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DNA Repair and Repair Fidelity in Metastatic Variants of the B16 Murine Melanoma

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Abstract We have examined the concept of genomic instability in relation to the metastatic progression of low (F1) and high metastasis (BL6, ML8) clones of the B16 mouse melanoma, by using a mutation assay, and DNA strand break repair and repair fidelity assays. The frequency of induced ouabain resistant colonies between the variant cell lines was consistent with the difference between their metastatic properties. Survival data for X-irradiation and bleomycin were similar among the 3 cell lines. When X-rays or bleomycin were used to induce strand breakage, no difference was detectable in either the rate or extent of DNA repair using the techniques of alkaline unwinding and alkaline elution for total strand breaks, and neutral elution for double strand breaks. DNA repair fidelity was measured using the PMH16 plasmid. A Kpn I restriction site was used to introduce a break within the *gpt* gene of the plasmid, prior to transfection. We found that ~100% and ~65% of the highly metastatic ML8 and BL6 clones, respectively, religated the gene with the required fidelity, compared with only ~25% of the low metastasis F1 clones. In summary, the metastatic variants show similar sensitivities to X-irradiation and bleomycin, but a differential response to EMS. This difference is not reflected in any subsequent DNA strand break religation, but the variants do differ in their fidelity of repair. However, although the fidelity of DNA religation is related to metastatic potential, it is not consistent with the mutation frequency data.

Key words: DNA repair, repair fidelity, genomic instability, metastasis

The Nowell hypothesis for tumour progression [1976] postulated that the generation of variants with increased invasive and metastatic abilities from the primary tumour cell population was due to a genetic variability within the developing neoplasm. This increased genetic instability could then allow for the selection of more "aggressive" or metastatic sublines from the same neoplastic mass under environmental selective pressure [Fidler et al., 1976]. Therefore, direct evidence for differences in genomic instability between variants with low and high malignancy has been sought. Cifone and Fidler [1981] presented evidence showing that mouse tumours derived from a UV-2237 fibrosarcoma and with a high metastatic potential had a higher spontaneous mutation rate for 6-thiopurine resistance or ouabain resistance than did their low metastatic cell controls. Subsequently Fisher and Cifone [1981] showed that on exposure to high doses of mutagenising UV irradiation, the mutation frequency leaning to ouabain resistance increased tenfold, and in parallel these tumour cells showed a greater metastatic capability than their unirradiated counterparts. On the other hand, however, Kaden et al. [1989] have found no correlation between spontaneous mutation rates, using 6-thioguanine selection, and the malignant phenotype in a series of Chinese hamster embryo fibroblasts (CHEF) derived tumour cell lines.

There is some evidence which indicates that altered DNA repair properties may be associated with genomic instability. For example, elevated SCE levels have been found in mouse B16 melanoma cells and in cells from patients with Bloom's syndrome [Kohn, 1983; Kihlman and Andersson, 1985; Sherbet et al., 1986]. Willis and Lindahl [1987] have shown that this instability in Bloom's syndrome is due to a defective repair enzyme, DNA ligase I. DNA strand breaks do occur naturally during normal cellular activities of DNA synthesis and gene expression. For example, the induction of differentiation in mu-

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rine erythroleukaemia cells [Terada et al., 1978; Scher and Friend, 1978] and in mitogen-induced human lymphocyte differentiation [Johnstone and Williams, 1982] has been shown to involve the appearance of DNA strand breaks. Since a broad spectrum of structural damage to the DNA helix results either directly or indirectly, in transient breaks associated with repair enzyme activity, it is reasonable to investigate if differences existed between normal tissues and malignant tissues in their ability to repair DNA strand breaks. In the past, alkaline techniques have provided evidence for the total strand break repair capacity of cells [Kohn et al., 1981; Hesslewood, 1978]. There is now a large body of data to suggest that the double strand break alone may be the critical lesion in determining differences in response to DNA damaging agents between cell lines [Thacker, 1989; Bryant, 1985; Radford, 1985]. More recently, techniques to measure the fidelity of double strand break repair have been established using plasmid transfection techniques [Debenham et al., 1988].

The aim of this work was to determine if any intrinsic differences existed in mutation frequency, DNA repair properties, and fidelity of the repair process between metastatic variants of the B16 murine melanoma, which could be correlated with their metastatic ability and provide a mechanistic model for implicating increased mutation frequency as the basis for the rapid generation of variants in highly metastatic tumour lines.

