Studies on Resistance against 5-Fluorouracil

I. Enzymes of the Uracil Pathway during Development of Resistance*

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SUMMARY

Four different 5-fluorouracil (FU)-resistant tumor lines were developed by treating a drug-sensitive Ehrlich ascites tumor, ELD, with FU during 25–30 passages. Acetone powder extracts of the tumor lines were assayed for the following enzymes: uridine phosphorylase and kinase, deoxyuridine phosphorylase and kinase.

In all four tumor lines significant decreases in uridine kinase activity occurred during FU treatment, whereas the other enzymes were not affected. The decrease in uridine kinase activity commenced around the tenth tumor passage and showed either a continuous or stepwise character. The enzyme values after twenty passages were between 5 and 15 per cent of the original values. From the available evidence it is proposed that the enzyme changes were caused by a multistep process, probably of genetic origin.

After 25–30 passages the FU-resistant lines were cross-resistant against 5-fluorouridine and 5-fluorodeoxyuridine. Furthermore, two tumor lines, after eight and nine passages, respectively, were resistant against FU before a decrease in uridine kinase activity had occurred. One of these early tumors was also resistant against 5-fluorodeoxyuridine, but not against 5-fluorouridine.

The loss of uridine kinase activity is thus only one biochemical factor which contributes to the resistance of the tumors against fluorouracil, and it is evident that one or several other unknown factors exist which explain the early resistance against the drug.

The resistant tumor lines showed no significant changes in permeability for the drug.

The pyrimidine, 5-fluorouracil (FU), originally synthesized and studied by Heidelberger and Duschinsky and their co-workers (4, 7), greatly inhibits the growth of a variety of tumor cells (7, 11). However, after prolonged treatment with FU a population of cells is obtained which shows a greatly increased resistance toward the growth-inhibiting effect of the drug (1, 6, 12).

We have attempted to study some of the changes in enzyme activities which occur in tumor cells developing resistance against FU. In an earlier study (12) we compared the capacity of FU-sensitive and -resistant cells to transform FU to the nucleotide level. It was found that extracts from two resistant lines contained considerably less of several enzymes involved in this process, as compared with extracts from the original FU-sensitive lines.

The present study represents a continuation of our earlier experiments. Four FU-resistant lines of Ehrlich ascites tumor were developed by prolonged FU treatment of tumor cells during serial transplantations. For each passage assays were made of the enzymes of the “uracil pathway” of pyrimidine biosynthesis. These enzymes catalyze
the formation of UMP and deoxyUMP from uracil by the following reactions:

(a) Uracil + ribose 1-phosphate ⇌ uridine + phosphate (uridine phosphorylase).

(b) Uridine + ATP → UMP + ADP (uridine kinase).

(c) Uracil + deoxyribose 1-phosphate ⇌ deoxyuridine + phosphate (deoxyuridine phosphorylase).

(d) Deoxyuridine + ATP → deoxyUMP + ADP (deoxyuridine kinase).

It was earlier shown that FU can substitute for uracil in reactions (a)–(d) and is thus transformed by these enzymes to FUMP and F-deoxyUMP (15).

![Chart 1](chart1.png)

CHART 1.—Derivation of R₀, R₁, B₀, and R₂ lines from the ELD tumor. The figures indicate the number of serial intraperitoneal transplantation passages. During each passage the tumor-bearing animals were treated with intraperitoneal FU injections on alternate days.

MATERIALS AND METHODS

**Mice**—Heterozygous albino mice of both sexes obtained from a commercial breeder were used. The animals were between 2 and 4 months old and weighed between 16 and 26 gm. They were kept on a standard pellet diet. Food and drinking water were available ad libitum.

