RESISTANCE OF MOUSE LYMPHOMA L5178YAII
CELLS TO ALKYLATION WITH METHYLMETHANE
SULPHONATE RESIDES IN A LATE STEP OF
EXCISION REPAIR

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SUMMARY

The mutant mouse lymphoma cell line (L5178YAII), resistant to X-rays, ultraviolet light and alkylating agents, was reinvestigated in an attempt to establish the nature of the mutation. These cells were compared with P388 mouse lymphoma cells, which exhibit normal sensitivity to these mutagens. A series of studies was conducted to compare DNA alkylation and strand breakage with cell survival after exposure of the two cell lines to methylmethane sulphonate.

It was found that neither the degree of alkylation nor the removal of the common alkylation products was correlated with the different sensitivities observed in these cell lines. A correlation was established between cell killing and the production of long-lived strand breaks. P388 cells were found to accumulate twice as many long-lived strand breaks compared to L5178YAII cells, at equal levels of alkylation. This suggested that long-lived strand breaks were the major toxic lesions.

Further experiments indicated that these long-lived strand breaks were produced by a process consistent with excision repair. Evidence is also presented that indicates that the mutation in L5178YAII cells that is responsible for their resistance may occur in ligase activity or its associated ADP-ribosyl transferase system.

INTRODUCTION

L5178YAII mouse lymphoma cells were originally selected as a mutant clone of L5178Y cells that was resistant to X-rays (Courtenay, 1969). Subsequent studies revealed that these cells were also resistant to a wide range of DNA-damaging agents including ultraviolet (u.v.) light and alkylating agents (Fox & Fox, 1973a,b,c,d; Fox, 1974). From these studies it was suggested that the mutation to resistance affected a part of the DNA repair pathway common to the repair of diverse types of damage. In an attempt to clarify this hypothesis the repair of alkylation damage in L5178YAII cells compared to P388 cells was reinvestigated using methylmethane sulphonate (MMS) as the alkylating agent. Unlike other studies, which have identified DNA repair deficiencies associated with hypersensitivity, the present study is concerned with acquired resistance to damage (Setlow, Regan, German & Carrier, 1969; Cleaver & Bootsma, 1975; Miller & Heflich, 1982; Amacher, Elliott & Leiberman, 1977; Maher, Birch, Otto & McCormick, 1975).

In the present study we have investigated the relationships between: DNA alkylation and dose; DNA alkylation and toxicity; and DNA strand breakage and cell
survival. This has been done to ascertain the nature of the lesion in DNA that is responsible for toxicity, and to examine the role of strand breaks in the repair process.

**MATERIALS AND METHODS**

**Cells**

Lymphoma cell lines P388 and L5178YAII were gifts from Dr M. Fox (Paterson Laboratories, Manchester, U.K.). Both cell lines were originally derived from lymphomas in DBA/2 mice. L5178YAII cells are a radiation-resistant clone of the L5178Y cell line (Courtenay, 1969). Cells were maintained in growth medium consisting of: Fischer's medium with 4 mM-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 1:125 g/l sodium bicarbonate and 20% (v/v) horse serum. Cultures containing 10^5 cells/ml in growth medium grew logarithmically with mean generation times of 11–14 h at 37°C in a humidified CO₂ incubator (4% CO₂ in air) (Flow Labs, Irvine, Scotland). Logarithmic growth continued until cultures reached approximately 4×10^5 to 6×10^5 cells/ml.

**Survival assays**

Logarithmically growing cells (10⁶–10⁷/ml) were exposed in growth medium to MMS in the concentration range from 0 to 260 μg/ml for 1 h at 37°C in an atmosphere of 4% CO₂ in air. After dosing, a 1 ml sample of cell suspension was pelleted at 300 g for 5 min, washed with 10 ml ice-cold phosphate-buffered saline (PBSA), pH 7.4, and resuspended in 10 ml of growth medium to give approximately 10⁷–10⁸ cells per ml; 1 ml of this suspension was further diluted in 4 ml of growth medium containing 0.37% (w/v) agar, plated into 50 mm vented Petri dishes (Nunc, Biocult Europe, Longbridge, U.K.) and incubated at 37°C in a CO₂ incubator for up to 2 weeks until colonies were of visible size. Colonies were then quantified with an automatic colony counter (Artek Instruments, Farmingdale, U.S.A.) and the ratio between colony numbers in treated cultures and their respective controls was determined. This ratio is defined as the fraction surviving, and is taken to reflect cell viability.

