Collateral Methotrexate Resistance in Cultured Human Head and Neck Carcinoma Cells Selected for Resistance to cis-Diaminedichloroplatinum(II) \(^1\)

Andre Rosowsky, Joel E. Wright, Carol A. Cucchi, Jennifer L. Flatow, Dorothy H. Trites, Beverly A. Teicher, and Emil Frei III

Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

A human head and neck squamous cell carcinoma line (SCC25) derived from a patient with no prior history of radiotherapy or chemotherapy was made resistant to cis-diaminedichloroplatinum(II) (CDDP) by continuous exposure of weekly 30-min pulses of the CDDP from 0 to 0.2 mM over 20 months and then cloned and pulsed weekly with 0.2 mM CDDP for another 20 months. This afforded a resistant subline, SCC25/CP[1], with an IC\(_{50}\) for CDDP 12-fold higher than that of the parental cells. The SCC25/CP[1] cells unexpectedly proved to be cross-resistant to methotrexate (MTX) (24-fold for 30-min treatment and 8-fold for continuous treatment). Resistance was associated with a modest (about 2-fold) increase in the dihydrofolate reductase (DHFR) content according to radioligand-binding assay, and in the rate of cell division. In addition there was a 4-fold decrease in the fraction of long-chain MTX polyglutamates MTX(G\(_m\)) in the cell after 24 h exposure to either 0.2 or 2.0 \(\mu\)M MTX. When the SCC25/CP[1] cells were kept out of CDDP for 8–9 months and 12 months to give the sublines SCC25/CP[2] and SCC25/CP[3], respectively, MTX sensitivity to continuous exposure returned to normal. The SCC25/CP[3] cells still exhibited a slightly elevated DHFR level, but their generation time became shorter than that of the parental SCC25 line. In addition the SCC25/CP[3] cells had an initial uptake velocity (\(V_0\)) for MTX that was 9-fold greater than the \(V_0\) of the SCC25 cells. The SCC25/CP[1] cells were resistant to MTX but not to doxorubicin, vincristine, or 5-fluorouracil. The extent of MTX resistance was most evident in survival curves, which revealed that exposure to 500 \(\mu\)M MTX for 1 h produced a 3-log kill in the parental cells whereas similar treatment of the SCC25/CP cells gave only a 1-log kill. Since the SCC25 cell line was initially established from the tumor of a patient who had received no prior MTX therapy, MTX resistance had to be due to the CDDP treatment alone. We were unaware of any precedent for collateral MTX resistance in CDDP-resistant human tumor cells and recognized that such collateral resistance might have important clinical implications for head and neck carcinoma patients who are treated with combinations of CDDP and MTX (4–7). In order to clarify this phenomenon, we sought to determine whether the phenotypic changes that were observed earlier in SCC cells treated with MTX also occurred after treatment with CDDP. We compared the SCC25 and SCC25/CP cells with respect to several biochemical parameters, and followed the changes that occurred in these phenotypic traits when the SCC25/CP cells were allowed to revert to MTX sensitivity in the absence of CDDP and when CDDP resistance was reestablished by resumption of CDDP treatment. As had been observed earlier with MTX-treated SCC cells, collateral MTX resistance in CDDP-treated cells was found to be associated with a complex pattern of changes in DHFR content, MTX influx, and MTX polyglutamylation.

MATERIALS AND METHODS

Cells and Chemicals. The origin of the SCC25 human head and neck squamous cell carcinoma line has been described previously (8, 9). The cells were grown in DME (Gibco, Grand Island, NY) supplemented with 20% FBS (M.A. Bioproducts, Walkersville, MD), penicillin (100 IU/ml), streptomycin (100 \(\mu\)g/ml), l-glutamine (2 mM), and hydrocortisone (0.4 \(\mu\)g/ml). Incubations were at 37°C in an 8% CO\(_2\) humidified atmosphere. CDDP was a gift from Johnson-Matthey, Malvern, PA, and MTX was from the National Cancer Institute, Bethesda, MD. [3',5',7-\(\text{H}\)]MTX, hereafter referred to as [\(\text{H}\)]MTX, was prepared by Amersham (Arlington Heights, IL) from 3',5',7-\(\text{H}\)-dichloromethotrexate and purified by HPLC as described earlier (8, 11). The specific activity of various batches was determined to be 17–20 Ci/mmol by UV spectrophotometry and scintillation counting.