**Tumor**—An Ehrlich ascites tumor, designated ELD, was used. Several properties of this tumor line have been studied previously and are summarized in a recent publication (18). The main line of this tumor was maintained by serial, intraperitoneal transfer of about 9×10⁶ cells in 0.1 ml. ascites, diluted tenfold with Ringer’s solution, containing 100 I.U. penicillin and 100 µg streptomycin/ml. During several periods of varying length, the cells were preserved by frozen storage at −70°C in a tumor bank as described previously (10).

FU-treated lines, denoted R₀ and R₁, respectively, were derived from the ascites of a mouse that had been given inoculations of ELD. Two further lines, R₂ and R₃, were derived from R₁, after its third and sixth passage, respectively (see Chart 1). Each of these lines was propagated by serial, intraperitoneal transfer of 0.1 ml. diluted ascitic fluid containing 10⁶ cells. At each transfer, fifteen mice were given inoculations. Starting the day after inoculation, ten mice were treated by the intraperitoneal injection of 20 mg/kg fluorouracil every 2d day, while five were left as untreated controls. For a subsequent transfer the ascites was used which developed in any one of the animals of the FU-treated group. During the early passages ascites did not become available until 3–4 weeks after inoculation, and in only a few of the FU-treated animals; during the later passages almost all the inoculated animals developed ascites within 10 days. On a few occasions samples of the ascites were frozen in several tubes and preserved viable in the tumor bank for various tests at a later date. The survival time of each of the inoculated animals was registered.

**Compounds**—Nonlabeled FU, FUR, and FUdR were generous gifts from the Hoffmann LaRoche Company; FU-2-C¹⁴ and uracil-2-C¹⁴ were obtained from the California Corporation for Biochemical Research. Uridine-2-C¹⁴, deoxyuridine-2-C¹⁴ and orotic acid-2-C¹⁴ were prepared as described earlier (14).

**Enzyme assays**—Tumors were collected from groups of mice (usually ten mice per group) on the 9th–12th day after intraperitoneal transplantation of ascites taken either from material in the course of an in vivo passage, or from a frozen sample. The enzymes catalyzing reactions (a)–(d) were measured in acetone powder extracts, as described earlier (14). In this paper one unit of enzyme activity is defined as the amount of enzyme which, under the conditions of our assays, gave rise to the formation of 1 mmole of product per mg. of acetone powder.

**Biochemical experiments with whole cells**—The tumors from ten mice were pooled 9–10 days after transplantation. For the experiments each vessel received 3 ml. of the pooled ascites, 3 ml. of Tyrode solution, 0.5 ml. of 0.25 M glucose and FU-2-C¹⁴ (0.04–0.46 mmoles). Incubations were carried out at 37°C for 30 minutes, with shaking, in an O₂:CO₂ (94:6) atmosphere.

The reaction was terminated by cooling in ice. All the following manipulations before chromatography were carried out at temperatures slightly above 0°C. The incubation mixture was centrifuged for 5 minutes at 2000 × g, and the sedimented cells were washed twice with 5 ml. of saline. The cells were then homogenized with 2 ml. of 0.6 M HClO₄, centrifuged, and washed...
twice with 2 ml of 0.6 M HClO₄. The combined acid supernatant solutions were neutralized with 4 M KOH (phenol red) and centrifuged after about 1 hour. The supernatant solution was then passed through a column of Dowex-9-formate (0.9 X 4 cm.). The column was first washed with 20 ml. of 0.05 M HCOOH. This fraction contained FU, FUR, and FUdR. Next the column was washed with 30 ml. of M ammonium formate; 4 M formic acid in order to elute FU-nucleotides.

The total radioactivity present in each fraction was determined by measuring the radioactivity of aliquots at infinite thinness with a windowless gas-flow counter.

