Altered Aconitase Activity in Hamster Cells Selected for Resistance to Fluorocitrate

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Summary: Two independent Chinese hamster ovary cell lines have been isolated in cell culture which exhibit resistance to the cytotoxic effects of fluorocitrate. Although the oxidation of citrate by wild type cell suspensions was markedly inhibited by 1 mM fluorocitrate drug-resistant cells oxidized citrate at approximately normal rates in the presence of the drug. The aconitase activity from the resistant cells was less sensitive to the inhibitory action of fluorocitrate *in vitro* and showed altered heat stability properties when tested in heat inactivation experiments at 3 different temperatures. These results are consistent with the view that the resistant cell lines contain a structural gene mutation.

Although many phenotypic variants have been selected in animal cell culture systems some reports question the genetic basis for these phenotypic changes (1, 2, 3). In general, alterations in phenotype may be due to one of two fundamental processes : mutation which results in a stable change in the DNA base sequence and phenotypic variation which involves changes within the range of a given genotype. Although it is often difficult to identify the process underlying a phenotypic change a clearer understanding of somatic cell genetics requires that attempts be made to distinguish between these two alternatives. It can be predicted that a mutation will lead to the formation of an altered gene product since the change in the DNA base sequence is usually translated into the amino acid sequence of an altered protein. Evidence supporting the view that somatic cell variants with altered gene products can be selected in cell culture is rapidly accumulating (4-11). In this report the selection of hamster cell variants with an increased resistance to the cytotoxic effects of fluorocitrate, an inhibitor of the aconitase enzyme, is described. These variants contain an altered aconitase activity.

Materials and Methods: Chinese hamster ovary (CHO) cells were routinely grown in suspension or monolayer cultures at 37° in α-minimal essential medium (Flow Laboratories, Inc.) supplemented with antibiotics and 10% fetal bovine serum (Grand Island Biological Co.). Cells were grown and plating efficiencies were determined by standard techniques (12). Isocitrate dehydrogenase and the various chemicals were purchased from the Sigma Chemical Co.

Aconitase activity was determined from cells grown exponentially in 4 liter batches at 37°. Cells were collected by centrifugation, washed with phosphate buffered saline, suspended in 2 volumes of 0.1 M Tris-Cl buffer, pH 7.4, and disrupted in a Tri-R homogenizer (Tri-R Instruments). The resulting suspension was centrifuged at 48,000 g for 30 min and the supernatant was dialysed for 1 hr against 500 volumes of 0.02 M Tris-Cl buffer, pH 6.5, containing 1 mM citrate. The dialysed preparation was centrifuged at 27,000 g for 15 min and the supernatant was used as a source of aconitase activity. For heat inactivation studies the pH of the homogenized preparation was reduced to pH 5.5 and the precipitate was removed by centrifugation. The supernatant was adjusted to pH 7.4, dialysed as described above and used as a source of aconitase activity. The protein content varied between 3 and 4 mg/ml as determined by the method of Lowry et al (14). Aconitase was activated and assayed by the method of Morrison (13) at 25° in the presence of isocitrate dehydrogenase by measuring the reduction of NADP on a Gilford recording spectrophotometer at 340 mμ in aqueous solution containing: tris-Cl, pH 7.4 (0.03 M); $MnSO_4$ (1.3 mM); NADP (1.5 mM); isocitrate dehydrogenase (1 unit); enzyme preparation (10 μl); Na_3 citrate (1 mM). The aconitase enzyme had a K_m of 0.25 mM for citrate as determined by varying the substrate at 5 different concentrations over a 20-fold range, including the K_m, and examining the data as double reciprocal plots.

D,L-fluorocitrate was purchased as a barium salt, $(C_6H_4O_7F_2)_2Ba_3$. For the selection of variant cells the drug was dissolved in growth medium with the aid of vigorous shaking and the barium was replaced with sodium by precipitating barium from solution with the appropriate amount of Na_2SO_4. The precipitate was removed by passing the medium through a filter.

The oxidation of citrate or isocitrate by cell suspensions was followed in a Gilson Oxygraph (Clark Oxygen Electrode) at 37°. Cells growing exponentially in suspension were centrifuged, washed with phosphate buffered saline, pH 7.0, and resuspended in the buffer at about 10^6 cells/ml. Endogenous activity was substantially reduced by first incubating the cell suspension for 30 min at 37° prior to addition of 1.5 ml to the reaction vessel. In experiments involving fluorocitrate the cells were first suspended in phosphate buffered saline containing 1 mM drug for 30 min before being analysed.

