Studies on Resistance against 5-Fluorouracil*

II. Thymidylate Synthetase from Drug-resistant Tumor Lines

AGNETA HÄGGMARK†

(Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden)

SUMMARY

A method is described for the assay of thymidylate synthetase activity in crude extracts of Ehrlich ascites tumor. The method involves the use of tritiated deoxyuridine-5'-phosphate as substrate.

The inhibition of thymidylate synthetase by 5-fluorodeoxyuridine-5'-phosphate was measured in extracts from one 5-fluorouracil-sensitive and four drug-resistant lines of Ehrlich ascites tumor. A high degree of inhibition was observed in all cases, and no difference between the drug-sensitive and -resistant lines was apparent.

Two different types of biochemical changes have been observed in cells treated with 5-fluorouracil (FU) over a prolonged period of time: (a) a decreased capacity to transform FU to the nucleotide stage (2, 13, 14) and (b) a decreased affinity of the enzyme TMP synthetase for F-deoxyUMP (7). Cells changed in either of these two ways would be expected to be less sensitive to the growth-inhibiting influence of FU, and it thus seems reasonable to see a causal relationship between either of these biochemical changes and the development of resistance against FU.

In a previous report from this laboratory (14) the derivation of four FU-resistant lines of Ehrlich ascites tumor was described, and some of the biochemical changes accompanying the development of resistance were studied. All four lines showed changes corresponding to mechanism (a) above. Several lines of reasoning led to the conclusion, however, that the observed changes could not alone be responsible for the development of resistance. The present investigation was therefore started in order to investigate whether mechanism (b) might also contribute to the resistance of the four lines. The results presented in this paper show that this was not the case.

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†Present address: Institute of Medical Chemistry, Uppsala University, Uppsala, Sweden.

MATERIALS AND METHODS

The tumor lines were identical with those used in the preceding paper (14). This paper also described the derivation of the four FU-resistant lines (Ro, Rb, Re, and Rj) from the FU-sensitive ELD tumor. All tumors were kept either in a tumor bank (10) or by serial intraperitoneal transfer of 2 X 10⁶ cells in 0.1 ml. ascites diluted tenfold with Ringer solution, containing 100 IU of penicillin and 100 µg streptomycin/ml.

Compounds.—FU and F-deoxyUMP were generous gifts from the Hoffman LaRoche Company and Dr. C. Heidelberger, respectively. Uracil-2-C¹⁴ was obtained from the California Foundation for Biochemical Research, orotic acid-2-C¹⁴ was prepared according to Nyc and Mitchell (12), DPNH and ATP were purchased from the Sigma Chemical Company. Tetrahydrofolic acid was obtained from Nutritional Biochemicals Corporation. It was kept in water solution (500 mg/ml) at 2° C. in a Mantoux syringe. A cork was attached on the tip of the needle to minimize oxidation by air. Snake venom (Crotalus adamanteus), containing 5-nucleotidase activity, was obtained from Ross Allen's farm, Silver Springs Fla. Before use the powder was dissolved in water (2 mg/ml), and the pH was adjusted to 8.5 with NaOH.

DeoxyUMP-H³ was prepared by deamination of deoxyCMP-H³ (Schwarz Bioresearch Inc.). For this purpose 0.4 ml. of a solution of NaNO₂ (406 mg/ml) was added slowly to a centrifuge tube containing 3.5 µmoles of deoxyCMP-H³ (2.2 X 10⁷ counts/min/µmole) dissolved in 0.4 ml. of 2 M acetic acid. After 2 hours at room temperature
0.4 ml of NaNO₂ was added again, and the reaction was allowed to proceed for another 5 hours. During that time the tube was shaken occasionally. Urea (282 mg) was then added, and the reaction mixture was left overnight. The solution was then evaporated in vacuo, the residue dissolved in a few ml of 0.1 M HCl and again evaporated to dryness in a vacuum. The residue was dissolved in a small amount of water and neutralized with NaOH (phenol red). The neutralized solution was then chromatographed on a column of Dowex-2-formate (15 X 0.9 cm) according to Hurlbert et al. (9). Fractions containing deoxyUMP were localized by their UV absorption and pooled. Formic acid was removed in vacuo, and the residue was dissolved in 5-10 ml of water and neutralized with 0.1 M NaOH. A total of 3.3 μmoles of deoxyUMP-H³ was recovered.

Incorporation experiments with whole cells.—The tumor containing ascites fluid from seven to ten mice was pooled at 0°C. 9-11 days after inoculation of the mice. Bloody ascites was omitted. The conditions of incubation were as follows: 3 ml of ascites fluid, 3 ml of Tyrode solution, 1.26 μmoles of uracil-C¹⁴ (5.5 X 10⁶ counts/min) or 4.8 μmoles of orotic acid-C¹⁴ (5.5 X 10⁶ counts/min) were shaken in 25-ml Erlenmeyer flasks for 2 hours at 37° C. in a CO₂:O₂ (6:94) atmosphere. The solution could be stored frozen for several weeks without appreciable loss of TMP synthetase activity.

Assay of TMP-synthetase.—The activity of TMP-synthetase was measured as the amount of TMP-H³ formed from deoxyUMP-H³ under standard conditions. TMP-H³ was measured as thymidine-H³. For this purpose the nucleotides were dephosphorylated with snake venom phosphatase, and thymidine-H³ was separated from deoxyuridine-H³ by paper chromatography. The radioactivity of the thymidine spot was a measure of thymine activity.
M. The paper was developed with butanol-water (87:13) for 36–40 hours. The lower end of the paper was cut in zig-zag to allow the eluent to flow through the paper into the container. After completion of the chromatogram the nucleosides were localized with a mineralight lamp, the appropriate spots were cut out and eluted with 3 ml. of water (37° C., 1 hr). Aliquots of the eluates (in duplicates) were then counted at infinite thinness in a Nuclear Chicago windowless gas-flow counter. From these values the total radioactivity present in each nucleoside could be calculated, and the percentage of radioactivity present in the thymidine spot was taken as a measure of TMP synthetase activity.

