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Reversal of Drug Resistance in Human Tumor Xenografts by 2′-Deoxy-5-azacytidine-induced Demethylation of the *hMLH1* Gene Promoter[1]

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ABSTRACT

Loss of DNA mismatch repair because of hypermethylation of the *hMLH1* gene promoter occurs at a high frequency in a number of human tumors. A role for loss of mismatch repair (MMR) in resistance to a number of clinically important anticancer drugs has been shown. We have investigated whether the demethylating agent 2′-deoxy-5-azacytidine (DAC) can be used *in vivo* to sensitize MMR-deficient, drug-resistant ovarian (A2780/cp70) and colon (SW48) tumor xenografts that are MLH1 negative because of gene promoter hypermethylation. Treatment of tumor-bearing mice with the demethylating agent DAC at a nontoxic dose induces MLH1 expression. Re-expression of MLH1 is associated with a decrease in *hMLH1* gene promoter methylation. DAC treatment alone has no effect on the growth rate of the tumors. However, DAC treatment sensitizes the xenografts to cisplatin, carboplatin, temozolomide, and epirubicin. Sensitization is comparable with that obtained by reintroduction of the *hMLH1* gene by chromosome 3 transfer. Consistent with loss of MMR having no effect on sensitivity *in vitro* to Taxol, DAC treatment has no effect on the Taxol sensitivity of the xenografts. DAC treatment does not sensitize xenografts of HCT116, which lacks MMR because of *hMLH1* mutation. Because there is emerging data on the role of loss of MMR in clinical drug resistance, DAC could have a role in increasing the efficacy of chemotherapy for patients whose tumors lack MLH1 expression because of *hMLH1* promoter methylation.

INTRODUCTION

The majority of sporadic tumors with loss of DNA MMR[3] in colon (1), gastric (2), and endometrial (3) cancers are MLH1 deficient and exhibit promoter hypermethylation. Experimental evidence suggests that for some cytotoxic drugs, MMR proteins provide a link between recognition of DNA damage and downstream effectors of an apoptotic response, such as p53 and p73 (4–6). Loss of MMR proficiency results in resistance *in vitro* to a number of clinically important anticancer drugs, including cisplatin and doxorubicin (7–9), and has been associated with selection for drug-resistant breast and ovarian tumors during chemotherapy (8, 10). Reintroduction of the *MLH1* gene into the MLH1 null mouse cells leads to sensitization to DNA-damaging agents (11). This supports a direct involvement of MMR in drug sensitivity and provides evidence that re-expression of MLH1 can partially overcome MMR-related drug resistance.

In ovarian cancer, a higher frequency of *hMLH1* promoter methylation is observed in postchemotherapy tumors compared with pre-chemotherapy tumors (12). We have reported that the majority of cisplatin-resistant derivatives of the ovarian tumor cell line A2780 lack MLH1 expression because of methylation of the *hMLH1* gene promoter (12). Re-expression of MLH1 by chromosome 3 transfer or by treatment with the demethylating agent 5-azacytidine results in sensitization of resistant variants to cisplatin *in vitro* (12, 13). Thus, our observation that demethylation of the *hMLH1* gene promoter results in drug sensitization *in vitro* raised the exciting possibility that MMR-related drug resistance could be overcome clinically. 5-Azacytidine and DAC have been used widely as demethylating agents in cell lines *in vitro*, and both are used clinically in the treatment of acute myeloid leukemia and myelodysplastic syndromes (14, 15). Of the two, DAC is the most potent in terms of DNA demethylation and gene activation and is the least carcinogenic (16). Although the antitumour activity of DAC has been evaluated in non-small cell lung cancer (17), the properties of DAC as a demethylating agent have not been addressed specifically in clinical trials. We have, therefore, investigated the effect of DAC treatment on MLH1 expression and the drug sensitivity of human tumor xenografts that lack MLH1 expression because of gene promoter methylation. Our results show clearly that DAC can be used *in vivo* at nontoxic doses to induce MLH1 expression. In addition, we show that DAC treatment sensitizes drug-resistant ovarian and colon human tumor xenografts to a number of clinically important cytotoxic drugs, raising the possibility that drug resistance mediated by methylation of *hMLH1* could be overcome.

MATERIALS AND METHODS

Materials. DAC was obtained from Sigma (Poole, Dorset, United Kingdom). It was dissolved in PBS at a concentration of 0.5 mg/ml and filter sterilized. Standard sterile clinical formulations of cisplatin, carboplatin, and epirubicin were obtained from the Western Infirmary Pharmacy (Glasgow, United Kingdom). Taxol (paclitaxel) was obtained from Sigma and was dissolved in cremophor EL:ethanol (1:1) at a concentration of 25 mg/ml and then diluted 10-fold in 10% dextrose to give a final concentration of 2.5 mg/ml Taxol and 5% each of cremophor and ethanol. Temozolomide was a gift from Professor Malcolm Stevens (CRC Experimental Cancer Chemotherapy Research Group, University of Nottingham, United Kingdom). It was dissolved in DMSO and then further diluted in PBS.

