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Advances in Brief

In Vitro and *in Vivo* Resistance to Cisplatin in Cells That Have Lost DNA Mismatch Repair[1]

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Abstract

***In vitro* studies have shown that loss of DNA mismatch repair due to lack of either hMSH2 or hMLH1 activity results in low-level resistance to cisplatin but not to oxaliplatin, an analogue that produces a different type of DNA adduct. No information is currently available on whether this low-level resistance is sufficient to result in enrichment of mismatch repair-deficient cells during drug exposure *in vitro* or to account for clinical failure of treatment *in vivo*. Mixed populations of cells containing a minority of DNA mismatch repair-deficient cells constitutively expressing green fluorescence protein were exposed repeatedly *in vitro* to cisplatin and oxaliplatin. Treatment with cisplatin resulted in a gradual enrichment for DNA mismatch repair-deficient cells, whereas treatment with oxaliplatin did not. $MSH2^{-/-}$ and $MSH2^{+/+}$ embryonic stem cells were established as xenografts in athymic nude mice. Animals were treated 48 h after tumor implantation with a single LD_{10} dose of either cisplatin or oxaliplatin. $MSH2^{-/-}$ tumors were significantly less responsive to cisplatin than $MSH2^{+/+}$ tumors, whereas there was no difference in sensitivity to oxaliplatin. These results demonstrate that the degree of cisplatin resistance conferred by loss of DNA mismatch repair is sufficient to produce both enrichment of mismatch repair-deficient cells during treatment *in vitro* and a large difference in clinical responsiveness *in vivo*. The results identify loss of DNA mismatch repair as a mechanism of resistance to cisplatin but not oxaliplatin.**

Introduction

Cisplatin and carboplatin are widely used chemotherapeutic agents, but their clinical effectiveness is frequently limited by the emergence of drug-resistant tumor cell populations. For example, the majority of human ovarian carcinomas respond initially to treatment with these agents, but most of these tumors recur, and, when they do so within 6 months, most are resistant to treatment with cisplatin (1).

Loss of DNA mismatch repair occurs very frequently in hereditary nonpolyposis colon cancers and has been observed in a wide variety of sporadic human cancers as well (reviewed in Ref. 2). We have recently reported the novel finding that loss of DNA mismatch repair produces 2-fold resistance to both cisplatin and carboplatin, drugs that form the same adducts in DNA, but not to analogues such as oxaliplatin that produce several other types of adducts (3). In addition to intrinsic resistance to cisplatin, DNA mismatch repair-deficient cells have high mutation rates not only in noncoding microsatellite sequences but also in genes such as *HPRT* (4) and *APRT* (5) and at the locus controlling ouabain resistance (6).

No information is currently available on whether loss of DNA mismatch repair causes resistance to cisplatin *in vivo*, and in fact there is little information about how much resistance is required to account for clinical failure of treatment. This laboratory has previously reported data suggesting that only very small degrees of resistance (<2-fold) are sufficient for a tumor to lose *in vivo* responsiveness to cisplatin (7). To investigate this issue further, we have examined the ability of cisplatin and oxaliplatin to enrich for repair-deficient cells during treatment *in vitro* and have tested the *in vivo* sensitivity of DNA mismatch repair-proficient and -deficient cells in a xenograft model.

