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Co-amplification of *dhfr* and a homologue of *hmsh3* in a Chinese hamster methotrexate-resistant cell line correlates with resistance to a range of chemotherapeutic drugs

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Abstract *Purpose*: To characterize a methotrexate-resistant Chinese hamster cell line, designated as M5, which had previously been shown to be resistant to gamma radiation, at the cellular and molecular levels. *Methods*: Sensitivity towards a number of chemotherapeutic drugs was determined by colony-forming ability and compared with that of parental V79 cells. Expression of the hamster homologue of the human mismatch repair gene *hmsh3* was also determined by RT-PCR. *Results*: Induced killing by chemotherapeutic agents cis-diamminedichloroplatinum II (cisplatin), the antimetabolite 6-thioguanine (6-TG), camptothecin, a topoisomerase I inhibitor, and 4-(9-acridinyl-amino)-methanesulfon-*m*-anisidide (mAMSA), an inhibitor of topoisomerase II, was less in M5 cells than in the parental V79 cells. The IC_{50} values, defined as the concentration of the drug that reduced the survival to 50% that of the untreated control, in V79 cells for mAMSA and camptothecin treatment were 0.35 ± 0.02 μg/ml and 84.3 ± 16.0 ng/ml, respectively. For M5 cells, equivalent values were 0.52 ± 0.10 μg/ml and 186 ± 40.8 ng/ml. Treatment with 30 μ*M* cisplatin reduced the survival of V79 cells to 0.09 ± 0.07, whereas the same treatment reduced the survival of M5 cells to 0.67 ± 0.16. Treatment of M5 cells with 6-TG did not induce appreciable killing up to the concentrations studied. However, for V79 cells, 6-TG was very toxic. We further observed that the dihydrofolate reductase (*dhfr*) gene as well as the hamster homologue of the human mismatch repair gene *hmsh3* was amplified in the methotrexate-resistant M5 cells. *Conclusion*: Resistance to this group of chemotherapeutic drugs observed in M5 cells could be due to the amplification of the hamster homologue of hMSH3, which in turn possibly sequesters all the hMSH2 making M5 cells functionally deficient in the mismatch repair system.

Keywords Drug resistance · hmsh3 gene amplification · Chinese hamster V79 cells

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Introduction

Cellular resistance to chemotherapeutic drugs and radiation is a major problem in tumour treatment. Depending on the drug, such resistance could arise for several reasons. For example, resistance to cisplatin may be due to reduced drug uptake, increased cellular thiol/folate levels, increased DNA repair or expression of certain oncogenes such as ras (reviewed in reference 14). It has been reported that cells with mismatch repair (MMR) defects are resistant to many drugs such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [11, 26, 28], cisplatin [1, 3, 16, 19] and 6-thioguanine (6-TG) [4, 23]. How MMR defects lead to resistance to the drugs is not clear. The MMR system might be involved in recognition of primary DNA adducts or the mismatches produced after the replication of altered DNA. All the agents mentioned above modify the guanosine residues of DNA which after replication produce *G-T mismatches, where *G represents the modified base. These mismatches are recognized and repaired by the MMR system [36]. Since the modified base *G remains in the old strand, attempts to repair these mismatches would provoke incomplete repair and thus lead to cell death. Failure to recognize such mismatches would thus decrease cell death, but also lead to a higher frequency of mutation induction. Similarly, defects in MMR have been shown to increase the bypass of cisplatin-induced damage [36] and the MMR system also interacts with the recombination as well as excision repair systems [7, 17]. Further, it has been re-

ported that cell lines with MMR defects are resistant to ionizing radiation, although to a lesser extent [20, 29], but the mechanism is unclear.

