Comparison of Folate Reductases of Sarcoma 180 Cells, Sensitive and Resistant to Amethopterin

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SUMMARY

Folate reductases of sublines of mouse Sarcoma 180 cells grown *in vitro*, sensitive and resistant (AT/3000) to amethopterin were compared. For this purpose the pattern of gel filtration on Sephadex G-75, the electrophoretic mobility on cellulose acetate strips, and the ratio of specific activities when folate or dihydrofolate were used as substrates were determined. No differences between these enzymes were noted. This, together with previous studies, leads to the conclusion that the only change which occurred when these cells became resistant to amethopterin by 3000-fold was a 300-fold increase in the cellular content of folate reductase.

INTRODUCTION

Previously we had shown that Sarcoma 180 (S-180) cells, made resistant to amethopterin *in vitro*, contain greatly increased amounts of folate reductase, also termed dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP-oxido-reductase, EC 1.5.1.3) (1, 2). The enzymes from sensitive and resistant cells had the same K_m value for folate, and the turnover numbers per amethopterin binding site for these enzymes were also identical (1). The permeability of resistant and sensitive sublines to amethopterin was shown to be identical and very slow (3, 4). Thus, it was suggested that only the quantity of folate reductase per cell had increased in the resistant cells and that structural or other changes in the enzyme molecule were unlikely. Later studies by other investigators with L-1210 cells of mouse, sensitive and resistant to amethopterin, have led to similar conclusions (5, 6).

In contrast, among bacteria differences in properties, such as pH-optimum, heat sensitivity, amethopterin binding capacity, or molecular weight, have been observed for dihydrofolate reductases of sensitive and resistant *Streptococcus faecalis* and *Diplococcus pneumoniae* (7–10). In one study on *S. faecalis* a 22-fold change was observed in the ratio of the specific activities when dihydrofolate or folate were used as substrates (8).

The observations with bacteria prompted further investigation on folate reductase of sensitive and resistant S-180 cells. This study revealed no differences in respect to electrophoretic mobility, molecular weight, or ratio of activities when folate and dihydrofolate were compared as substrates.

METHODS

Cell extract. The origin and maintenance of the parent and the amethopterin resistant subline (AT/3000) of S-180 cells have been described (1, 2). For these studies the cells were grown as a monolayer in Roux flasks; the parent cells in their maintenance medium and AT/3000 cells in hypoxanthine-thymidine-glycine (HTG) medium (11) for 10–12 days to free the cells of folate reductase-bound amethopterin. The cells were removed from glass using rubber policemen, washed twice with 4-fold volume of balanced salt solution, and homogenized

in 1–2 volumes of cold saline in a Potter-Elvehjem homogenizer. The homogenate was centrifuged in Spinco Model L ultracentrifuge for 15 min at 15,000 *g*, and the supernatant (S-15) was recentrifuged at 105,000 *g* for 1 hr. The last supernatant (S-100) is the cell extract mostly used. Protein was determined by the method of Lowry *et al.* (12) using bovine serum albumin as the standard.

Sephadex fractionation. Sephadex G-75, new bead form, was equilibrated with 0.1 M KCl in a column of 3.8 × 48 cm; 36 mg of cell extract protein was applied to this column in the form of S-15 for AT/3000 and of S-100 for the parent cells and eluted with 0.1 M KCl. The fractions containing folate reductase were combined, dialyzed overnight against 2 × 4 liters of cold 0.05 M sodium citrate, pH 8.0, lyophilized, and dissolved in a small amount of water.

Electrophoresis. Electrophoresis was performed using Reco paper electrophoresis instrument Model E 800-2 at 450–600 V and 15 mA per strip with running tap water for cooling. To label the enzyme, 450 μg of S-100 protein supplemented with tritiated amethopterin (methotrexate-3′,5′-T, Na-salt, 845 mC/mmole, Nuclear Chicago, Corp.) was applied on cellulose acetate strips, 1 × 12 inches (Sepraphore III, Gelman Instrument Co., Ann Arbor, Michigan). The electrophoresis was carried out for 4 hr in potassium phosphate buffer pH 6.5 of ionic strength 0.045. The strips were cut crosswise into 1-cm segments which were then counted in 20 ml of scintillation solution (13) using Packard Tri-Carb Scintillation Counter, Model 3002.