Production of Resistance. SCC25 cells (2 \(\times\) 10\(^5\)/100-mm dish) were treated for 30 min with an IC\(_{50}\) concentration (0.02 mM) of CDDP, washed twice with DME, and allowed to grow out in fresh medium. The cells were observed daily, and when they became nearly confluent (about 14 days) they were replated at the initial density and treated with the parental line (SCC25) (2, 3). Resistance to CDDP was accompanied by moderate cross-resistance to melphalan but minimal cross-resistance to other alkylating agents such as busulfan and nitrogen mustard. When the SCC25/CP cells were routinely tested for sensitivity to a group of standard anticancer drugs of the nonalkylating type, they were found to be cross-resistant to MTX but not to doxorubicin, vincristine, or 5-fluorouracil. The extent of MTX resistance was most evident in survival curves, which revealed that exposure to 500 \(\mu\)M MTX for 1 h produced a 3-log kill in the parental cells whereas similar treatment of the SCC25/CP cells gave only a 1-log kill.

Received 4/6/87; revised 8/10/87; accepted 8/14/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by a program grant (CA19589) from the National Cancer Institute, Department of Health and Human Services.

2 To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

3 The abbreviations used are: CDDP, cis-diaminedichloroplatinum(II) (cis-platin); MTX, N-(4-amino-4-deoxy-o-N\(^\text{4}\)-methyluronyl)-L-glutamic acid (methotrexate); DME, Dulbecco’s minimum essential medium; FBS, fetal bovine serum;
with a 1.5- to 2-fold higher concentration of CDDP. The dose escalation process was continued over a period of 20 months. As the CDDP dose increased, the cells took longer to reach near confluence. These slow-growing cultures were sometimes subjected to several weekly CDDP treatments at the same concentration before being split. At 20 months, when weekly treatment with 0.2 mM CDDP was well tolerated, cells were cloned from large colonies using cloning chambers. Several clones were cryopreserved for future study, and one (clone Pt2) was studied for another 20 months while being maintained at the same CDDP resistance level. The resulting cells, called SCC25/CP in our earlier papers (2, 3), will be referred to here as SCC25/CP[1]. Sublines SCC25/CP[2] and SCC25/CP[3] were obtained from SCC25/CP[1] cells by allowing them to grow in the absence of CDDP for 8–9 and 12 months, respectively. Subline SCC25/CP[4] was obtained by subjecting the SCC25/CP[2] cells to renewed CDDP treatment (0.2 mM, weekly 30-min pulses) for 4–6 months. The “generation time,” defined as the average number of days per generation divided by the number of generations per passage, was calculated for each cell line as described earlier (8).

Cytotoxicity. The level of CDDP and MTX resistance was determined for each subline on the basis of cytotoxicity assays as described earlier (2, 3, 8). Cells (10^5/60-mm dish) were plated in regular growth medium and allowed to attach overnight. After 24 h the medium was replaced with serum-free DME containing a range of drug concentrations. Approximately 2 weeks later the cells were fixed with phosphate-buffered 10% formalin, and colonies were stained with 0.2% methylene blue and counted microscopically. Fold resistance toward each drug was calculated by dividing the IC_{50} for the resistant cell line by the IC_{50} for the parental SCC25 line. Each IC_{50} was the mean of at least 3 separate experiments using the same lot of FBS.

DHFR Content. The amount of DHFR in each cell line was measured by radioligand-binding assay according to the method of Kamen et al. (12).

MTX Uptake. The ability of the various cells to take up [3H]MTX was measured as described earlier (8). Briefly, cells (5 x 10^5/100-mm plate) were grown to 75–85% of confluence in DME containing 20% dialyzed FBS, 2 mM L-glutamine, conditioned in the same fresh medium at 37°C for 30 min, and exposed to 0.2 µM MTX in folate- and serum-free improved minimum essential medium (Gibco, Grand Island, NY) containing 2 mM L-glutamine, conditioned in the same fresh medium at 37°C for 30 min, and exposed to 0.2 µM MTX in folate- and serum-free improved minimum essential medium for various intervals (up to 2 h). Washed cells were digested overnight in 1 N NaOH, aliquots of the digest were combined as described earlier (2, 3, 8). Cells (10^5/60-mm dish) were plated in regular growth medium and allowed to attach overnight. After 24 h the medium was replaced with serum-free DME containing a range of drug concentrations. Approximately 2 weeks later the cells were fixed with phosphate-buffered 10% formalin, and colonies were stained with 0.2% methylene blue and counted microscopically. Fold resistance toward each drug was calculated by dividing the IC_{50} for the resistant cell line by the IC_{50} for the parental SCC25 line. Each IC_{50} was the mean of at least 3 separate experiments using the same lot of FBS.

DHFR Content. The amount of DHFR in each cell line was measured by radioligand-binding assay according to the method of Kamen et al. (12).

