Sites of Inhibition of Mitochondrial Electron Transport in Macrophage-injured Neoplastic Cells

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ABSTRACT Previous work has shown that injury of neoplastic cells by cytotoxic macrophages (CM) in cell culture is accompanied by inhibition of mitochondrial respiration. We have investigated the nature of this inhibition by studying mitochondrial respiration in CM-injured leukemia L1210 cells permeabilized with digitonin. CM-induced injury affects the mitochondrial respiratory chain proper. Complex I (NADH-coenzyme Q reductase) and complex II (succinate-coenzyme Q reductase) are markedly inhibited. In addition a minor inhibition of cytochrome oxidase was found. Electron transport from α-glycerophosphate through the respiratory chain to oxygen is unaffected and permeabilized CM-injured L1210 cells oxidizing this substrate exhibit acceptor control. However, glycerophosphate shuttle activity was found not to occur within CM-injured or uninjured L1210 cells in culture hence, α-glycerophosphate is apparently unavailable for mitochondrial oxidation in the intact cell. It is concluded that the failure of respiration of intact neoplastic cells injured by CM is caused by the nearly complete inhibition of complexes I and II of the mitochondrial electron transport chain. The time courses of CM-induced electron transport inhibition and arrest of L1210 cell division are examined and the possible relationship between these phenomena is discussed.

Macrophages activated by lymphocyte-derived mediators and by bacterial components such as lipopolysaccharide are cytotoxic for neoplastic cells in vitro (see reference 1 for review). Binding of the macrophage to the target cell is required for cytotoxicity (2), but phagocytosis does not necessarily occur. Cytotoxic macrophages (CM) inhibit target cell DNA synthesis within 4–6 h after initial contact (3, 4). In the case of some neoplastic cell lines death occurs in hours or days following CM-induced cytostasis (5, 6), but other cell lines exhibit remarkable resistance to cytolysis (7).

Recently it was found that neoplastic cells in contact with CM lose their capacity for mitochondrial respiration (8). This is not a secondary effect of target cell death because it occurred in cell lines that resist cytolysis under conditions of the in vitro assay. For example, a line of lymphoblastic leukemia cells (L1210) injured by CM remain viable and amitotic for several days in culture (8). During this time they derive adenosine triphosphate (ATP) from glycolytic conversion of glucose to lactate at a very high rate (8). CM-injured L1210 cells eventually recover their capacity for cell division and this event correlates with resumption of mitochondrial respiration (9).

At the outset of this study it was known that CM-injured L1210 cells did not respire when suspended in either buffered saline (endogenous respiration) or in complete culture medium containing precursors of substrates for mitochondrial oxidation (8). The lack of respiration under these conditions could be explained by an extramitochondrial defect, for example, inhibition of plasma membrane transport for a necessary substrate, or by a mitochondrial effect, either enzyme inhibition or loss of a critical substrate, cofactor, or ion. An obvious approach to resolving these two possibilities would involve isolating mitochondria from CM-injured L1210 cells and examining respiratory chain redox reactions in the presence of added substrates, cofactors, etc. This avenue proved technically difficult because of the limited number of L1210 cells that could be injured by contact with CM in cell culture. Instead, we applied a method for measuring mitochondrial functions in whole cells permeabilized with digitonin (10–12). Because the molar ratio of cholesterol to phospholipid in eukaryotic plasma membranes is significantly greater than in mitochondrial membranes, binding of membrane cholesterol by digitonin leads to a selective increase in permeability of the plasma membrane (13, 14). Under defined conditions the acceptor control ratio (ACR) of digitonin-permeabilized hepatocytes and Ehrlich ascites cells was equal to or greater than that obtained with mitochondria isolated from the same cells using standard methods (15). An

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added advantage of this method for comparing CM-injured L1210 cells with uninjured cells was that measurements could be expressed on a per cell basis. This obviated errors inherent in the isolation process due to loss of mitochondria as well as inclusion of nonmitochondrial protein in the two types of samples.

In the experiments that follow several mitochondrial functions were measured in digitonin-permeabilized L1210 cells before and after injury by CM. The results show that CM-injured L1210 cells exhibit blocks in the respiratory chain in at least two loci. The CM-induced inhibition is selective since other mitochondrial functions remain intact.

MATERIALS AND METHODS

Reagents

Rotenone (K and K Laboratories, Plainview, NY), antimycin A, and oligomycin (Sigma Chemical Co., St. Louis, MO), and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Dupont Co., Wilmington, DE) were dissolved in absolute ethanol. Digitonin (Grand Island Biological Co., Grand Island, NY) was prepared according to Kun et al., (16) and dissolved in dimethyl sulfoxide. The small amounts of these solvents alone added to cell suspensions had no effect on any of the measurements made. Potassium tetramethylphenylenediamine (TMPD) (Sigma Chemical Co.) and KCN were prepared fresh and neutralized just before use. Ferricytochrome c (Sigma Chemical Co.) stock solution was stored frozen and the concentration was determined spectrophotometrically after reduction with dithionite ($\epsilon = 27.6\ mM^{-1}$ at 550 nm).

Murine Cytotoxic Macrophages

Outbred Swiss female mice 2-mo old (Microbiological Associates, Walkersville, MD) served as the source of macrophages. Mice were immunized intraperitoneally with 10^7 colony forming units of *Mycobacterium bovis*, strain BCG, Pasteur (TMC No. 1011, Trudeau Institute, Inc., Saranac Lake, NY). 2 wk later an intraperitoneal shot of 1.0 ml 10% proteose peptone (Difco Laboratories, Detroit, MI) was given. 3 d later peritoneal exudate cells were harvested and plated in petri dishes (Costar, Cambridge, MA) at 1.5×10^6 cells per cm^2 surface area for in vitro culture. The method used for preparing adherent monolayers of murine CM has been described in detail (8). CM were cultured at 37°C under 95% humidified air, 5% CO_2 atmosphere in Dulbecco's Modified Eagles Medium (Grand Island Biological Co.) supplemented with 10% horse serum (Sterile Systems, Inc., Logan, UT), additional glucose (15.5 mM final concentration), HEPES, 20 mM, pH 7.4, Penicillin G, 100 U/ml, and streptomycin, 100 μg/ml (referred to as: culture medium). Endotoxin (ET) (phenol extract of *Escherichia coli* 0.128:B12 from Sigma Chemical Co.), 10 ng/ml, was added to culture medium as the final stimulus to activate macrophages to the cytotoxic state (17). At this concentration ET had no effect on the doubling time or on mitochondrial respiration of the neoplastic target cells used in these experiments.

