Isolation of Tumor Cells from Patients with Osteosarcoma and Analysis of Their Sensitivity to Methotrexate

José Mordoh, Reinaldo D. Chacón, and Jorge Filmus


ABSTRACT

Many clinical studies have been conducted on the role of high-dose methotrexate (MTX) in human osteosarcoma, but information about the in vitro effect of MTX on human osteosarcoma cells is lacking. In this paper, the effect of MTX on tumor cells derived from seven patients with osteosarcoma has been studied in an attempt to correlate clinical and in vitro sensitivity to the drug. Isolation of the cells from the primary tumors (four patients) or metastasis (three patients) was carried out with a collagenase treatment followed by purification through a density gradient. The osteosarcoma cells were identified by electron microscopy and histochemical reactions. The cellular sensitivity to MTX was measured by the inhibitory effect of MTX on [3H]deoxyuridine incorporation into DNA. This incorporation was 50% inhibited in primary tumors at concentrations from 3 x 10^{-7} to 3 x 10^{-6} M. The metastatic cells isolated from patients that were clinically resistant to high-dose MTX had a 50% inhibition ranging from 1.5 x 10^{-7} to 4 x 10^{-6} M. Human stimulated lymphocytes, Sarcoma 180 cells, and Ehrlich ascitic mouse cells had a 50% inhibition of about 1.5 x 10^{-7} M. When [3H]thymidine incorporation into DNA of human osteosarcoma cells was studied, it was observed that MTX increased its incorporation up to 4-fold. This increase was stable for at least 6 hr and was only slightly enhanced by the addition of hypoxanthine. The stimulation by MTX of [3H]thymidine incorporation into DNA in stimulated lymphocytes and Ehrlich cells is much smaller, between 40 and 60%. A hypothesis to explain these results is that osteosarcoma cells build their deoxythymidine monophosphate pool largely through the de novo pathway, the salvage pathway being less important. It is suggested that the importance of the de novo pathway for deoxythymidine monophosphate synthesis is a biochemical characteristic of the osteosarcoma cells which could be related to the initial sensitivity of this tumor to MTX and that an activation of the salvage pathway could constitute an additional mechanism of resistance to this drug.

INTRODUCTION

High-dose MTX followed by leucovorin rescue has been used extensively in the treatment of osteosarcoma (4, 13, 19). This type of treatment appears to be especially effective as a coadjuvant to surgery and only when subclinical metastases are present. When overt metastatic disease develops, high-dose MTX appears to be less effective since the development of clinical resistance is frequent (18). The mechanism of MTX action has been reviewed recently (1). It is an analog of folic acid which acts essentially through the binding to DHFR, an enzyme which catalyzes the reduction of dihydrofolate acid to tetrahydrofolic acid. Goldman (8) has also demonstrated that a certain amount of free intracellular MTX is necessary in order to achieve a complete inhibition of DNA synthesis. The main effect of MTX is a cellular depletion of reduced folates, essential for the de novo synthesis of dTMP and purines. The resulting "thymineless state" or "purineless state" would lead to cell death, it being at present uncertain which is the predominant mechanism in a given cell type (2, 10, 12). Some authors have also described a direct inhibitory effect of MTX on thymidylate synthetase, which catalyzes the synthesis of dTMP from dUMP (22).

Several mechanisms have been pointed out to explain the development of cellular resistance to MTX. The most frequent are: (a) an increase in the levels of DHFR (11, 15, 21); (b) a diminished transport of MTX into the cell (7, 9, 14); (c) an altered DHFR with less affinity for MTX (1); (d) the presence of a large amount of tumor cells in a nonproliferative state (G0) which would make them refractory to the cytotoxic effect of MTX (2).

The rational basis for the utilization of therapeutic regimens including high-dose MTX was that the higher drug concentrations in blood could overcome cellular resistance. However, to our knowledge, no study has been conducted to analyze MTX resistance at a cellular level in patients with osteosarcoma.

The work reported in this paper was intended to verify if the metastatic osteosarcoma cells isolated from patients clinically resistant to MTX were also resistant in vitro to this drug, as compared with cells isolated from primary tumors. A method developed to dissociate the osteosarcoma cells is also reported. Some aspects of the origin of the dTMP pool were also analyzed since its changes appear to play a fundamental role in the lethal effect of MTX.

