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SHORT COMMUNICATIONS

Increased levels of folic acid reductase as a mechanism of resistance to amethopterin in leukemic cells

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STUDIES concerning amethopterin-resistance in murine leukemic cells (L5178Y) have been described.[1] Using a medium developed to permit the clonal reproduction of single isolated leukemic cells,[2] it has been established that independently derived "first-step" mutants (presumed to be the result of single independent mutational events), had separate "low" levels of resistance. The concentration of amethopterin required for 50% inhibition of growth of 20 first-step mutant clones ranged from $1 \cdot 4$ to $4 \cdot 3 \times 10^{-8}$ M, or 1·5 to 4·8 times that concentration ($0 \cdot 9 \times 10^{-8}$ M) required for comparable inhibition of the parent strain. This finding suggests that, with respect to this enzymic activity, a multiplicity of mutable sites or alternate stable forms of a single mutable site may be present in these cells. With one such first-step mutant clone, selected in an amethopterin-containing medium which completely prevented the reproduction of sensitive cells, approximately twice the concentration of amethopterin ("2-fold mutant") was needed for 50% inhibition of growth, as was the case with the parent strain. From this first-step clone, a "second-step" clone ("16-fold mutant") was isolated. These first and second-step clones had a 2- and 17-fold increase, respectively, in their levels of folic acid reductase activity (Table 1).[6] Increased folic acid reductase activity has been reported in amethopterin-resistant cells from (a) sublines of mouse leukemia L5178 selected in culture[1, 6] (b) various leukemic lines selected in mice,[7] (c) cultured sarcoma-180 cells,[8] and (d) human leukemic individuals treated with the drug.[9]

TABLE 1. FOLIC ACID REDUCTASE LEVELS OF AMETHOPTERIN-SENSITIVE AND AMETHOPTERIN-RESISTANT CELLS

Line tested	Relative level of resistance*	Relative activity of folic acid reductase†
Sensitive	1·0	1·0
"First-step" clone	2·3	2·0
"Second-step" clone	16·0	17·0

A mixture containing 0·02 M phosphate buffer (pH 6·0), $1 \cdot 8 \times 10^{-2}$ M isocitric acid, $1 \cdot 8 \times 10^{-2}$ M magnesium chloride, 2×10^{-4} M triphosphopyridine nucleotide and a preparation of isocitric dehydrogenase[3] was incubated for 5 min at 37 °C. Of this mixture, 0·15 ml was added to 0·30 ml of a preparation of folic acid reductase (this enzyme was prepared by the addition of 4 volumes of water to packed cells from culture, dialysis for 18 hours against 1,000 volumes of 0·002 M phosphate buffer (pH 6·0), centrifugation at 100,000 **g** for 30 min, recovery of the supernatant fraction, and adjustment of the phosphate concentration to 0·02 M (pH 6·0)); the, 0·05 ml of 0·0012 M folic acid was added. After 20 min the reaction was terminated by the addition of 0·2 ml of 4 N hydrochloric acid, 0·8 ml of acetone and 0·5 ml of water. The diazotizable product was determined by the method of Bratton and Marshall.[4]

* Concentration required for 50% inhibition of sensitive line = $0 \cdot 9 \times 10^{-8}$ M.

† Activity of sensitive line = 2·6 units of folic acid reductase activity per mg of protein.[5] One unit of folic acid reductase activity = 1×10^{-3} μmoles of product (measured as diazotizable amine) per 30 min.

"Pseudo-irreversible"[10] complete inhibition of one unit of folic reductase activity (see Table 1) required the same amount of amethopterin ($2{\cdot}9 \times 10^{-12}$ moles) whether the enzyme was prepared from drug-sensitive or drug-resistant cells. Since amethopterin is bound to the catalytic site of folic acid reductase,[11, 12, 13] and apparently to no other cell component, it is suggested that an increased number of such catalytic sites, with identical turnover number and "affinity" for amethopterin, are present in the resistant cells.

Resistant cells, grown in the presence of tritium-labeled amethopterin (103 μc/mole) bound the antagonist in a manner which suggested that the entry of amethopterin into the cell was less rapid than that of the synthesis of folic acid reductase (Table 2). From such "bound" enzyme, a minimum of 80% of the ^{3}H-amethopterin was liberated by dialysis against 100 volumes of 0·2 M phosphate buffer (pH 6·0) and enzyme activity was regenerated. Although the amount of bound radioactivity (sufficient for the binding of 8 units of enzyme activity per 10^{7} cells) which was release by dialysis was not in close agreement with the units of enzyme recovered after dialysis (15 units per 10^{7} cells), it is conceivable that, under the conditions of the experiment, some tritium may have been lost from the amethopterin. In any case, the increased levels of enzyme activity found in the resistant lines correlated directly with the degree of resistance, a circumstance which appears to reflect an increased amount of enzyme synthesis in the resistant cells. It is suggested that such synthesis provides excess enzyme which "binds" nearly all of the amethopterin which is capable of entering the leukemic cells under the conditions described. The enzyme which remains "unbound" under these circumstances is sufficient to reduce folic acid to coenzyme forms essential for cell reproduction.