Results: *Drug resistance at the cellular level* - Cells derived from 2 independent clones of the wild type population were used separately in the selection of 2 variant cell clones. 2×10^5 cells derived from each clone were added separately to 15 x 60 mm plastic plates (Falcon Plastics) containing 5 ml growth medium supplemented with either 1.5 mM or 5.5 mM fluorocitrate. After 2 weeks in the presence of the drug surviving cells (approximately 1×10^{-5} at 5.5 mM) formed an incomplete monolayer and were removed from the plates and cloned again by distributing the cells at limiting dilution into the wells of a Linbro plastic tray (IS-FS96-TC). Clones were selected from wells containing only one colony. One clone was selected from the culture originally grown at 1.5 mM fluorocitrate and another from the culture grown in the presence of 5.5 mM drug. These cell lines were named FLC^R-1 and

$FLC^{R}-2$ respectively. As can be seen in Table 1 the drug-resistant cells were significantly more successful than the wild type cells at forming colonies in the presence of fluorocitrate with the variant cell line selected in the presence of the highest concentration of drug ($FLC^{R}-2$) exhibiting the greatest reduction in sensitivity. The resistant property was stable; cells cultured for more than 50 generations in the absence of the drug exhibited the expected level of resistance to fluorocitrate when retested.

The ability of suspensions of wild type and $FLC^{R}-2$ cells to oxidize citrate and isocitrate is shown in figure 1. Both wild type and FLC^{R-2} cell suspensions (1.5×10^{6} cells) oxidized these compounds at a rate of about 0.05 μmoles O_2 utilized/min in the presence of either 1 mM citrate or 1 mM isocitrate. When the wild type cells were incubated in the presence of 1 mM fluorocitrate for 30 min prior to the addition of 1 mM citrate the rate of oxygen utilization was decreased to about 20% of the rate that occurred in the absence of the drug (figure 1a). However, when isocitrate was added to the suspension of cells in which citrate oxidization was blocked by fluorocitrate the rate of oxygen consumption returned to the level that was observed in the absence of drug (figure 1a). Isocitrate, which can enter the tricarboxylic acid cycle as a substrate for the isocitrate

TABLE 1

Colony-forming ability of CHO cells grown in the presence of fluorocitrate.*

fluorocitrate concentration	wild type	$FLC^{R}-1$	$FLC^{R}-2$
1.5 mM	6	78	97
3.5 mM	0.02	0.3	8

* colony-forming efficiency of treated cells relative to untreated cells X 100.

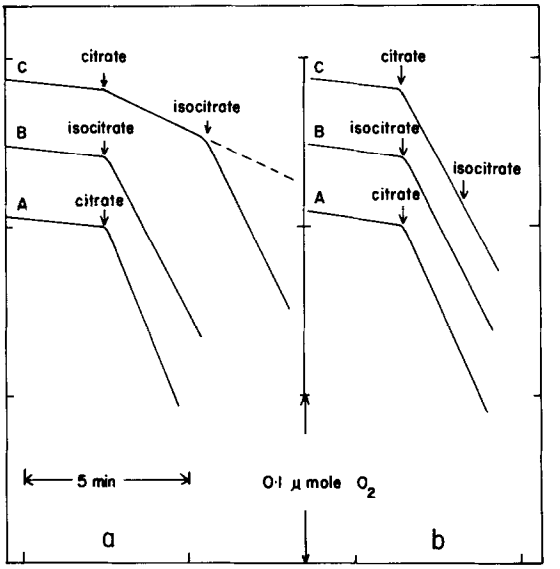

Figure 1. Oxidation of citrate and isocitrate by 1.5 x 10^6 wild type (1a) and FLC^R-2 (1b) cells. Oxygen consumption was followed in a Gilson Oxygraph as described in Materials and Methods. At the times indicated by arrows additions were made as follows: (A) 1.0 mM citrate, (B) 1.0 mM isocitrate, (C) 1.0 mM citrate or 1.0 mM isocitrate added to cells previously incubated for 30 min in the presence of 1.0 mM fluorocitrate.

dehydrogenase enzyme, can bypass the fluorocitrate block. This result supports the idea that fluorocitrate specifically inhibits aconitase activity in the wild type CHO cells.

One of the several ways in which cells could exhibit resistance to fluorocitrate would involve the formation of aconitase activity which was less sensitive to the inhibitory activity of the drug. If the drug-resistant cells contained an altered aconitase activity citrate oxidation would proceed at a rate that was greater than wild type in the presence of the drug. In good agreement with this prediction it was found that oxygen utilization with FLC^R-2 cells in the presence of 1 mM fluorocitrate occurred at a rate greater than 90% of the normal rate obtained in the absence of the drug (figure 1b). Also, citrate oxidation by FLC^R-1 cell suspensions continued at a rate of about 75% of the normal in the presence of 1 mM fluorocitrate (results not shown).

Drug resistance at the enzyme level - The effect of fluorocitrate on aconitase activity is shown in figure 2. Enzyme preparations from cell lines selected for resistance to fluorocitrate showed significantly more activity than wild type preparations in the presence of various concentrations of the drug. For example the wild type activity was inhibited by 50% with 0.008 mM fluorocitrate, whereas aconitase activity from FLC^R-1 and FLC^R-2 were inhibited by about 50% in the presence of 0.025 mM and 0.07 mM fluorocitrate respectively. Enzyme activity from the wild type preparation and the FLC^R-2 preparation were mixed in various proportions and assayed at different fluorocitrate concentrations. These mixing

