of normal and leukemic human lymphocytes obtained from small volumes of circulating blood. Mitochondrial respiratory functions were measured by polarographic techniques, and active calcium uptake was measured by a spectrofluorometric procedure utilizing the calcium-sensitive dye muromexide. The results indicate that the rates of active oxygen consumption of normal human lymphocyte mitochondria are very low in comparison to values obtained from a number of animal tissue sources. Mitochondria extracted from the lymphocytes of leukemic patients and from cultured, and phytohemagglutinin-transformed human lymphocytes exhibited higher respiratory rates than did normal controls. Although respiratory rates were relatively low in all preparations, the efficiency of energy coupling (ADP:O₂ ratio of nmol of adenosine diphosphate phosphorylated to nanoatoms of O₂ consumed) was within normal limits. The mitochondria extracted from leukemic, cultured, and phytohemagglutinin-transformed lymphocytes exhibited high rates of respiratory substrate-supported calcium uptake compared to controls.

INTRODUCTION

One of the problems encountered in conducting metabolic studies of neoplastic tissues is the lack of appropriate controls (25). This has been the case with regard to mitochondrial studies as well as other biochemical systems. In some instances, appropriate control tissues have been available to compare to neoplastic tissues. For example, normal liver mitochondria have been available for comparison to hepatomas (4, 17, 24), and normal mammary tissues have been available in studying mammary carcinomas (15).

Studies of both normal and leukemic human lymphocyte oxidative functions have been sparse (12). Foster and Terry (6) demonstrated respiratory activity with a variety of substrates of isolated normal human leukocyte mitochondria, but they were unable to show direct respiratory stimulation by ADP or respiratory control in their preparations. Castelli et al. (3) assayed a variety of respiratory enzymes in isolated human leukocyte mitochondria and found the specific activities of these enzymes to be lower than those of yeast and bovine heart mitochondria. Kirschner et al. (12) have pointed out that the difficulties in obtaining good yields of mitochondria from normal lymphocytes have caused most of our information on lymphocyte respiration to be obtained from leukemic and lymphoma cell types.

A large number of studies have been aimed at determining if significant functional changes have occurred in mitochondria extracted from neoplastic tissues. The rationale for these studies is predicated on the fact that many types of tumors have high glycolytic activity, and the assumption has been made that significant changes in the oxidative metabolism associated with the mitochondria could underlie or constitute a primary cause in the etiology of tumorigenesis (7, 25). To date, no substantial information exists to implicate alterations in tumor mitochondrial respiration as playing a role in the altered metabolism of neoplastic tissues (7). However, recent studies suggest that significant changes in the calcium transport properties of mitochondria derived from certain tumors may have a role in the transformation of these cells (1, 5, 14, 19, 26).

In the studies reported here, a method (2) developed in this laboratory is used to isolate a purified lymphocyte fraction from small volumes of circulating human blood. Mitochondria are subsequently isolated from the normal and leukemic lymphocytes. The methods involve microassay techniques in order to assess mitochondrial functions from the small amounts of cells obtained for study. The significant feature of these studies is the higher rates of respiration and calcium uptake found in mitochondria from leukemic and transformed lymphocytes.

MATERIALS AND METHODS

Approximately 120 to 150 ml of heparinized whole blood were obtained from healthy donors and leukemic patients prior to any therapeutic intervention. A purified lymphocyte preparation was obtained by a previously described technique (2). This procedure resulted in recovery of approximately 70% of the viable lymphocytes found in the initial sample of whole blood. Other cell types, including platelets, were absent or negligible (2).

The pellet of lymphocytes (200 to 350 x 10⁶ cells) was resuspended in an approximately 10% homogenate (v/v) in a medium consisting of 0.25 M sucrose, 5 mM Tris-HCl, 5 mM EGTA,³ and 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.; Fraction V) (pH 7.2). This cell suspension was homogenized with a specially machined Teflon pestle designed to fit a 12-ml heavy-walled Sorvall glass centrifuge tube. The pestle was attached to a motor rotating at approximately 6000 rpm, and 20 up-and-down strokes were performed to effect cell rupture. In some experiments, the cells were homogenized with a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio) at a rheostat setting of 40 for 5 sec. The homogenate was then centrifuged at 480 x g for 10 min to remove nuclei and heavier cellular elements. The heavy-walled Sorvall glass tubes were centrifuged in a Sorvall SS-34 rotor fitted with rubber adapters.