CM-injured Neoplastic Cells

A murine lymphoblastic leukemia cell line (L1210) was used as target for CM. L1210 cells were maintained in culture medium in suspension and passaged twice weekly. For coculture with CM, log phase L1210 cells were washed by centrifugation and added to freshly explanted CM at a final cell density of 2.5×10^5 per cm^2 culture dish surface area. Culture medium volume was 0.5 ml/cm^2. After a suitable incubation period L1210 cells were rinsed from CM monolayers as previously described (8). Experiments with radiolabeled cells showed that essentially all L1210 cells are removed from CM using this procedure. L1210 cells cocultured with CM are termed injured cells. Control or uninjured L1210 cells were incubated in culture medium + ET without CM for the same length of time.

Cell Counts

Both measurements of cell growth and oxygen consumption rates were based on the number of viable L1210 cells per ml. Cells were counted electronically (Model ZBI, Coulter Electronics Inc., Hialeah, FL) and viability was assessed microscopically by trypan blue (0.04% wt/vol) exclusion. L1210 cells rinsed from CM monolayers were invariably contaminated with some macrophages that dislodged from the plastic surface during rinsing. Cell volume distribution curves of L1210 cells alone and macrophages alone, rinsed from CM monolayers, were generated using the Coulter counter. The two cell populations showed very little overlap based on the larger volume of L1210 cells compared to CM. Thus, by adjusting the cell counter lower threshold to a setting corresponding to the nadir between the two populations, most contaminating CM were excluded from the electronic cell counts. Cell counts using appropriate controls revealed that L1210 cell suspensions rinsed from CM monolayers could be enumerated with <2% error due to contaminating macrophages and consequently no corrections were made.

Respiration Measurements

L1210 cells rinsed from CM were washed twice by centrifugation (4°C, 500 *g*) with 0.25 M sucrose containing 20 mM HEPES (pH 7.1), and 10 mM $MgCl_2$. The pellet of packed cells was then suspended in a small volume of the same medium and stored on ice. Oxygen consumption was measured with a Clark electrode in a water jacketed chamber (Gilson Medical Electronics, Inc., Middleton, WI) stirred with a magnetic flea. The electrode signal was fed through suitable amplification into a strip chart recorder (Sargent Welsh Model DSRG-2, Sargent-Welch Co., Skokie, IL) that was calibrated over a 0–100% scale. The temperature was 37°C in a medium consisting of air-saturated 0.25 M sucrose, 20 mM HEPES, pH 7.1, 2 mM KP_i, 10 mM $MgCl_2$, and 1.0 mM ADP (respiration medium). The oxygen concentration under these conditions was taken to be 390 ng atoms/ml. 50–100 μl of concentrated cell suspension were added to 1.2 ml respiration medium and then three small aliquots were removed from the electrode chamber for cell counts. The chamber was sealed with a stopper containing a capillary port for additions. Oxygen consumption rates are expressed in nanogram atoms per minute per million L1210 cells based on the mean of three separate cell counts for each trace.

Control experiments were performed to determine whether contaminating macrophages dislodged from CM monolayers during removal of L1210 cells contributed to oxygen consumption measurements. Their contribution was found to be negligible.

Mitochondrial Oxidation of NADH

Uninjured or CM-injured L1210 cells were washed by centrifugation in cold 0.25 M sucrose containing 20 mM HEPES, pH 7.1, and 10 mM $MgCl_2$, and resuspended in 10 ml of this medium. A cell count was made and a small aliquot of 10% digitonin (5 $\mu l/10^7$ cells) was added while mixing. Then 40 ml of sucrose medium was added and the cells were pelleted (4°C, 800 *g*) and resuspended in 0.5–1.0 ml sucrose medium to give $\sim 5 \times 10^7$ cells/ml. Counts were made on this stock suspension of permeabilized cells. This procedure resulted in >95% trypan blue emitting L1210 cells that retained their spherical morphology. Permeabilization followed by centrifugation effectively eliminated endogenous respiratory substrates. These cells did not consume oxygen without added substrate but addition of malate or succinate, but not NADH, initiated respiration. Ferricytochrome c, 25 μM final concentration, was added and the permeabilized cells were sonicated (Model W185, Branson Sonic Power Co., Danbury, CT) with three 6-s bursts at an output of 50 W. Known aliquots of the sonicated cell suspension were added to equilibrated respiration medium for oxygen consumption measurements. Counts of the stock cell suspension before sonication were used to calculate the rates. Permeabilized cell sonicates prepared by this method using both uninjured and CM-injured L1210 cells oxidized α-glycerophosphate at equal rates (~4 ng atoms oxygen per minute per 10^6 cells).

RESULTS

Mitochondrial Respiration in L1210 Cells Permeabilized with Digitonin

Before comparing mitochondrial functions of CM-injured and uninjured L1210 cells, it was necessary to establish conditions for selective permeabilization of the plasma membrane with digitonin. Increasing concentrations of digitonin were added to L1210 cells in the presence of succinate, rotenone, ADP, and P_i (state 3 conditions), and the rate of O_2 consumption was measured (Fig. 1). Without digitonin the rate is very low, due primarily to low permeability of the plasma membrane to succinate. Maximal state 3 respiratory rate was obtained with as little as 0.0025% digitonin. Significant inhibition of the state 3 rate does not take place on further increases in digitonin, up to 0.025%. Identical titration curves were obtained with malate and α-glycerophosphate as substrates, suggesting that the site of digitonin-induced inhibition at high concentrations