MATERIALS AND METHODS

Cells

A low metastasis variant, F1, and high metastasis variants, BL6 and ML8, of a mouse B16 murine melanoma were used. F1 shows a low ability to metastasize to the lungs, BL6 has a high metastatic ability, and ML8 metastasizes even more extensively to the lungs. The metastasizing abilities of the metastatic variants has been reported by Lakshmi et al. [1988].

The F1 and BL6 cell lines were obtained from E.G. & G. Mason Research Institute (Worcester, MA). The variant ML8 line was isolated in our laboratory from metastatic lung deposits arising from a BL6 primary tumour grown subcutaneously in C57BL6/J mice.

Routine tissue culture has not revealed any difference in the growth rate of the three cell lines [Sherbet, 1987; Hallouche et al., 1992].

Culture Conditions

Cells were grown in Eagles MEM containing 10% FCS (NBL) glutamine (2 mM) and 1% non-essential amino acids at 37°C in a 5% CO_2 atmosphere. The cells were grown as monolayers and maintained in exponential culture throughout these experiments.

G418 medium contained Geneticin (Gibco) at a final concentration of 1 mg/ml. XHATM medium contained xanthine (Sigma) 250 μg/ml, hypoxanthine (Sigma) 15 μg/ml, adenine (Sigma) 25 μg/ml, thymidine (Sigma) 10 μg/ml, and mycophenolic acid (Sigma) 10 μg/ml.

DNA Damaging Agents

Bleomycin was obtained as a freeze-dried preparation in 15 mg ampoules (containing 15 units of activity) from Lundbeck Ltd., Luton, Beds.

DNA damage was induced in cells by irradiation from a 240KeV output Marconi X-ray machine (with aluminium filter).

Survival Curves

Bleomycin. Exponentially growing cells were trypsinized and seeded into 10 cm tissue culture petri dishes at 300 cells/plate. Cells were allowed at least 4 h for attachment before exposure to a range of concentrations of the drug for 1 h. Cells were then washed twice with Dulbecco A (phosphate buffered saline) and incubated in normal medium at 37°C for 10 days to allow visible colonies to form. Surviving colonies were expressed as a fraction of the total number of cells plated.

X-rays. Exponentially growing cells were plated out as above. Prior to irradiation, most of the medium was aspirated off, leaving only 1 ml to cover the cells. The cells were exposed to a range of X-ray doses between 0 and 1,200 rads at 0°C, and a dose rate of 75 rads/min. The cells were then incubated in medium at 37°C for 10 days to allow visible colonies to form. Counts were expressed as a surviving fraction.

Ethylmethane sulphonate (EMS) (Sigma). Exponentially growing cells were plated out at 5×10^2 cells/plate for low concentrations of the drug and 5×10^3 cells/plate for higher concentrations. Cells were exposed for 4 h to a range of concentrations, washed twice with Dulbecco A, then incubated in normal growth medium for 8 days at 37°C. Surviving fractions were plotted on a semi-log scale.

Mutation Assay

Exponentially growing cells were exposed for 4 h to a mutagenic dose (1 mg/ml) of the alkylating agent EMS. The cells were washed with Dulbecco A and transferred to normal growth medium for 4–5 days to allow for expression of any cell mutations. Prior to ouabain exposure, the cells were transferred to medium containing 1% foetal calf serum for 3–4 days (this step allows for a more rapid emergence of resistant colonies under selective conditions). Finally, the cells were seeded onto plastic 10 cm petri dishes at 10^6 cells/plate and exposed to a selective concentration of ouabain (3 mM) for 10 days. The number of mutant colonies able to grow was stained with a 0.4% crystal violet stain and counted. Mutation frequencies were expressed as the mean number of ouabain-resistant mutants ± S.E.