Cell Lines. The human ovarian carcinoma cell line A2780 and a cisplatin-resistant subline, A2780/cp70, were originally obtained from Dr. R. F. Ozols (Fox Chase Cancer Center, Pennsylvania, PA). A2780 is MMR proficient and expresses MLH1, whereas A2780/cp70 is MMR deficient and does not express MLH1 protein because of hypermethylation of the *hMLH1* gene promoter (12). CP70-ch3 is a derivative of A2780/cp70 that has chromosome 3 introduced by microcell-mediated chromosome transfer (13). CP70-ch3 contains a wild-type copy of the *hMLH1* gene, which restores MMR proficiency and MLH1 expression. Cells were maintained in RPMI 1640 containing glutamine (2 mM) and FCS (10%), and the chromosome transfer lines were grown in the presence of hygromycin B (200 units/ml).

The MMR-deficient human colon tumor cell lines SW48 and HCT116 were obtained from American Type Culture Collection (Rockville, MD). SW48 lacks MLH1 expression because of hypermethylation of the *hMLH1* gene promoter (1). The gene promoter is unmethylated in HCT116, but it lacks MLH1 expression because of a mutation in the *hMLH1* gene (18). Cells were maintained in DMEM medium containing glutamine (2 mM) and FCS (10%).

Human Tumor Xenografts. Monolayer cultures were harvested with trypsin/EDTA (0.25%/1 mM in PBS) and resuspended in PBS. About 10^7 cells were injected s.c. into the right flank of athymic female nude mice (MF1 *nu/nu* mice from Harlan Olac). After 10–15 days when the mean tumor diameter was at least 0.5 cm, animals were randomized into groups of six for experiments. Cytotoxic drugs were administered on day 0 either i.p. or i.v. via a tail vein as specified. Where specified, mice were pretreated with DAC 6 days before the cytotoxic drug, when tumors were just visible. DAC (5 mg/kg) was adminis-

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[3] The abbreviations used are: MMR, mismatch repair; DAC, 2′-deoxy-5-azacytidine.

tered i.p. at 10:00, 13:00, and 16:00 (total dose, 15 mg/kg/mouse). For the combination studies with DAC, all cytotoxic drugs were used at lower than the maximum tolerated dose to identify possible interactions in the CP70-ch3 xenografts that could not be explained by MLH1 expression. Mice were weighed daily, and tumor volumes were estimated by caliper measurements assuming spherical geometry (volume = $d^3 \times \pi/6$). Tumor doubling times were estimated as the time taken for the tumor volume to reach twice the initial volume. Significant differences between groups were identified by ANOVA, and the significance level of individual differences was determined by Student's *t* test.

Immunohistochemistry. At specified times, mice were killed, and tumors were removed and fixed in 10% neutral buffered formalin. Tissue was embedded in paraffin, and 5-μm sections were cut. Sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation for 30 min in hydrogen peroxide (0.5% in methanol). The slides were washed in water and placed in a pressure cooker containing boiling citrate buffer (0.01 M, pH 6) and brought to full pressure for 90 s. They were then washed in water and then in Tris-buffered saline containing Tween 20 (0.05%, TBST). Sections were blocked with TBS containing normal rabbit serum (1%) for 30 min and then incubated overnight at 4°C with monoclonal anti-MLH1 mouse IgG (Cambridge Bioscience) at a dilution of 1:200 in TBS containing BSA (0.1%) and sodium azide (0.01%). Slides were washed three times in TBST (5 min/wash). They were then processed with streptavidin and biotin reagents, according to the manufacturer's instructions (StreptABC; Dako, Cambridge, United Kingdom). Slides were counterstained with hematoxylin, dehydrated, cleared, and mounted.

***hMLH1* Gene Promoter Methylation.** The methylation status of the *hMLH1* gene promoter was determined by Southern blotting. Tumor tissue was frozen in liquid nitrogen immediately after removal from the mouse. For DNA extraction, the frozen tissue was crushed with a pestle and mortar and then powdered in a Mikro-dismembrator II (Braun). It was then added to 10 ml of lysis buffer [0.3 M sodium acetate (pH 8.0), 0.5% SDS, 5 mM EDTA, and 50 μg/ml proteinase K] and shaken at 37°C overnight. Samples were then extracted with phenol and chloroform:isoamyl alcohol (24:1) and then precipitated in one tenth volume of 8 M sodium acetate and two volumes of ethanol.