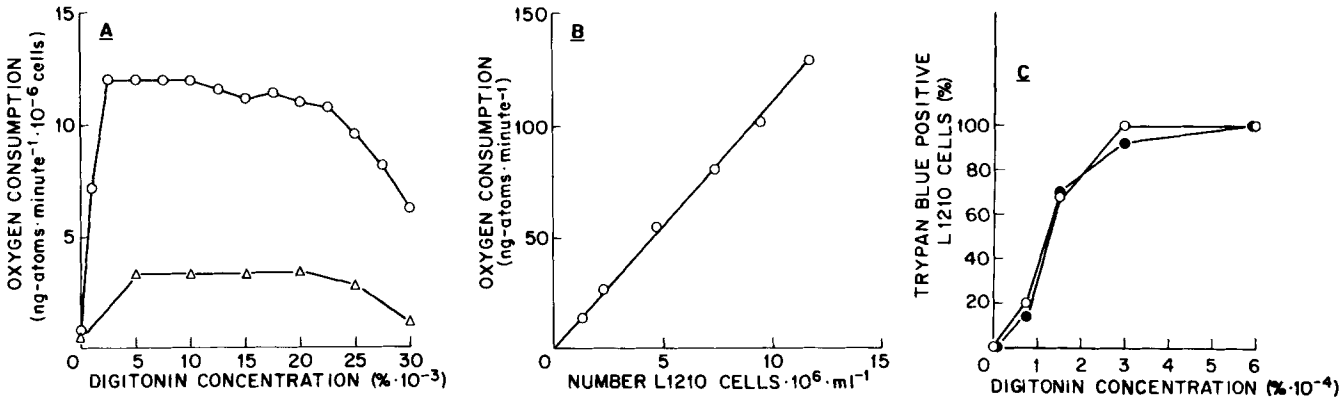

FIGURE 1 Permeabilization of L1210 cells to mitochondrial substrates with digitonin. (*A*) Increasing concentrations of digitonin were added to L1210 cells (5×10^6 cells per ml) suspended in respiration medium containing 5.0 mM succinate and 100 nM rotenone with (△) or without (○) oligomycin, 100 nM. (*B*) Digitonin, 0.005% final concentration, was added to suspensions of L1210 cells in respiration medium containing 5.0 mM succinate and 100 nM rotenone. Each point represents the rate of oxygen consumption per ml for a suspension of L1210 cells at a given density. (*C*) L1210 cells incubated alone (●) or with CM (○) for 20 h were washed by centrifugation and suspended in respiration medium at 5×10^5 cells per ml. Varying concentrations of digitonin were added to aliquots of the cell suspensions and then trypan blue, 0.04% final concentration, was added to each tube. The proportion of stained cells was determined microscopically.

is not at the level of the individual dehydrogenases, which catalyze the oxidation of these substrates.

Digitonin at concentrations up to 0.02% did not increase the state 4 respiratory rate (Fig. 1 *A*). At higher digitonin concentrations some inhibition was produced, parallel to the effect observed in state 3. The acceptor control ratio (state 3 rate/state 4 rate) at 0.005% digitonin in such experiments was ~3.7, comparable to that observed in mitochondria isolated from L1210 cells using standard methods (18). Use of digitonin to permeabilize the plasma membrane thus has an ample safety margin before mitochondrial functions are affected.

L1210 cell density was then varied, with digitonin concentration held constant at 0.005%, and the rates of O_2 consumption in state 3 were measured. A linear relationship was observed within the range of cell densities tested (Fig. 1 *B*). Based on the results shown in Fig. 1 *A* and *B* the standard permeabilization procedure adopted for the experiments that follow employed 0.005% digitonin with an approximate cell density of 5×10^6/ml.

It was also important to determine whether CM-injured and uninjured L1210 cells were permeabilized by similar concentrations of digitonin. This was examined by measuring entry of trypan blue (molecular weight = 961) at increasing digitonin concentrations. The dose-titration curves for L1210 cells before and after injury by CM were essentially identical (Fig. 1 *C*). In this experiment lower concentrations of digitonin sufficed presumably because L1210 cell densities were more than 10 times less than those used for O_2 uptake measurements as in Fig. 1 A. Thus CM-induced injury did not appear to alter the properties of the plasma membrane of L1210 cells that render the cells susceptible to permeabilization by digitonin.

Addition of Mitochondrial Substrates and Cofactors to CM-injured L1210 Cells

At the top of Fig. 2 a simplified scheme of the mitochondrial respiratory chain is presented for reference. The oxygen electrode trace *B* in Fig. 2 shows that when uninjured L1210 cells are permeabilized with digitonin in the presence of malate, ADP, and P_i (state 3 conditions) rapid linear O_2 consumption occurs. Malate enters the mitochondrial matrix via the electroneutral dicarboxylate carrier (19) and, in L1210 cells, is largely oxidized by malic enzyme to yield NADH, pyruvate, and CO_2 (20). The NADH formed is in turn oxidized by the respiratory chain. Rotenone, which inhibits complex I (NADH-coenzyme Q reductase), halts oxygen consumption. The rate of respiration by an equal number of CM-injured L1210 cells under the same conditions is markedly reduced (Fig. 2, trace *A*). Addition of succinate, which bypasses the rotenone block, leads to resumption of respiration in uninjured L1210 cells, but again, in CM-injured cells there was only a slight response (Fig. 2 traces *B* vs. *A*). Succinate respiration was stopped entirely by addition of antimycin A; oxygen consumption resumed when tetramethylphenylenediamine (TMPD) was added to donate electrons to cytochrome c. The rate of oxidation of TMPD by CM-injured L1210 cells was slightly reduced when compared to uninjured L1210 cells. A series of experiments was performed to check the consistency of the results shown in Fig. 2. These data are listed in Table I. They show that extensive inhibition of NAD-linked and succinate respiration is a constant finding when L1210 cells are cocultured with CM for 20 h. Other NAD-linked substrates (pyruvate, citrate, isocitrate, glutamate, and α-ketoglutarate), added alone or in combiantion with malate, were not oxidized by CM-injured L1210 cells (data not shown). NADH, which would not be expected to enter the mitochondrial matrix when added to permeabilized L1210 cells, likewise had no effect on CM-injured cells (data not shown). Under these conditions added malate and endogenous mitochondrial substrates are responsible for NAD-linked respiration of uninjured cells. In the absence of added malate the O_2 uptake rate slows as endogenous substrates are depleted. A linear rate of respiration is restored on addition of excess malate. If uninjured L1210 cells are first permeabilized and then repeatedly washed in sucrose medium by centrifugation, endogenous respiration is reduced to zero. Under these conditions NAD-linked respiration is totally dependent on added substrate. Parallel treatment of CM-injured L1210 cells failed to restore NAD-linked respiration. Therefore, the inhibition of electron transport induced by CM cannot be "washed away" from the cells.