Alkaline Unwinding Assay

The DNA strand unwinding method in weak alkali coupled with hydroxylapatite chromatography was essentially as reported by Hesslewood [1978]. Two DNA damaging agents were used: X-irradiation (4 Krads) at 0°C and bleomycin (250 μg/ml for 1 h). Cells to be treated were routinely seeded from exponential cultures into 3.5 cm petri dishes at 10^4 cells/dish, yielding $\sim 10^5$ cells (subconfluent) at the end of a 2 day growth period. Prior to damage, the cells were labelled with 0.2 μCi/ml tritiated thymidine [^{3}H] (Amersham) for 2 h, then "chased" for 3 h in warm unlabelled medium. After exposure to the DNA damaging agent, the cells were allowed to repair in a CO_2 incubator at 37°C. At set time intervals, the cells were harvested and resuspended in ice-cold lysis buffer (0.03 M NaOH, 0.97 M HCl, 1 mM EDTA) for 30 min at 4°C (in the dark) to allow the DNA to unwind. The cell suspension was neutralised with 0.02 M NaH_2PO_4, 1 mM EDTA, and the mol. wt. of the DNA reduced by sonication for 10–15 sec at half power with a small probe. These prepared samples were then loaded onto hydroxylapatite columns maintained at 60°C. Single stranded (SS) DNA was eluted out with a 0.125 M phosphate buffer and double stranded (DS) DNA with a 0.5 M phosphate buffer. The quantity of DNA in the respective fractions was assessed by measuring the amount of incorporated labelled thymidine by scintillation counting. The ratio of SS:DS material is a measure of the extent of DNA strand breakage.

Alkaline Elution Assay

A modification of the method of Kohn et al. [1981] was used to measure SS breaks and alkali labile sites. The technique utilizes the mechanical impedance of filters to discriminate between long DNA strands (few breaks) and shorter DNA fragments (many breaks). Exponentially growing cells were seeded at 4×10^4/3.5 cm petri dish and labelled with [^{14}C]-thymidine (0.02 μCi/ml) (Amersham) for 48 h. After exposure to DNA damage (X-rays (1000 rads) at 0°C; EMS for 4 h at 37°C), the cells were deposited on filters by force of gravity, lysed with a detergent solution, and the "naked" DNA strands treated with proteinase K to digest any bound protein and to reveal any protein associated strand breaks. After washing, an alkaline solution (pH 12.1) was pumped through the filters at a flow rate of 2 ml/h for 24 h, with fractions being collected every 90 min (10 fractions). The amount of radiolabelled DNA eluted was counted in a Beckman β-counter and compared with the amount of labelled DNA remaining on the filter. The rate of elution of the incorporated label is a measure of the total breaks present, both single and double strand breaks.

The precision of the assay was increased by the use of internal standard cells labelled with [^{3}H]-thymidine (Amersham). The [^{3}H]-labelled cells were irradiated with an X-ray dose of 300 rads at 0°C, which results in an almost linear elution curve in semi-log plots. These internal standard cells were mixed with the sample [^{14}C]-labelled cells at the same cell density and the mixture carried through the alkaline elution procedure. [^{3}H] and [^{14}C] radioactivity levels were determined using a double-isotope counting programme on the Beckman scintillation counter. Elution curves normalized with respect to internal standards were plotted on a double log scale.

Neutral Elution Assay

The method of Bradley and Kohn [1979] has provided a modification of the alkaline elution above and is based on the rate at which DNA double strands elute through a membrane filter under non-denaturing conditions, at pH 9.6. Measurements of double strand breaks were carried out on test cells which had been irradiated with 10 Krad X-rays, and internal standard

(reference) cells irradiated with 5 Krad X-rays. Data are expressed as [^{14}C]-retained vs. [^{3}H]-retained on a log-log plot.

Repair Fidelity Assay

This approach used a restriction enzyme to introduce a double strand break within a selectable marker gene, so that the fidelity of the religation event could be assessed. The PMH16 [Debenham et al., 1988] plasmid used was first cut with the restriction enzyme KpnI, which produces a single cut within the *gpt* gene. The linearised plasmid was transfected onto the B16 cells using the $CaPO_4$/DNA co-precipitation method [Graham and Van der Eb, 1973]. Cells (5×10^5) were exposed to 10 μg DNA precipitate per 10 cm dish and the precipitate was left overnight at 36°C and 3% CO_2 [Chen and Okayama, 1987]. Initially, non-transfectants were eliminated by selecting for the intact neomycin resistance gene in G418 medium containing geneticin over a 10 day growth period (at the end of 10 days, the plasmid is fully integrated into the host genome). Transformant colonies were ringed on the underside of the dishes and their religated *gpt*$^+$ function assessed by refeeding the colonies with 3 changes of XHATM medium. Any clones with a functional (correctly religated) *gpt* gene continued to grow past the previously ringed boundary, and crystal violet staining readily distinguished between viable (*gpt*$^+$) and non-viable (*gpt*$^-$) colonies.