Growth curves.—A suspension of 1 X 10⁶ cells of the tumor to be tested and taken either from material in the course of an in vivo passage or from a frozen sample was injected intraperitoneally into each of a group of 80 mice of the same sex and similar weight. Starting the day after inoculation and every 92d day thereafter the animals were treated by intraperitoneal injections of either 0.1 ml. of a solution of one of the fluorinated compounds (20 mg/kg of FU and FUdR, 3 mg/kg of FUR) in physiological saline, or 0.1 ml. of a Ringer solution. On alternate days the size of the tumor cell population was determined in four randomly chosen animals. A quantitative rinsing procedure was used in combination with quantitative and differential cell counts. The details of this technic have been described previously (9).

RESULTS

Changes in enzyme activities during treatment with FU.—The activities of the enzymes catalyzing reactions (a)-(d)—uridine phosphorylase and kinase, and deoxyuridine phosphorylase and kinase—were determined in extracts of four lines (Rₐ, R₉, R₉, and R₉) of ELD ascites tumor during the course of treatment with FU. The analyses were carried out for almost every passage, and the results, recorded in Charts 2–5, were obtained over a period of about 1 year.

It is evident that the two phosphorylases and also deoxyuridine kinase showed considerable, rather irregular variations during this period. The changes are probably explained by physiological variations, at least in part caused by the inhomogeneity of the animals used for the experiments.

Although the variations of these three enzyme activities are of a random nature the results for uridine kinase, as represented in Chart 3, clearly show a general trend downward, starting around the eighth passage in all four tumor lines. This decrease of uridine kinase activity was slow and spread out over many passages. In two of the lines (R₉ and R₉) the final enzyme values observed after 25–30 passages were around 4 units, whereas lines R₉ and R₉, after about the same number of passages, had reached levels of around 10 units. The starting values were around 60. The decrease in activity after the eighth passage was apparently more or less continuous for lines R₉ and R₉, until the lowest values of 4 and 10 units, respectively,
were reached. On the other hand, line R showed a stepwise decrease. In this line the enzyme values, which started around 65 units, reached a level of around 30 units between the tenth and eighteenth passage and finally fell to 4 units after the twentieth passage.

From line R (after the thirteenth passage) and line R (fourteenth passage), respectively, separate lines were developed and transferred during ten more passages without treatment with FU ("no FU" in Charts 4–5). Under such conditions no further changes in uridine kinase activity occurred.

Similarly, line R (after 31 passages with FU) was transferred during ten more passages without the drug. The uridine kinase level remained unchanged at 4 units.

As a further general control the activities of the enzymes involved in the transformation of orotic acid to UMP were determined occasionally in all four lines. Considerable variations were observed, but no general trend was apparent in any line. The values are not given here.

**Chart 4.**—Activity of deoxyuridine phosphorylase during treatment with FU.

Finally, two separate lines of Ehrlich ascites tumor were treated with uracil (same dose as FU) during 23 passages, and enzyme activities were determined. No changes in any of the enzymes catalyzing reactions (a)–(d) were observed.

**Determination of resistance against fluorouracil compounds.**—The mean survival time of the FU-treated mice decreased considerably (from about 60 days to about 12 days) in the course of subsequent intraperitoneal transfers of the different sublines of the ELD tumor. This indicated that, during prolonged treatment, a marked increase in resistance of the tumor cells developed against the growth-inhibiting effect of FU. A similar trend for a decreased survival time was apparent if mice were given inoculations of FU-resistant cells and subsequently treated with FUR or FUDR, as compared with the survival of animals bearing previously untreated ELD cells and treated with the same fluorinated compounds.

The survival time of the tumor-bearing animals appeared, however, to be a rather unreliable index of sensitivity (and resistance) of the tumor for treatment. Inconsistent and often contradictory results were obtained. The poor reproducibility of the survival data may have been due to the immunizing action of the tumor cells upon their immunogenetically foreign, homologous hosts. The strength of such a homograft reaction is an unknown variable, and may be superimposed upon the inherent sensitivity of the graft toward a certain treatment (18). Furthermore, the host survival may also be influenced unspecifically by the accumulated toxic effects of the drug during prolonged use.