MATERIALS AND METHODS

Patients. Seven patients were included in this study, and some clinical data relevant to this study are detailed in Chart 1. Several therapeutic regimens were administered to the patients: (a) LOMA: MTX, 3, 5, and 7 g/sq m on Days 1, 8, and 15 with previous administration of vincristine (1, 4 mg/sq m) and followed by leucovorin rescue (10 mg/sq m i.m. starting 2 hr after MTX infusion; every 6 hr during 72 hr). Adriamycin (60 mg/sq m) was given on Day 18.

This treatment was administered every 6 weeks. (b) VIMELECIDA: vincristine (1.4 mg/sq m) on Days 1, 8, 15, 22, 29, and 36; MTX, 1.5...
Tumor Cell Separation. The tumor samples were obtained during surgery performed on primary or metastatic osteosarcoma. The material was collected under sterile conditions in MEM with the following additions: 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2; 1 mM glutamine; penicillin (100 units/ml); streptomycin (100 µg/ml); and mycostatin (50 units/ml). In the laboratory, the tissue was minced with a scalpel and resuspended in the above-described medium at 0.2 to 0.3 g/ml. Different concentrations of collagenase were added, and the suspension was incubated at 37° under magnetic stirring (see “Results”). The turbid supernatant was afterwards filtered through gauze, and the tumor cells were either used at this step or further submitted to a density gradient centrifugation. The gradient was performed by gently layering 10 ml of the filtered cell suspension on top of a cushion obtained by mixing 7.2 ml of 8% Ficoll and 3 ml of 33% Hypaque, and the suspension was centrifuged in an HS-5 International centrifuge at 2500 rpm for 30 min. The fractions obtained are described in the text. The interphase containing the tumor cells was aspirated with a Pasteur pipet, diluted with MEM, and centrifuged at 2000 rpm for 10 min. The pellet was resuspended at a concentration of 1 x 10^6 cells/ml in the culture medium described above with the addition of 10% FCS.

The cell viability was measured with the trypan blue exclusion test and ranged from 60 to 95% in different experiments. Alternatively, the cells were resuspended at a concentration of 10 x 10^6 cells/ml in MEM-10% FCS-10% dimethyl sulfoxide and frozen in liquid nitrogen. After thawing, the viability of the cells ranged from 40 to 60%.

Lymphocyte Purification and PHA Stimulation. The method used for lymphocyte purification from normal peripheral blood has been described (6). Purified lymphocytes were resuspended at a concentration of 1 x 10^6 cells/ml in MEM containing 10% FCS, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 1 mM glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Mitogenic stimulation was achieved by adding 0.1 ml of PHA (PHA-P resuspended in 5 ml of bidistilled water) per 100 ml of culture. The cultures were incubated at 37° for 72 hr.

Ehrlich Ascitic Mouse Cells. These were obtained from the peritoneal cavity of Swiss mice, which had been inoculated 5 days before with 0.2 ml of a suspension containing 3 x 10^6 cells. The cells were washed with phosphate-buffered saline (NaCl, 8 g/liter; KCl, 0.2 g/liter; NaHPO_4·2H_2O, 1.15 g/liter; MgCl_2·6H_2O, 0.1 g/liter; phenol red, 10 mg/ml) and were resuspended at 1 x 10^6 cells/ml in the same medium used for lymphocyte culture.

Mouse Sarcoma 180 Cells. This tumor was grown in BALB/c mice, inside a small glass rod inserted s.c. After 10 days of growth, the tumor was extracted, and the cells were dissociated with collagenase as described for osteosarcoma. They were then resuspended at 1 x 10^6 cells/ml in the medium described above.