To allow methylation status of the *hMLH1* gene promoter to be determined, 10 μg of genomic DNA was first digested overnight with *Eco*RV and *Xba*I (Life Technologies) at 37°C to release an 884-bp fragment of the *hMLH1* promoter. The digested DNA was then further digested with either *Hpa*II or *Msp*I (Life Technologies) overnight at 37°C. Blotting of the digested DNA and hybridization with a probe specific for the *hMLH1* promoter was carried out as described before (12).

Global DNA Methylation. Mice were killed, and blood was removed by cardiac puncture and placed in tubes containing heparin anticoagulant. The Wizard Genomic DNA Purification kit (Promega UK Ltd.) was used to isolated DNA from mouse blood. RNA was removed by treatment of the samples with RNase A (Roche Diagnostics Ltd.). DNA from 600 μl of blood was rehydrated with 100 μl of distilled water at 4°C overnight. It was then denatured by heating at 95°C for 5 min and then cooled on ice to prevent religation. DNA was then digested with P1 nuclease (5 u/sample; Pharmacia Biotech) in the presence of alkaline phosphatase (4 units/sample) at 37°C for 24 h. Two volumes of ethanol were added, and samples were centrifuged for 15 min at 13,500 rpm (Eppendorf centrifuge) to pellet the proteins, and the supernatant was dried in a Speedivac.

Deoxynucleotides were separated and quantified by HPLC. The system consisted of a Hypersil ODS column (Jones Chromatography) with a μBondapak C18 GuardPak precolumn and a photodiode array detector set at 254 nm (Waters). The mobile phase contained 50 mM sodium dihydrogen phosphate at pH 4 and 2.5% methanol, and the flow rate was 1 ml/min. Retention times were 4.5 min for deoxycytosine, 9 min for methyldeoxycytosine, 14 min for deoxyguanosine, 17 min for deoxythymidine, and 31 min for deoxyadenosine.

Global DNA methylation is quantified as the amount of methyldeoxycytosine expressed as a percentage of the total deoxycytosine present (deoxycytosine + methyldeoxycytosine).

RESULTS AND DISCUSSION

Loss of MMR and Drug Resistance of Xenografts. The cisplatin sensitivities of xenografts of the ovarian cell line A2780 and derivatives directly correlate with their MMR status (Fig. 1). Thus, xenografts of the parental MMR-proficient ovarian cell line A2780 show a growth delay in response to cisplatin treatment (doubling time of control, 2.17 days, and of 8 mg/kg cisplatin treated for 5.52 days; $P < 0.001$) and the growth delay is dose dependent (Fig. 1*A*). In contrast, xenografts of the cisplatin-selected, MMR-deficient derivative A2780/cp70, which is methylated at the *hMLH1* promoter, are resistant to the maximum tolerated dose of cisplatin (8 mg/kg; Fig. 1*B*). Human chromosome 3 has been reintroduced into the A2780/cp70 line, leading to re-expression of MLH1, restoration of MMR activity, and sensitization *in vitro* to cisplatin and doxorubicin (13). Re-expression of MLH1 in the resistant A2780/cp70 cells by chromosome 3 transfer (CP70-ch3) is sustained in xenografts (results not shown), and these show a growth delay in response to cisplatin (Fig. 1*C*; doubling time of control, 2.1 days, and of cisplatin treated, 4.8 days; $P < 0.001$). Sensitization of A2780/cp70 by introduction of *hMLH1* demonstrates that MMR is directly involved in drug sensitivity rather than loss of MMR, causing higher mutation rates at drug-resistant genes, because in the latter case, reintroduction of MLH1 will not lead to sensitization. Although A2780 and A2780/cp70 are matched lines, it might be anticipated that during drug selection and growth, these lines may have diverged and may differ in a number of mechanisms that affect cisplatin sensitivity. However, the effect of cisplatin on the growth of xenografts of CP70-ch3 is comparable with that obtained for the parental A2780 cell line, which suggests that MLH1 is a major determinant of the resistance of A2780/cp70 to cisplatin *in vivo*, although we cannot exclude effects of other genes present on chromosome 3.

Re-expression of MLH1-induced by DAC. We then determined whether it is possible to use a demethylating agent such as DAC *in vivo* at nontoxic concentrations to induce re-expression of MLH1 in xenografts with *hMLH1* promoter methylation. The most effective and nontoxic schedule is treatment of mice with three doses of DAC (5 mg/kg, i.p.) at intervals of 3 h (Fig. 2, compare *A* with *B*). At this dose,