MTX Uptake. The ability of the various cells to take up [3H]MTX was measured as described earlier (8). Briefly, cells (5 x 10^5/100-mm plate) were grown to 75–85% of confluence in DME containing 20% dialyzed FBS, 2 mM L-glutamine, conditioned in the same fresh medium at 37°C for 30 min, and exposed to 0.2 µM MTX in folate- and serum-free improved minimum essential medium for various intervals (up to 2 h). Washed cells were digested overnight in 1 N NaOH, aliquots of the digest were combined as described earlier (2, 3, 8). Cells (10^5/60-mm dish) were plated in regular growth medium and allowed to attach overnight. After 24 h the medium was replaced with serum-free DME containing a range of drug concentrations. Approximately 2 weeks later the cells were fixed with phosphate-buffered 10% formalin, and colonies were stained with 0.2% methylene blue and counted microscopically. Fold resistance toward each drug was calculated by dividing the IC_{50} for the resistant cell line by the IC_{50} for the parental SCC25 line. Each IC_{50} was the mean of at least 3 separate experiments using the same lot of FBS.

DHFR Content. The amount of DHFR in each cell line was measured by radioligand-binding assay according to the method of Kamen et al. (12).

Table 1: Collateral MTX resistance in human head and neck SCC lines with acquired resistance to CDDP

<table>
<thead>
<tr>
<th>Treatment (mo)</th>
<th>CDDP IC_{50} (µM)^a</th>
<th>MTX R S Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCC25/CP[1]</td>
<td>20 16 12 0.050 0.006 8.3</td>
</tr>
<tr>
<td></td>
<td>SCC25/CP[2]</td>
<td>20 8 9 0.016 0.011 1.4</td>
</tr>
<tr>
<td></td>
<td>SCC25/CP[3]</td>
<td>20 12 5.8 0.016 0.015 1.1</td>
</tr>
<tr>
<td></td>
<td>SCC25/CP[4]</td>
<td>20 8 4.6 0.046 0.010 4.6</td>
</tr>
</tbody>
</table>

a CDDP was given as a 30-min pulse and MTX as a continuous treatment (about 2 weeks). Ratio = resistant (R) cells/sensitive SCC25 controls (S) in the same experiment.

b Resistant cells were grown in drug-free medium for the indicated time.

RESULTS

MTX Cross-Resistance in CDDP-resistant Cells. The concomitant acquisition of CDDP and MTX resistance in SCC25 cells exposed during 20 months to increasing CDDP selection pressure is shown in Table 1. The IC_{50} of CDDP (30-min pulse) for the SCC25/CP[1] cells was 0.2 mM as compared with 0.016 mM for the parental SCC25 line. CDDP resistance in the SCC25/CP[1] line was therefore 12-fold. No substantial changes in CDDP resistance were observed when the cells were grown in the absence of drug for up to 1 year or when weekly CDDP treatment was resumed. The IC_{50} of MTX for the SCC25/CP[1] cells following continuous (14-day) drug exposure was 0.05 µM as compared with 0.006 µM for the parental SCC25 line. For the SCC25/CP[2] and SCC25/CP[3] cells, which were kept out of CDDP, the IC_{50} of MTX (14-day treatment) declined to 0.016 µM. For the SCC25/CP[4] cells, which were obtained by treatment with CDDP, the IC_{50} rose to 0.046 µM. The levels of resistance to MTX (14-day treatment) in the SCC25/CP[1] and SCC25/CP[4] cells were 8- and 5-fold, respectively. In the SCC25/CP[2] and SCC25/CP[3] cells, on the other hand, MTX resistance was negligible. There was thus an apparent congruence between MTX resistance and CDDP resistance in these cells.

Generation Time. In order to estimate the proliferative rate of the various cells, we compared their generation times (Table 2). The parental SCC25 cells had a generation time of 44 h, while this value for the SCC25/CP[1] cells was 58 h. For the SCC25/CP[3] and SCC25/CP[4] cells, however, the generation times were only 22 and 28 h, respectively. Thus, MTX resistance was associated with a decreased proliferative rate in the SCC25/CP[1] cells but an increased rate in the SCC25/CP[4] cells. Moreover, restoration of MTX sensitivity in the SCC25/CP[3] cells was not accompanied by a change in proliferation relative to the SCC25/CP[1] cells.