Under the conditions used for the experiments in Fig. 2 and Table I, inhibition of malate and succinate oxidation could be

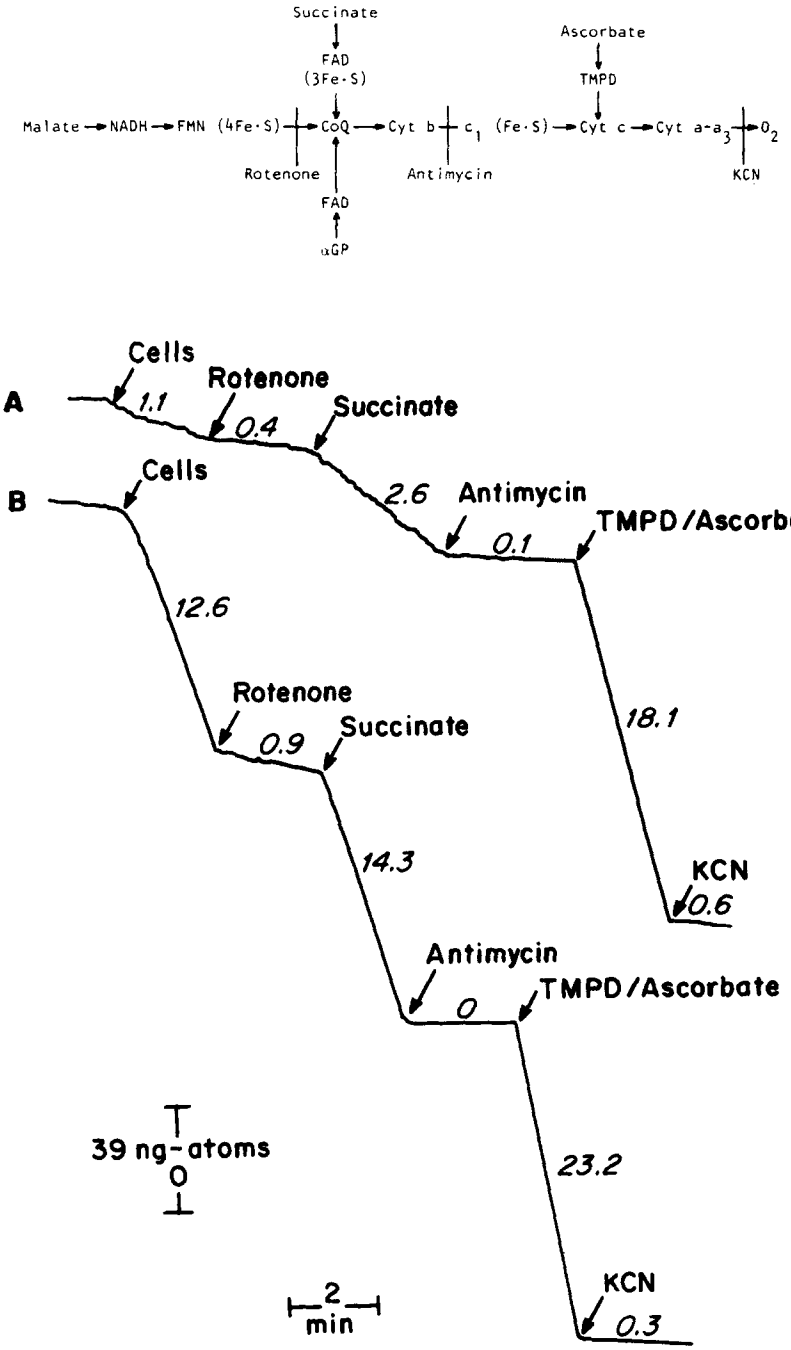

FIGURE 2 Addition of mitochondrial substrates to CM-injured L1210 cells permeabilized with digitonin. *Top*: A simplified scheme of the respiratory chain with sites of action of some mitochondrial inhibitors is shown for reference. Trace *A*: L1210 cells following an 18-h incubation with CM. Trace *B*: Uninjured L1210 cells. Respiration medium contained 5.0 mM malate and cells were permeabilized with 0.005% digitonin. Substrates and inhibitors were added at the final concentrations given in Table I. KCN was 1.0 mM. Traces were made with ~5 × 10^6 cells per ml. The reaction vessel contained 1.2 ml. Numbers are rates of oxygen consumption in nanogram atoms per minute per 10^6 L1210 cells.

due to either a block in translocation of ADP and/or P_i or inhibition of the F_1-ATPase complex. The data in Fig. 3 show that neither of these possibilities is the case. When the protonophore p-trifluoromethoxyphenylhydrazone (FCCP) is added to injured L1210 cells in the presence of malate, there is only an insignificant increase in O_2 consumption (trace *A*). As a control the effect of FCCP on NAD-linked respiration by uninjured L1210 cells in the presence of oligomycin is shown (trace *B*).

Also shown in Table I are the state 3 rates for another mitochondrial substrate, α-glycerophosphate. Electrons from α-glycerophosphate bypass the rotenone block as shown at the top of Fig. 2. Unlike the inhibited malate and succinate respiration, α-glycerophosphate is oxidized by CM-injured cells at the same state 3 rate observed with uninjured cells. This is an important finding because it shows that the respiratory chain between coenzyme Q and O_2 is functional in L1210 cells following CM-induced injury. It is evident that CM-induced injury leads to selective defects in the mitochondria of L1210 cells. Oxidation of the major mitochondrial substrates gener-

TABLE I

*State 3 Respiration Rates of L1210 Cells Injured by Cytotoxic Macrophages**

Substrates and inhibitors	Concentration‡	O_2 Consumption		Percent inhibition
		Uninjured cells	Injured cells	
		ng atoms · min $^{-1}$ · 10 $^{-6}$ cells§		
Malate	5.0 mM	12.0 ± 0.3	1.0 ± 0.2	92
Succinate	5.0 mM	13.9 ± 0.7	2.6 ± 0.5	81
+ rotenone	100 nM			
α-Glycerophosphate	10.0 mM	6.1 ± 0.3	5.9 ± 0.3	3
+ rotenone	100 nM			
TMPD	0.2 mM	27.1 ± 2.0	22.9 ± 2.2	15
Ascorbate	1.0 mM			
+ rotenone	100 nM			
+ antimycin A	20 nM			

* L1210 cells had been incubated with or without cytotoxic macrophages for ~20 h. State 3 rates were initiated by adding digitonin, 0.005%, to L1210 cells in respiration medium containing substrates and inhibitors.