In humans, the MMR genes identified are *hmsh2, hmsh3, hmsh6,* and *hmlh1*, and they are homologous to the bacterial MMR genes *mutS* and *mutL*. There are several MutL homologues in yeast such as MLH1, MLH2, MLH3, PMS1, and PMS2. These proteins function interdependently. hMSH3 and hMSH2 dimerize to form MutSβ, which recognizes small loops generally formed at the repeat DNA by slippage. On the other hand, hMSH2 and hMSH6 dimerize to form the complex MutSα, which recognizes base-base mismatches. Once MutSβ or MutSα recognizes the defect, other proteins such as hMLH1 or PMS2 come into play and complete the repair. In humans and mice, the *msh3* gene is located upstream of the dihydrofolate reductase (*dhfr*) gene and shares a common promoter region. These genes have been shown to transcribe in the opposite direction [32]. Overexpression of the *dhfr* gene increases the expression of *msh3* in *dhfr*-amplified human cells. Thus, hMSH3 possibly interacts with all hMSH2 to form the MutSβ complex, making the cell functionally deficient in MutSα [15, 27]. Even though hMSH3-amplified cells exhibit a higher rate of spontaneous mutation [15], the cellular response to different drugs in such cells remains unknown.

We have characterized a cell strain resistant to methotrexate (MTX) derived from Chinese hamster V79 cells [30, 31]. It has been observed that this cell strain, designated as M5, is resistant to killing by gamma radiation and is therefore termed radioresistant. The cell strain M5 is also resistant to MNNG exposure as well as H_2O_2 treatment. The mutation frequency induced by gamma irradiation and MNNG exposure is also higher in M5 cells compared to the parental V79 cells [30]. Furthermore, we have reported that this strain is resistant to the induction of apoptosis by gamma irradiation [22].

We report here the results of experiments designed to investigate whether these MTX-resistant M5 cells, which also exhibit resistance to ionizing radiation, are also resistant to other chemotherapeutic agents. We chose two groups of drugs, one that induces single-strand and double-strand breaks and thus mimics ionizing radiation and the other comprising two drugs that produce mismatches in DNA after replication and mimic MNNG with respect to mismatches. It is known that among various lesions produced by MNNG, *O*-6-methylguanine, if not removed, produces G*-T mismatches after replication. 6-TG after phosphorylation is incorporated into DNA. During subsequent replication G*-T mismatches are formed. Cisplatin can form 1,2-intrastrand crosslinks with G residues. During replication C is incorporated opposite the first cisplatin-bound G residue and T opposite the second G. Thus G*-T mismatches are produced [33, 35] which are recognized by MutSα.

We have observed that M5 cells are also resistant to camptothecin, 4-(9-acridinyl-amino)-methanesulfon-*m*-anisidide (mAMSA), cisplatin and 6-TG. The extent of resistance is higher for cisplatin and 6-TG than for camptothecin and mAMSA. The Chinese hamster homologue of the human MMR gene *hmsh3* was found to be co-amplified with the *dhfr* gene in this cell strain compared to the parental V79 cells.

Materials and methods

Cell culture

Chinese hamster V79 male lung fibroblast cells were obtained from the National Institute of Virology, Pune, India. Cells were routinely cultured as monolayers in plastic petri dishes in Eagle's minimal medium supplemented with dialysed goat serum (complete medium) as described previously [9]. Isolation and characterization of the radioresistant cell strain M5 has been reported previously [30, 31], and the cells were grown regularly as described above.

Cell survival assay

The method used to determine cell survival in terms of colony-forming ability was essentially the same as that described previously [30, 31]. In brief, exponentially growing cells were trypsinized, counted and plated in 55-mm diameter plastic petri dishes in triplicate at low density (200–20,000). Cells were treated with various concentrations of the drug in complete medium for different times. The cells were treated with camptothecin and mAMSA (Sigma Chemicals, St Louis, Mo.) for 6 h, and with various concentrations of cisplatin (Sigma) for 1 h. At the end of the treatment, the medium was removed, the cells were washed with serum-free medium and fresh complete medium was added followed by incubation until colony formation. For 6-TG, the drug was present throughout colony formation.