DHFR Content. In order to determine whether MTX resistance in the CDDP-resistant cells was associated with an increase in amount of [3H]MTX binding, lysates of the resistant cells and of the parental SCC25 line were compared (Table 2). The ratio of [3H]MTX (pmol DHFR/mg protein) bound in lysates of the SCC25/CP[1] cells relative to the SCC25 cells was 2.4 ± 0.3 (SD) (n = 4). The corresponding ratios with the SCC25/CP[3] and SCC25/CP[4] cells were 1.6 ± 0.3 (n = 3) and 1.6 ± 0.2 (n = 3), respectively. MTX resistance in the SCC25/CP[1] cells was therefore associated with a small (<2-fold) increase in DHFR content in relation to the SCC25 cells, as measured by the ligand-binding assay. There was only partial return to the parental DHFR level in the SCC25/CP[3] cells even though these had lost MTX resistance (see above). We concluded from these results that, although some change in DHFR content did occur when SCC25 cells were treated with

Table 1: Collateral MTX resistance in human head and neck SCC lines with acquired resistance to CDDP

<table>
<thead>
<tr>
<th>Cells</th>
<th>+CDDP (0–0.2 mM)</th>
<th>−CDDP</th>
<th>+CDDP (0.2 mM)</th>
<th>CDDP IC_{50} (µM)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC25/CP[1]</td>
<td>20</td>
<td>16</td>
<td>12</td>
<td>0.050 0.006 8.3</td>
</tr>
<tr>
<td>SCC25/CP[2]</td>
<td>20</td>
<td>8</td>
<td>9</td>
<td>0.016 0.011 1.4</td>
</tr>
<tr>
<td>SCC25/CP[3]</td>
<td>20</td>
<td>12</td>
<td>5.8</td>
<td>0.016 0.015 1.1</td>
</tr>
<tr>
<td>SCC25/CP[4]</td>
<td>20</td>
<td>8</td>
<td>4.6</td>
<td>0.046 0.010 4.6</td>
</tr>
</tbody>
</table>

a CDDP was given as a 30-min pulse and MTX as a continuous treatment (about 2 weeks). Ratio = resistant (R) cells/sensitive SCC25 controls (S) in the same experiment.

b Resistant cells were grown in drug-free medium for the indicated time.
The DHFR content measured in cell lysates by radioligand-binding assay using \[^{3}H\]MTX as described (7). Each value is the mean ± range for \(n\) experiments (in parentheses) on separate days. The relative DHFR content represents the pmol DHFR/mg protein in an extract of the resistant cells divided by the pmol DHFR/mg protein in an extract of the parental SCC25 cells determined in the same experiment. The following ranges of values for the DHFR content (pmol/mg protein) were obtained: SCC25, 0.39-1.8; SCC25/CDDP[1], 0.89-2.0; SCC25/CDDP[3], 0.98-3.6; SCC25/CDDP[4], 1.2-3.1. The variability in DHFR content determined on different days is thought to be due, in part, to the fact that the assays were done with different batches of charcoal.

Cells were incubated with 0.2 \(\mu\)M [\(^{3}H\)]MTX for 2 h in folate-free medium containing 2 mM L-glutamine and no serum (7).

CDDP, this change was not sufficient to explain the level of resistance.

MTX Uptake. Velocities of unidirectional MTX influx were measured in the various cell lines in order to ascertain whether collaterally acquired MTX resistance was associated with impaired transport. Experiments were performed in folate-free medium containing no serum in order to maximize uptake. As shown in Table 2, the resistant SCC25/CP[1] cells took up MTX with a \(V_0\) of 0.014 ± 0.004 pmol/mg protein/min, a value identical with the \(V_0\) of the wild-type SCC25 line. The SCC25/CP[3] and SCC25/CP[4] cells, on the other hand, had \(V_0\) values of 0.093 ± 0.008 and 0.042 ± 0.006 pmol/mg protein/min, respectively. It thus appeared that MTX resistance collaterally developed in the wild-type SCC25 cells was not associated with a decreased rate of MTX influx. In contrast, reestablishment of resistance in the SCC25/CP[4] cells was accompanied by a 2-fold decrease in MTX influx rate.

Plateau levels of MTX calculated from a first-order exponential model (Table 2) were also compared for the various cells in order to determine whether there was a correlation with the DHFR content measured in cell lysates by radioligand-binding assay. The calculated plateau level for the parental SCC25 cells was 0.75 ± 0.11 pmol/mg protein, whereas for the other cells this level was 3- to 4-fold higher.