‡ Concentrations used were those required to produce maximal respiration rates (substrates) or maximal inhibition under the conditions described in Materials and Methods.

§ Values are mean ± SEM for at least five separate experiments.

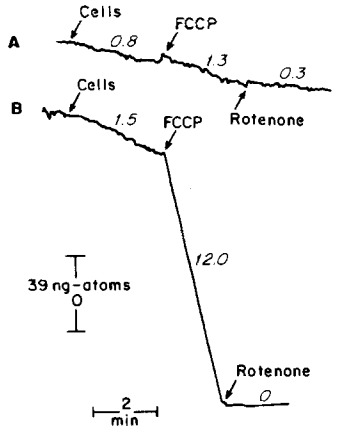

FIGURE 3 Effect of uncoupler (FCCP) on CM-injured L1210 cells. Trace *A*: L1210 cells had been cocultured with CM for 21 h. Respiration medium contained 5.0 mM malate. L1210 cells were permeabilized with 0.005% digitonin. FCCP concentration was 80 nM. Numbers are rates of oxygen consumption (nanogram atoms per minute per 10^6 L1210 cells). Trace *B*: Uninjured L1210 cells. Conditions the same as in trace *A* except that respiration medium contained in addition, oligomycin, 100 nM.

ated by the tricarboxylic acid cycle reactions (NAD-linked and succinate) is blocked. However, electrons pass from α-glycerophosphate to oxygen at an unimpaired state 3 rate. The terminal segment, from cytochrome c to O_2 via cytochrome oxidase, can promote electron flow at a high rate, well above the state 3 rates for oxidation of NAD-linked substrates. The rate-limiting step(s) for oxidation of NAD-linked substrates or of succinate in mitochondria of injured L1210 cells must therefore be located before the point of coenzyme Q reduction.

Correlation between CM-induced Cytostasis and Inhibition of Mitochondrial Respiration

The time course of inhibition of malate and succinate oxidation was measured and these results are plotted together with the induction of cytostasis in L1210 cells injured by CM (Fig. 4). At the times shown L1210 cells were removed from the CM, counted, permeabilized, and the state 3 rates of NAD-linked and succinate respiration were measured. Cessation of L1210 cell division occurs between 4 and 6 h after exposure to CM (Fig. 4*A*). This delayed onset has been observed by others measuring inhibition of DNA synthesis (3, 4). Inhibition of NAD-linked respiration begins between 2 and 4 h whereafter a steady decline in the state 3 rate occurs over the ensuing 10 h. Thus mitochondrial inhibition progresses slowly over a period of hours. This is in contrast to DNA synthesis, which attains maximal inhibition within 2 h of onset (4). With succinate as substrate, again, a progressive inhibition is seen but at a lower rate compared to NAD-linked respiration (Fig. 4*C*). Also plotted in Fig. 4*C* are the state 4 rates of CM-injured and uninjured cells in the presence of succinate and oligomycin. These rates are equal and constant for both uninjured and injured cells at each time point measured. During the period in which state 3 NAD-linked and succinate respiration is undergoing progressive inhibition the state 4 rate does not increase, suggesting that respiration remains coupled to phosphorylation.

In Fig. 5 the time course of inhibition of cell division and respiration is plotted over a 3-d period. In this experiment L1210 cells were incubated with CM for 23 h and then removed and cultured in fresh medium for an additional 47 h (Fig. 5*A*). Like the cytostatic effect, inhibition of both NAD-linked and succinate-linked respiration is not reversed by separating target cells from CM (Fig. 5). Oxygen consumption on α-glycerophosphate, which is unaffected at 20 h, remains at control levels on days 2 and 3 as well. This is the case for L1210 cells separated from CM on day 1 (Fig. 5*D*) or for L1210 cells cocultured with CM during the entire 3-d incubation (not shown).

At 70 h uninjured L1210 cells have reached the plateau phase of their growth curve (Fig. 5*A*). This has no effect on the rate of state 3 respiration in the presence of any of the substrates tested. Thus cessation of L1210 cell division in itself is not sufficient to explain the effects produced by CM on the respiratory chain of these neoplastic cells.

Activities of Selected Components of Mitochondrial Electron Transport

One or more of three possibilities could account for the inhibited respiration in CM-injured L1210 cells. First is inhibition of the dicarboxylate transport system of the inner mitochondrial membrane. Second, it is possible that either the