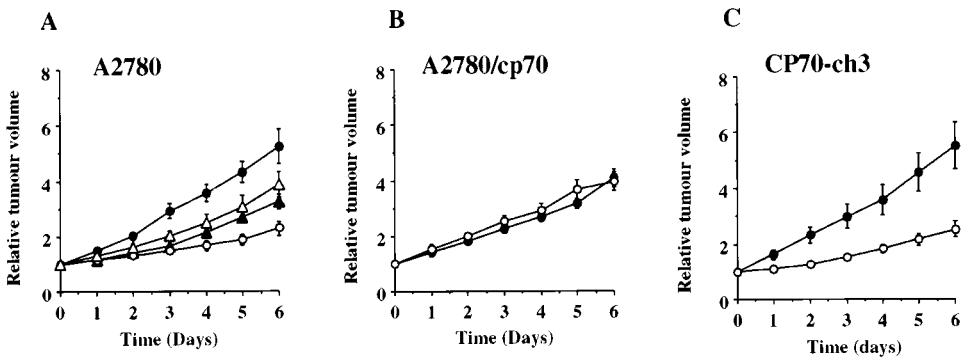

Fig. 1. Effect of cisplatin on the growth rate of human ovarian tumor xenografts established from cell line A2780 (*A*), A2780/cp70 (*B*), and CP70-ch3 (*C*). Mice received a single i.p. injection of either PBS (●) or cisplatin 4 mg/kg (△), 6 mg/kg (▲), and 8 mg/kg (○) on day 0 when the mean tumor diameter was ~0.5 cm. Results are the means of six mice; *bars*, SE.

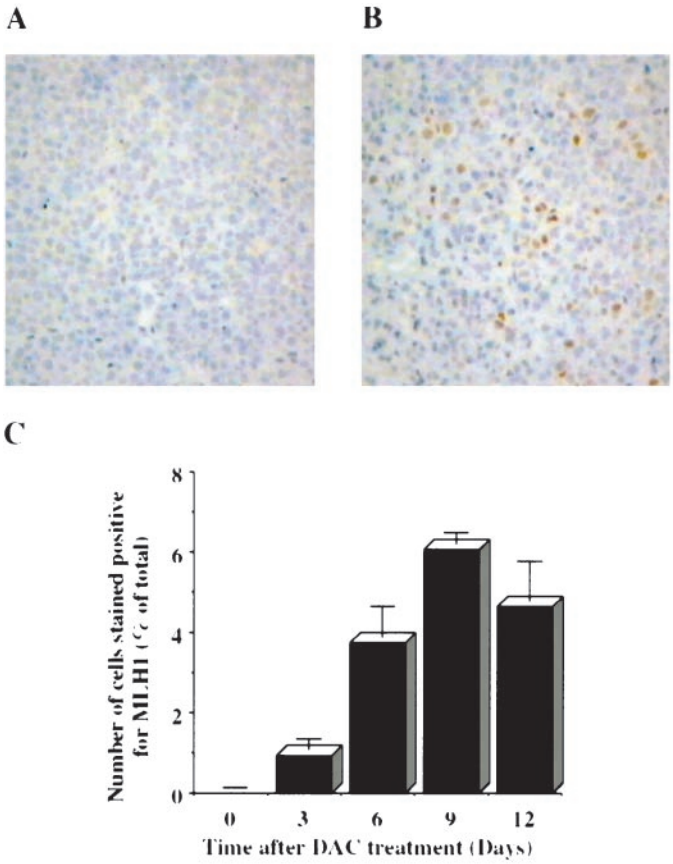

Fig. 2. Expression of MLH1 in A2780/cp70 xenografts (*A*) and in A2780/cp70 6 days after DAC treatment (*B*) determined by immunohistochemistry (*brown* staining cells are positive for MLH1 and *blue* is the counterstain). *C*, expression is also quantified as the percentage of cells that stain positive for MLH1 in sections of A2780/cp70 tumors taken at various times after treatment with DAC (means of three tumors; bars, SE).

there is no weight loss or any other sign of toxicity. Lower doses or a single bolus dose failed to induce MLH1 expression. MLH1 expression is apparent after 3 days and is maximal by 9 days after treatment with DAC (5 mg/kg × 3; Fig. 2*C*). Southern blot analysis of DNA from the tumors demonstrates that DAC treatment results in the appearance of MLH1 hybridizing bands at 349 and 569 bp after *Hpa*II digestion, which indicates reduced *hMLH1* gene promoter methylation in the tumors (Fig. 3 *A* and *B*). It has been shown that total demethylation of gene promoters is not essential for gene re-expression because transcriptional repression depends on CpG methylation density rather than complete hypomethylation (19). The time course of promoter demethylation closely reflects re-expression of MLH1 (compare Fig. 3*B* with Fig. 2*C*), although MLH1 expression is sustained for longer than hypomethylation of the promoter. This may be explained by the half life of the MLH1 protein. Between 60 and 90% of cytosines in CpG dinucleotides of normal cells are methylated (20), and we were able to demonstrate a significant decrease in global DNA methylation ($P < 0.05$) in peripheral mononuclear cells in blood removed from mice treated with DAC (Fig. 3*C*). Doses of DAC that do not induce MLH1 expression have no effect on global DNA methylation (results not shown). Thus, DAC treatment clearly induces MLH1 expression *in vivo*, and there is a dose response to DAC both in terms of MLH1 expression and global DNA demethylation. These observations suggest that measurement of global DNA methylation in blood samples could be used in a clinical trial as a surrogate marker of demethylation, where direct access to tumor DNA is not possible.