MTX Polyglutamylated. The distribution of radioactive MTX polyglutamates in extracts of cells treated for 24 h with 0.2 or 2.0 \(\mu\)M [\(^{3}H\)]MTX was analyzed by high-performance liquid chromatography to determine whether a polyglutamylated defect was associated with CDPD-induced MTX resistance. As shown in Table 3, the parental SCC25 cells efficiently converted MTX to the noneffluxing long-chain polyglutamates MTX(G4-6) and there was a commensurate increase in the latter were less efficient in producing long-chain polyglutamates MTX(G4-6) and there was a commensurate increase in the MTX(G3) and MTX(G2-3) fractions.

Polyglutamylation of MTX in the revertant SCC25/CP[3] cells was not exactly the same as in the SCC25/CP[1] line, although the difference was evident only at 0.2 \(\mu\)M MTX. The long-chain species MTX(G4-6) accounted for 73% of the total intracellular drug pool in the revertant cells. Thus, the revertants appeared to have the same ability to polyglutamylate MTX as the original MTX-sensitive SCC25 cells. At 2.0 \(\mu\)M MTX, the differences in polyglutamylation between SCC25/CP[3] and SCC25/CP[1] cells were minor, and the pattern of polyglutamylation did not return to that of the parental line. The total drug pool in SCC25/CP[3] cells was 4-fold higher than in SCC25 or SCC25/CP[1] cells at the lower extracellular MTX concentration, whereas at the higher concentration this ratio was only 2-fold.

Finally, examination of the polyglutamylation pattern in SCC25/CP[4] cells revealed that the MTX(G4-6) fraction was diminished in comparison with SCC25 or SCC25/CP[3] cells at 0.2 \(\mu\)M extracellular MTX and was similarly decreased in comparison with SCC25 cells, but not SCC25/CP[3] cells, at 2.0 \(\mu\)M extracellular MTX. Total intracellular pools in the SCC25/CP[4] cells were comparable to those in SCC25 and SCC25/CP[1] cells and clearly lower than in SCC25/CP[3] cells.
DISCUSSION

Previous work in our laboratory on low-level (<20-fold) MTX resistance induced in human squamous cell carcinoma lines by MTX selection pressure showed resistance to be multifactorial (8, 15). Furthermore, there appeared to be heterogeneity with respect to the biochemical determinants of MTX sensitivity even among parent lines. While studies performed to date with these lines have by no means dealt with all the possible modes of MTX resistance, some of the cellular properties that changed when resistance was induced with MTX were the generation time, DHFR content, rate of MTX influx, uptake plateau levels, and ability to form long-chain polyglutamates. The basis of resistance to CDDP in the SCC25/CP[1] line is likewise multifactorial, reflecting a combination of decreased drug uptake, increased protein sulfhydryl content, and increased glutathione S-transferase activity (3). Perhaps not surprisingly in view of this heterogeneity of biochemical phenotype, resistance to CDDP in SCC25/CP[1] cells appears to be accompanied by subtle variations in alkylating agent sensitivity, as illustrated by the fact that the cells were 5-fold resistant to melphan and 3-fold resistant to 4-hydroperoxycyclophosphamide, but not appreciably resistant to nitrogen mustard, bis(chloroethyl)-nitrosourea, and several other drugs of the alkylating agent type (2).

The results reported here suggest that a complex phenotypic pattern exists when MTX resistance is induced with CDDP rather than MTX. Thus, in comparison with the parental SCC25 cells, the SCC25/CP[1] cells showed an increase in generation time, an increase in DHFR content, and, depending on the extracellular MTX concentration, a decrease in formation of long-chain polyglutamates MTX(G4-e). The rate of unidirectional MTX transport remained unchanged, but, as expected from the DHFR content, an increased steady-state drug level was observed.

An interesting feature of the SCC25/CP[3] cells was that MTX sensitivity was regained despite the fact that the DHFR level did not completely return to that of the SCC25 cells. The SCC25/CP[3] cells, on the other hand, showed a striking increase in their V0 for MTX uptake and also in their rate of proliferation. This suggested that these "revertant" cells may have a high requirement for reduced folates to sustain their one-carbon metabolism. Rapid proliferation could therefore make the cells vulnerable to MTX even though their DHFR level remained somewhat higher than normal. Scanlon et al. recently reported that CDDP causes a decrease in methionine-active transport (16) and an increased amount of reduced folates in human ovarian carcinoma cells in culture (17). A byproduct of this expanded folate pool is decreased MTX sensitivity. The change in reduced folate pool size was postulated to be due to the attempt of the cell to compensate for a decreased ability to take up methionine. A corollary to this would be that when cells conditioned to grow in the presence of CDDP are taken out of CDDP, reduced folate pools and MTX sensitivity should both return to normal. Experiments to determine whether collateral MTX resistance in CDDP-resistant SCC25 sublines is similarly associated with an increase in cellular reduced folates are in progress in our laboratory.