Assay of folate reductase activity. This was done at 37° by measuring the diazotizable amine released from tetrahydrofolate (14). The reaction mixture (0.5 ml) contained 0.045 mM folic acid, 0.4 mM NADPH, 10 mM $NaHCO_3$, and 100 mM sodium citrate buffer pH 6.0. The amount of S-100 protein used for the assay was 1.04 mg for the parent cells and 10.6 μg for AT/3000. When the enzyme, partially purified on Sephadex, was used the amount of protein was 123 μg and 9.2 μg for the parent and AT/3000 cells, respectively. The time of incubation was 1–4 hr for the enzyme from the parent and 10 min for AT/3000 cells. The reduction rate under these conditions was found to be linear, and therefore the values represent initial velocities. The specific activity was calculated from the optical density at 560 mμ using the molar extinction coefficient 55,000 M^{-1} cm^{-1} (14).

Assay of dihydrofolate reductase activity. In this case the decrease in absorbance at 340 mμ at 37° (15) was measured using Zeiss Spectrophotometer PMQ II equipped with Haake thermostat. The reaction mixture (1.05 ml) contained 0.105 mM dihydrofolic acid, 1.35 mM β-mercaptoethanol, 0.095 mM NADPH, and 0.0475 M potassium phosphate buffer pH 7.0. In case of S-100 extract, 1.63 mg of parent and 74 μg of AT/3000 protein were used; and in case of Sephadex fractionated extract, 0.62 mg of parent and 9.2 μg of AT/3000 protein. β-Mercaptoethanol and dihydrofolate were omitted from the blank. The specific activity was calculated from the molar extinction coefficient 13,400 M^{-1} cm^{-1} (16).

RESULTS

Reduction of Folate and Dihydrofolate

Using purified folate reductase of AT/3000 cells it has been demonstrated previously that the turnover number for dihydrofolate at pH 6.0 and 30° is 31 times larger than that for folate (16). In the present study the rate of reduction of folate at pH 6.0 and 37° was compared with that of dihydrofolate at pH 7.0 using enzyme preparations of sensitive and resistant cells. Under these assay conditions one would expect somewhat larger difference, and indeed in both cases dihydrofolate was reduced 32 to 46 times faster than folate (Table 1). Thus, toward these two substrates the reductases from sensitive and resistant cells are similar. The capacity of the crude extract (S-100) of the resistant AT/3000 cells to reduce dihydrofolate and folate was 240 and 268 times greater than that of the parent cells. Previous studies had shown that AT/3000 cells when grown in maintenance medium contained folate

TABLE 1

Specific activities for reduction of folate and dihydrofolate in the sensitive and resistant sublines of Sarcoma 180 cells grown in vitro

S-180 subline	Source of enzyme	Specific activity (mU/mg protein[a])		
		For folate	For H_2-folate	H_2-Folate:folate
Sensitive parent	S-100	0.019	0.871	45.6
	Sephadex peak	0.14	4.48	32.1
Resistant, AT/3000	S-100	5.13	208	40.6
	Sephadex peak	29.4	1032	35.2
Ratio, resistant:sensitive	S-100	268	240	—

[a] As suggested by the Enzyme Commission (18).

reductase equal to 121 ± 11.5 μmolar equivalents of amethopterin per kilogram, which is 320 times more than the parent cells which contained only 0.38 ± 0.065 μmolar equivalents (1, 2). One must take into consideration that the reductase content of the resistant cells is reduced by one half when these cells are grown in amethopterin-free medium for 30–40 days (1). Therefore, the agreement is very good indeed between the present data (254-fold difference) obtained five years later with AT/3000 cells, which were grown for 10–12 days in amethopterin-free medium, and the old data.