RESULTS

Survival

Bleomycin, X-ray, and EMS survival data for the F1, BL6, and ML8 metastatic variants are shown in Figure 1A–C, respectively. The 3 variant lines are similar in their degree of sensitivity to ionising radiation and the radiomimetic drug, bleomycin, but differ in their cross-sensitivity to a third DNA damaging agent, EMS. The survival data are based on 3 separate experiments for each agent used. EMS toxicity was calculated to kill > 40% of the plated cells (mutagenic dose). A uniform dose of 1 mg/ml EMS was used for all 3 variant cell lines in determining frequency of EMS induced mutations.

Rate of Mutation

In an effort to determine the spontaneous and induced mutation rates for the high (BL6, ML8) and low (F1) metastatic variants of the mouse B16 melanoma, we used ouabain to select for resistant mutants, and the strong alkylating agent EMS as the mutagenic source. Ouabain resistant mutants have a point mutation in the gene which codes for the plasma membrane associated ATPase. This affects the property of the Na^+/K^+ transport of the cell; therefore, any enzyme without an alteration is specifically inhibited. The results shown in Table I confirm that EMS was mutagenic to the melanoma variant cell lines (i.e., the number of ouabain resistant colonies increased with exposure to EMS in

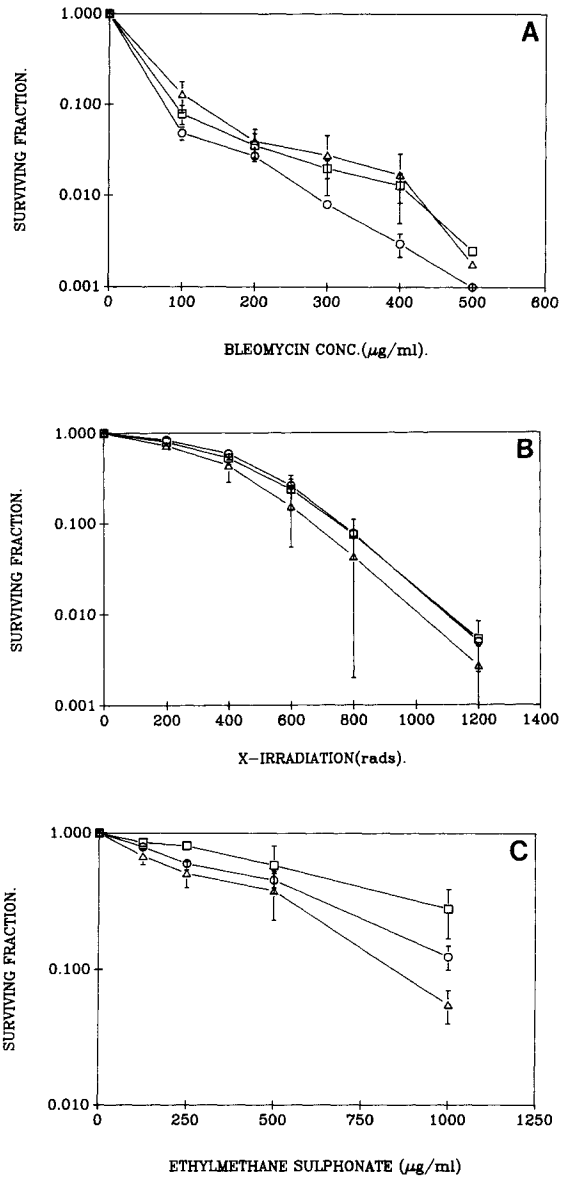

Fig. 1. Dose effect of bleomycin, X-irradiation, and EMS on cell survival of metastatic variants. F1 (low metastasis), ○—○; BL6 (high metastasis), □—□; ML8 (high metastasis), △—△.