Materials and Methods

Cell Lines and Materials. The hMLH1-deficient human colorectal adenocarcinoma cell line HCT116 was obtained from the American Type Culture Collection (ATCC CCL 247); the subline complemented with chromosome 3 (clone HCT116/3–6, identified here as HCT116+ch3) was obtained from Drs. C. R. Boland and M. Koi (8). HCT116 and HCT116+ch3 cell lines were maintained in Iscove's modified Dulbecco's medium (Irvine Scientific, Irvine, CA) supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum. The chromosome-complemented subline was maintained in medium supplemented with geneticin (400 μg/ml; Life Technologies, Inc., Gaithersburg, MD). The isogenic wt-2 ($MSH2^{+/+}$) and the double knockout dMsh2–9 ($MSH2^{-/-}$) embryonic stem cell lines were obtained from Dr. H. te Riele (9) and maintained in a 5% CO_2 atmosphere at 37°C on irradiated mouse embryonic fibroblast feeder layers in DMEM (Irvine Scientific) supplemented with nonessential amino acids, sodium pyruvate, penicillin/streptomycin, leukemia inhibitory factor (10^3 units/ml; Life Technologies, Inc.), and 10% heat-inactivated fetal bovine serum. The absence and presence of expression of hMLH1 in HCT116 and HCT116+ch3 as well as expression of MSH2 in dMsh2–9 and wt-2 ES[3] cells was verified by immunoblot analysis (data not shown). All cell lines tested negative for contamination with *Mycoplasma* spp. Cisplatin was kindly provided by Bristol-Myers Squibb Co. (Princeton, NJ). Oxaliplatin [(*trans*-(L)-1,2-diaminocyclohexane)oxalatoplatinum(II)] was a gift from Debiopharm (Lausanne, Switzerland).

Preparation of GFP Expressing DNA Mismatch Repair-deficient Cells. pCLNCGFP was constructed by removing the GFP cycle three-mutant cDNA from the Alpha+GFP vector (Maxygen, Palo Alto, CA) with *Xba*I and *Cla*I and filling in the *Xba*I cut ends with Klenow DNA polymerase. The resulting 746-bp fragment was cloned into the *Cla*I and Klenow-filled *Hin*dIII sites behind the cytomegalovirus promoter in pCLNCX (10, 11). Amphotropic retrovirus was produced by cotransfecting 2×10^6 late-passage 293 cells with 20 μg of vector, either pCLNCX or pCLNCGFP, and the pCL-Ampho packaging vector as described by Naviaux *et al.* (11). Viral supernatant was harvested 24 and 48 h after transfection. Viral titers were determined on BALB/c 3T3 cells by G418-resistant colony formation. HCT116 cells were infected with viral supernatant three times over a 12-h period in the presence of polybrene (8 μg/ml). Infected cells were selected for 9 days with G418 (400 μg/ml), and the resulting population was identified as HCT116-GFP; GFP was

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[3] The abbreviations used are: ES, embryonic stem; GFP, green fluorescence protein.

expressed in high levels in 90–95% of these cells. Cells were subjected to flow cytometric analysis on a Becton Dickinson FACScan using an argon ion laser tuned to 488 nm to identify GFP-positive cells. Fluorescence was observed with a 515/545 bandpass filter.

***In Vitro* Enrichment Experiments.** HCT116+ch3 and HCT116-GFP cells were mixed in a 95:5 ratio and analyzed by flow cytometry to document that the population contained 5% GFP-expressing cells. The cell populations were exposed in suspension to cisplatin (25 μM or 50 μM) and oxaliplatin (25 μM or 50 μM) for 1 h, washed with PBS, resuspended in media, and maintained in culture. Flow cytometric analysis was repeated 5 days later, at which time a second cycle of drug exposure was initiated. A total of four treatment cycles of 5 days each was completed. Each experiment was performed three separate times for each drug concentration.

Cytotoxicity Assays. Cisplatin and oxaliplatin were dissolved immediately before use in a 0.9% NaCl solution to produce a stock solution of 1 mM. The ES cells were separated from the feeder layer by trypsinizing all cells and then depleting the fibroblasts from the suspension by allowing them to reattach to a plastic surface for 1 h. Before seeding, ES cells were exposed in suspension to appropriate amounts of the drugs for 1 h and then washed once with PBS. Clonogenic assays were performed by seeding 350 cells from a single-cell suspension into 60-mm gelatin-coated plastic dishes in 5 ml of drug-free media. Colonies of at least 50 cells were scored visually after 5 days. Each experiment was performed three times using triplicate cultures for each drug concentration. IC_{50} values were estimated using logarithmic interpolation at a relative plating efficiency of 0.5.