Effect of DAC on Drug Sensitivity. DAC treatment alone had no effect on tumor growth (Table 1). Although A2780/cp70 xenografts were resistant to the maximum tolerated dose of cisplatin (8 mg/kg), treatment with cisplatin at a lower dose (6 mg/kg), either 6 or 12 days after treatment with DAC (5 mg/kg × 3), results in a clear growth delay (Fig. 4*A* and Table 1; $P < 0.001$). The drug combination is ineffective if the sequence is reversed and DAC is administered 6 days after cisplatin (Fig. 4*B*). Sensitization of A2780/cp70 by DAC was not limited to cisplatin. We also observe sensitization of A2780/cp70 xenografts to carboplatin (80 mg/kg i.p.), temozolomide (200 mg/kg i.p.), and epirubicin (10 mg/kg i.v.; Fig. 4), and MMR is known to be involved in sensitivity to these agents (21). Thus, growth of the xenografts was unaffected by treatment with carboplatin or temozolomide alone, but a clear growth delay was apparent when mice were treated with DAC 6 days before treatment with the cytotoxic drug (Table 1). Interestingly, although xenografts of A2780/cp70 were sensitive to treatment with epirubicin alone (Fig. 4 and Table 1; $P < 0.005$), pretreatment with DAC was still able to sensitize the

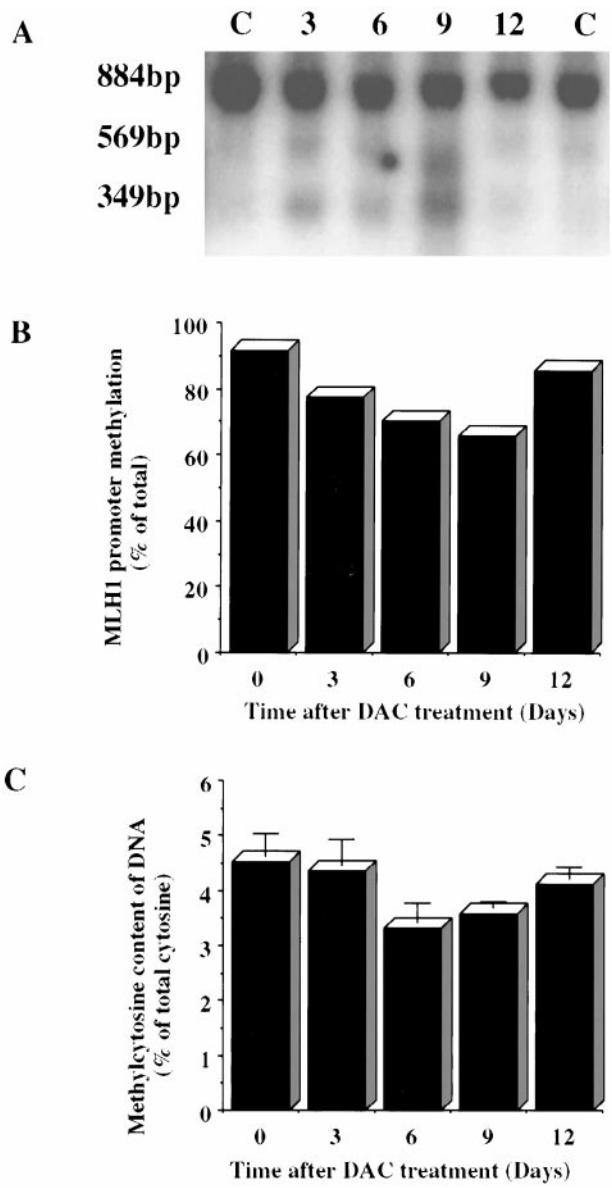

Fig. 3. *A*, Southern blot of DNA from A2780/cp70 tumors and from tumors taken 3, 6, 9, and 12 days after treatment of mice with DAC (5 mg/kg × 3) showing a methylated *hMLH1* promoter (884 bp) and appearance of a nonmethylated promoter (349 and 569 bp). *B*, quantification of the Southern blot by densitometry (mean of two tumors). *C*, changes in global DNA methylation determined by HPLC analysis of P1 nuclease-digested DNA extracted from blood taken from DAC-treated mice (means of three mice; *bars*, SE).

