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DEVELOPMENT AND CHARACTERISTICS OF A SUBLINE OF EHRLICH ASCITES CARCINOMA CELLS PERSISTENTLY RESISTANT TO 5-FLUORO-2'-DEOXYURIDINE

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A subline of Ehrlich ascites carcinoma (EAC) cells resistant to 5-fluoro-2'-deoxyuridine (FdUrd) was developed by continuous exposure to progressively increasing concentrations of the drug (35 - 75 mg/kg per day) during 15 passages through mice. Since then, the EAC cells have been retransplanted more than 80 times through drug-untreated mice and continue to be resistant. After adaptation to growth in suspension culture the drug-adapted cells were 1000 times more resistant to FdUrd in comparison with parental ones, and remained near-tetraploid with doubling time longer than in parental line. The activity of thymidine kinase was deeply depressed (100-fold) whereas that of thymidylate synthetase several-fold increased in the resistant EAC cells, both grown in vivo and in vitro.

Resistance to antitumour drugs is one of the limiting factors in chemotherapy of cancer. Fluorinated pyrimidines, such as 5-fluoro-2'-deoxyuridine (FdUrd) and its free base (FUra) often used in experimental chemotherapy can also provoke resistance in various transplantable tumours and in cultured neoplastic cells (e.g. Reichard et al., 1959; Morse & Potter, 1965; Ardalan et al., 1980; Priest et al., 1980; Mulkins & Heidelberger, 1982a). Since FdUrd is less toxic for mice and more effective against Ehrlich ascites carcinoma (EAC) cells than FUra (e.g. Heidelberger et al., 1958; Kessel et al., 1971; Kanazawa et al., 1980), this nucleoside was used in our study on the biochemical basis of drug resistance in Ehrlich ascites carcinoma cells. This paper reports the development and characteristics of the subline of EAC cells persistently resistant to FdUrd when grown in vivo or in vitro in the absence of the drug. In particular, the resistant cells exhibit deeply depressed activity of thymidine kinase which catalyzes phosphorylation of thymidine, and several-fold increased activity of thymidylate synthetase which catalyzes thymidylate synthesis de novo. Purification and properties of the latter enzyme from our EAC resistant cells are described elsewhere (Jastreboff et al., 1983).
MATERIAL AND METHODS

EAC cells (about $5 \times 10^6$) in 0.5 ml of the ascites fluid diluted with phosphate buffered saline (PBS) were retransplanted by weekly intraperitoneal injections into recipient Swiss albino mice (females, 3 months old, about 30 g in weight).

To develop resistance in EAC cells, FdUrd was given intraperitoneally to mice, starting 1 day after inoculation, as 5 daily injections of 0.1 - 0.25 ml of its PBS solution; on the seventh day the EAC cells were passaged into new recipients. This procedure was repeated for 15 passages using the following doses of FdUrd: 35 mg/kg per day for the first four passages, followed by 40, 50, 60 mg/kg per day for two passages each and by 75 mg/kg per day for the final five passages. Survival times of tumour-bearing mice, expressed in days after inoculation with EAC cells of the particular lines, were recorded as their life span (LS). The mean survival time of 50% of EAC inoculated mice was denoted as LS$_{50}$. Prolongation of LS$_{50}$ after FdUrd treatment of the host-mice was considered as an indication of drug-sensitivity of EAC cells, whereas no change in LS$_{50}$ as FdUrd-resistance of the cells (Ardalan et al., 1980).

The EAC cells of both lines were adapted to growth in suspension cultures in Eagle’s minimal essential medium with Earle’s salts, supplemented with 10 - 15% calf serum, glucose (5.5 mm), glutamine (2 mm) and antibiotics (streptomycin 50 µg/ml, penicillin 100 U/ml). During six weeks of adaptation the cells were only once passaged into other vials but the medium was renewed every other day. Susceptibility of the cells to FdUrd and FUra was checked by addition of the respective drug to 24 h cultures for 48 h to final concentrations in the medium of $10^{-9}$ - $10^{-4}$ M and then by processing the cells for estimation of their density, and by calculation of their EC$_{50}$ values (i.e. the FdUrd concentration effective in killing 50% of the cells).