Xenograft Experiments. ES cells in log phase were harvested by trypsinization, separated from the fibroblast feeder layer, washed in PBS, and resuspended in serum-free culture media at a concentration of 5×10^6 cells/ml before s.c. implantation into mice. Female BALB/c-*nu/nu* mice, 7–8 weeks old (22 ± 2 g; Simonsen, Inc., Gilroy, CA), were quarantined for 1 week before the study and allowed access to food and water *ad libitum*. A total of 36 mice were implanted with 10^6 cells bilaterally into the left and right axillary and flank regions (right side, dMsh2–9; left side, wt-2). Animals were randomized into treatment groups (cisplatin, oxaliplatin, and drug vehicle) 48 h after tumor implantation. Each group consisted of 12 animals bearing two $MSH2^{+/+}$ and two $MSH2^{-/-}$ tumors/animal. LD_{10} doses of either cisplatin (10 mg/kg) or oxaliplatin (12 mg/kg; Ref. 12) were administered i.p. 48 h after tumor implantation. Animal weights were recorded daily, and the tumor volume of each of the four tumors/animal was determined daily by measuring crossed diameters with Vernier calipers and estimating volume from the formula (volume = length × $(\text{width})^2/2$; Ref. 13). All animals of a group were euthanized when calculated tumor volume reached 1000 mm^3. The care and use of the animals were in accordance with institutional guidelines.

Data Analyses. Mean ± SD values are indicated for all data sets. ANOVA for repeated measures was used for analyzing the extent of enrichment during the *in vitro* selection. The mean volumes of the two $MSH2^{+/+}$ and the two $MSH2^{-/-}$ tumors of each animal were used for the calculations. Two-sided paired *t* tests with Bonferroni adjustment for multiple comparisons were performed to compare the effect of MSH2 on drug sensitivity. The drug effects on tumor growth were compared using one-way ANOVA, and *post-hoc* comparisons were performed using the Scheffé procedure.

Results

Effect of Loss of DNA Mismatch Repair on Enrichment *in Vitro*. Parental DNA mismatch repair-deficient HCT116 cells were infected with a retrovirus encoding the *GFP* gene driven by a cytomegalovirus promoter, and a population that stably expressed GFP was selected. The HCT116 and HCT116-GFP cells, as well as the HCT116 cells with an empty pCL vector, were tested using the clonogenic assay, and no difference in sensitivity to cisplatin or oxaliplatin was found (data not shown). A population containing 5% DNA mismatch repair-deficient GFP-expressing cells and 95% DNA mismatch repair-proficient HCT116+ch3 cells was prepared by mixing and subjected to four cycles of a 1-h exposure to cisplatin or oxaliplatin followed by a 5-day recovery period. Fig. 1 shows that 5 days after a single 1-h exposure to an IC_{50} concentration of cisplatin (25 μM for HCT116 cells) the treated population contained 53% more