**Alkylation of DNA**

One-litre cultures of lymphoma cells in growth medium were incubated until a cell density of approximately 10⁶ cells/ml was achieved at which point they were labelled with 0.2 μCi/ml of [³H]thymidine (24 Ci/mmol) for 15 h. At the end of this period the medium was changed for fresh growth medium without radiolabel and the cultures were incubated for a further 18 h before exposure to MMS. This regime provided approximately 10⁷ cells in each culture that were still in exponential growth. Cultures were then exposed to [¹⁴C]MMS (Radiochemical Centre, Amersham, U.K.) in the dose range from 0 to 260 μg/ml (sp. act. 0.02–0.7 μCi/μg) for 1 h in growth medium at 37°C in a CO₂ incubator. Before exposure, 2×10⁶ cells were sampled to determine the specific activity of [³H]DNA. The amount of DNA present was determined by the method of Burton (1956) and radioactivity was measured by scintillation counting. After exposure to MMS the cells were pelleted by centrifugation at 300 g for 5 min, and resuspended in 10 ml ice-cold PBSA (pH 7.4). This procedure was repeated twice. The resultant pellets were taken up in 2 ml 0.15 M NaCl, 0.015 M sodium citrate (SSC) and frozen at −20°C to await DNA extraction. The pellets were thawed and nuclei were isolated according to the method of Kuchler (1977). The DNA was then extracted from the nuclear pellet by the method of Kirby & Cook (1967). In order to improve recovery of the DNA, 500 μg of calf thymus DNA was added as carrier. The DNA obtained was resuspended in 1 ml of SSC and treated for 1 h with 50 μl RNase A (1 mg/ml; Sigma Chemicals, St Louis, U.S.A.) and further treated with 150 μl Protease (10 mg/ml; Sigma Chemicals, St Louis, U.S.A.) for 2 h; both incubations were conducted at 37°C. The DNA was re-extracted and the specific activity of the ¹⁴C label in DNA was determined from the ¹⁴C/³H ratio. This was expressed as pg MMS equivalents per pg DNA. In preparations for high-pressure liquid chromatography (HPLC) analysis, 10⁶ cells were used and therefore no calf thymus DNA was added as carrier.
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HPLC analysis of methylpurines

Approximately 200 µg of DNA extracted from cells treated with equitoxic doses of [14C]MMS (P388, 40 µg/ml, 0.131 μCi/µg; and L5178Y/AII, 180 µg/ml, 0.033 μCi/µg) was suspended in 1 ml of 0.1 M HCl. This solution was then heated at 70 °C for 30 min to release purines and HPLC analysis was then conducted by the method of Beranek, Weis & Swenson (1980). A Partisil 10SCX (Whatman, Springfield Mill, Kent) column was used with a Perkin-Elmer series 3B HPLC system (Perkin Elmer, Beaconsfield, Bucks, U.K.). Solvent programme 4 (exponential gradient) starting with 0.02 M ammonium formate/6% methanol (pH 4.0), and finishing with 0.2 M ammonium formate/8% methanol (pH 4.0) at a flow rate of 1 ml/min for 25 min was used for the analysis. Absorbance at 260 nm was monitored, and fractions equivalent to 1 min of flow were collected directly into scintillation vials.

Strand breaks

Cultures were labelled in growth medium at a cell density of about 10⁴ cells/ml with [3H]-thymidine (0.2 μCi/ml, 24 Ci/nmol) or [14C]thymidine (0.02 μCi/ml, 56 mCi/mmol) for 15 h at 37 °C. The medium was then changed for fresh growth medium and the cultures were incubated for a further 24 h before exposure to MMS in the dose range from 0 to 260 µg/ml. After exposure of 10 ml of 3H-labelled culture for 1 h at 37 °C (or in some experiments 11.5 °C) the cells were pelleted by centrifugation at 300 g for 4 min and washed twice with ice-cold PBSA. The cells were then resuspended in 1 ml ice-cold PBSA and mixed with a similarly prepared 1-ml sample of unexposed 14C-labelled cells. This mixture was assayed for DNA single-strand breaks by the method of alkaline elution (Swenberg, Petzald & Harbach, 1976). The difference in % retention between 14C- and 3H-labelled DNA at fraction 10 of the alkaline phase of the analysis was termed % relative loss and was proportional to the degree of strand breakage.

DNA polymerase inhibition

A combination of cytosine arabinoside and hydroxyurea (Sigma Chemical Co.) was used to inhibit DNA polymerase activity (Collins & Johnson, 1981). In these experiments cells were labelled as described for strand breaks, and exposed to 65 µg/ml MMS for 1 h at 37 °C; 10-ml samples were taken at intervals of 1 h, starting at the beginning of exposure and continuing for 3 h. This regime was repeated with 2 × 10⁻³ M-hydroxyurea (HU) and 10⁻⁴ M-cytosine arabinoside (araC) included in the medium 30 min before exposure and present for the duration of the experiment. The samples were treated and assayed for strand breaks as described above.