The surviving fraction was determined by dividing the plating efficiency of treated cells by the plating efficiency of untreated control cells. The plating efficiencies of untreated control Chinese hamster V79 cells were in the range 55–65%, and of M5 cells were in the range 65–75%. IC_{50} values were determined from survival curves from individual experiments and the results were analysed using Student's *t*-test (http://intrepid.mcs.kentedu/~blewis/stat/tTest.html).

Southern hybridization and dot blot analysis

Genomic DNA was isolated from V79 cells and M5 cells using a standard protocol. DNA (10 μg) was digested with *EcoRI* (Invitrogen, San Diego, Calif.) and *BamHI* (Invitrogen) according to the manufacturer's protocol. Digested DNA was separated on 0.8% agarose gel, transferred to Hybond N^+ membrane (Amersham, Little Chalfont, UK) in the presence of 0.4 *M* NaOH. The membrane was neutralized with 2×SSC and was then hybridized with ^{32}P-labelled *dhfr* probe in 6×SSC, 10×Denhardt's and 1.0% SDS at 60°C for 16 h. The membrane was washed twice with 2×SSC and 0.1% SDS at 60°C for 15 min and then exposed to X-ray film (Kodak, New Delhi, India) and kept at –70°C. For dot blot analysis, equal amounts of DNA from V79 and M5 cells were denatured with 0.4 *M* NaOH and spotted onto Hybond N^+ membrane at different concentrations. After neutralizing with 2×SSC, membranes were hybridized with labelled *hmsh3* probe as described above. Human *dhfr* cDNA used as the probe, cloned in pSP64 vector, was obtained from Dr. Rakesh Sharma, Stanford University (Stanford, Calif.), and *hmsh3* cDNA, cloned in pBluescript was a generous gift from Prof. R. Fishel, Thomas Jefferson University (Philadelphia, Pa.). Autoradiographs obtained by dot blot analysis were scanned using an Image Master Video Documentation System (Pharmacia Biotech, Stockholm, Sweden) and quantified. The

ratios of the intensities of the dots were calculated to provide an indication of the relative abundance of these genes in the two cell lines.

Expression of *msh3* gene by reverse transcription-polymerase chain reaction (RT-PCR) amplification

Chinese hamster full-length *msh3* gene expression was detected using PCR primers and the methods described by Hinz and Meuth [24]. Total RNA was isolated from exponentially growing cells following a standard protocol. Concentrations of RNA were determined spectrophotometrically. First-strand cDNA was synthesized with 2 μg total RNA using reverse transcriptase (Life Technologies, USA) and random hexamer (Pharmacia Biotech, Sweden) following the protocols provided by the suppliers. This product was then amplified using the primers described by Hinz and Meuth [24]. A control experiment to check the presence of equal amounts of RNA in both the cases was done using hamster-specific mdm2 primers [12].

Microsatellite instability

Microsatellite instability was studied following the methods described by Bhattacharyya et al. [8] at three microsatellite loci (22.1, 11.1 and 10.1) having $(CA)_n$ repeats described by Aquilina et al. [5]. In brief, DNA was isolated from several independent clones derived from M5 cells and V79 cells. PCR was done with the primers described above. Amplified products were run on 6% denaturing polyacrylamide gels, transferred to Hybond N^+ (Amersham, Little Chalfont, UK) and probed with one of the PCR primers labelled with ^{32}PdCTP (Brit, Hyderabad, India). The membrane was washed with 2×SSC, 0.1% SDS at 37°C and exposed to X-ray film (Kodak, New Delhi, India).