[3H]Thd and [3H]Durd Incorporation into DNA. After the cells were resuspended in the culture medium, they were divided in aliquots of 2 ml (1 x 10^6 viable cells/ml) and incubated for 1 hr at 37°. Unless otherwise stated, the cells were pulsed with 5 µCi of [3H]Thd (specific activity, 40 to 60 Ci/mmol) or with 2 µCi of [3H]Durd (specific activity, 15 to 30 Ci/mmol) for 60 min at 37°. The reaction was stopped by centrifugation, and the cell pellet was resuspended in 1.2 ml of water. After the preparation was mixed and allowed to stand in ice for 5 min, 0.1 ml of 0.1 M sodium pyrophosphate, 0.2 ml of heat-denatured DNA (2.5 mg/ml), and 0.25 ml of 12% perchorlic acid were added. The mixture was centrifuged at 5000 rpm for 10 min, and the precipitate was dissolved in 0.5 ml of 0.2 N NaOH. After 1 ml of water was added, the solution was precipitated with 0.25 ml of 12% perchloric acid. This procedure was repeated once more, and the precipitate was finally dissolved in 0.2 ml NCS, added to 10 ml of Toluene-Omni fluor scintillation mixture, and counted in a Nuclear Chicago liquid scintillation counter.

Materials. Tissue culture media were obtained from Grand Island Biological Co. (Grand Island, N. Y.), MTX was from Lederle Laboratories (Pearl River, N. Y.), collagenase was from Worthington Biochemical Co. (Freehold, N. J.), and PHA-P was from Difco Laboratories (Detroit, Mich.). Radioactive materials and Omnifluor were from New England Nuclear (Boston, Mass.), and NCS was from Amersham, The Radiochemical Centre, (Buckinghamshire, England).

Electron Microscopy. The cells recovered from the gradient interphase were centrifuged at 1000 rpm for 5 min, fixed with 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4° for 2 hr, and rinsed overnight in the same buffer containing 0.1 M sucrose. The cells were then postfixed in 1% OsO_4 in the same buffer at 4° for 2 hr. The specimens were then dehydrated in graded ethanol and embedded in Epon. Ultrathin sections were cut with a Porter-Blum MT-2B ultramicrotome, mounted on copper grids, doubly stained with uranyl acetate followed by lead citrate, and observed with a Siemens Elmiskop 101 electron microscope.

RESULTS

Cellular Disaggregation of Osteosarcoma. Several methods were assayed to release the osteosarcoma cells from the surgical sample. Since mechanical dissociation with needles released only negligible amounts of cells, experiments were performed in which the tissue was incubated with several enzymes. Pronase, trypsin, and collagenase were tested, and it was found that the last enzyme was the most efficient in releasing tumor cells. In order to determine the optimal conditions, several collagenase concentrations (0.1, 0.5, and 1.0 mg/ml) and incubation times were assayed. A sample from a primary osteosarcoma (Patient 3) was resuspended at 0.2 g/ml of culture medium as described previously (see “Materials and Methods”). After addition of collagenase, the number of released viable cells was determined at different times. It was observed that without collagenase there was practically no release of cells and that 1 mg/ml was the most efficient concentration tested, a plateau being reached after 3 hr of incubation at 37°. These conditions were afterwards used for the experiments described below.

Characterization of Dissociated Tumor Cells. After the collagenase treatment, phase-contrast microscopy revealed the presence of large cells with irregular nuclei, often binucleated, and lymphocytes infiltrating the tumor at a variable degree according to the patient. RBC were also found in variable amounts, mainly depending on the histological variety of osteosarcoma. The cellular suspension obtained was filtered through gauze, and the cells were used at this step in some experiments described below. However, in order to verify if some of the observed biochemical characteristics could be truly ascribed to the osteosarcoma cells, a further purification step was attempted. For this purpose, the cell suspension was layered on the top of a gradient and centrifuged as described in “Materials and Methods.” The interphase contained virtually pure large cells (Fig. 1), whereas the pellet contained cell debris, lymphocytes, and RBC.

The recovery of the tumor cells after the gradient centrifugation varied between 40 and 67% in different experiments. Electron microscopy performed on the cells recovered in the interphase revealed cytological features which have been described as characteristic of osteosarcoma cells (26), i.e., nu-
Action of MTX on Human Osteosarcoma

DNA synthesis by cells isolated from different zones of an osteosarcoma metastasis

A lung metastasis (3 x 2 cm) from Patient 5 was placed in the medium described in "Materials and Methods." The cells from 3 different zones were dissociated with collagenase (see "Materials and Methods") and divided into aliquots of 3 ml, and 10 μCi of [3H]dThd were added. After 2 hr at 37°, the radioactivity incorporated into DNA was measured as described in "Materials and Methods."