³ The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetra-acetic acid; PHA, phytohemagglutinin.
The supernatant was then centrifuged at 10,000 x g for 10 min to obtain the mitochondrial pellet. This pellet was resuspended in a small volume (200 to 500 μl) of the above-mentioned isolation medium minus EGTA. The resulting "washed" mitochondrial pellet was resuspended in 100 to 200 μl of a 0.25 M sucrose-5 mM Tris-HCl-0.5% bovine serum albumin solution (pH 7.2). Siliconized transfer pipets were used in all manipulations, and size 0 glass homogenizing vessels with Teflon pestles (A. H. Thomas & Co., Philadelphia, Pa.) were used to "wash" and resuspend the mitochondrial pellets. The entire mitochondrial isolation procedure was done at 4°C.

Mitochondria were also isolated from 2 types of cultured lymphocytes. One group consisted of normal human lymphocytes originally cultured by Dr. George E. Moore (16). The culture medium was (Grand Island Biological Co., Grand Island, N.Y.) Roswell Park Memorial Institute medium 1640 fortified with 10% fetal calf serum. The cells were cultured for 3 days; they were then harvested, and mitochondria were isolated by the techniques described above. Approximately 500 x 10⁶ cells were used for each preparation. The second group consisted of normal lymphocytes obtained from a healthy donor (data not shown). A great deal of variability in the respiratory activities being measured is apparent, or malate in the presence or absence of added NAD₃. Mitochondria were isolated from leukemic human lymphocytes; C, mitochondria isolated from normal human lymphocytes grown in tissue culture; D. mitochondria isolated from PHA-stimulated normal human lymphocytes. ADP:O, ratio of nanomoles oxygen consumed to nanomoles ADP added; RCI, respiratory control index, ratio of resting rate of respiration to active respiration during ADP phosphorylation. Numbers to left of traces, rates of oxygen uptake during phosphorylating respiration (State 3) expressed in nanomoles/min/mg. Succinate is the substrate. Additional details in text.

Protein determinations were made by a biuret method (10). The above-described isolation procedure usually yielded a mitochondrial suspension of 12 to 15 mg protein per ml with a range of 9 to 22 mg per ml. The percentage of recovery of mitochondria was determined by cytochrome oxidase measurements made on initial homogenates and mitochondrial suspensions using a spectrophotometric method (27). Mitochondrial assays were conducted immediately after isolation had been accomplished.

Mitochondrial respiratory activity was measured in a 1.0-ml chamber with a Clark oxygen electrode utilizing standard polarographic techniques (25). The assay medium consisted of: 0.25 M sucrose; 1.0 mM Tris-HCl (pH 7.2); 75 mM KCl; 2 mM P₃; 5 mM MgCl₂; 2 mM glutamate-malate, pyruvate-malate, or succinate as substrate; and 1 to 2 mg of mitochondrial protein. Rotenone (2 μg) was included in the assay with succinate as substrate. Temperature was maintained at 30°C.

Mitochondrial calcium transport was continuously monitored by a dual-beam spectrophotometric method (21, 23) utilizing the calcium-sensitive dye murexide at the wavelength pair 541–507 nm. The assay medium was the same as described above for the respiratory studies, with the inclusion of MgCl₂ and the inclusion of 50 μM murexide. The total volume of the assay system was 1.0 ml, and the temperature was 30°C. Antimycin A and 2,4-dinitrophenol were used in 70% ethanolic solutions.

RESULTS

Chart 1 illustrates representative oxygen electrode traces of mitochondrial respiration in normal, leukemic, cultured, and PHA-stimulated human lymphocytes. Succinate is the substrate. The addition of ADP results in a rapid increase in respiration (State 3) associated with the phosphorylation of ADP (downward deflection of traces). When all the ADP is phosphorylated, the respiratory rates return to a slower rate (State 4). The efficiency of oxidative phosphorylation (ADP:O) is essentially the same in all preparations. The significant features are the low rates of phosphorylating respiration (State 3) in the control preparations compared to the leukemic, cultured, and PHA-stimulated lymphocyte mitochondria. Addition of the uncoupler 2,4-dinitrophenol (Chart 1, U) results in a stimulation of respiration in all preparations. The subsequent addition of the respiratory chain inhibitor antimycin A (Chart 1, I) results in complete cessation of oxygen consumption. These data indicate that the respiratory activities being measured are mitochondrial, based on the response of the system to classic mitochondrial uncouplers and inhibitors.