Cell viability and count were estimated by the trypan blue exclusion test (McLamins et al., 1957). The doubling time of cell populations was evaluated from the straight line portions of the growth curves. The chromosome number of the cells was estimated by the method of Hsu (1973).

To prepare enzyme extracts for enzyme assays, the cells (10 - 15 mg wet weight/ml) were centrifuged at 300 g for 3 - 5 min at 4°C, washed twice with PBS and suspended in 0.05 M-Tris/HCl buffer, pH 7.5, containing 0.01 M-2-mercaptoethanol, then disintegrated ultrasonically by use of the MSE ultrasonic power unit (1.5 A, 5 times for 20 s). Protein content was determined according to Sedmak & Grossberg (1977) using bovine albumin as a standard. The activity of thymidine kinase, ATP: thymidine 5’-phosphotransferase (EC 2.7.1.21) was estimated by the method of Münch-Petersen & Tyrsted (1977), based on measuring of the radioactivity of phosphorylated [methyl-3H]thymidine. The activity of thymidylate synthetase, 5,10-methylene-tetrahydrofolate: dUMP 1-methyltransferase (EC 2.1.1.45) was estimated by the method of Roberts (1966) as described in detail by Jastreboff et al. (1982). The method is based on measurements of tritium released from the substrate, [5-3H]2’-deoxyuridine-5’-monophosphate; release of 1 µg equivalent of tritium corresponds with formation of 1 µmole of thymidylate. All assays were performed in triplicate.
Chemicals used were of analytical grade. [methyl-\(^3\)H]Thymidine (spec. act. 50 Ci/mmol) and [5-\(^3\)H]-2'-deoxyuridine-5'-monophosphate (spec. act. 12.7 Ci/mmol) were purchased from Amersham, U.K.

RESULTS AND DISCUSSION

Five sublines of EAC cells capable of growth in mice treated with FdUrd were developed, but only one remained resistant on omitting drug-treatment of the hosts. Since then, this subline has been retransplanted for more than 80 passages through FdUrd-untreated mice. The life span (Fig. 1) of the mice inoculated with EAC cells of the parental line was much shorter (LS\(_{50}=14\) days) than that of the mice bearing the FdUrd-adapted EAC cells (LS\(_{50}=20\) days), thus indicating that the latter were less invasive. However, 5 daily injections of FdUrd prolonged the life span of the mice with FdUrd-sensitive parental EAC cells (LS\(_{50}=24\) days), whereas the life span of the mice with FdUrd-adapted EAC cells remained unchanged (Fig. 1). This shows that the FdUrd-adapted cells retained their resistance, and this was also the case in cells examined at their late passages (34th, 46th, 59th) through drug-untreated mice.

![Life span of host mice inoculated with EAC cells](image)

Fig. 1. Life span of host mice inoculated with EAC cells of the parental line (circles) or of the FdUrd-adapted subline examined at the 8th - 20th passages through drug-untreated mice (triangles). Full marks refer to animals untreated with the drug, open ones refer to animals treated with FdUrd (75 mg/kg per day for 5 days). Experimental series of untreated animals consisted of 161 mice with the parental EAC cells and of 150 mice with the drug-adapted ones whereas series of FdUrd-treated animals consisted of 32 and 43 mice with the parental or drug-adapted cells, respectively.