Table 1
DNA synthesis by cells isolated from different zones of an osteosarcoma metastasis

<table>
<thead>
<tr>
<th>Cells obtained from</th>
<th>[3H]dThd incorporated into DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral zone</td>
<td>6.150 ± 653% ± 100</td>
</tr>
<tr>
<td>Intermediate zone</td>
<td>4.560 ± 602% ± 74.1</td>
</tr>
<tr>
<td>Central zone</td>
<td>2.810 ± 262% ± 42.4</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

The viability of the cells from the peripheral part of the metastasis are more actively synthesizing DNA. The cells recovered from the peripheral layer of the metastasis are more actively synthesizing DNA.

Correlation between in Vivo and in Vitro Sensitivity to MTX In Patients with Osteosarcoma. As a first approach to the analysis of the possible mechanisms responsible for clinical resistance to MTX, it was decided to study in vitro the cellular sensitivity to MTX in patients in which metastatic disease continued to develop despite repeated courses of MTX. As a control, cells from primary tumors were also analyzed. The clinical evolution of the patients, the administered treatments, and the moment at which the surgical samples were obtained are indicated in Chart 1. The sensitivity to MTX was measured through the inhibition of [3H]dUrdd incorporation into DNA. The tumor cells used for these experiments were obtained after purification through a density gradient (see "Materials and Methods"). In some cases, the results were compared with those obtained using the tumor cells without purification and were found to be closely similar (data not shown). In Chart 2, A to C, the results obtained with 3 patients are presented. Two of them displayed similar sensitivity to MTX (Chart 2, A and B) whereas the inhibition results obtained with Patient 6 (Chart 2C) revealed the presence of cells more resistant to MTX. The shoulder observed in the inhibition curve could suggest the existence of 2 different cell populations. The same experiment was also performed with different cell types, neither of which had been previously exposed to MTX (Chart 2D). In order to quantify the results, the sensitivity to MTX is expressed as 100, i.e., the concentration of MTX which inhibits the incorporation of [3H]dUrd into DNA to 50% of the controls without MTX. The sensitivity of the cells isolated from all the primary tumors and metastases is shown in Table 2. Four patients with primary tumors were analyzed, and it may be observed that the 100 ranges between 3 x 10^{-7} and 3 x 10^{-6} M. With respect to the sensitivity of the tumor cells isolated from metastases, the results were variable. In the case of Patient 5, the lung and s.c. metastases were obtained during different surgical procedures separated by a period of 19 months (see Chart 1). In the meantime, the patient received 20 courses of high-dose MTX with leucovorin rescue. It may be seen that the sensitivity of the cells isolated from both metastases is similar to that of the primary tumors, with 100 of 5 x 10^{-7} and 1.5 x 10^{-7} M, respectively. The cells from Patient 6 were isolated from a lung...
patent

10

0

5

10

15

20

25

30

35

40

45

TIME (MONTHS)

PATIENT N°1, G.P, MALE, 18 y
OSTEOSARCOMA FROM TIBIA

PATIENT N°2, J.C., MALE, 18 y
OSTEOSARCOMA FROM TIBIA

PATIENT N°3, O.G.P, MALE, 19 y
OSTEOSARCOMA FROM TIBIA

PATIENT N°4, O.J, MALE, 12 y
OSTEOSARCOMA FROM FEMUR

PATIENT N°5, O.F, MALE, 22 y
OSTEOSARCOMA FROM FEMUR

PATIENT N°6, Ch G MALE, 15 y
TELANGIECTATIC OSTEOSARCOMA FROM FEMUR

PATIENT N°7, C.S FEMALE, 14 y
OSTEOSARCOMA FROM HUMERUS

chart 2. effect of MTX on [3H]dUrd incorporation. The osteosarcoma cells were obtained as described in "Materials and Methods." and 30 min after addition of MTX at the above-indicated concentrations a pulse of [3H]dUrd was given. The radioactivity is expressed as percentage of the control without the addition of MTX. A, Patient 1. B, Patient 5. C, Patient 6. In D, the following cells were used: 72-hr PHA-stimulated lymphocytes (Φ); Ehrlich ascitic tumor cells (Ω); and mouse Sarcoma 180 cells (Δ). [3H]dUrd pulses were performed as described above.