TABLE I. Spontaneous and Induced Rates of Mutation to Ouabain Resistance*

Cell line	Metastatic potential	No. of ouabain resistant colonies: No EMS	No. of ouabain resistant colonies: 4 h exposure to EMS (1 mg/ml)
F1	low	1	29.03 ± 3.19
BL6	high	0	55.13 ± 3.01
ML8	high	2	60.40 ± 7.07

*B16 melanoma variants of high (BL6, ML8) and low (F1) metastatic potential were seeded at 10^6 cells/plate in 10 × 10 cm plastic petri dishes Selection for mutants was performed in 3 mM ouabain for 14 days The dose of EMS (ethylmethane sulfonate) was calculated to kill > 40% of the plated cells (mutagenic dose [Fig 1C]) Induced mutation data are expressed as the mean number of ouabain resistant colonies ± S E Mutation data are given in terms of per cell survivor

all 3 cell lines). The rate of EMS induced mutations was significantly higher in the highly metastatic BL6 and ML8 variants compared to the low metastasis, F1, cell line.

DNA Strand Break Repair

Alkaline unwinding of DNA coupled with hydroxylapatite chromatography was used to measure the ability of cells to repair DNA single strand damage induced by ionising radiation and bleomycin. No difference in either the rate or extent of DNA single strand break religation was detected between the low (F1) and high (BL6, ML8) metastasis variants (Fig. 2A,B).

The high and low metastatic lines were next treated with 1 Krad X-ray irradiation and their subsequent repair measured by the more sensitive alkaline elution technique. Results were similar to those obtained by alkaline unwinding with most of the repair being completed within the first 15 min. A comparison of the relative retention values [Fornace and Kohn, 1976] did not reveal any significant difference between the mean and S.D. of 5 experiments for all 3 cell lines (Fig. 3). Subsequent measurements of double strand break repair were made on cells irradiated with 10 Krad X-rays and using a neutral filter elution technique, at pH 9.6. Elution profiles of [^{14}C] DNA labelled cells were essentially the same in 3 separate experiments, for the 3 metastatic variants (Fig. 4A–C).

Alkaline elution was also used to measure DNA strand break religation after EMS treatment to see if the observed differential sensitivity to the drug (Fig. 1C) was projected in the

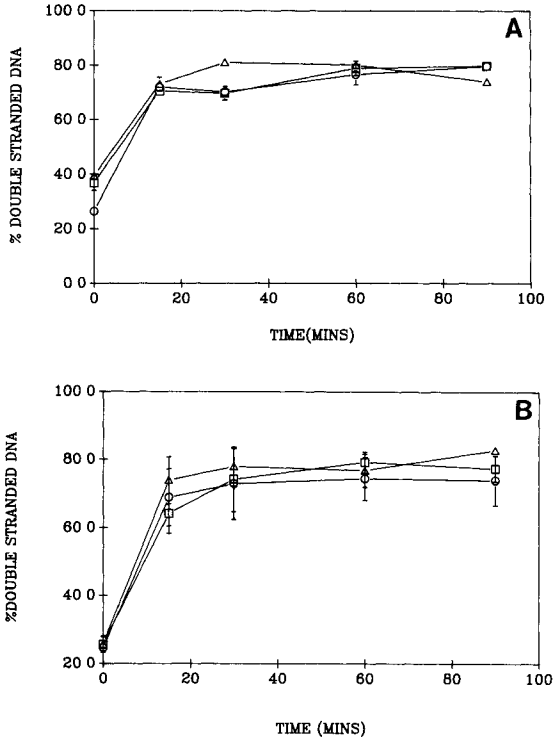

Fig. 2. DNA strand break repair time courses for lesions induced by ionising radiation (40 Gy X-rays) and bleomycin (250 μg/ml for 1 h), respectively The percentage of double stranded DNA remaining after controlled DNA unwinding in weak alkali is assessed by hydroxylapatite chromatography and used as a measure of DNA strand breaks The time shown is that of post-treatment incubation at 37°C before sampling for analysis Cell lines ○—○, F1, □—□, BL6, △—△, ML8

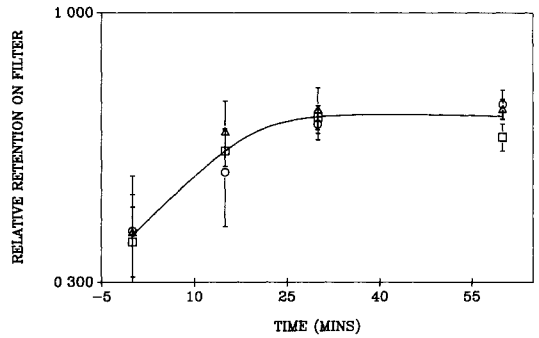

Fig. 3. Measurement of DNA strand break repair by alkaline elution in the metastatic variants *Relative retention* is defined as the fraction of test cell DNA retained on the filter when 40% of the internal standard DNA has eluted Cell lines ○—○, F1, □—□, BL6, △—△, ML8

capacity for subsequent repair of its damage. The resultant profiles revealed no difference in repair capacity between the high (BL6, ML8) and low (F1) metastasis lines (Fig. 5A–C).