TABLE 2. BINDING OF FOLIC ACID REDUCTASE DURING THE REPRODUCTION IN CULTURE OF A "16-FOLD" AMETHOPTERIN-RESISTANT MUTANT

Molar concentration ($\times 10^{-8}$) of ^{3}H-amethopterin in growth medium	cpm/10^{7} cells		Units of enzyme/10^{7} cells	
	cpm	Indicated units bound	Before dialysis	After dialysis
14	290	11		
7·0	190	8	1·6	17
3·5	160	6		
1·8	80	3		
0·9	20	0·8		
0·0	0	0	21	20

For each level of ^{3}H-amethopterin (103 μc/μmole), $2{\cdot}5 \times 10^{7}$ cells were collected after seven generations in culture. The enzyme was prepared from washed cells and the radioactivity was measured in a liquid scintillation counter. The enzyme activity, expressed in units (see Table 1), was measured by determination of the product formed after incubation for 10 min at varying concentrations of the enzyme. The inhibited enzyme was regenerated by equilibrium-dialysis against 100 volumes of 0·2 M phosphate buffer (pH 6·0) for 18 hr.

In the absence of amethopterin the resistant lines have retained their levels of resistance quantitatively for a period of 6 months. Furthermore, no gross chromosomal abnormalities have been detected in the resistant lines, when these were compared with the near-diploid clone of origin. Comparative nutritional studies of various aspects of the metabolism of folic acid have disclosed no other differences between amethopterin-sensitive and -resistant cells. Thus, the requirement for very high levels of folic acid, which characterizes the drug-sensitive cells,[1, 14] remains elevated in the amethopterin-resistant cells. Also, in the presence of very high levels of amethopterin (10^{-6} M), the same levels of thymidine, hypoxanthine and serine are required to support the growth of either the sensitive or the resistant cells. These findings suggest that the increase in folic acid reductase activity may reflect a single mutational event, associated with a single biochemical alteration in the resistant cell (possibly affecting the extent to which a single enzyme is formed).

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The inhibition of norepinephrine and epinephrine synthesis *in vitro*

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The biogenesis of norepinephrine involves the oxidation of phenylalanine to tyrosine, the catalyzed conversion of tyrosine to 3,4-dihydroxyphenylalanine by tyrosinase, the decarboxylation of 3,4-dihydroxyphenylalanine to dopamine (3,4-dihydroxyphenylethylamine) by DOPA decarboxylase, and the β-hydroxylation of dopamine to norepinephrine by dopamine β-oxidase. Epinephrine is formed from norepinephrine by N-methylation of the latter. However, the β-hydroxylation of epinine (3,4-dihydroxyphenylethylmethylamine) may be considered as another possible route for the formation of epinephrine.[1]

The enzyme dopamine β-oxidase, which converts dopamine to norepinephrine, has been shown to be non-specific for dopamine.[5] Among other structural analogs of dopamine, epinine in high concentrations inhibits the conversion of dopamine to norepinephrine. The present communication will show that dopamine β-oxidase catalyzes the conversion of epinine to epinephrine, but at a much lower efficiency than at which dopamine is converted to norepinephrine. Under the same conditions in which dopamine is converted to norepinephrine to the extent of 40–50 per cent, only 5–10 per cent of epinephrine is formed from epinine. It will also be shown that amphetamine and *p*-hydroxyamphetamine inhibit both the conversion of dopamine to norepinephrine and the conversion of epinine to epinephrine.

The enzyme dopamine β-oxidase was prepared by the method of E. Y. Levin *et al.*[2], but the purification on calcium phosphate gel was omitted. Either dopamine or epinine were added in the same concentrations to a mixture which contained the following components (in μmoles): potassium phosphate buffer, pH 6·4, 100; (1-methyl-2-phenyl)-ethyl hydrazine hydrochloride, 1·3; ascorbic acid, 6; fumaric acid, 10; ATP, 12·5. To this mixture, 0·2 ml of the enzyme was added and the final volume was adjusted to 1 ml with phosphate buffer, pH 6·4. The reaction mixture was incubated for 20 min at 37 °C, using air as a gas phase. At the end of the period of incubation the reaction was stopped by the addition of 0·5 ml of 3% trichloroacetic acid, the precipitated proteins were removed by centrifugation, and the supernatant fluid was adjusted to pH 6 and diluted to 10 ml with water. Depending upon the precursor used, an aliquot was then analyzed either for epinephrine or norepinephrine, by a modification of the fluorometric method of von Euler.[3] The compounds used in the present experiments have not been found to interfere with the specificity of the trihydroxyindole fluorometric method. However, an excess of potassium ferricyanide was ued in order to oxidize the ascorbic acid which was present in the incubation mixture.[4] The relative inhibition rate of dopamine β-oxidase by amphetamine and *p*-hydroxyamphetamine was determined by a comparison of the