Table 2

<table>
<thead>
<tr>
<th>Patient</th>
<th>Source of cells</th>
<th>[3H]dUrd inhibition by MTX (IC50) (m)</th>
<th>Maximal stimulation of [3H]dThd incorporated into DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary tumor, tibia</td>
<td>8.7 x 10^-7</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Primary tumor, tibia</td>
<td>2 x 10^-4</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>Primary tumor, tibia</td>
<td>3 x 10^-7</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>Primary tumor, femur</td>
<td>3 x 10^-4</td>
<td>205</td>
</tr>
<tr>
<td>5</td>
<td>Lung metastasis</td>
<td>5 x 10^-7</td>
<td>310</td>
</tr>
<tr>
<td>6</td>
<td>S.c. metastasis</td>
<td>1.5 x 10^-7</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>Lung metastasis</td>
<td>1.3 x 10^-4 (2)</td>
<td>249 (2)</td>
</tr>
<tr>
<td></td>
<td>Lung metastasis</td>
<td>4 x 10^-8</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Human peripheral lymphocytes, PHA stimulated</td>
<td>1.4 ± 0.6 x 10^-7 (3)</td>
<td>52.3 ± 8.3 (9)</td>
</tr>
<tr>
<td></td>
<td>Ehrlich mouse ascitic cells</td>
<td>2.1 ± 0.1 x 10^-7 (4)</td>
<td>54.7 ± 2.7 (3)</td>
</tr>
<tr>
<td></td>
<td>Sarcoma 180 cells</td>
<td>1.5 ± 0.2 x 10^-7 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND, not determined.

a Numbers in parentheses, number of experiments.

b Mean ± S.D.
is completely inhibited by MTX at high concentrations (Chart 2), the dTMP pool should contract, being solely constituted via the salvage pathway. If it is assumed that neither the enzymes involved in the salvage pathway of dTMP synthesis nor those directly involved in DNA replication are affected by MTX in our experimental conditions, when tracer amounts of \([3H]dThd\) are given to the cells, the increase in the \([3H]dThd\) incorporated into DNA would reflect directly the higher specific activity of the dTMP pool due to the suppression of the de novo synthesis.

The results obtained with osteosarcoma cells from 2 patients (Patients 5 and 6) in typical experiments are shown in Chart 3A. It may be observed that in the presence of MTX \([3H]dThd\) incorporation into DNA is highly increased, 310 and 249%, respectively. The inverse relationship existing between the \([3H]dUrd\) and \([3H]dThd\) incorporation into DNA strongly suggests that the contraction of the de novo dTMP pool is responsible for the increase of \([3H]dThd\) incorporation into DNA [i.e., Patient 5 (Charts 2A and 3A)]. In the case of Patient 6, whose pattern of inhibition by MTX of \([3H]dUrd\) incorporation demonstrates the presence of MTX-resistant cells (Chart 2C), the \([3H]dThd\) incorporation into DNA in the presence of MTX appears to increase more gradually and at an MTX concentration of \(5 \times 10^{-5}\) M has not yet reached a plateau.

This effect was also tested in nonrelated cells, i.e., human PHA-stimulated lymphocytes and Ehrlich ascitic cells. In Chart 3B, it is shown that the increase in \([3H]dThd\) incorporation in the presence of MTX is much smaller, 52.3 and 54.7%, respectively.

Table 2 shows the data of maximal stimulation by MTX of \([3H]dThd\) incorporation into DNA in the osteosarcoma cells isolated from primary tumors and metastases and in nonrelated cells. The smaller increase in osteosarcoma was obtained in Patient 7, whose cells were isolated from a lung metastasis.

In order to test whether the collagenase treatment to which osteosarcoma cells were submitted could in some way alter the effect of MTX on \([3H]dThd\) incorporation into DNA, lymphocytes and Ehrlich cells were submitted to an identical collagenase treatment without any change being observed in the degree of stimulation by MTX (data not shown). From the data shown in Chart 3 and Table 2, it may be concluded that in osteosarcoma cells (with the exception of Patient 7) the dTMP pool is size primarily through the de novo pathway, whereas in Ehrlich cells and human stimulated lymphocytes its origin is mainly through the salvage pathway.