Sephadex Fractionation

Using Sephadex-fractionation the molecular weight of folate reductase of AT/3000 cells has already been estimated to be 21,000 (16). Figure 1 shows the fractionation pattern of the cell extract of both the resistant and the sensitive S-180 cells. In both cases the folate reductase activity was

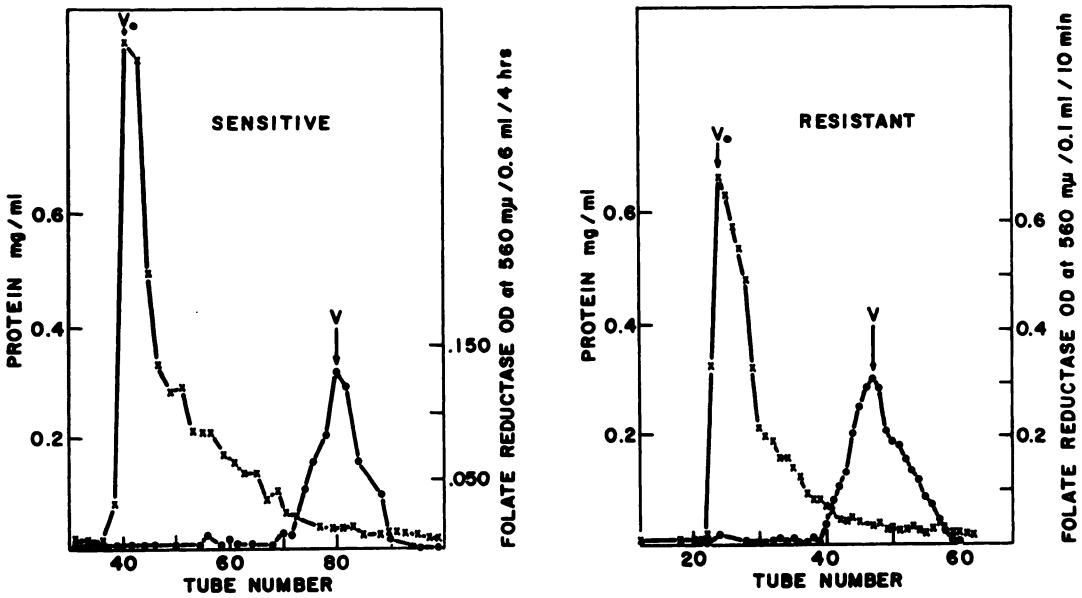

FIG. 1. *Fractionation on Sephadex G-75 column of cell extracts of S-180 cells, sensitive and resistant to amethopterin*

Size of fractions 5.1 ml for the resistant and 3.0 ml for the sensitive cells. ×, protein, mg/ml; ●, folate reductase activity. For experimental details see Methods.

found only within one peak. When the elution volume of the protein peak is taken as V_0 and that of folate reductase peak as V, one finds that V/V_0 for both folate reductases is 2.0. On the basis of the relationship of V/V_0 to molecular weight (17), one can estimate that a dimer of folate reductase would have V/V_0 in the range of 1.5–1.6, which in the column for sensitive cells would fall into fractions nos. 58–65 (Fig. 1). When the folate reductase in both cell extracts was tagged with tritiated amethopterin, single peaks were also obtained. Also the fractionation of cell extract of another sensitive subline of S-180 cells, AH/S (2), gave one single peak of folate reductase having V/V_0 equal to 2.0.

Electrophoresis

It has been shown previously that purified folate reductase of the resistant AT/3000 cells in free boundary electrophoresis migrates as a single peak toward the anode at pH 8.7 (16). When the mobilities of folate reductase amethopterin complex of the sensitive and resistant cells were compared at pH 6.5 on cellulose acetate strips (Fig. 2) no difference was noted.