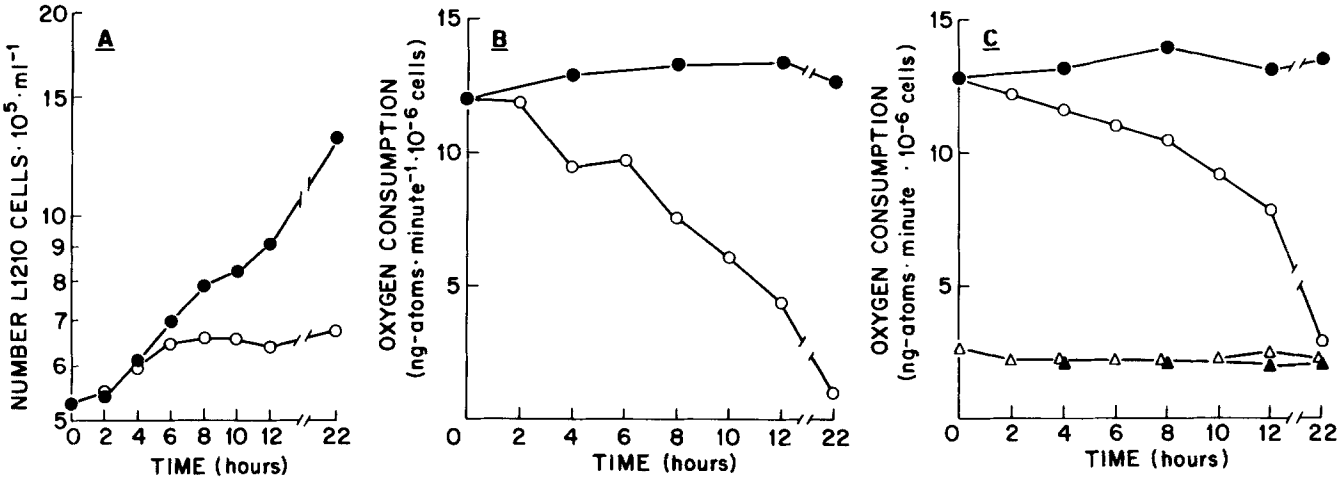

FIGURE 4 Time course of CM-induced cytostasis and inhibition of mitochondrial respiration. L1210 cells were cultured alone (●), or with CM (○), and at the times shown the cells were removed from culture for the following measurements. (*A*) Cell counts. Viability in both populations was >95% during the course of observation. (*B*) NAD-linked state 3 respiration. Respiration medium contained 5.0 mM malate and 0.005% digitonin. (*C*) State 3 (○) and state 4 (△) rates in the presence of succinate, 5.0 mM, rotenone, 100 nM, and digitonin, 0.005%. State 4 measured after addition of oligomycin, 100 nM.

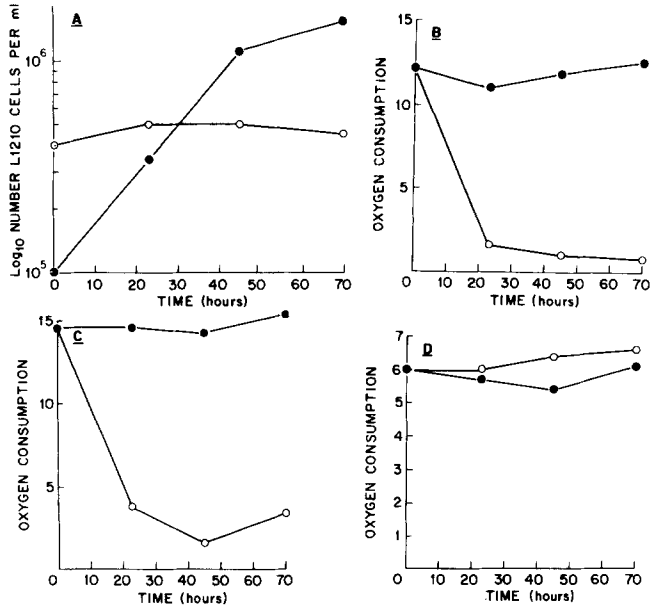

FIGURE 5 Pattern of CM-induced mitochondrial inhibition is unchanged during prolonged culture. L1210 cells were incubated with CM (○) for 23 h and then they were removed and cultured in fresh medium for an additional 47 h. Uninjured L1210 cells (●) were treated in the same way except that there were no macrophages present during the first incubation (hours 0–23). At the times shown measurements were made on: cell counts, *A*; state 3 respiration, rates for NAD-linked substrates, *B*; succinate, *C*; and α-glycerophosphate, *D*. Conditions for state 3 were the same as given in Table I.

TABLE II

*Mitochondrial Oxidation of NADH in L1210 Cells Injured by Cytotoxic Macrophages**

	O_2 Consumption			
	Rotenone sensitive‡		Rotenone insensitive§	
Experiment	Uninjured cells	Injured cells	Uninjured cells	Injured cells
	ng atoms · min $^{-1}$ · 10 $^{-6}$ cells			
1	3.7	0.2 (95)‖	1.1	1.2
2	2.8	0.3 (89)	1.1	0.9
3	2.9	0.5 (83)	1.0	0.9

* L1210 cells cultured alone (uninjured cells) or with cytotoxic macrophages (injured cells) for ~20 h before measurements.

‡ That portion of the total oxygen consumption initiated by addition of NADH, 0.1 mM, and inhibited by rotenone, 100 nM.

§ The rate of oxygen consumption remaining after addition of rotenone, 100 nM.

‖ Numbers in parentheses are percent inhibition.

matrix dehydrogenases are inhibited or their cofactors are missing or unreactive. The third possibility would be inhibition of both complex I (NAD-coenzyme Q reductase) and complex II (succinate-coenzyme Q reductase) of the electron transport chain. The third possibility was tested first.

Oxidation of NADH by complex I is difficult to measure in digitonin-treated cells because the inner mitochondrial membrane is impermeable to the pyridine nucleotides and because there are numerous other enzymes in the cells for which NADH is a reductant. These difficulties were circumvented by first disrupting the cells, including the inner mitochondrial membrane, by sonication and then assessing NADH oxidation by two criteria: the NADH oxidation must be linked to oxygen consumption and it must be inhibited by rotenone. Although the sonicated preparations of uninjured L1210 cells gave relatively low rates of NADH oxidation, the method was useful for comparison with CM-injured L1210 cells (Table II). In each experiment rotenone-sensitive NADH oxidation was markedly inhibited in L1210 cells injured by CM. The degree of inhibition (mean = 89%) was similar to that observed with NAD-linked substrate (i.e., malate, 92%, Table I). Rotenone-insensitive NADH oxidase activity was not affected in L1210 cells injured by CM (Table II).

The activity of succinate dehydrogenase (SDH) in complex II was measured using the artificial electron acceptor dye, phenazine methosulfate (PMS). Reduced PMS formed upon reaction with SDH is rapidly autoxidized and this reaction can be followed with an oxygen electrode (21). Although this assay may not detect the full activity of SDH (22), it was found to be

suitable for the purpose of comparing CM-injured L1210 cells with uninjured cells (Table III). When coenzyme Q is acceptor, electrons pass from reduced SDH through the respiratory chain to cytochrome oxidase. With PMS as electron acceptor the respiratory chain is blocked with antimycin A and cyanide. In both cases CM-induced injury results in significant loss of activity. Not presented in Table III are control experiments in which a known inhibitor of SDH, malonate, was found to inhibit succinate oxidation in uninjured L1210 cells using either assay method.

A small but consistent inhibition of the terminal segment of the respiratory chain from cytochrome c to oxygen was noted (TMPD plus ascorbate as substrate, Table I). Assay of cytochrome oxidase (23) was performed and the activity was calculated on a per cell basis. A consistent but relatively weak inhibition of cytochrome oxidase (range = 15–20%) was found when CM-injured L1210 cells were compared to uninjured cells. The activity of cytochrome oxidase in injured L1210 cells was 35 ng atoms oxygen per minute per 10^6 cells (mean of seven experiments).