**Effect of Time and Hypoxanthine on MTX Stimulation of \([3H]dThd\) Incorporation into DNA.** The high-dose MTX infusions performed on patients maintain high blood levels (\(10^{-5}\) M) for about 24 hr. It was therefore important to verify if the dTMP pool contraction due to DHFR inhibition obtained with 30 min of MTX treatment was maintained for longer periods. Also, since it has been reported that the dTTP pool is larger than the dCTP, dATP, and dGTP pools in many different types of cells (5, 24), it was important to verify if purine nucleotides, the synthesis of which is also blocked by MTX, were rate limiting for \([3H]dThd\) incorporation into DNA. If this were the case, addition of a purine precursor like hypoxanthine should increase \([3H]dThd\) incorporation into DNA. The result of this experiment performed with osteosarcoma cells is shown in Chart 4. It may be observed that the stimulatory effect of MTX on \([3H]dThd\) incorporation remains quite stable during at least 6 hr at 37°C and that hypoxanthine produces a small stimulatory effect.

When the experiment was performed with stimulated lymphocytes, it was observed that the stimulatory effect of MTX was maximal after 30 min of MTX action and then became very small. The stimulatory effect of hypoxanthine was again not very important. Control experiments demonstrated that hypoxanthine alone did not alter \([3H]dThd\) incorporation into DNA. These experiments strongly suggest that the dATP and dGTP pools are size mainly through the de novo pathway.
pools are not seriously rate limiting for \[^{3}H\]dThd incorporation into DNA, at least during the first 6 hr of MTX action.

**DISCUSSION**

Further advances in the treatment of human osteosarcoma will probably be obtained if some characteristics of the osteosarcoma cells, such as cell growth kinetics, growth requirement factors, and sensitivity to chemotherapeutic agents, may be estimated. To perform many of these experiments, it is desirable to obtain isolated cells from this tumor, and in this paper a method is described which makes it possible to obtain a large number of highly purified osteosarcoma cells. This method involves an enzymatic treatment with collagenase, and similar treatments have been reported to preserve the cloning capabilities of human melanoma and sarcoma (16).

The question of whether the clinical resistance to MTX was accompanied by in vitro cellular resistance to the drug was also analyzed in this paper. Two methods are commonly used to measure drug sensitivity of tumor cells: long-term cultures where the ability of drugs to diminish cellular reproduction and colony formation is measured (20); or short-term cultures where the effect of drugs on the incorporation of metabolic precursors into macromolecules is determined. The results of both methods have been reported to be correlated with the clinical response (20, 25). Since attempts performed in our laboratory to develop colonies in vitro from osteosarcoma using the semisolid agar technique (20) have until now been unsuccessful, the inhibition by MTX of \[^{3}H\]dUrd incorporation into DNA was therefore analyzed. For the cells isolated from primary tumors, \( I_{50} \) ranged between 3 \( \times 10^{-7} \) and 3 \( \times 10^{-6} \) M. The results obtained with cells isolated from clinically resistant patients were variable. In one patient (Patient 5), clinical resistance to MTX was not accompanied by in vitro cellular resistance to the drug, even in tumor cells obtained from a metastasis 2 months before the patient's death. In other patients, however (Patients 6 and 7), the \( I_{50} \) for MTX was considerably higher. It seems clear, therefore, that clinical resistance to MTX may or may not be accompanied by increased in vitro cellular resistance to the drug. In the case of tumor cells with an increased \( I_{50} \), it could be assumed that they developed resistance mechanisms such as higher amounts of DHFR, an altered DHFR, or a lesser permeability to MTX, which could be bypassed by higher MTX concentrations. In the second case, where the cells have normal sensitivity to MTX in our assay system, clinical resistance could be due to other mechanisms, such as a high number of resting cells or diminished drug access to the tumor. Another possible mechanism to circumvent the inhibition by MTX is suggested by the observations in this paper on the different contribution of the salvage and the de novo pathways to form the dTTP pool in cells of different origins. Since the "thymineless state" appears to be a main factor in the cell death caused by MTX (12), it could be assumed a priori that cells with a relatively important de novo pathway for dTMP synthesis would be more sensitive to MTX than were those with a predominant salvage pathway. In the course of studies on the intrinsic sensitivity to MTX of different cell lines, Tattersall et al. (24) observed that: (a) a higher sensitivity to MTX appeared to be associated with smaller dTTP pools; (b) after 24 hr of MTX treatment, the only deoxyribonucleoside triphosphate pool consistently diminished was the dTTP pool, the reduction again being highest in the most sensitive cells; and (c) after the addition of dThd to the extracellular medium, there was a greater increase in the dTTP pool in the most resistant cells. It is reported here that in human osteosarcoma cells the de novo pathway for dTMP synthesis is predominant and this could explain the reported efficacy of MTX as a coadjuvant chemotherapeutic agent in this tumor. These observations could lead to the speculation that an activation of the salvage pathway would make it possible to acquire a certain degree of resistance to MTX. Partial support for this hypothesis has been obtained in only one patient (Patient 7) whose de novo pathway for dTMP synthesis was considerably smaller than usual (Table 2). It is interesting to note that in this patient the increase in the salvage pathway coexisted with an increased \( I_{50} \) for \[^{3}H\]dUrd incorporation into DNA. This observation would suggest that 2 resistance mechanisms could combine in a particular patient. In the case of Patient 6, in whom osteosarcoma cells resistant to MTX were detected (Chart 2), the stimulation of \[^{3}H\]dThd incorporation by MTX had peculiar characteristics. Although the maximal increase obtained was high (249%), the activation curve had several inflection points and was still rising at the higher MTX concentration tested. This pattern could be compatible with a heterogeneous cellular population as regards the MTX ability to inhibit the de novo pathway.