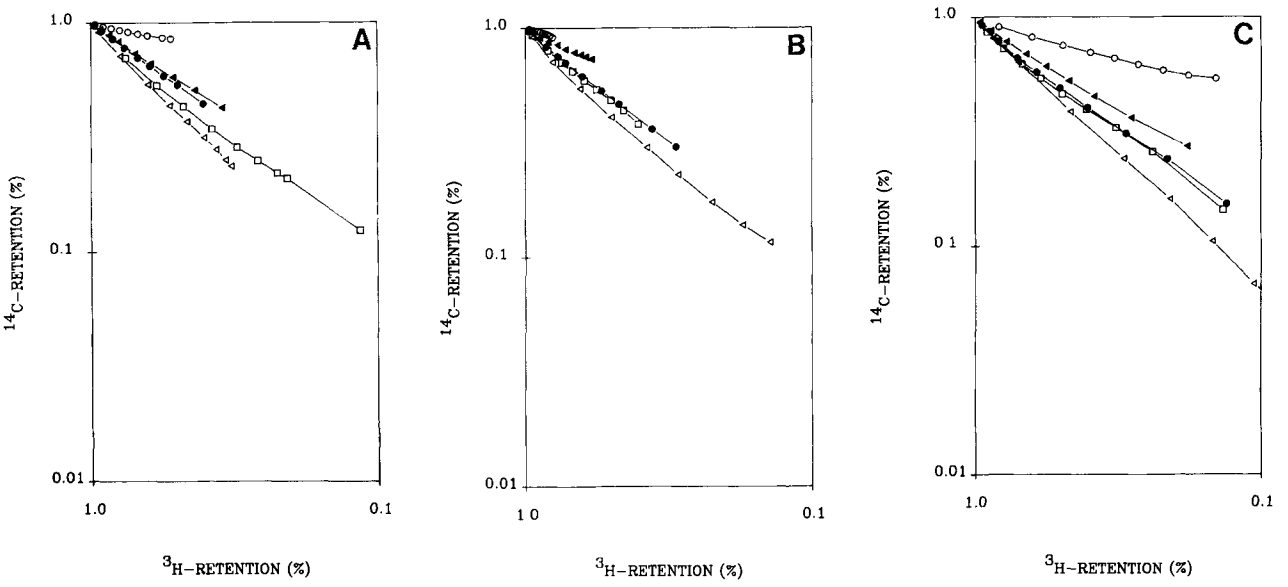

Fig. 4. Neutral elution measurements of DNA double strand breaks produced in B16 melanoma cells by X-rays (100 Gy) and their repair. The fraction of [^{14}C]-DNA retained on the filter is plotted against the internal standard [^{3}H]-DNA retained, on a log-log plot. **A:** Low metastasis F1. **B:** High metastasis BL6. **C:** High metastasis ML8. Time 0, △—△; time 15, □—□; time 30, ●—●; time 60, ▲—▲; control (undamaged), ○—○.

Repair Fidelity

The transfection efficiency for the low metastasis variant was consistently higher than that for the high metastasis lines (Table II) (using the alternative polybrene transfection procedure of Kawai and Nishizawa [1984], no difference in transfection efficiency was found in our variant cell lines between the 2 processes [data not shown]. However, the two stage selection procedure corrects for such differences. The more metastatic variants showed a greater fidelity of religation of the *gpt* gene with ~100% of ML8 clones growing in XHATM medium and ~65% of BL6 clones continuing to grow at the second stage selection. Of the low metastasis F1 clones, only ~25% had a religated and functional *gpt* gene (Table II).