After adaptation to growth in suspension culture, the EAC cells of both lines were compared as regards their susceptibility to FdUrd and FUra present in the medium (Fig. 2). The cells of the parental line were 1000-fold more sensitive to FdUrd (EC\(_{50}=10^{-7}\) M) than to FUra (EC\(_{50}=10^{-4}\) M), whereas the cells of our subline resistant to FdUrd (EC\(_{50}=10^{-4}\) M) continued to exhibit low sensitivity to FUra (EC\(_{50}=10^{-4}\) M), as was recently reported for various leukemic clones (Mulkins & Heidelberger, 1982a). It seems only a question of chance that the acquired resistance to FdUrd of our FdUrd-adapted cells was of the same order of magnitude as the intrinsic resistance of the cells to FUra. The shape of the concentration curve
for the parental EAC population (Fig. 2) suggests that it contained a large fraction of FdUrd-resistant cells. Very likely therefore, the development of the FdUrd-resistant resistance in our EAC cells had mostly a selective basis. This process, probably, was a multistep one, since the subline was established by continuous exposure of the parental EAC cells to progressively increasing \textit{in vivo} concentrations of FdUrd (Mulkins \& Heidelberger, 1982a).

![Graph showing concentration effects of FdUrd and FUra on EAC cell growth](image)

Fig. 2. Concentration effects of FdUrd (full marks) and of FUra (open marks) on EAC cell growth \textit{in vitro} as compared in the FdUrd-sensitive parental line (circles) and the drug-resistant descendant subline (triangles). The drug was added to 24 h cultures for 48 h, then the cells were processed for cell count. The data in the figure are means from 5 separate experiments with 3 parallel cultures.

Comparison of basic biological characteristics of our EAC cells indicates that the cells of more invasive parental line grew in mice faster (doubling time 17 h) than those of the resistant subline (doubling time 25 h). A similar case were the EAC cells of the parental line (doubling time 25 h) and those of the FdUrd-resistant subline (doubling time 29 h) adapted to growth in culture. Both these EAC lines were near-tetraploid either \textit{in vivo} or \textit{in vitro} (73 - 77 chromosomes), being polyploid (116 - 120 chromosomes) in not more than 6\% (with exception of the resistant cells grown \textit{in vitro} which were polyploid in 12\%).

Since cytotoxicity of FdUrd (Kanazawa \textit{et al.}, 1980) depends mostly on its intracellular phosphorylation by thymidine kinase to FdUMP, which is a strong inhibitor of thymidylate synthetase, we estimated the activity of these enzymes in our cells. As shown in Table 1, the specific activity of thymidine kinase, rather high in extracts of the parental FdUrd-sensitive cells, was hardly detectable in those of the drug-resistant ones, whereas the activity of thymidylate synthetase in extracts of the resistant cells was 5-fold higher than in those of the parental line, and this was the case both for cells grown in mice and in culture. Thymidylate synthetase of our FdUrd-resistant EAC cells differs in some kinetic properties from the enzyme of the parental sensitive ones (Jastreboff \textit{et al.}, 1983). An altered thymidylate synthetase in FUra-resistant EAC cells was already reported, although its detailed characteristics were not examined (Heidelberger \textit{et al.}, 1960). In contrast to unstable overproduction of thymidylate synthetase in FdUrd-resistant cells of various 3T6
derived clones (Rossana et al., 1982), the increased activity of this enzyme in
our resistant EAC cells grown in the absence of FdUrd has been a stable
phenomenon.

In FdUrd-resistant neoplastic murine cells other than our EAC cells, only a line
of Novikoff hepatoma (Wilkinson et al., 1977) exhibited a simultaneous deficiency
of thymidine kinase activity and an increase in thymidylate synthetase activity.
More frequently, either a deficiency of thymidine kinase (Morris & Fischer, 1960;
Umeda & Heidelberger, 1968; Kessel & Vodinsky, 1970; Slack et al., 1976; Baskin
et al., 1977; Mulkins & Heidelberger, 1982b) or an increase in activity of thymidylate
synthetase (Baskin et al., 1975; Baskin & Rosenberg, 1975; Priest et al., 1980)
in FdUrd-resistant variants have been reported. Neither our FdUrd-resistant EAC
cells nor those of Wilkinson’s et al. (1977) hepatoma line were cloned. We wonder,
therefore, whether our resistant subline and that of Wilkinson et al. were mixed
populations of cells either lacking thymidine kinase or with increased activity of
thymidylate synthetase, or else whether they consisted of cells with both these
enzymic deviations.