Results

Resistance to cisplatin and 6-TG

The colony-forming abilities of V79 cells and M5 cells after treatment with various concentrations of cisplatin were determined as described in Materials and methods. Both cell types were treated with 10, 20 and 30 μ*M* cisplatin for 1 h. For both V79 cells and M5 cells there was a dose-dependent decrease in colony-forming ability. However, M5 cells were more resistant to killing than the parental V79 cells. For example, treatment with 30 μ*M* cisplatin for 1 h reduced survival of V79 to 0.09 ± 0.07, but reduced the survival of M5 cells to only 0.67 ± 0.16 ($P = 0.022$). The results of these experiments are shown in the Fig. 1A. The IC_{50} for V79 cells was

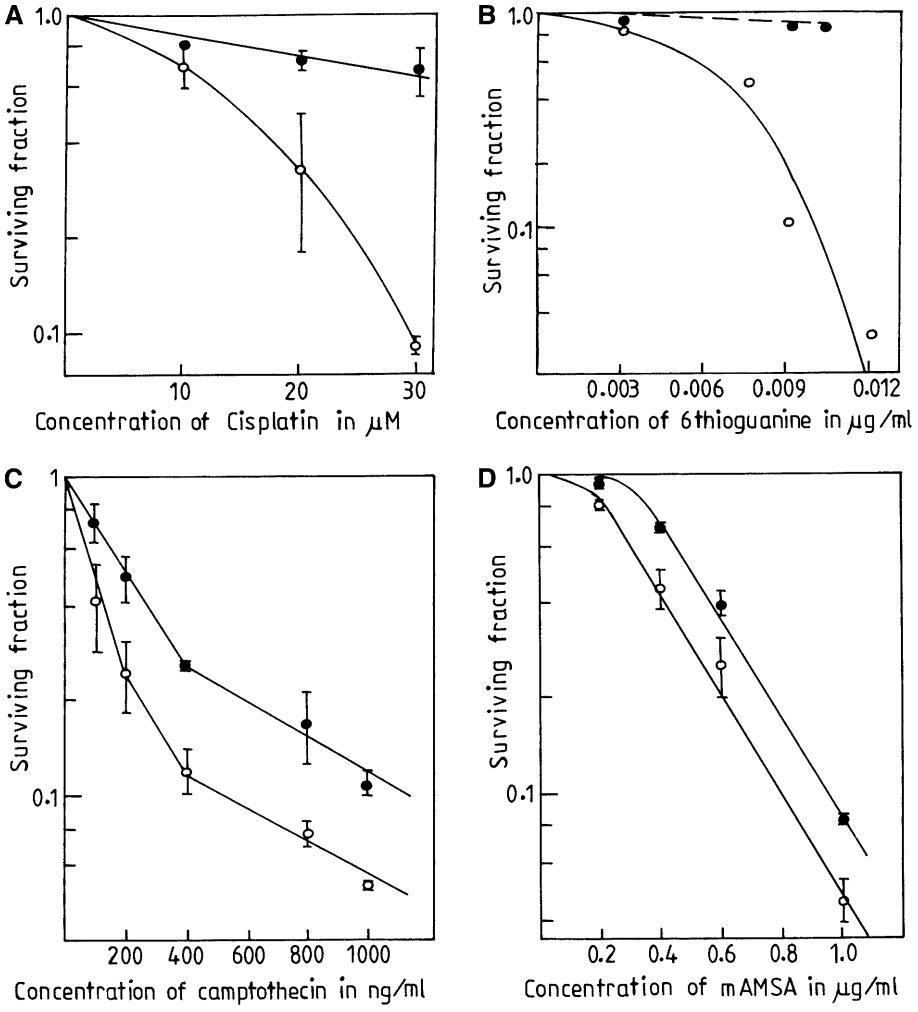

Fig. 1A–D Survival curves of V79 cells (*open circles*) and M5 cells (*closed circles*) treated with cisplatin (**A**), 6-TG (**B**), camptothecin (**C**) and mAMSA (**D**). Each data point was derived from two to four independent experiments and the standard deviations are denoted by the vertical bars

calculated as 15 ± 5.9 μ*M* and for M5 cells was determined by linear extrapolation of the survival curves (graph not shown) as 43.8 ± 12.4 μ*M* ($P = 0.048$).