The reestablishment of MTX resistance when SCC25/CP[3] cells were treated again with CDDP was interesting because it suggested that our initial observation of resistance in the cloned cells was probably not just an isolated event. It should be noted that, while the SCC25/CP[1] cells were of clonal origin, the SCC25/CP[4] cells obtained from SCC25/CP[3] cells upon retreatment with CDDP were not cloned. Therefore, the resistance phenotype observed in this case was evolving in a population of cells which, by then, had probably become heterogeneous. The SCC25/CP[4] cells showed some properties similar to those of the clonally derived MTX-resistant line SCC25/CP[1], but other properties which were distinctly different. The DHFR level remained elevated and polyglutamylation again was somewhat impaired, but now the generation time and MTX uptake rates were also higher. Indeed, with respect to the latter two parameters, the SCC25/CP[4] cells more closely resembled sensitive SCC25/CP[3] cells.

A question relating to the original isolation of the MTX-resistant SCC25/CP[1] cell line by cloning at 20 months (see "Materials and Methods") was whether other clones isolated at the same time would also give rise to MTX-resistant cells on further treatment with CDDP. We examined a number of these clones for CDDP resistance and MTX cross-resistance (data not shown) but did not find any with the same level of CDDP or MTX resistance as the SCC25/CP[1] cells when MTX sensitivity was assayed with continuous drug treatment. A human lymphoma cell (Raji) with 7-fold CDDP resistance was also tested and found not to be cross-resistant to continuous MTX treatment (2,3). Since it was possible that MTX cross-resistance might be more prominently expressed in cells exposed to MTX for a shorter time, we also tested several of our original CDDP-resistant clones for resistance to 30-min, rather than continuous, MTX treatment. Support for this idea came from our earlier observation that SCC25/CP[1] cells (from clone Pr2) were approximately 3-fold more resistant to a 30-min pulse than to continuous 14-day MTX treatment (2). Two other CDDP-resistant clones (Pi1A1 and Pi5A2) were tested after 20 months of continuous drug escalation and were found to be 3- to 6-fold resistant to a 3-min pulse of MTX (data not shown). It thus appears that the production of CDDP resistance may give rise to multiple clones with varying degrees of collateral resistance to MTX pulse treatment. Since the survival of these clones need not be based on the same favorable phenotype, it is likely that MTX resistance acquired during CDDP treatment is biochemically heterogeneous. Moreover, resistance appears to be greater when the cells are exposed to MTX for a short time. Treatment of cultured cells to MTX for short periods may be considered to approximate a pulse dose in the clinical setting where continuous exposure may be viewed as being representative of continuous infusion.

The fact that a decreased MTX(G4-e) fraction was observed in the SCC25/CP[1] cells in spite of normal uptake kinetics and a 24-h intracellular drug pool at least 20-fold higher than the DHFR level suggests that deficient MTX polyglutamylation was not simply due to inadequate substrate concentration in the cell. A possible explanation for decreased formation of MTX(G4-e) in the SCC25/CP[1] cells was that they had a lower proliferation rate than the parental line (Table 1). MTX polyglutamylation in cultured human breast carcinoma lines has been found to be highly dependent on the state of confluence (18). It was therefore possible that we were observing another example of this phenomenon. We also considered the possibility that diminished polyglutamylation in SCC25/CP[1] cells was due to differences in cellular GSH level. An elevation in cellular GSH has been seen in a human ovarian carcinoma line made resistant to CDDP in culture (19), and cellular GSH has been reported to influence MTX uptake and polyglutamylation in hepatocytes (20). This explanation seems unlikely, however, because a difference in cellular GSH between SCC25 and SCC25/CP[1] cells was not observed (21). Other plausible explanations for the apparent defect in MTX(G4-e) production
in SCC25/CP[1] and SCC25/CP[3] cells are that these cells have low cellular polyglutamate synthetase activity (21) or high cellular polyglutamate hydrolase (conjugase) activity (22), or that there is competition by endogenous reduced folates as polyglutamylation substrates (23). We are currently undertaking experiments to address these possibilities.