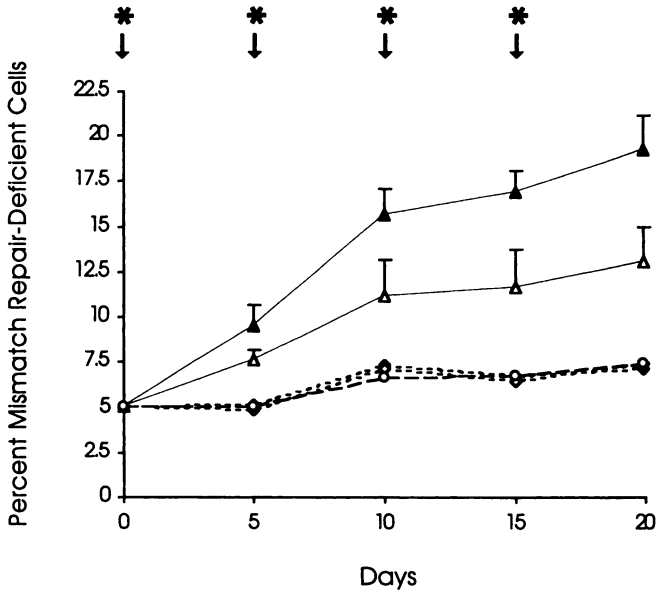

Fig. 1. Effect of loss of DNA mismatch repair on enrichment *in vitro* in a population containing 5% DNA mismatch repair-deficient GFP-expressing cells and 95% DNA mismatch repair-proficient HCT116+ch3 cells subjected to four cycles of a 1-h exposure (↕) to either cisplatin (△, 25 μM; ▲, 50 μM) or oxaliplatin (◇, 25 μM; ◆, 50 μM). Each data point represents the mean of three experiments compared with the untreated control population (○). *Bars*, SD.

GFP-expressing mismatch repair-deficient cells than the untreated population; after the second, third, and fourth cycles there were 68, 72, and 77% more GFP-expressing cells in the treated population, respectively. Enrichment was more dramatic when the cells were exposed to an IC_{90} concentration of cisplatin (50 μM for HCT116 cells), and after the fourth cycle of exposure there were 163% more GFP-expressing cells in the treated than in the untreated control population ($P < 0.0001$, ANOVA). In contrast, when the cells were treated with either an IC_{50} (25 μM) or IC_{90} (50 μM) concentration of oxaliplatin, there was no progressive enrichment for the GFP-expressing DNA mismatch repair-deficient cells. Thus, treatment with cisplatin, to which the repair-deficient cells were 2-fold resistant, resulted in rapid enrichment of the population for the resistant cells, whereas treatment with oxaliplatin, to which the deficient cells were not resistant, produced no enrichment.

Effect of Loss of DNA Mismatch Repair on Sensitivity *in Vivo*. Fig. 2 shows the *in vitro* survival of isogenic DNA mismatch repair-proficient and -deficient ES cells as a function of drug concentration following a 1-h exposure to either cisplatin or oxaliplatin. The MSH2-knockout dMsh2–9 cells were 2.1-fold more resistant to cisplatin than the DNA mismatch repair-proficient wt-2 cells [IC_{50}, 12.2 ± 1.3 μM *versus* 5.9 ± 0.5 μM (SD); $n = 3$; $P < 0.05$ in a two-sided *t* test]. In contrast, there was no significant difference in sensitivity to oxaliplatin [IC_{50}, 6.4 ± 0.6 μM *versus* 6.8 ± 0.7 μM (SD); $n = 3$; $P = 0.48$ in a two-sided *t* test].

BALB/c-*nu/nu* mice were inoculated s.c. with either the DNA mismatch repair-proficient wt-2 $MSH2^{+/+}$ or -deficient dMsh2–9 $MSH2^{-/-}$ ES cells. Fig. 3 shows tumor volume as a function of time following a single i.p. injection of either isotonic saline or an LD_{10} dose of cisplatin or oxaliplatin given 48 h after tumor implantation. In the untreated control animals, the doubling time was similar for the $MSH2^{+/+}$ and $MSH2^{-/-}$ tumors (2.85 ± 0.31 d *versus* 2.90 ± 0.31 d, respectively; $P = 0.71$ in a two-sided *t* test). The LD_{10} dose of cisplatin produced a greater response in the $MSH2^{+/+}$ than in the $MSH2^{-/-}$ tumors; the DNA mismatch repair-proficient tumors shrank in size over the first 10 days after treatment, reaching a nadir