In an attempt to study the effect of the different compounds on tumor cell growth more directly than by observing the survival of the host, the multiplication of the cells of the different lines was followed quantitatively after inoculation. During the first 10 days, before an efficient host
response develops, the growth curves can be considered to be influenced to only a small, if any, extent by the homograft reaction or by an accumulated toxic effect of the compound on the host, and may thus reflect a direct influence of the drug on tumor cell multiplication.

Chart 6 gives the growth curves of tumor lines R0, Rb, R0, and Rj after 25, 29, 23, and nineteen passages with FU treatment, respectively. Each line was tested not only for its sensitivity to FU treatment, but also for cross-resistance against FUR and FUdR (Chart 7). For comparison, Chart 8 shows similar experiments with previously untreated ELD cells.

The results represented by Charts 6–8 can be summarized by stating that lines R0, Rb, R0, and Rj were not only resistant to treatment with FU but also to treatment with FUR and FUdR. In most cases there was probably a very small inhibition of tumor growth as compared with that in the nontreated controls, indicating a slight antitumor effect of the different drugs. These effects, however, were of doubtful significance and can in no way be compared with the growth-inhibiting effect of the three compounds on the sensitive tumor, as demonstrated by Chart 8.

The growth curves described above were ob-
tained with tumors which had been treated extensively with FU and which showed a large decrease in their uridine kinase activity. In two of the tumor lines (R₀ and Rₙ) the effect of FU on tumor growth was investigated already after nine and eight passages, respectively—i.e., at a stage when there was very little change in uridine kinase activity (cf. Chart 3). Chart 9 demonstrates that already at this point the two tumors were highly resistant to treatment with FU.

With line R₀, treated during eight passages with FU, we also investigated cross-resistance of the tumor to FUR and FUdR (Chart 10). In two different experiments it was found that the tumor was resistant to FUdR, whereas there was still a considerable growth inhibition by FUR.

Biochemical experiments with whole cells.—Further information about the state of the four FU-resistant tumor lines, as compared with the original FU-sensitive ascites tumor, was obtained in experiments in which tumor cells from the different lines were incubated in vitro with radioactive FU. After a brief incubation the amounts of radioactive acid-soluble compounds inside the cells were determined. These experiments were primarily designed to test whether there existed a difference in permeability to FU between the resistant lines and the original tumor.

The tumor cells were incubated for 30 minutes with different amounts of radioactive FU, and the cells obtained after centrifugation were washed twice with saline. The washings were considered necessary to remove all extracellular FU.

Chart 11 shows the results from such experiments. The curves for each tumor correspond to the amounts of radioactivity in (a) the intracellular FU + FU-nucleoside fraction; (b) the intracellular FU-nucleotide fraction; and (c) the last saline wash. The latter values are included, since it is believed that a large part of the radioactivity of this fraction may correspond to FU, originally located within the cells.

It is seen that the radioactivity in (a) above (= intracellular FU) from two of the resistant tumors (R₁ and R₉) showed higher values as compared with the FU-sensitive tumor (ELD), whereas the two other resistant tumors (R₀ and Rₙ) showed lower values. On the other hand, the latter two tumors showed the largest amounts of radioactivity in the last saline wash (= fraction [c]). When the radioactivity of fractions (a) and (c) are added together all five tumor lines show almost identical values. It therefore appears that there is little difference in permeability for FU between the normal and the four resistant tumors.

Chart 11 also gives the amounts of radioactivity corresponding to FU nucleotides (= fraction [b]). These values were considerably higher in the sensitive tumor than in any of the resistant lines.
DISCUSSION

In connection with a search for agents useful for the chemotherapy of malignant tumors a large variety of different pyrimidines and purines inhibiting cell growth have been synthesized (5). Most of these substances are structural analogs of the purines and pyrimidines which form part of the nucleic acids, and the biological effect of the synthetic bases depends on their interference with the function of the normal bases. In most cases the analogs must first be transformed to the nucleotide stage (mononucleotide or polynucleotide) before they exert their growth-inhibiting influence (5). These transformations occur through pyrophosphorylase, phosphorylase and kinase reactions, during which the analogs substitute for the “normal” purines and pyrimidines.