DISCUSSION

The generation of metastatic variants has been attributed to an increased level of genomic instability [Nowell, 1976]. This instability is believed to be the direct result of the capacity acquired by malignant cells to undergo limited differentiation and establish a variability at the genetic level [Alexander, 1984]. Mutation assays are a direct measure of the stability of the genome, and elevated mutation frequencies have been measured in highly metastatic tumours [Cifone and Fidler, 1981]. As a measure of the inherent genomic instability within the metastatic variants, we have used a strong alkylating agent, EMS (mutagenic source), to induce mutations at the Na^+/K^+-ATPase locus. Any alterations in this membrane associated enzyme confer resistance to the drug, ouabain. Our results (Table I) show that the frequency of emerging mutants resistant to the drug ouabain was higher in the high metastasis BL6, ML8 variant cell lines compared with the low metastasis variant, F1. Thus, the induced mutation frequency was consistent with the Nowell hypothesis for metastatic progression.

However, the mechanism for the generation of elevated mutation frequencies has not been fully understood. One possibility is that these arise as a result of a reduced capacity for DNA repair. Clinical and genetic studies have already shown that a relationship exists between mutagenesis and a deficiency in DNA repair mechanisms. For example, XP cells are defective in excision repair of UV damage [Lehmann and Norris, 1989]. Fanconi's anaemia cells are defective in cross-link repair of pyrimidine dimers [Fujiwara et al., 1977]. Bloom's syndrome cells have a defective repair enzyme, DNA Ligase I, suggesting a possible association of altered DNA repair properties with genomic instability [Willis and Lindahl, 1987]. The present studies were designed to determine whether the Nowell hy-

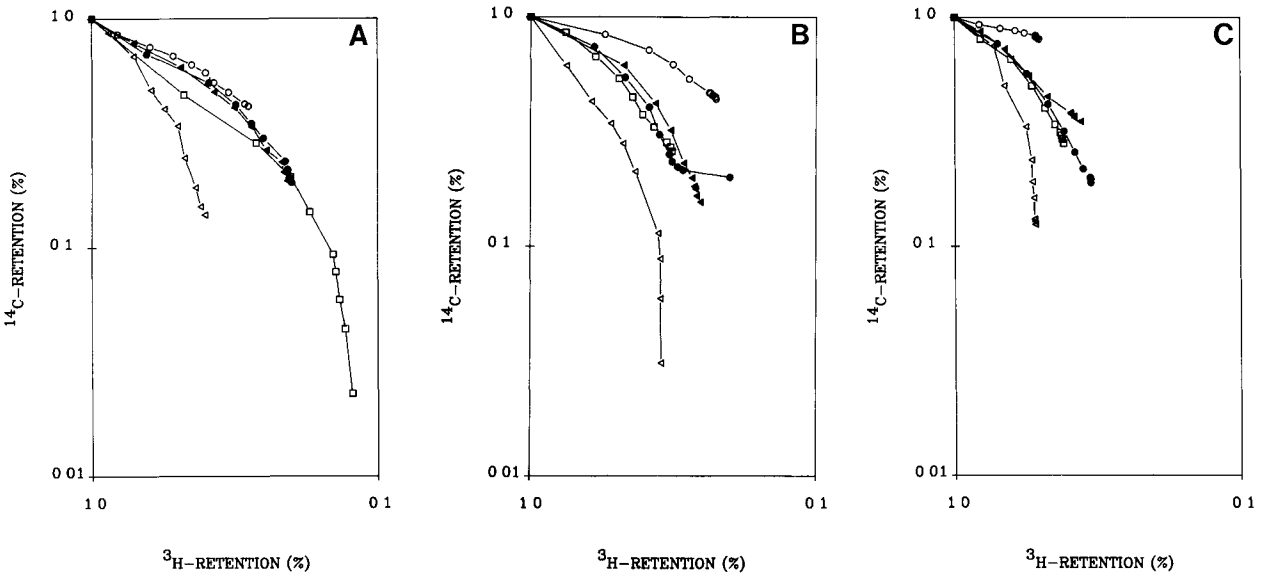

Fig. 5. Time repair courses of DNA strand breaks induced by EMS (1 mg/ml) as measured by alkaline filter elution The alkaline technique measures total strand breaks, including both single and double strand breaks, together with alkali-labile sites **A:** Low metastasis F1 **B:** High metastasis BL6 **C:** High metastasis ML8 Time 0, △—△, time 15, □—□, time 30, ●—●, time 60, ▲—▲, control (undamaged), ○—○