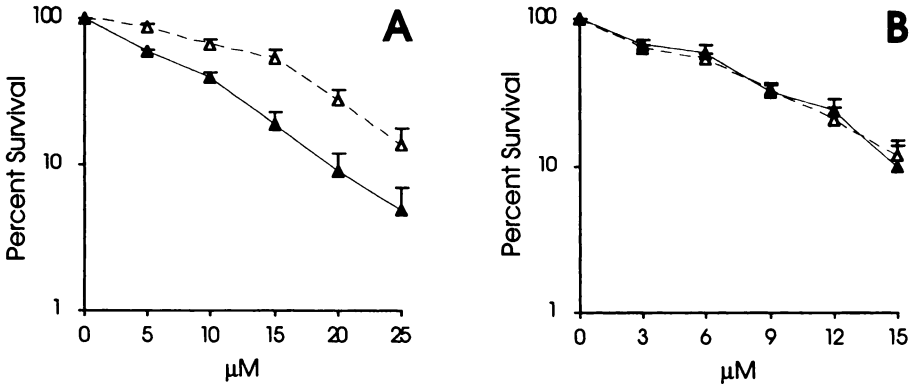

Fig. 2. Clonogenic survival curves for cisplatin (*A*) and oxaliplatin (*B*) for the MSH2-deficient (△, dMsh2–9) and -proficient (▲, wt-2) ES cells. Each data point represents the mean of three experiments performed with triplicate cultures. *Bars*, SD.

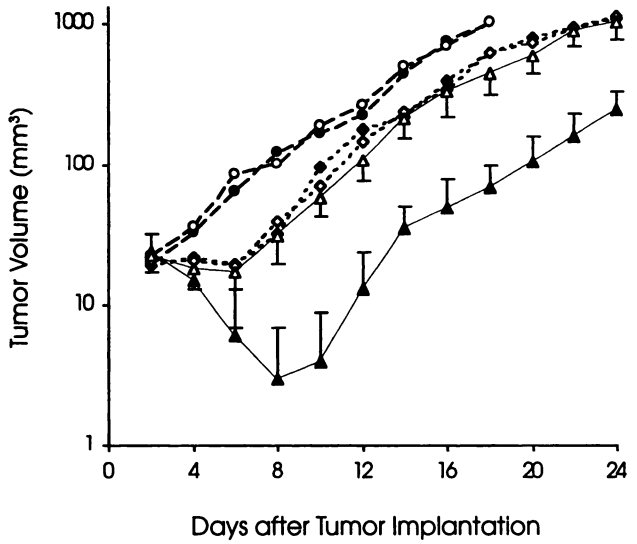

Fig. 3. Tumor volume as function of time. ●, ▲, and ◆, $MSH2^{+/+}$; ○, △, and ◇, $MSH2^{-/-}$. ○, untreated controls; △, cisplatin; and ◇, oxaliplatin. Data are the means of experiments. *Bars*, SD. $n = 12$ for each data point.

volume that averaged only 13% of their starting volume before growth resumed. In contrast, the DNA mismatch repair-deficient tumors suffered a growth delay of only 4 days before resuming growth. The LD_{10} dose of oxaliplatin produced a 4-day delay in growth for both the repair-proficient and -deficient tumors.

The mean tumor volumes on day 18 after tumor implantation for each group are shown in Table 1. Fig. 4 shows the difference between the mean volumes of the tumors on each side for each animal on day 18, the last day for which data for all of the tumors were available. The mean volumes of the $MSH2^{-/-}$ tumors treated with cisplatin were statistically significantly greater than those of the $MSH2^{+/+}$ tumors ($P < 0.0001$). There was no difference in either the control ($P = 0.86$) or in the oxaliplatin-treated group ($P = 0.87$). In none of the groups was there any treatment-related mortality. Thus, the 2.1-fold difference in cisplatin sensitivity between the $MSH2^{+/+}$ and $MSH2^{-/-}$ cells measured *in vitro* translated into a marked difference in tumor responsiveness *in vivo*.

Discussion

Two major conclusions can be drawn from the results of these studies. First, loss of DNA mismatch repair due to loss of MSH2 results in low-level resistance to cisplatin. Second, this low-level resistance is sufficient to produce progressive enrichment for mismatch repair-deficient cells during treatment *in vitro* and a major impairment in responsiveness of the tumor *in vivo*.