Once one accepts the view that the action of an analog depends on its transformation to a nucleotide, then it follows that a cell which loses this capacity will be resistant to the action of the analog. By now many cases have been established in which enzyme activities necessary for the formation of nucleotides disappear on prolonged exposure of bacterial or neoplastic cell populations to different types of antipurines or antipyrimidines (5). Such losses of enzyme activities actually seem to be a general rule, albeit with some exceptions, and it is therefore tempting to see a causal relationship between the enzyme changes and the development of resistance.

In our earlier experiments with one FU-resistant line of Ehrlich ascites tumor and one line of lymphoma L1210 we found in both cases a decreased capacity of the resistant cells to transform uracil (and therefore FU) to the nucleotide level required prolonged treatment with FU. The first definite changes were observed after the eighth passage in all four tumor lines. This would correspond to about 100 cell generations. On continued treatment with the drug a further decrease of uridine kinase activity occurred, until, after a total of 25–30 passages, the enzyme activity may be as low as 5 per cent of the original (lines $R_o$ and $R_j$). This further decrease in enzyme activity was always slow and occurred either as an apparently continuous decrease (e.g., line $R_o$), or in a stepwise fashion (most pronounced in $R_j$).

When the tumor cells were transplanted in the absence of the drug no further changes in enzyme activity occurred. This was true when treatment was stopped either at intermediate or at the lowest levels of enzyme activities and demonstrates that in both instances a permanent change had occurred. The results speak against enzyme
repression as the cause of the decreased uridine kinase activity. Against enzyme repression is also the observation that treatment with uracil in place of FU did not cause any changes in enzyme activities.

For the interpretation of our results it was important to demonstrate the extent of selective advantage which the resistant cells possess in the presence of FU. In experiments to be reported later a mixture consisting of one resistant cell per $10^8$ sensitive cells was transplanted in mice which were treated with FU. Already after one or two passages the resulting cell population showed a uridine kinase activity characteristic of the resistant cells, demonstrating that the resistant cells very rapidly overgrew the sensitive cells in the presence of FU.

These results rule out one type of mechanism which might be considered as an explanation for the slow decrease in uridine kinase activity during treatment with FU. This would be that a single event around the eighth passage had resulted in a tumor cell with very low uridine kinase activity, and that the changes in enzyme activity observed during subsequent passages depended on a gradual increase of the relative proportion of this type of cell in the total cell population. Against such a concept also speak the results obtained with line R1, where one can clearly observe a stepwise decrease of uridine kinase activity.

Instead, we would like to explain our results by a mechanism which involves a series of events, probably of genetic origin, which, each in a stepwise fashion, decreased the amount of uridine kinase activity present in the tumor cells. During each event one cell with a decreased amount of enzyme activity arose. The presence of FU on subsequent passages conferred a selective advantage to such a cell, and during a relatively short time period it gave rise to a new cell population with a decreased content of uridine kinase activity.

It is not yet clear whether the changes in enzyme activity reflect an actual loss of enzyme protein or a change in the structure of the protein, resulting in a less active enzyme. A reproducible and fairly simple method is now available for the extensive purification of uridine kinase from Ehrlich ascites tumor, and experiments are at present under way to differentiate between these two possibilities.

The second question to be discussed is whether the loss in uridine kinase activity may be considered as the cause of the resistance against the drug. At the bottom of this page are summarized the enzyme reactions which lead to the formation of nucleotides from FU.