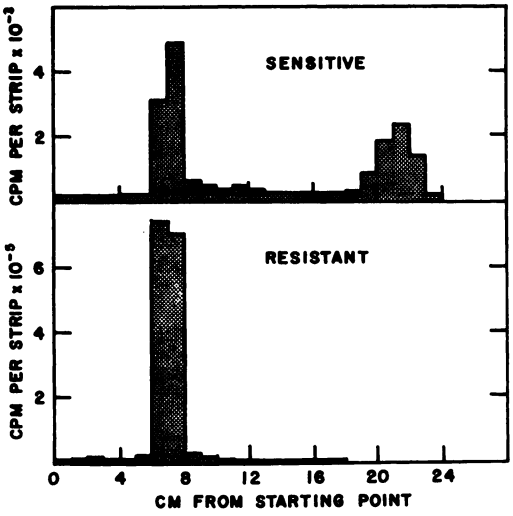

FIG. 2. *Comparison of the electrophoretic mobility of folate reductases of S-180 cells sensitive and resistant to amethopterin on cellulose acetate strips at pH 6.5*

S-100 of sensitive cells was supplemented with an excess of tritiated amethopterin while that of AT/3000 cells was supplemented with half of the amount required for saturation of folate reductase in the extract. The labeling at 19–24 cm is caused by the excess of free amethopterin. Details in Methods.

DISCUSSION

The present study was prompted by recent publications concerning amethopterin resistance in bacteria (7–10). These reports suggest several changes in folate reductase associated with resistance involving not only the quantity but also the size and other characteristics of the enzyme molecule. In contrast, the only change yet discovered in mammalian folate reductase as a result of amethopterin resistance has been the quantity (1, 2, 5, 6). It is possible that certain subtle changes might have escaped the experimental scrutiny. Therefore, S-180 cell system which has been thoroughly investigated in many respects (1–4, 11, 16, 19, 20) was investigated further. In this cell system, developed *in vitro*, the most resistant subline (AT/3000) tolerates 3000 times higher concentration of amethopterin than the parent cells and contains 320 times more folate reductase than the parent. Moreover, K_m values for folate and turnover numbers per amethopterin binding site are identical. The enzyme from AT/3000 cells has been purified (mol. wt. 21,000) and electrophoretic mobility, kinetics and energetics of activation have been investigated (16). This enzyme was shown to be specifically protected against SH reagents, metal chelators, and proteolytic enzymes by substrates, coenzymes, and inhibitors (19). In the present study the enzyme from the sensitive parent cells was compared with the enzyme from the 3000-fold resistant strain. No differences in the electrophoretic mobility, relative reaction rates with folate and dihydrofolate or molecular size were noted.

These results, together with previous observations, strongly suggest that amethopterin-resistant mammalian cells produce greater number of enzyme molecules identical with those in the drug-sensitive cells. It leads to the conclusion that the enzyme-producing systems are qualitatively the

same in both cell lines and that there is probably no change in the structural gene for folate reductase. Theoretically, the increased amount of folate reductase in the drug-resistant cells could be the result of: (a) a mutation in the regulatory gene of folate reductase either pre-existing or caused by amethopterin; (b) differences in the amounts of regulatory substances in the two cell lines; (c) prevention of the intracellular proteolytic breakdown of folate reductase by the drug.

Four aspects of amethopterin resistance which are pertinent to this interpretation have been experimentally determined in S-180 cells. (A) Exposure of cells to amethopterin (in absence of selection) causes a relatively rapid increase in cellular content of folate reductase (20). However, this increase is small (maximum 3-fold in a week) compared with the 300 to 400-fold increase involved here. (B) Amethopterin stabilizes the enzyme against proteolytic digestion (19). (C) The resistant cells are the offspring of a small fraction of the parent cell population, not killed by amethopterin (2). (D) Resistance and folate reductase content decrease slowly and in parallel fashion when cells are grown in the absence of the drug (half-life about 30–40 days) (1). These facts suggest that induction (A) of folate reductase or its protection by amethopterin (B) apparently have some role in development of resistance. However, the drastic elimination of cells during this process (C) proves that we are dealing here also with a resistant mutant either preexisting or presently evolved (a). Slow loss of resistance (D) would then indicate spontaneous back-mutation which is favored in the absence of the drug.

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