Oxidative Phosphorylation in CM-injured L1210 Cells Respiring on α-Glycerophosphate

The finding that CM-injured L1210 cells can oxidize α-glycerophosphate at the control state 3 rate raised two important questions: can mitochondrial ADP phosphorylation occur in the presence of this substrate and if so, is it available to intact L1210 cells under the conditions of in vitro culture? Measurement of the P:O ratio during oxidation of α-glycerophosphate would provide quantitative data on the efficiency of energy coupling. However, a disadvantage of the permeabilized cell method used here precluded direct ATP measurements. Multiple ATPase activities remain active in digitonin-permeabilized cells. Hence the efficiency of mitochondrial ATP synthesis cannot be accurately measured due to concomitant hydrolysis at extra-mitochondrial sites. Instead the ACR of CM-injured and uninjured L1210 cells oxidizing α-glycerophosphate were determined. This provides indirect evidence for coupling of electron transport to ATP synthesis. In a coupled system under state 3 conditions inhibition of the mitochondrial ATPase by oligomycin leads to an increased protonmotive force which depresses the rate of electron transport (24). An experiment of this type is shown in Fig. 6. Here the state 3 and 4 rates of CM-injured and uninjured L1210 cells were similar. However, when this experiment was repeated several times, a small but significant difference emerged (Table IV). The state 4 rate of CM-injured cells was significantly elevated and consequently the ACR of these cells was significantly decreased. State 3 rates of injured versus uninjured L1210 cells were not significantly different. The presence of acceptor control in CM-injured L1210 cells oxidizing α-glycerophosphate is strong evidence that electron transport is coupled to phosphorylation of ADP; however, the relative efficiencies cannot be quantitated. The increased state 4 rates of injured L1210 cells suggest that coupling efficiency may be reduced, although direct measurement of ATP synthesis would be required to substantiate

TABLE III

*Oxidation of Succinate by Macrophage-injured L1210 Cells**

	Electron Acceptor‡			
	Coenzyme Q§		Phenazine Methosulfate (PMS)‖	
Experiment	Uninjured cells	Injured cells	Uninjured cells	Injured cells
1	15.4	5.3 (66)	24.0	4.0 (83)
2	16.5	4.5 (73)	25.5	5.1 (80)
3	13.4	1.7 (87)	23.3	1.1 (95)

* Incubation of L1210 cells with CM or alone was for ~20 h.

‡ Data are expressed as rates of succinate oxidation in nanomoles per minute per 10^6 L1210 cells. Numbers in parentheses are percent inhibition.

§ Measured by oxygen consumption rate of L1210 cells suspended in respiration medium containing digitonin, 0.005%, succinate 0.5 mM, rotenone, 100 nM, and FCCP 80 nM.

‖ Measured by initial oxygen consumption rate of L1210 cells suspended in respiration medium containing digitonin, 0.005%, succinate 5.0 mM, rotenone, 100 nM, antimycin A, 20 nM, KCN, 1 mM, phospholipase A_2 from *Naja naja* (Miami Serpentine, Miami, FL), 3.0 μg per ml, $CaCl_2$ 1.0 mM, and PMS, 1.0 mM. The reaction was started by addition of PMS after a 10-min incubation to activate the enzyme (22). The rates have been corrected for the slow autoxidation of PMS without cells present.

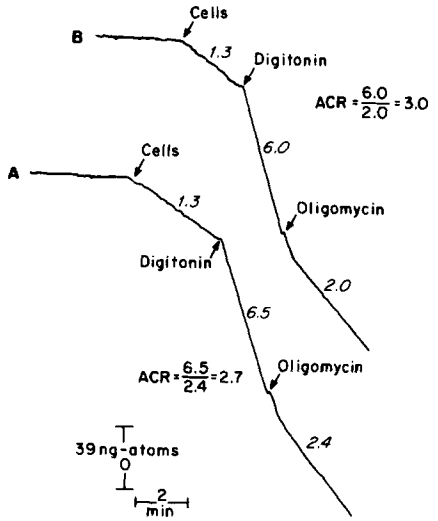

FIGURE 6 Acceptor control of CM-injured and uninjured L1210 cells oxidizing α-glycerophosphate. Trace *A*: L1210 cells, previously incubated with CM for 23 h. Respiration medium contained 10 mM DL-α-glycerophosphate and rotenone, 100 nM. State 3 rate initiated by addition of digitonin, 0.005%, final concentration. Transition to state 4 was produced by addition of 100 nM oligomycin. Trace *B*: same conditions as for trace *A* except that uninjured L1210 cells were used. Traces were made with 8 × 10^6 cells per ml. Acceptor control ratio (ACR) equals state 3 rate divided by state 4 rate. Rates are given in nanogram atoms oxygen per minute per 10^6 L1210 cells.

TABLE IV

*Acceptor Control Ratio (ACR) of Macrophage-injured L1210 Cells Oxidizing α-Glycerophosphate**

	O_2 consumption		
Conditions‡	Uninjured L1210 cells	Injured L1210 cells	p§
	ng atoms · min $^{-1}$ · 10 $^{-6}$ cells‖		
State 3	5.9 ± 0.2	6.3 ± 0.3	>0.05
State 4	2.1 ± 0.02	2.9 ± 0.2	<0.001
ACR	2.8 ± 0.1	2.2 ± 0.2	<0.001

* L1210 cells were incubated in the presence or absence of CM for ~20 h before respiration measurements.

‡ Addition of digitonin, 0.005%, to L1210 cells in respiration medium containing α-glycerophosphate 10 mM, and rotenone 100 nM (state 3). State 4 rate is same conditions as state 3 but with addition of oligomycin 100 nM.

§ Determined by Student's t test.

‖ Values are means ± SEM for seven separate experiments.

this. Elevation of state 4 rate presumably occurs following the initial inhibition of complexes I and II, because during the first 12 h of CM-L1210 cell interaction state 4 respiration on succinate remains at the control level (Fig. 4).