The effects of continuous exposure to low concentrations (0.003–0.012 μg/ml) of 6-TG on V79 cells and M5 cells are shown in the Fig. 1B. As expected, 6-TG was highly toxic to V79 cells as indicated by a sharp decrease in colony-forming ability. For example, in comparison with untreated controls, treatment with 0.01 μg/ml 6-TG reduced survival of V79 cells to 7% and survival of M5 cells to about 90% ($P < 0.0001$). Thus, M5 cells were highly resistant to killing by 6-TG compared to the parental V79 cells.

Resistance to camptothecin and mAMSA

The sensitivity of MTX-resistant M5 cells and the parental V79 cells was determined using the methods described in above. Both camptothecin and mAMSA killed both cell types in a dose-dependent manner. For camptothecin, the survival curve was biphasic without a shoulder. At any particular dose, the survival of M5 cells was higher than that of V79 cells. For example, treatment with 200 ng/ml camptothecin for 6 h resulted in about 75% killing of V79 cells (25% survival) and about 55% killing of M5 cells (45% survival). The results of these experiments are shown in the Fig. 1C. The IC_{50} values were 84.3 ± 16 ng/ml for V79 cells and 186.0 ± 40.8 ng/ml for M5 cells ($P = 0.007$).

The sensitivity of V79 cells and M5 cells to mAMSA are shown in Fig. 1D. For both cell types, the survival curves had shoulders at low concentrations and then killing increased with increasing dose. At any particular concentration of mAMSA the survival of M5 cells was higher than that of V79 cells. For example, treatment with 0.4 μg/ml mAMSA for 6 h reduced survival of V79 cells to about 42% and survival of M5 cells to 70%. The IC_{50} values were 0.35 ± 0.02 μg/ml for the V79 cells and 0.52 ± 0.10 μg/ml for the M5 cells ($P = 0.029$).

Amplification of *dhfr* gene

To determine whether higher DHFR activity and resistance to MTX were due to amplification of the *dhfr* gene, we determined the copy number of the *dhfr* gene by Southern hybridization and dot blot analysis. A representative autoradiograph is shown in Fig. 2. As is evident, the relative intensity of the 4.5-kb band obtained with *Eco*RI-digested M5 DNA (lane 1) was about sevenfold higher than the intensity obtained with V79 cells (lane 2). In the dot blot autoradiographs, the intensity was higher (the ratios of the intensity of signals obtained with M5 cells to those with V79 cells were in the range 2.9–1.33) with all concentrations of M5 DNA (data not shown). This result indicates that the *dhfr* gene was amplified in the M5 cell strain.

Fig. 2 Southern blot analysis of hamster genomic DNA probed with human *dhfr* cDNA (*lane 1* M5 DNA, *lane 2* V79 DNA)

Dot blot analysis for amplification of the hamster homologue of the *hmsh3* gene

As described in the Introduction, in mouse, human and hamster the *msh3* gene is located adjacent to the *dhfr* gene [24, 32]. Dot blot analysis was used to determine whether the hamster homologue of *hmsh3* has co-amplified along with the *dhfr* gene. Various amounts of DNA (100 ng to 10 μg) from both cell types were spotted and probed with human *msh3* gene. A representative result is shown in Fig. 3. The ratios of the intensities of the signals obtained using DNA from M5 and V79 cells varied from 1.5 to 2.5. The same blot was stripped and probed with labelled $(CAC)_5$. The signals obtained in this case were the same (data not shown).