Table 1 *Analysis of the effects of DAC pretreatment on the drug sensitivity of MMR-deficient A2780/cp70 and MMR-proficient CP70-ch3 xenografts*

Treatment	Time to double initial tumor volume (days)[a]			
	A2780/cp70		CP70-ch3	
Control	2.4 ± 0.2	NS	2.9 ± 0.2	NS
DAC (5 mg/kg × 3)	2.5 ± 0.3		3.3 ± 0.4	
Carboplatin (80 mg/kg)	2.9 ± 0.2	[b]	5.4 ± 0.2	NS
DAC + carboplatin	6.1 ± 0.5		5.6 ± 0.2	
Cisplatin (6 mg/kg)	2.9 ± 0.2	[b]	5.1 ± 0.2	[c]
DAC + cisplatin	6.0 ± 0.3		6.1 ± 0.2	
Temozolomide (200 mg/kg)	2.1 ± 0.2	[b]	4.7 ± 0.4	NS
DAC + temozolomide	3.6 ± 0.2		4.6 ± 0.4	
Epirubicin (10 mg/kg)	4.3 ± 0.4	[c]	4.8 ± 0.5	NS
DAC + Epirubicin	6.0 ± 0.6		5.3 ± 0.7	
Taxol (15 mg/kg)	4.5 ± 0.2	NS	5.2 ± 0.4	NS
DAC + Taxol	4.8 ± 0.6		5.2 ± 0.03	

[a] Growth delay is quantified as the time taken for the tumor to double the initial volume (day 0), and the results are the mean ± SE of six mice. NS, not significant.
[b] $P < 0.001$.
[c] $P < 0.05$.

tumors to this drug. Sensitization of A2780/cp70 xenografts to the cytotoxic drugs is comparable with that achieved by reintroduction of the *hMLH1* gene by chromosome transfer (Table 1), although the level of protein expression as detected by immunohistochemistry is low (~6% of cells). Immunohistochemistry is relatively insensitive and may not detect low level re-expression. However, this level of gene reactivation is comparable with that reported for the *FMR1* gene in cells treated with DAC *in vitro* (22).

If the synergy we observe *in vivo* in the A2780/cp70 xenograft is not related to MLH1 re-expression but to some other interaction between DAC and the cytotoxic drug, then it might be expected that synergy would also be observed in xenografts of A2780/cp70 that have *hMLH1* reintroduced by chromosome 3 transfer. Indeed, there is a small but significant increase in sensitivity of CP70-ch3 to cisplatin (Fig. 4*G* and Table 1; $P < 0.01$), but this is a very small effect compared with the sensitization observed in A2780/cp70. DAC did not sensitize MMR-proficient CP70-ch3 xenografts to the other three cytotoxic drugs examined, carboplatin, temozolomide, and epirubicin (Table 1). This suggests that the small sensitization that we observe for cisplatin is attributable to a specific interaction between these two agents, which may be related to the synergy reported in cell lines *in vitro* (23). A2780/cp70 and CP70-ch3 xenografts are equally sensitive to Taxol (15 mg/kg i.v.), and DAC has no effect on sensitivity to this drug (Fig. 4*F* and Table 1). Because MMR is not thought to play a direct role in sensitivity to Taxol (21), these results support our hypothesis that DAC acts through re-expression of MLH1.

We have also confirmed our observations in a colon tumor xenograft model. The colon tumor cell line SW48 lacks MLH1 expression because of *hMLH1* gene promoter methylation (1). These tumors have a much longer volume doubling time (7.4 days) than the ovarian tumors, and this allowed us to treat with the cytotoxic drug on day 0 and again on day 7. Xenografts of this cell line are resistant to cisplatin, carboplatin, and temozolomide. However, when mice are