We have recently documented that mismatch repair-deficient sublines of the HCT116 human colon carcinoma and HEC59 human endometrial cells demonstrate 2.1- and 1.8-fold resistance to cisplatin, respectively, relative to their repair-proficient counterparts (3, 14). These results suggested that loss of mismatch repair due to either loss of hMLH1 function, such as occurs in the HCT116 cells, or loss of MSH2 function, such as occurs in the HEC59 cells, is sufficient to produce this low-level resistant phenotype. However, the proficient and deficient members of each of these pairs of cell lines were not truly isogenic since to restore DNA mismatch repair activity a whole copy of chromosome 3 carrying a wild-type copy of MLH1 was inserted into the HCT116 cells (8), and a whole copy of chromosome 2 carrying a wild-type copy of MSH2 was inserted into the HEC59 cells (15). Thus, it is conceivable that the differences in cisplatin sensitivity observed in these pairs could have been due to one of the many genes on the inserted chromosome other than the wild-type

Table 1 *Mean tumor volumes on day 18 after tumor implantation*

	Tumor volume (mm^3), mean ± SD	
	$MSH2^{-/-}$	$MSH2^{+/+}$
Control	$1010 \pm 365^{a,b}$	$1029 \pm 338^{c,d}$
Cisplatin	$434 \pm 125^{b,e}$	$68 \pm 31^{d,f}$
Oxaliplatin	$617 \pm 151^{a,e}$	$611 \pm 135^{c,f}$

[a] $P < 0.0001$.
[b] $P < 0.0001$.
[c] $P < 0.0001$.
[d] $P = 0.0001$.
[e] $P = 0.08$.
[f] $P < 0.0001$. $n = 12$ for each group.

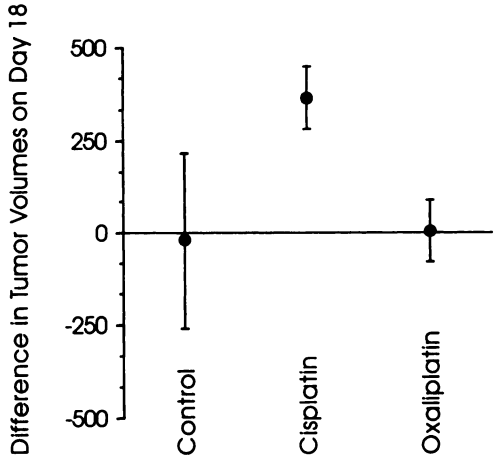

Fig. 4. Differences between the mean volumes of the tumors on each side for each animal on day 18. Each data point represents the mean difference ± 95% confidence interval. $P = 0.86$ for controls, $P < 0.0001$ for cisplatin, and $P = 0.87$ for oxaliplatin.

copy of the missing DNA mismatch repair gene. The mouse pluripotent ES cells, in which both *MSH2* alleles have been knocked out (9), provide a more rigorous test of whether loss of the function of just this one gene is sufficient to produce low-level cisplatin resistance. The 2.1-fold resistance observed in the $MSH2^{-/-}$ ES cells is identical in magnitude to the resistance observed in the hMLH1-deficient HCT116 cells and close to that observed in the hMSH2-deficient HEC59 cells. The similarity in the degree of resistance between three such disparate types of cells suggests a lack of cell line-specific factors that can modulate the impact of loss of mismatch repair.

It has been suggested that the DNA mismatch repair proteins serve as a detector system for the presence of DNA damage (16, 17). Indeed, human MutSα (18), a heterodimer of hMSH2 and GTBP/p160 (19, 20), has been shown to bind to DNA-containing adducts produced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, 6-thioguanine, and cisplatin, and we demonstrated recently that DNA adducts formed by oxaliplatin are not recognized by the DNA mismatch repair system (3). The paradigm is that loss of the detector function of the DNA mismatch repair system results in failure to generate signals that contribute to the cytotoxic effect of cisplatin; since the mismatch repair system appears not to serve as a detector for oxaliplatin adducts, there is no consequence to the loss of mismatch repair with regard to the triggering of apoptosis. From the enrichment experiments we can conclude that the binding of hMSH2-GTBP or hMSH2-hMSH3 dimers alone is not sufficient for detector function because cells containing normal amounts of hMSH2 but lacking hMLH1 (HCT116) were still resistant to cisplatin. This is consistent with the hypothesis that assembly on the damaged DNA of at least either hMSH2-GTBP (19) or hMSH2-hMSH3 (21) and the hMLH1-hPMS2 heterodimer (22) are required before a damage signal can be generated.