TABLE II. Analysis of Repair Fidelity in B16 Melanoma Metastatic Variants*

Cell line	Metastatic potential	Plasmid PMH16	No. of colonies: 1st stage selection *neo*$^+$	No. of colonies: 2nd stage selection *neo*$^+$ *gpt*$^+$	Repair efficiency (%)
F1	low	uncut	400	217	54 (100)
		cut	429	58	13 (24)
BL6	high	uncut	109	68	62 (100)
		cut	142	57	40 (64.5)
ML8	high	uncut	35	16	45 (100)
		cut	56	25	44 (97.7)

*The fidelity of strand break repair was assessed by transfection with the plasmid PMH16 which carries two selectable genes, *neo* and *gpt* Prior to transfection, enzymatic breaks were introduced within the *gpt* coding region Following transfection, cells were subjected to sequential selection for intact *neo* and *gpt* genes This selects first for cells which have taken up the plasmid and subsequently for those which have successfully religated and integrated a functional *gpt* gene into the host chromosome The proportion of *neo*$^+$ cells which are also *gpt*$^+$ provides a measure of the repair fidelity for the enzymatically induced DNA double strand breaks A Kpn I restriction enzyme is used to produce breaks within the *gpt* coding region and uncut plasmid is used as the control The two stage selection procedure corrects for any differences in transformation frequency between the cell lines

pothesis implicating an elevated mutation frequency in tumour progression would be compatible with any perceived differences in repair of induced DNA damage within the metastatic variants of the B16 mouse melanoma.

With respect to the DNA damaging agents used we found that the high (BL6, ML8) and low (F1) metastasis variants were equally sensitive to X-irradiation and bleomycin, but differentially sensitive to EMS. Furthermore, within the limits of sensitivity of the alkaline techniques used to measure DNA strand break repair, no differences were detectable in either the rate or extent of DNA repair between these variant lines, for each of the DNA damaging agents used.

It is now well established that DNA double strand breaks are induced by ionising radiation [Radford, 1985; Bryant, 1985], and cell lines specifically hypersensitive to ionising radiation have been reported to be deficient in the repair of DNA double strand breaks [Kemp et al., 1984; Giaccia et al., 1985]. In our studies of repair of double strand breaks produced by X-irradiation

in the metastatic variants, their repair as measured by neutral elution revealed no difference. Surprisingly, however, when we measured the fidelity of the double strand break repair process, we found that between 60 and 100% of the high metastasis variants were able to religate a functional *gpt* gene in the repair fidelity assay, compared with only 25% of cells with a low metastatic phenotype. This difference in the fidelity of repair might be an important phenotypic difference between the metastatic variants. For example, A-T cells (ataxia telangiectasia) which are known to be radiation sensitive have been found to have a poor religation fidelity relative to human cells with normal radiation sensitivity [Cox et al., 1986], suggesting that the sensitivity to ionising radiation may be due to a reduced religation fidelity and not incompetent rejoining of the double strand breaks. However, the B16 variant cell lines do not show any differences in sensitivity to X-rays, but do reveal a different fidelity of repair. A similar phenomenon has been observed between two radiation sensitive V79 Chinese hamster cell lines, called *irs1* and *irs2* [Debenham et al., 1988]. These mutant cell lines were similar in their degree of hypersensitivity to ionising radiation, but differed in their fidelity of repair and in their cross-sensitivities to other DNA damaging agents. We have also shown a difference in cross-sensitivity among the metastatic variants with respect to the strong alkylating agent, EMS (i.e., they are genetically distinct). Therefore, the differences seen in double strand break repair fidelity among the cell lines is not due to a difference in ionising radiation sensitivity, or bleomycin sensitivity, but might instead reflect a difference in a hierarchy of degradative enzymatic processes which compete to rejoin or degrade DNA on different double strand termini. This "competition" hypothesis has recently been favoured by Costa and Bryant [1991], who observed that the kinetics of double strand breaks in mammalian cells subjected to restriction enzyme treatment are the result of the competing processes of enzymatic incision and repair of double strand breaks. It is well established that tumour cell lines are heterogeneous in nature with respect to different cellular and biochemical functions. Therefore, it might not be unreasonable to suggest that the high metastasis cell lines, which are more genetically unstable, have a lower abundance of regular degradative enzymes which act at enzyme-mediated double strand break termini. Such an imbalance would explain an increased fidelity of religation in highly metastatic cells.

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