CDDP is known to be mutagenic to mammalian cells (24–28). In one study (24), for example, treatment of Chinese hamster ovary cells in suspension culture with 2 μM CDDP for 20 h (7 population doublings) resulted in 78% survival and a mutation frequency of $1.5 \times 10^{-4}$ with respect to AG resistance. This mutation frequency was 5–7-fold higher than the spontaneous mutation frequency, and a comparable increase was observed when the selecting agent was TG. Similar results with hamster cell lines were reported by other workers (25–28). Resistance to AG and TG is associated with a decrease in activity of HGPRTase, the enzyme responsible for bioactivation of these drugs as their 5'-nucleotides, and with a large increase in sister chromatid exchange. The effect of CDDP in mammalian cells is believed to involve primarily base substitutions (point mutations) rather than frameshift mutations or base deletions. Substitutions involving the HGPRTase gene are considered to have a relatively high likelihood of producing viable mutants because HGPRTase is not essential to the growth of most cells. This is not true for mutations involving genes for essential enzymes such as ATPase because these mutations tend to be lethal. In a recent investigation (28), CDDP was found to be only 5-fold less potent than nitrogen mustard in inducing TG resistance, and sister chromatid exchange was again found to be commensurate with mutagenicity. These results leave little doubt that, if MTX resistance were to arise via a mutational event triggered by another drug, CDDP could certainly play the role of the mutagen. However, a distinction between resistance to MTX and resistance to AG or TG is that, while mutations of the gene for HGPRTase result in loss of enzyme activity, resistance involving DHFR most often occurs via an increase in enzyme level (29). In the present work we found that MTX resistance induced collaboratively during selection for CDDP resistance was associated, at least in part, with an increase in DHFR content that occurred without active selection for this trait. It thus appears that CDDP treatment does not necessarily give rise only to cells with decreased levels of a nonessential enzyme such as HGPRTase but can also produce cells with an increase of an enzyme which is essential for proliferation, such as DHFR.

The manner in which CDDP induces increased DHFR levels in cells is unknown but may involve a mechanism analogous to the one recently proposed by Schimke et al. (29–34; for general discussions of DHFR gene amplification, see Refs. 29 and 34) to explain how MTX resistance and augmented DHFR production occur in cells treated during passage through a narrow window of the G2-S interface of the cell cycle with DNA-damaging agents or DNA synthesis inhibitors. According to the Schimke model, if DNA synthesis is transiently blocked during this discrete part of the cycle, when the DHFR gene is transcriptionally most active, the result is overtranscription of the DHFR message within a single cycle of replication. To account for this, Schimke postulates that there may occur concomitant overexpression of certain unidentified regulatory proteins the normal role of which is to stimulate transcription. The extent to which the proposed mechanism operates may vary with the cytokinetic properties of the cell and the precise timing of the arrest of DNA synthesis. Treatments used successfully thus far to test the model have included UV and 2-(N-acetoxy-N-acetylaminofluorene (29–30), hydroxyurea (31, 35, 36), N-methyl-N'-nitro-N-nitrosoguanidine (35), phosphor esters (36), aphidicolin (cited in Ref. 29), and hypoxia (33). Moreover, it now seems likely that many other agents can have a similar mode of action in inducing MTX resistance via DHFR overproduction, and it is possible that the findings reported here with CDDP are an example of this effect. It would be of interest to determine how well CDDP can induce DHFR reexpression within a single cell cycle in comparison with other agents, including especially alkylating agents. It should be stressed, however, that there is a major difference between our treatment of the cells and the one used by Schimke et al. in their studies. Whereas these workers selected cells for MTX resistance after a single round of DNA arrest, in producing CDDP-resistant SCC25 cells we exposed them once a week to a 30-min pulse of CDDP for up to 40 months before testing them for MTX collateral resistance.

The present finding that treatment of human head and neck squamous cell carcinoma in culture with CDDP produces collateral MTX resistance is novel and potentially of clinical significance if it can be shown to occur in vivo. There is continuing interest in the use of CDDP and MTX with bleomycin and other drugs in chemotherapy and combined radiotherapy-chemotherapy of head and neck cancer (4–7, 37–40). While resistance is known to develop in patients treated with these regimens, there have been no studies to determine how much, if any, of the MTX resistance is a secondary effect associated with prior treatment with other drugs or radiation. Since CDDP and MTX are normally given together or at close time intervals, opportunities to clinically observe the type of collateral resistance that can be produced in culture are limited. Nonetheless, the finding that MTX resistance is inducible with CDDP in cultured cells not previously exposed to MTX is of interest because it demonstrates that this has the potential to occur, if only in rare instances, in the clinic.