Expression of *msh3* gene by RT-PCR

Expression of the Chinese hamster homologue of the *hmsh3* gene in V79 and M5 cells was determined by PCR with specific primers using cDNA derived from the two cell types. cDNA was synthesized using 2.0 μg RNA and PCR was carried out for different numbers of cycles (22 to 30 cycles). In each case, the PCR yield was higher with RNA derived from M5 cells than with that obtained from V79 cells. For example, the ratio of the yield of PCR product obtained after 22 cycles of amplification using RT product from M5 cells to that obtained from V79 cells was 5.1. This value varied from experiment to experiment, but the yield was always higher with M5 cells. Representative results of these experiments are shown in Fig. 4A.

cDNA was also synthesized using different concentrations (0.2–3.0 μg) of RNA, and PCR was carried out for 30 cycles. For each concentration of RNA, the PCR

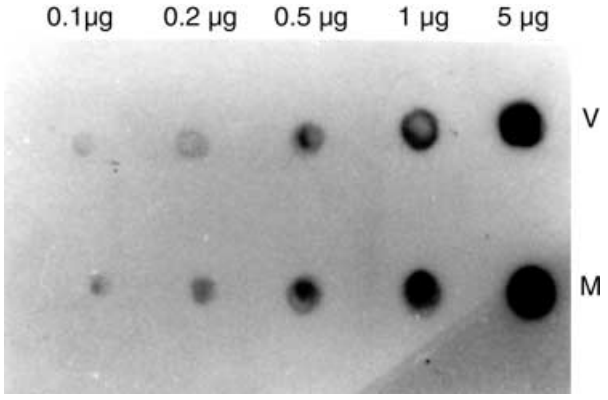

Fig. 3 Dot blot analysis for detection of hamster homologue of *hmsh3* gene

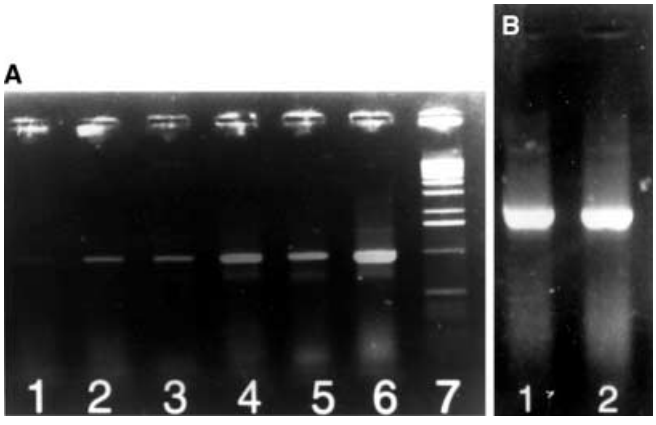

Fig. 4 **A** Expression of hamster homologue of *hmsh3* in V79 and M5 cells by RT-PCR. Using the same amount of V79 and M5 cDNA, PCR was done for 22 cycles (*lane 1* V79, *lane 2* M5), 27 cycles (*lane 3* V79, *lane 4* M5) and 30 cycles (*lane 5* V79, *lane 6* M5); *lane 7* 1-kb ladder. **B** Expression of *mdm2* in V79 cells (*lane 1*) and M5 cells (*lane 2*) by RT-PCR

yield was higher with RNA derived from M5 cells than with that obtained from V79 cells (data not shown). To check that this differential gene expression was specific for the *msh3* gene, a control experiment was performed for the expression of *mdm2* with the same RT product [12]. In all cases the PCR yield was the same. A representative result is shown in Fig. 4B.

Microsatellite instability

Dinucleotide repeat sequences $(CA)_n$ in Chinese hamster V79 cells were amplified using specific primers flanking the regions. Independent clones from V79 and M5 were isolated and the genomic DNA from these clones was used for PCR amplification and the detection of alleles. Nine V79 colonies and 38 M5 colonies were analysed for all three loci. For all the three loci there was no shift in position of the two alleles and none of them was missing (data not shown). Thus, microsatellite instability, an indication of defect of MutSβ, was absent in M5 cells.