The activation of the salvage pathway has been reported in other cells. Thus, Cooper et al. (3) found in leukemic cells that the proportion of dThd from the extracellular medium incorporated into DNA varies between 13 and 87% when dThd concentration in the extracellular medium increased from 0.03 to 300 \( \mu M \). This finding would be particularly important in patients being treated with chemotherapy, since due to cellular destruction large amounts of degradation products are probably released to the extracellular medium. These nucleotides could supply the lack of thymidylate produced by the inhibition of the de novo pathway. It is interesting to note that Pinedo et al. (17) observed that it was not possible to obtain consistent blockage of colony-forming units with MTX when nondialyzed FCS and L-cell supernatant were used. This effect was probably due to the presence of nucleic acid precursors which after dialysis were reduced to undetectable levels. These authors have also found that the amount of nucleic acid precursors varied between different batches of FCS. This source of variability was considered in the experiments described in this paper; therefore, the same batch of FCS was used throughout.

It seems therefore that the cellular proportion of the de novo and salvage pathways for dTMP synthesis is variable, depending on several factors. One of them is the nature of the cell itself, since it has been shown in this paper that under identical culture conditions osteosarcoma cells have a de novo pathway more important than that of lymphocytes or Ehrlich cells. Another factor appears to be the dThd concentration in the extracellular medium (3). Once the de novo pathway for dTMP synthesis in a cell has been interrupted by MTX, the ability of the cell to survive will probably depend on 2 factors, the size of the remaining dTMP pool and the ability of the cell to replenish it through a higher activity of the salvage pathway. It has been shown in this paper that the remaining dTMP pool after exposure to MTX is different in osteosarcoma cells from that in lymphocytes. The capacity of these different cells to activate their salvage pathways is...
unknown, although it has been shown in this paper that the contraction of the dTMP pool by MTX in osteosarcoma is stable for at least 6 hr under our culture conditions (Chart 4). It has already been mentioned that some cells increase their salvage pathway when dThd extracellular concentration increases. Apparently, this effect can be therapeutically exploited, since it has been reported that in mice bearing L1210 leukemia administration of dThd could prevent MTX toxicity without affecting its antitumor action (23). In the case of osteosarcoma, it is reasonable to assume that these cells would be less effectively rescued by dThd than would normal tissues with more important salvage pathways.

The methodology and data reported in this paper could be useful for further studies on the biological behavior of osteosarcoma cells including the biochemical basis of the response and resistance to MTX and other agents.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 2. Electron microscopy of disaggregated osteosarcoma cells (Patient 4). A, osteosarcoma cell containing an irregular nucleus, mitochondria (mi), and abundant and dilated rough endoplasmic reticulum (rer). × 10,500. B, high magnification of A showing filamentous structures (f) irregularly arranged or in bundles and atypical configuration of mitochondria (mi). × 40,500. C, detail of portion of malignant cell with mitochondria (mi), Golgi complex (g), and dilated rough endoplasmic reticulum (rer). × 19,500.
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