A more thorough study was made with soluble TMP synthetase. This enzyme was described and studied by Friedkin and Kornberg (4) and Cohen (3) in extracts of E. coli and by Greenberg and co-workers in mammalian extracts (5, 8). Cohen found that the bacterial enzyme was inhibited by very low concentrations of F-deoxyUMP, and Heidelberger et al. (7) showed that this also applied to TMP synthetase from a FU-sensitive ascites tumor.

As an assay method for TMP synthetase these different groups of investigators used the form-

### TABLE 1

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific activity of thymine in counts/min/μmole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELD Exp 1</td>
</tr>
<tr>
<td>Uracil-C¹⁴ +FU</td>
<td>151</td>
</tr>
<tr>
<td>Orotic acid-C¹⁴ +FU</td>
<td>1770</td>
</tr>
</tbody>
</table>

Experimental conditions: uracil-C¹⁴, 2X10⁻⁴ M conc. (1.16X10⁴ counts/min/μmole) or orotic acid-C¹⁴, 7.8X10⁻⁴ M conc. (1.14X10⁴ counts/min/μmole) was incubated with ascites fluid and Tyrode solution with and without FU (10⁻⁴ M conc.) for 2 hr. at 37° C. in a CO₂-O₂ atmosphere.

RESULTS AND DISCUSSION

Two experimental approaches were chosen to investigate whether a difference existed in the inhibition of TMP synthetase by F-deoxyUMP between the drug-sensitive and drug-resistant tumor lines. In the first type of experiments a tumor cell suspension was incubated in vitro with radioactive orotic acid or uracil, and the influence of FU on the incorporation of these precursors into DNA-thymine was determined. In the second approach the inhibition of TMP synthetase by F-deoxyUMP was measured directly in crude extracts of the FU-sensitive and -resistant lines.

The results with whole cells are presented in Table 1. These experiments are considered exploratory experiments only, and no significance is attached to minor differences in the results among the various tumor lines. It may be seen, however, that FU considerably inhibited the incorporation of both labeled precursors into DNA-thymine (cf. 1). At the concentration of FU used in our experiments no major differences existed in the inhibition of labeled TMP from formaldehyde-C¹⁴ + deoxyUMP. Because of different side reactions and of the low activity of TMP synthetase in mammalian tissues the application of this method to our problem would have required laborious chromatographic procedures for the purification of TMP. A method has, therefore, been chosen, where labeled TMP was formed from deoxyUMP-H³ + nonlabeled formaldehyde. At the end of the reaction TMP was dephosphorylated, and the nucleoside was then purified by paper chromatography with butanol-water. With this solvent thymine and thymidines show identical Rf values, and the method therefore picks up all thymine compounds formed from deoxyUMP. An almost identical assay method was independently worked out by Heidelberger's group.¹

The conditions for the incubation were based upon those described by Humphreys and Greenberg (8). With the tumor extract optimal forma-

¹ C. Heidelberger, personal communication to Dr. Peter Reichard.
tion of TMP was observed at 4 mM concentration of formaldehyde. Similarly, the concentration of deoxyUMP and tetrahydrofolic acid used during the incubation were found to be optimal. Omission of DPNH resulted in a drop of activity to about 30 per cent, whereas addition of ATP did not result in a definite effect.

As can be seen from Charts 1 and 2, TMP synthetase from the drug-sensitive line was strongly inhibited by F-deoxyUMP. A 50 per cent inhibition was observed at concentrations between $10^{-4}$ and $10^{-7}$ M. These results are similar to those reported earlier by Heidelberger et al. (7).

Charts 1 and 2 also show similar experiments with the four drug-resistant tumor lines. There was no apparent difference between the inhibition of TMP synthetase from these lines and the inhibition of the enzyme from the drug-sensitive tumor. The results thus clearly demonstrate that the enzymes from the four resistant lines were also strongly inhibited by F-deoxyUMP.

These results sharply contrast to those of Heidelberger et al. (7) with one FU-resistant line of Ehrlich ascites tumor, where these authors had found that TMP synthetase from the resistant tumor showed a 50 per cent inhibition with concentration of F-deoxyUMP about a thousandfold higher. However, recently Heidelberger found that his drug-resistant line again showed the same high degree of inhibition by F-deoxyUMP as the original sensitive tumor line, without losing the resistance against FU.\(^1\)

The present results, together with Heidelberger's recent results, demonstrate that a change in the affinity of TMP synthetase for F-deoxyUMP is not a phenomenon which regularly accompanies the development of drug resistance against FU. The only regular change in enzyme pattern accompanying the development of drug resistance which has been observed by several groups of workers is a decreased capacity of the drug-resistant cells to transform FU to the nucleotide level. Such changes may actually be observed also in paper by Heidelberger et al. (7).

As outlined in the introduction, however, a decreased capacity to transform FU to the nucleotide level cannot be the only reason for the development of resistance in these four tumor lines. In a previous paper from this laboratory (14) it was demonstrated that at an early stage other biochemical changes had contributed to the development of resistance. The present paper demonstrates that a changed affinity of TMP synthetase for F-deoxyUMP did not contribute to

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\(1\) Heidelberger et al. (7)
the development of resistance. The question as to the nature of these other biochemical changes is thus left open at the present time.

REFERENCES

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Agneta Häggmark


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