The question as to whether intact L1210 cells, injured or not, actually carry out oxidation of α-glycerophosphate was explored. The α-glycerophosphate shuttle for oxidation of cytosolic NADH (25) is active in some normal tissues and also certain neoplastic cells (26). The occurrence of the α-glycerophosphate shuttle in intact L1210 cells may be detected by measuring the increase in oxygen consumption upon addition of glucose in the presence of rotenone, which prevents the malate-aspartate shuttle from operating (26). The increase in oxygen consumption so produced should be completely inhibited by antimycin A. When this experiment was performed using CM-injured or uninjured L1210 cells there was only a slight increase in oxygen consumption (0.79 to 0.85 ng atoms oxygen per minute per 10^6 cells). This indicates that glucose is an insignificant source of α-glycerophosphate for intact L1210 cells. A second possible source of cellular α-glycerophosphate is from phosphorylation of glycerol by glycerol kinase and ATP (27). Here again oxygen uptake did not increase when glycerol was added to injured or uninjured L1210 cells in the presence of rotenone. These experiments indicate that intact injured or uninjured L1210 cells do not oxidize α-glycerophosphate at a significant rate despite the fact that they contain mitochondrial glycerophosphate dehydrogenase activity as shown in Table I.

DISCUSSION

In a previous report it was shown that incubation of a variety of neoplastic cell types with CM led to inhibition of target cell mitochondrial respiration by 80–90% (8). The results presented here confirm this observation and provide information on the sites in the mitochondrial respiratory chain at which inhibition occurs. In CM-injured cells state 3 respiration of NAD-linked substrates was inhibited by ~90% and this effect is due in large part to inhibition of electron flow in complex I, from NADH to coenzyme Q. Under in vitro culture conditions maximal inhibition of complex I by rotenone reduces L1210 cellular respiration by 88% (Granger, D. L., unpublished results). Because most of the electron flow to oxygen proceeds through NADH and complex I, an effect at this site alone could account for most of the respiratory inhibition in CM-injured cells. A second major site of inhibition occurs in complex II, effectively reducing state 3 succinate oxidation by ~80–90%. Together these effects close the two major entry sites for electrons into the respiratory chain. A third potential pathway for electron transport in L1210 cells is through the mitochondrial α-glycerophosphate dehydrogenase. However, we found no evidence for glycerophosphate shuttle activity either in uninjured or injured L1210 cells, despite the fact that the mitochondrial dehydrogenase is functional and unaffected by macrophage cytotoxicity.

A host of macrophage-derived cytotoxins have been identified (28–33). It is not clear from our data which, if any, of these products lead to the respiratory chain inhibitions shown here. One possibility is mitochondrial dysfunction resulting from accumulation of calcium and phosphate as a consequence of Ca^{2+} influx into the cytosol (34, 35). Several factors weigh against this type of mitochondrial damage in CM-injured L1210 cells: (*a*) Macrophage-induced injury is not abrogated in calcium-poor (26 μM) medium (Granger, D. L., unpublished results) as it is in cell injury induced by plasma membrane-directed agents (36). (*b*) The pattern of inhibition of mitochondrial functions upon Ca^{2+} loading is different from CM-induced mitochondrial injury (34, 35, 37, 38). (*c*) Cellular Ca^{2+} content of CM-injured L1210 cells is not increased (Granger, D. L., unpublished results) whereas this is a striking feature of ischemia/re-perfusion liver cell injury (39).

A more likely mechanism of pathogenesis may involve peroxidation of mitochondrial inner membrane phospholipids. Recently Narabayashi et al. (40) have characterized the sites of electron transport inhibition in heart submitochondrial particles subjected to NADPH-dependent lipid peroxidation in the presence of ADP-Fe^{3+}. Under these conditions >90% of mitochondrial linoleic and arachidonic acids were converted to oxygenated products. The site most sensitive to inhibition was complex I, which lost >90% activity. Complex II activity was inhibited by ~80% but at a lower rate compared with complex I. Cytochrome c oxidase was inhibited to a minor degree (20%). In addition the activity of complex III (coenzyme Q-cytochrome c reductase) was inhibited by ~50%. These results are very similar to the pattern of inhibition present in CM-injured L1210 cells with the exception that in our system using permeabilized cells we have, as yet, been unable to assay complex III under conditions of maximal activity. Conceivably a moderate inhibition at this site could go undetected at the relatively low rate of electron transport limited by the activity of glycerophosphate dehydrogenase.

In another system enzymatic peroxidation of mitochondrial membrane lipids leads to similar effects on electron transport. During red blood cell maturation at the reticulocyte stage a cytosolic lipoxygenase appears that inhibits the mitochondrial respiratory chain when tested on heart submitochondrial particles (41). Again, complex I is most sensitive to inhibition, followed by complex II. Cytochrome c oxidase is inhibited to a minor degree and in this system electron flow from coenzyme Q to cytochrome c is not inhibited (41, 42). Presumably the reticulocyte lipoxygenase is involved in the involution of mitochondria that occurs during erythrocyte differentiation. This enzyme appears to be distinct from lipoxygenases of platelets, neutrophiles, and macrophages in that it oxygenates arachidonic acid at carbon 15 (43).

The foregoing examples raise the possibility that contact of L1210 cells with CM may lead to peroxidation of the unsaturated fatty acids of the inner mitochondrial membrane lipids. The resulting changes in membrane lipids might be expected to affect adversely the catalytic activities of complexes I and II of the respiratory chain. Prime candidates as mediators of lipid peroxidation are CM-generated oxygen intermediates (30, 44) although other possibilities exist.

The question as to whether CM-induced mitochondrial dysfunction is causually related to target cytostasis remains open. Inhibition of electron transport begins shortly before L1210 cell division stops (Fig. 4). At the time of target cell division arrest, oxidation of NAD-linked substrates in state 3 is inhibited by only ~25%. It is possible, however, that this effect could upset the ATP/ADP ratio and/or the NADH/NAD ratio of injured L1210 cells. These alterations in turn could decrease the metabolic flux of substrates required for DNA polymerization, namely, the deoxyribonucleotides. Such an effect is commensurate with the findings of Krahenbuhl (4), which show complete abortion of S phase in synchronized target cells in contact with CM. However, at what level inhibition of DNA synthesis occurs is not known. The potential

relationship of the mitochondrial effects shown here and the cessation of cell division deserves further study. Measurements of the phosphorylation potential and the ratio of reduced:oxidized pyridine nucleotides during the early hours of CM–L1210 cell interaction are required.

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