Discussion

We showed that the MTX-resistant cell strain M5 was more resistant to killing by the chemotherapeutic drugs cisplatin, antimetabolite 6-TG, topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor mAMSA than the parental Chinese hamster V79 cells. It has also been shown that this cell strain is also resistant to killing by gamma irradiation, MNNG treatment and hydrogen peroxide exposure. Also, the induction of mutation by gamma irradiation and MNNG treatment is higher in M5 cells than in the parental V79 cells. This cell strain has a homogeneously staining region (HSR) on the long arm of chromosome 2 (location of the *dhfr* gene in Chinese hamster), indicating amplification of the *dhfr* gene. Besides, DHFR activity measured by ^{3}H-folate reductase assay is about 13-fold higher in M5 cells than in the parental V79 cells [30, 31]. We found by Southern blot analysis that the *dhfr* gene copy number was at least sevenfold higher in M5 cells than in V79 cells. The cytological, biochemical and molecular data taken together, and the fact that this cell strain grows continuously in the presence of 2.4 μM MTX, indicate that the *dhfr* gene has been amplified in the M5 cell strain.

Dot blot analysis using hmsh3 probe and RT-PCR revealed that the M5 cell strain also had an amplified hamster homologue of *hmsh3*. The human and mouse *msh3* gene is upstream of the *dhfr* gene [21]. It has been reported that in a Chinese hamster cell strain with deletion of the *dhfr* gene, the *msh3* gene is also deleted indicating that the hamster homologue of *msh3* is located adjacent to the *dhfr* gene [24]. In human MTX-resistant cells with an amplified *dhfr* gene, *hmsh3* is co-amplified [27]. In the present study we found that the Chinese hamster V79 cell-derived M5 cells were resistant to cisplatin and 6-TG, and also had an amplified *msh3* gene. As suggested previously, the observed resistance in M5 cells to these drugs could thus be due to defects in the MMR system involving MutSα [27]. Absence of alterations in the microsatellite loci in M5 cells indicates that there was no defect in the MutSβ.

Resistance to cell death as determined by colony-forming ability induced by diverse DNA-damaging agents observed in the MTX-resistant M5 cells can be divided into two groups: high resistance (MNNG, cisplatin and 6-TG) and low resistance (radiation, camptothecin, and mAMSA). Sensitivity to ultraviolet light, which predominantly induces thymine dimers and is repaired by nucleotide excision repair was similar in both the cell types (data not shown). Even though cisplatin-induced cell killing can be modulated by nucleotide excision repair [10, 18], the above result argues against such a scenario. Tumour cells with defects in the MMR system are resistant to many drugs such as cisplatin, MNNG and 6-TG [11, 26]. On the basis of this observation, it has been proposed that failure to recognize the damage produced by cisplatin, MNNG or 6-TG

leads to a failure to generate the signal for cell death, thus providing resistance to these drugs [19, 27]. Low levels of resistance to camptothecin or mAMSA could also be due to clonal variation. We cannot exclude this possibility.

However, it is to be mentioned that resistance to MNNG-induced killing in MTX-resistant cells correlates with DHFR activity [31] and several independent MTX-resistant clones exhibit resistance to MNNG (M. Roy, unpublished observation). It has been observed recently that mammalian cells defective in MMR are slightly resistant to etoposide, an inhibitor of topoisomerase II [2], and also ionizing radiation [20, 29]. Interestingly, it has been suggested that the MMR system interacts with a "cleavable complex" formed by topoisomerase inhibitor [13]. The MMR system has also been reported to participate in recombination, possibly by acting as a barrier (reviewed in reference 6) to recombination. Thus, the suggested absence of MMR in M5 cells could render the cells resistant to agents that are thought to be repaired by recombination [25, 34]. In this connection, it is also known that spontaneous as well as induced sister chromatid exchanges, an indicator of recombination, are higher in M5 cells than in the parental cells [31]. In conclusion, we observed that M5 cells were resistant to cisplatin, 6-TG and topoisomerase inhibitors and the hamster homologue of *hmsh3* was co-amplified with the *dhfr* gene. We hypothesize that such resistance may be due to a defect in the MutSα-mediated MMR.

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