The biological significance of this small degree of resistance was a major question. In fact, the issue of how much resistance to cisplatin is required to account for clinical failure of the drug has vexed the field for some time. It has been difficult to answer because of the variability of *in vitro* drug sensitivity assays, and the fact that the opportunity to test fresh tumor tissue from the same patient both prior to treatment and again at recurrence is quite limited. Because of the fact that ES cells require a cisplatin-sensitive fibroblast feeder layer for prolonged propagation, studies of the extent to which low-level cisplatin resistance can mediate enrichment for mismatch repair-deficient cells during treatment could not be addressed in this isogenic system, and we were limited to using the less truly isogenic HCT116 and HCT116+ch3 pair of cells. Nevertheless, the results argue cogently that even a 2-fold difference in cisplatin sensitivity is sufficient to result in rapid enrichment for mismatch repair-deficient cells in a proliferating cell population.

In the xenograft model, tumors generated by the 2-fold resistant $MSH2^{-/-}$ cells were much less responsive than the $MSH2^{+/+}$ tumors, arguing that this degree of resistance is likely to have clinical significance. However, under these experimental conditions, all of the cells in the tumor were DNA mismatch repair-deficient, whereas in actual sporadic nonhereditary nonpolyposis colon cancer tumors the fraction of tumor cells that are deficient is not currently well defined. Under circumstances where loss of DNA mismatch repair occurs early in the expansion of the malignant clone, one would expect most of the cells to be deficient, in which case no further enrichment might occur during cisplatin treatment. However, if mismatch repair is lost later in the transformation process, only a fraction of the cells may be deficient, in which case the results reported here suggest that cisplatin treatment would be effective in selecting for the deficient cells and enriching the population. At the present time, nothing is known about the kinetics of this process nor the extent of enrichment that can occur *in vivo*. It is likely that clinical resistance will become manifest at relatively low levels of enrichment. In mixing experiments performed with the L1210 leukemia cells that are sensitive and resistant to cyclophosphamide, Skipper *et al.* (23) demonstrated that the presence of only 1% resistant cells was sufficient to cause clinical failure of treatment. Both we (14) and others (24) have reported the loss of DNA mismatch repair in cell lines selected with cisplatin. Whether mismatch repair is lost during the course of treatment with cisplatin *in vivo* is currently under investigation.

The ability of cisplatin to enrich tumor populations for DNA mismatch repair-deficient cells is of some concern over and above the issue of drug resistance. These cells have a very unstable genome and high mutation rates at many alleles including some, such as the transforming growth factor β receptor (25), that are known to influence tumor phenotype. It would not be unreasonable to expect that such enrichment would foster the additional mutations that underlie tumor progression (26).

Our earlier studies suggested that loss of DNA mismatch repair did not result in resistance to oxaliplatin, and the current results support this conclusion by showing that, at equivalent exposure intensities, oxaliplatin treatment did not enrich for repair-deficient cells *in vitro* and that there was no difference between the *in vivo* growth rate of tumors derived from $MSH2^{-/-}$ and $MSH2^{+/+}$ cells following treatment with a LD_{10} dose of oxaliplatin. The results further identify loss of DNA mismatch repair as a mechanism of resistance to cisplatin but not oxaliplatin and are supportive of the use of oxaliplatin rather than cisplatin under circumstances where avoiding enrichment for DNA mismatch repair-deficient cells is important in tumors of equivalent sensitivity to both agents.

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