REFERENCES

passage squamous cell carcinoma lines derived from patients with known
11. Wright, J. E., Rosowsky, A., Waxman, D. J., Trites, D., Cuochi, C. A.,
Flato, J., and Frei, E. III. Metabolism of methotrexate and γ-tert-butyl
methotrexate by human leukemic cells in culture and by hepatic aldehyde
radiochemical ligand binding assay for methotrexate. Anal. Biochem., 70:
measurements with the Folin phenol reagent. J. Biol. Chem., 193: 265–275,
1951.
15. Frei, E., III, Rosowsky, A., Wright, J. E., Cuochi, C. A., Lipkpe, J. A., Ervin,
T. J., Jolivet, J., and Haseltine, W. A. Development of methotrexate resist-
ance in a human squamous cell carcinoma of the head and neck in culture.
systems in K562 cells sensitive and resistant to cis-diaminedichloropla-
for cisplatin and fluorouracil synergism in human ovarian carcinoma cells.
18. Kennedy, D. G., Van Den Berg, H. W., Clarke, R., and Murphy, R. F. The
effect of the rate of cell proliferation on the synthesis of methotrexate poly-
yglutamates in two human breast cancer cell lines. Biochem. Pharmacol.,
19. Hamilton, T. C., Winker, M. A., Batist, G., Behrens, B. C.,
Tsuruo, T., Grotzinger, K. R., McKey, W. M., Young, R. C., and OzoIs, R. F.
Identification of a new human breast cancer cell line resistant to methotrexate.
transport and accumulation in hepatocytes—a consequence of variations in
562 cells with multiple defects, including diminished formation of methotrexate
M. Hydrolytic cleavage of methotrexate γ-polyglutamates by polyglutamy-
lyl hydrolase derived from various tumors and normal tissues of the mouse.
23. Balinska, M. Regulation of methotrexate polyglutamate formation in Ehrlich
ascites carcinoma cells by endogenous folate pool. Acta Biochim. Polon., 33:
31–37, 1986.
induced mutations to 8-azaguanine resistance in Chinese hamster ovary cells.
A., and Hise, A. W. A quantitative assay of mutation induction at the
hydroxanthine-guanine phosphoribosyl transferase locus in Chinese hamster
ovary cells (CHO/HGPRT system): utilization with a variety of agents.
26. Singh, B., and Gupta, R. S. Mutagenic responses of thirteen anticancer drugs
mutation induction at multiple genetic loci and on sister chromatid exchanges
27. Plooy, A. C. M., van Dijk, M., and Lohman, P. H. M. Induction and repair of
dNA cross-links in Chinese hamster ovary cells treated with various
platinum coordination compounds in relation to platinum binding to DNA,
2051, 1984.
Comparison of 6-thioguanine-resistant mutation and sister chromatid ex-
changes in Chinese hamster V79 cells with forty chemical and physical agents.
resistance and dihydrofolate reductase gene amplification by treatment of
31. Mariani, B. D., and Schirme, R. T. Gene amplification in a single cell cycle
32. Hill, A. B., and Schirme, R. T. Increased gene amplification in LS178Y
mouse lymphoma cells with hydroxyurea-induced chromosomal aberrations.
33. Reece, C. S., Hoy, C., and Schirme, R. T. Transient hypoxia enhances the
frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells.
34. Schirme, R. T., Sherwood, S. W., Hill, A. B., and Johnston, R. N. Overrep-
lication and recombination of DNA in higher eukaryotes potential conse-
2161, 1986.
resistance and dihydrofolate reductase amplification in Chinese hamster cells.
36. Barsoum, J., and Varshavsky, A. Mitogenic hormones and tumor promoters
greatly increase the incidence of colony-forming cells bearing amplified
hydrofolate reductase genes. Proc. Natl. Acad. Sci. USA, 80: 5330–5334,
1983.
randomized Phase I trial of cisplatin with or without methotrexate for
recurrent squamous cell carcinoma of the head and neck. A Northern Cali-
38. Kuden, A., Zidan, J., Cohen, Y., and Robinson, E. Multidrug chemotherapy
using bleomycin, methotrexate and cisplatin alone or combined with radio-
1983.
Intensive sequential chemotherapy with bleomycin, oncovin, mitomycin C,
and methotrexate followed by Adriamycin, cisplatin, and cyclophosphamide
51–58, 1983.
40. Espena, P., Smith, F., Abrams, J., Hairaid, D., Ueno, W., Weoley, P.,
and Schein, P. Phase II study of the cis-diammine-dichloroplatinum (cis-
platin), bleomycin, and methotrexate for advanced squamous cell carcinoma

5918

Downloaded from cancerres.aacrjournals.org on July 22, 2017. © 1987 American Association for Cancer Research.
Collateral Methotrexate Resistance in Cultured Human Head and Neck Carcinoma Cells Selected for Resistance to cis-Diamminedichloroplatinum(II)

Andre Rosowsky, Joel E. Wright, Carol A. Cucchi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/22/5913

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.