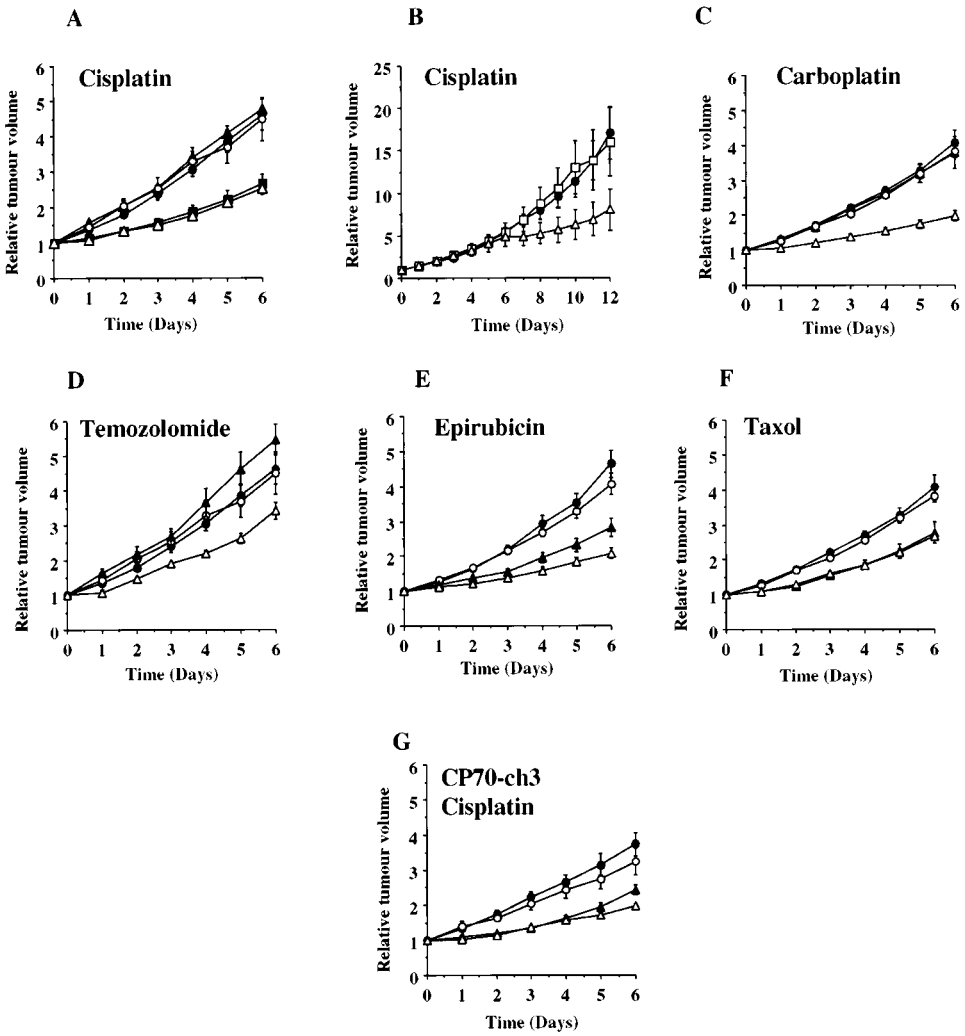

Fig. 4. The effect of DAC pretreatment on the drug sensitivity of A2780/cp70 (*A–F*) and CP70-ch3 (*G*) xenografts. Mice were treated with DAC (5 mg/kg i.p.) every 3 h for three injections or with PBS on day −6. They were then treated on day 0 with PBS or with a cytotoxic drug (●, PBS alone; ○, DAC alone; ▲, cytotoxic drug alone; △, DAC followed by cytotoxic drug). The drug was either cisplatin (*A*, 6 mg/kg i.p.), carboplatin (*C*, 80 mg/kg i.p.), temozolomide (*D*, 200 mg/kg i.p.), epirubicin (*E*, 10 mg/kg i.v.), or Taxol (*F* 15 mg/kg i.v.). In *A*, an additional group was treated with DAC on day −12 and then with cisplatin (6 mg/kg i.p. ■) on day 0. In *B*, the drug sequence is reversed. Mice were treated on days 0 and 6 with PBS (●); on day 0 with DAC (5 mg/kg × 3) and on day 6 with cisplatin (6 mg/kg i.p., △); on day 0 with cisplatin (6 mg/kg) and on day 6 with DAC (5 mg/kg × 3, □). Results are the means of six mice; *bars*, SE.