Table 1

Specific activity of thymidine kinase and thymidylate synthetase as determined in
extracts of EAC cells grown in vivo and in vitro

The standard reaction mixture for thymidine kinase in a total volume of 225 μl contained: 45 nmol
of [methyl-3H]thymidine, 2.25 μmol of MgCl2, 1.13 μmol of ATP, 9 μmol of NaF, 22.5 μmol of
Tris/HCl buffer (pH 8.0) and the enzyme extract or distilled water in the control samples. The
standard reaction mixture for thymidylate synthetase in a total volume of 40 μl contained: 2.0 μmol
of [5-3H]dUMP, 20 μmol of (+)tetrahydrofolate, 0.2 μmol of formaldehyde, 4 μmol of 2-mercaptoethanol, 2 μmol of NaF, 2 μmol of phosphate buffer (pH 7.5), 0.4 μmol of ascorbate buffer
(pH 7.5) and the enzyme extract or the buffer in the control samples. The data in the table are mean
values ± S.E.M., in parentheses numbers of experiments.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cells grown</th>
<th>Specific activity (pmol/min/mg protein)</th>
<th>R/P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Parental line (P)</td>
<td>Resistant line (R)</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>in vivo</td>
<td>289 ± 5.3 (6)</td>
<td>3.3 ± 0.48 (6)</td>
</tr>
<tr>
<td></td>
<td>in vitro</td>
<td>267 ± 4.5 (6)</td>
<td>2.2 ± 0.14 (6)</td>
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<tr>
<td>Thymidylate synthetase</td>
<td>in vivo</td>
<td>274 ±19.3 (10)</td>
<td>1586 ±57.8 (8)</td>
</tr>
<tr>
<td></td>
<td>in vitro</td>
<td>205 ±26.4 (7)</td>
<td>1005 ±60.5 (8)</td>
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</tbody>
</table>

It is of interest that in particular FdUrd-resistant lines of Ehrlich ascites car-
cinoma, lymphoma LS178Y, mast cell P5815, neuroblastoma or Novikoff hepatoma
(e.g. Heidelberger et al., 1958; Morris & Fischer, 1960; Kessel & Vodinsky, 1970;
Wolberg, 1970; Baskin et al., 1975; Wilkinson & Crumley, 1977) different patterns
of alterations in the activity of thymidine kinase and thymidylate synthetase were
observed. Thus, the different changes in the enzyme pattern may characterize FdUrd-
resistance, even in cells of common origin.
REFERENCES


**WYPRAWDZENIE I CHARAKTERYSTYKA LINII KOMÓREK RAKA WYSIĘKOWEGO EHRLICHACA TRWAŁE OPORNEJ NA 5-FLUORO-2'-DEZOKSYURYDYNĘ**

**Streszczenie**

Wyprowadzono linię komórek raka wysiękowego Ehrlicha (EAC) paszącą się 15-krotnie poprzez myszy traktowane dootrzewnowo wzrastającymi dawkami (35 - 70 mg/kg/dzień) 5-fluoro-2'-dezoksyurydyny (FdUrd). Komórki tej linii pozostawały oporne pomimo pasażowania ich ponad 80 razy poprzez myszy nietraktowane FdUrd. Po zaadaptowaniu do wzrostu w zawiesinie *in vitro*, komórki wyprowadzonej linii pozostawały tetraploidalne, czas podwojenia ich populacji był jednak dłuższy a oporność wobec FdUrd 1000 razy większa w porównaniu z komórkami macierzystymi. W komórkach oporowych aktywność kinazy tymi dynanowej była około 100-krotnie niższa, a aktywność syntez tymi dynanowej kilkakrotnie wyższa niż w komórkach wraźliwych rosnących *in vivo* i *in vitro*.

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