There are two pathways leading to the synthesis of F-deoxyUMP, the most important antimitabolite formed from FU (2, 8, 11). Only one of the pathways involves the participation of uridine kinase, whereas the other involves the sequential action of deoxyuridine phosphorylase and kinase. From the work of deVerdier and Potter (3) it seems probable that this latter pathway is of little importance for deoxynucleotide synthesis in vivo, since the substrate (= deoxyribose 1-phosphate) for the anabolic action of deoxyuridine phosphorylase does not seem to be available in the cell. It may therefore be assumed that a loss of uridine kinase activity would lead to a substantial decrease in the formation of F-deoxyUMP from FU and therefore would result in cells with a higher resistance against FU.

Even though it seems clear that a decrease of uridine kinase activity would result in an increased resistance against FU, we do not wish to infer that the enzyme loss is the cause of resistance. Significant for this question are our results on cross-resistance against FUR and FUdR. All four FU-resistant lines were also completely resistant against FUR and FUdR. From the above scheme it can be seen that the transformation of FUR to the nucleotide requires phosphorylation by uridine kinase. However, this does not apply to FUdR. This nucleotide is phosphorylated by deoxyuridine kinase, which did not change during treatment with FU. Therefore, the resistance of the tumor cells against the deoxynucleoside could not have been due to insufficient phosphorylation of FUdR, and at least one more change must have occurred which rendered the cells resistant against FUdR.

Further information on this point was obtained from experiments on the development of resistance.

\[ \text{F-uridine} \xrightarrow{\text{kinase}} \text{FUMP} \xrightarrow{\text{FUDP}} \]

\[ \text{FU} \]

\[ \text{F-deoxyuridine} \xrightarrow{\text{F-deoxyUMP}} \text{F-deoxyUDP} \]

1 A large part of the injected F-deoxyuridine is no doubt cleaved to FU, which then may be utilized for nucleotide formation. This utilization would involve the action of uridine kinase. Thus, if F-deoxyuridine would exert all its carcinostatic action only after cleavage to FU, a large decrease in uridine kinase might well be considered to give the observed cross-resistance against F-deoxyuridine. However, we do not consider this to be a likely possibility.
against FU, FUR, and FUdR in lines R9 and R0, respectively, during their early passages. After eight and nine passages, respectively, these lines did not yet show any significant decrease in uridine kinase activity. Nevertheless, both lines were clearly resistant against treatment with FU (Chart 9). Furthermore, after eight passages R9 was also cross-resistant against FUdR, but not against FUR (Chart 10).

Since these two tumors were resistant against FU (and FUdR) without showing a decreased uridine kinase activity, it is evident that a different biochemical change must be responsible for the resistance observed. However, the lack of resistance against FUR might indicate that the resistance against the ribonucleoside observed on further treatment with FU (Chart 7) was caused by the loss of uridine kinase.

Heidelberger et al. (8) have shown that the affinity of TMP-synthetase for F-deoxyUMP was greatly decreased in one FU-resistant line, and it was proposed that this was the biochemical change responsible for resistance. We have investigated the levels of TMP-synthetase and the inhibition of this enzyme by F-deoxyUMP in our lines and could not find any significant changes from the original FU-susceptible cells. Heidelberger also recently found that his FU-resistant line had lost the decreased affinity of TMP-synthetase for F-deoxyUMP, without losing the resistance against FU, and that therefore the altered TMP-synthetase could not have been responsible for the observed resistance against FU.

No gross changes in permeability for FU could be observed in any of the resistant lines, and such changes can therefore not be regarded as the cause of resistance.

Thus, we arrived at the conclusion that several changes did occur during prolonged treatment with FU and that all of them together express themselves in the final FU-resistant cell population. Relatively early during FU treatment (around the eighth to ninth passage) one or more unknown biochemical changes have taken place which render the cell populations resistant to FU and FUdR. Later on we observe a stepwise decrease in uridine kinase activity. We consider this to be a factor which further contributes to the resistance against FU and which also renders the tumor cells resistant to FUR.

REFERENCES

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