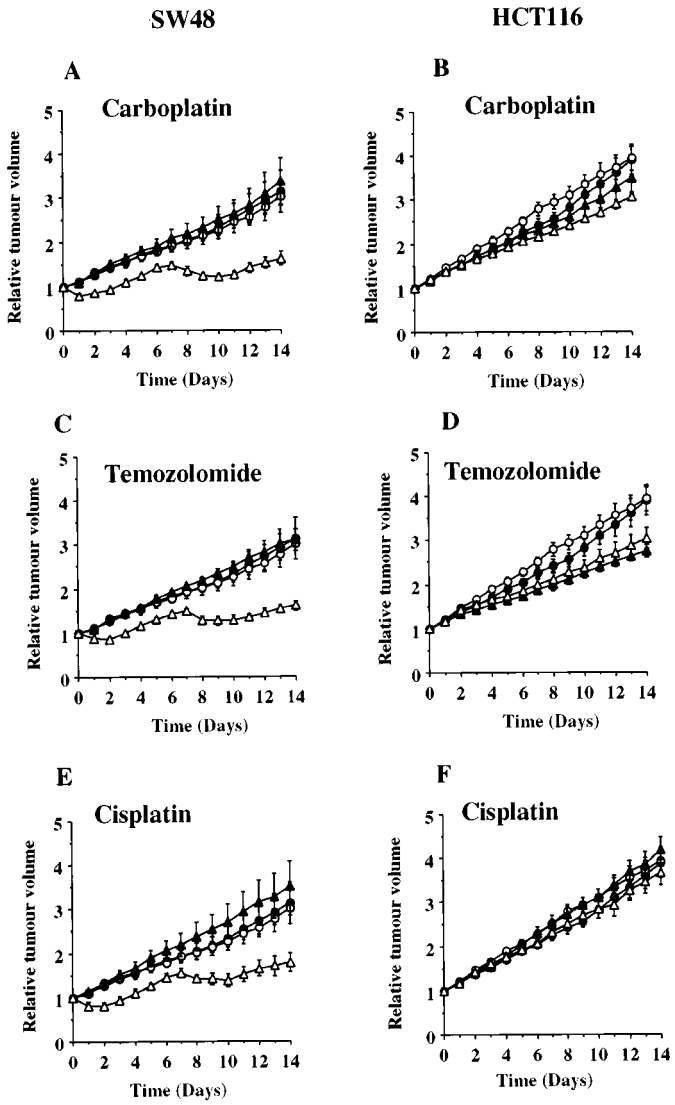

Fig. 5. Effect of pretreatment with DAC on the drug sensitivity of SW48 and HCT116 colon tumor xenografts. Mice were treated with DAC (5 mg/kg i.p.) every 3 h for three injections (*open symbols*) or with PBS (*closed symbols*) on day −6. They were then treated on Day 0 with PBS or with a cytotoxic drug (●, PBS alone; ○, DAC alone; ▲, cytotoxic drug alone; △, DAC followed by cytotoxic drug). The cytotoxic drug was either cisplatin (*A*, 6 mg/kg i.p.), carboplatin (*B*, 80 mg/kg i.p.), or temozolomide (*C*, 200 mg/kg i.p.). Results are the means of groups of six mice; *bars*, SE.

pretreated with DAC (5 mg/kg × 3 on day −6), the xenografts are sensitized to all three cytotoxic drugs and furthermore show a second response when retreated with the drug 7 days later (Fig. 5). Although the ovarian tumors show a growth delay in response to treatment, the colon tumors actually show an initial reduction in tumor volume after treatment with the cytotoxic drug. DAC is metabolized to 5-aza-deoxycytidinetriphosphate and then incorporated into DNA, where it forms a covalent adduct with DNA methyltransferase (24). It could be hypothesized that sensitization is attributable to differences in the incorporation of DAC into DNA in MMR-proficient and -deficient cells. However, DAC does not sensitize xenografts of cell line HCT116 (Fig. 5), which lacks MLH1 expression because of a mutation in the *hMLH1* gene (18). This demonstrates that sensitization by DAC is not attributable to MMR deficiency *per se* but is restricted to tumors in which *hMLH1* is inactivated by promoter hypermethylation and can thus be reactivated by inhibition of DNA methyltransferase activity.

Conclusions and Implications for Clinical Studies. Our results show clearly that DAC can be used *in vivo* to induce re-expression of MLH1 in MMR-deficient cells and that this in turn sensitizes xenografts to cisplatin, carboplatin, epirubicin, and temozolomide. Sensitization is observed only in tumors that lack MLH1 expression because of gene promoter methylation (A2780/cp70 and SW48). It is not observed in tumors that express MLH1 (CP70-ch3) or in tumors that lack MLH1 expression because of a mutation in the *hMLH1* gene (HCT116). A number of gene promoters are known to be frequently methylated in tumors, including the tumor suppressor genes *RB1* and *p16* (25). Indeed, DAC has been shown to induce expression of *p16 in vivo* in human T24 bladder tumors grown in *nu/nu* rats (26). DAC treatment alone had no significant effect on the growth rate of any of the tumors models over the time period of the study. This adds support to our hypothesis that sensitization by DAC is related to re-expression of MLH1 and not to re-expression of a tumor suppressor gene.

Induction of MLH1 expression in tumors could have a considerable impact on the efficacy of chemotherapy. We have shown that chemotherapy for breast cancer results in a significant reduction in MLH1 expression, which strongly associates with poor disease-free survival (10). Similarly, in ovarian cancer low MLH1 expression is associated with poor survival (27). In an analysis of the *hMLH1* gene promoter of ovarian tumor samples, we reported hypermethylation of the promoter in 9% of untreated tumors but increasing to 50% of tumors that had been exposed to chemotherapy (12). Expression of MLH1 was lost in the samples that exhibited promoter methylation while still being clearly detectable in the tumors without promoter hypermethylation.

There is one reported clinical trial that has evaluated the combination of DAC and cisplatin, which followed from an observation of synergy *in vitro*, although the mechanism was not understood (28). DAC was given on 3 successive days as a 30-min infusion with cisplatin given on day 4. According to our results, this is probably too soon to give the cytotoxic drug and the DAC treatment schedule is suboptimal, because we showed that a single bolus i.p. dose of DAC in mice was insufficient to induce expression of MLH1. The maximum tolerated dose of DAC in the combination was 50 mg/m^2, which gave peak plasma levels of 2 μM, which is more than adequate for demethylation based on our studies *in vitro* (12). Thus, DAC could have a role in increasing the efficacy of various forms of chemotherapy for patients with a wide range of tumors that lack MLH1 expression because of *hMLH1* promoter methylation.

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