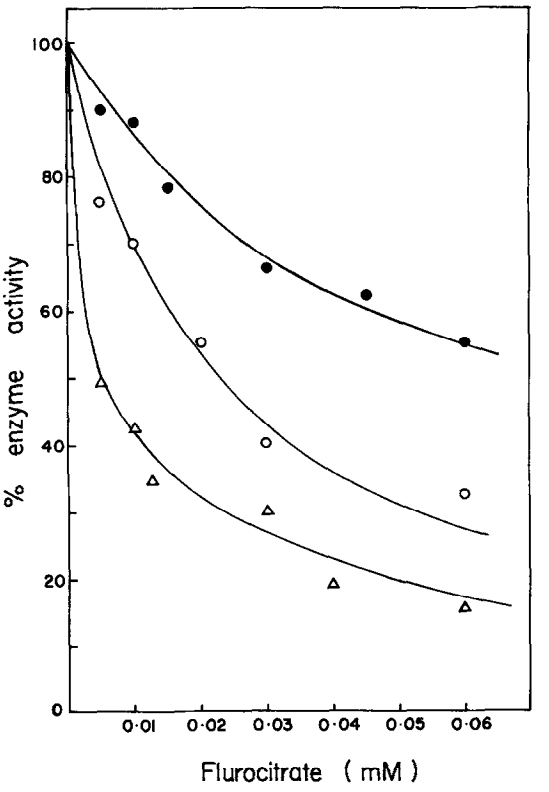

Figure 2. Inhibition of aconitase activity by fluorocitrate. The enzyme was assayed in the presence of varying concentrations of the drug. The points for each cell line are the average of duplicate values obtained in 3 different experiments utilizing separate enzyme preparations. Wild type (Δ), FLC^R-1 (○), FLC^R-2 (●).

experiments demonstrated that the increased resistance was not due to a trival property of the enzyme preparation but was due to actual differences in the enzyme itself. For example an equal mixture of wild type and FLC^R-2 enzyme activities exhibited the expected (see figure 2) 50% reduction in activity in the presence of 0.03 mM fluorocitrate.

Aconitase activity with altered heat stability - Heat inactivation properties of the aconitase preparations were examined by determining the time required to reduce the aconitase activity by 50% at 3 different temperatures. As shown in figure 3a the aconitase activity from the FLC^R-2 preparation was significantly more stable than the wild type activity at 50°, 53°, and 56°. At 53° the enzyme activity from FLC^R-2 was about 3 times more stable as compared to wild type enzyme

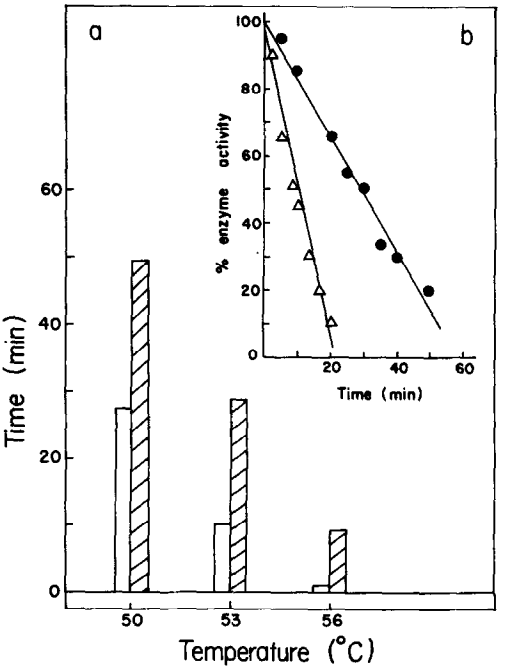

Figure 3. Heat inactivation of aconitase activity. (3a) Time required to reduce the activity by 50% at 50°, 53°, and 56°. Wild type (clear bars), FLC^R-2 (hatched bars) (3b) Inactivation of aconitase activity with time at 53°. Wild type (Δ), FLC^R-2 (●). Enzyme preparation was added to plastic tubes and placed into a water bath. At various times an aliquot was removed and assayed as described in Materials and Methods.

(figure 3b). Various mixing experiments demonstrated that the decreased sensitivity of FLC^R-2 was not due to an unusual property of the partially purified preparation but was due to a difference in the enzyme. For example, an equal mixture of wild type and FLC^R-2 enzyme activities were heated at 53^o for 15 min and the expected (see figure 3b) reduction in enzyme activity of 50% was obtained.

Discussion: CHO cells selected for resistance to fluorocitrate (1) exhibit a higher plating efficiency than wild type in the presence of the drug (2) oxidize citrate at approximately normal rates in the presence of 1 mM fluorocitrate (3) possess an aconitase activity which is less sensitive to fluorocitrate than the wild type activity and (4) contain an aconitase activity with altered heat stability properties. These results support the view that fluorocitrate is a suitable selective agent for the isolation of variant cell lines with altered aconitase activity. Although other explanations are possible the simplest one is that the resistant cell lines contain a structural gene mutation. These observations provide additional evidence to support the view that somatic cell variants with altered gene products can be selected in cell culture. These variants will be useful in further studies on the genetics of cells in culture. Also, a more detailed biochemical comparison of wild type and altered enzyme activities should provide important information on the mechanism of action of the aconitase enzyme.

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