All preparations of lymphocyte mitochondria failed to oxidize effectively NADH-linked substrates such as glutamate, pyruvate, or malate in the presence or absence of added NAD⁺ (data not shown). A great deal of variability in the respiratory activity of both the control and leukemic lymphocyte mitochondrial preparations was also found. The mitochondrial functions in the cultured and PHA-stimulated lymphocytes were generally more consistent from one preparation to the next. Measurements of cytochrome oxidase activity (27) in the initial total homogenates and in the final mitochondrial suspensions revealed a consistent yield of 18 to 20% for all preparations. The specific activities for cytochrome oxidase in total homogenates were 0.02 to 0.03 μmol/min/mg; mitochondrial specific activities ranged from 0.8 to 1.5 μmol/min/mg, indicating significant enrichment in mitochondrial protein.

Chart 2 illustrates representative dual-beam spectrophoto-
of traces). At the point where the system becomes anaerobic (Zero O₂), the Ca²⁺ is released back into the medium (upward deflection). Numbers to right of traces, initial rates of calcium uptake expressed in nmol/min/mg mitochondrial protein. Control: normal human lymphocyte mitochondria; △, leukemic lymphocyte mitochondria; △, cultured lymphocyte mitochondria; △, PHA-stimulated lymphocyte mitochondria. Additional details in text.

**DISCUSSION**

To our knowledge, this is the first direct demonstration of respiratory control in mitochondria isolated from circulating human lymphocytes. The respiratory activities are relatively low in all preparations compared to mitochondria isolated from animal tissues. This would appear to be consistent with the reported low mitochondrial enzymatic activity measured in human leukocytes by others (3, 6, 12). Low rates of respiration are not unusual for tumor mitochondria (24, 25), but we found the control lymphocyte preparations to be lowest of all (Chart 1A). It would appear that altered oxidative metabolism is associated with transformation of the lymphocyte cell. Both the leukemic and transformed cell lymphocyte mitochondria consistently exhibited higher rates of respiration (Chart 1, B to D). The greater variability between the control and leukemic lymphocyte mitochondrial preparations and those obtained from culture (cultured and PHA-stimulated) is not readily apparent. One possibility is that intrinsic differences exist in lymphocytes obtained from whole blood in the case of the control and leukemic cells compared to those obtained from tissue culture. However, the reasons for such differences are at present unclear. The possibility also exists that during preparation, particularly of the control mitochondria, some damage to the organelles occurred. However, electron micrographs of the mitochondrial suspensions revealed intact mitochondria with relatively little subcellular debris in all of the preparations studied (data not shown).

Our observation of enhanced metabolic activity in transformed and leukemic lymphocytes is in good agreement with the data of Silber et al. (22) and Kester et al. (11). The former reported higher NAD⁺ levels in acute leukemic cells than in normal leukocytes and higher levels of NAD⁺ and NADPH in human leukemic cells when compared to other types of solid tumors. Kester et al. (11) demonstrated a 2- to 3-fold increase in lymphocyte-glycolytic enzymes during blastogenesis.

Similar data were obtained on the calcium-transporting activities of the 4 types of lymphocyte mitochondria (Chart 2). Mitochondrial calcium transport increased in parallel with increasing respiratory function. This is not unexpected since calcium transport is regulated by the relative activity of the electron transport chain under substrate-driven conditions (9). A number of recent studies have suggested that mitochondrial calcium sequestration may be related to rapid cell growth associated with tumors (5, 14, 26). Reynafarje and Lehninger (19) found unusual stoichiometry for mitochondrial calcium transport in mouse ascites cells compared to normal liver. McIntyre and Bygrave (14) have reported prolonged calcium retention by Ehrlich ascites tumor cell mitochondria after their system became anaerobic. However, in our studies, the mitochondria of cultured, transformed, and leukemic cells do not retain accumulated calcium when the system becomes anaerobic (Chart 2).

There appears to be a correlation between the decreasing doubling times of the cell types used in our studies and the increased function of the mitochondria. The doubling times for the respective lymphocyte preparations used in these studies were: 60 to 70 hr for leukemic (20), 40 hr for the normal cultured cells (16), and 18 to 24 hr for PHA-stimulated cells (13). The respiration and calcium transport activity of their mitochondria increase in the same order, and this observation may suggest a relationship between the enhancement of the mitochondrial functions and the mitotic activity of cultured, transformed, and leukemic lymphocytes.

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**REFERENCES**


Respiratory and Calcium Transport Functions of Mitochondria Isolated from Normal andTransformed Human Lymphocytes

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