RESULTS

Differential toxicity of MMS

Survival was determined by cloning cells in agar after exposure to MMS for 1 h at 37 °C. The cell lines were markedly different in their sensitivities to the toxic effects of MMS, with P388 showing greater sensitivity than L5178Y/AII cells (Fig. 1). Linear regression analysis of the logarithmic portion of each curve produced the empirical equations shown in the legend to Fig. 1. Correlations were made in terms of MMS dose as this was shown to be directly proportional to the level of DNA alkylation (see Fig. 2). The constant term in each equation is equivalent to values of n (the extrapolation number at zero dose) of 1.05 and 9.6 for P388 and L5178Y/AII, respectively, and an approximately twofold difference in gradient for the logarithmic kill phase was observed between these cell lines.
Fraction surviving was determined as the ratio of test to undosed controls. The bar lines represent standard deviations. Empirical equations were generated by regression analysis for the linear sections of the curves and were as follows: (O) L5178YAI1 loge \( S = -0.0259(x) + 2.27, r = 0.9911 \) (5 df); (●) P388 loge \( S = -0.0556(x) + 0.056, r = 0.9942 \) (5 df), where \( x \) is MMS dose and \( S \) is viable fraction; df, degrees of freedom; \( r \), correlation coefficient.

**Alkylation of DNA**

Gross alkylation of DNA was determined after exposure of cells to \(^{14}\text{C}\)MMS for 1 h at 37°C. The dose response was found to be linear with respect to MMS concentration in the range from 0 to 260 \( \mu \text{g/ml} \) and had the same dependence for both cell lines (see Fig. 2). This relationship is described by a single empirical equation derived by linear regression of the data (see legend to Fig. 2). The distribution of alkyl purines and the rate of removal of these products were determined by HPLC analysis after doses of \(^{14}\text{C}\)MMS that were equally toxic to both P388 and L5178YAI1 cells. A typical separation and radioactivity profile is shown in Figs 3 and 4. No qualitative or quantitative differences were found in the distribution or type of alkylation products produced in the DNA of these cells. Approximately 80% of the alkylation in DNA was found as 7-methylguanine and 8% as 3-methyladenine, the remainder was found to be associated with the oligonucleotide fraction. No \( \beta \)-methylguanine was detected in either cell line.
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Fig. 2. The dosimetry of gross alkylation of DNA in lymphoma cell lines P388 (●) and L5178Y AII cells (○) labelled with 0.2 μCi/ml [3H]thymidine were exposed to [14C]MMS (0.02–0.07 μCi/μg) for 1 h at 37°C and the DNA was extracted by the phenol/cresol method. The purified DNA was then assayed for 14C and 3H radioactivity by scintillation counting. The 14C/3H ratio obtained was converted to 14C specific activity by the estimation of 3H specific activity in undosed control samples using the Burton (1956) method. The bar line represents the mean and standard deviation of eight determinations. An empirical equation for this relationship was derived and is as follows: DNA alkylation = 0.9638(x) − 7.59, r = 0.9955 (12 df), where x is MMS dose in μg/ml, and DNA alkylation is pg MMS equivalents/μg DNA.

Fig. 3. A typical HPLC separation of purines using a Whatman Partisil 10 SCX column with a Perkin-Elmer solvent programme 4 exponential gradient, starting at 0.02 M-ammonium formate/6% methanol (pH 4.0), and finishing at 0.2 M-ammonium formate/8% methanol (pH 4.0), with a flow rate of 1 ml/min for 25 min. The remaining time was in the finishing buffer at the same flow rate. Peaks 1, pyrimidine oligonucleotides; 2, guanine; 3, 7-methylguanine; 4, adenine; 5, O6-methylguanine; 6, 3-methyladenine.
Half-lives of 7-methylguanine and 3-methyladenine were determined by sampling cultures treated with equitoxic doses of \(^{14}\text{C}\)MMS for up to 24 h after exposure. It was found that 7-methylguanine and 3-methyladenine were removed in both cell lines at equal rates and had half-lives of 24 h and 2 h, respectively. In conclusion, no evidence was found for differential reaction or removal of the common MMS alkylation products in DNA from the cell lines studied.

DNA strand breaks

Alkaline elution analysis was used to determine the extent of strand breakage in cells exposed to MMS (Fig. 5). No significant differences between cell lines were found for DNA retention by the filter at fraction 10 of the alkaline phase of the elution in control samples. The mean retention was found to be 94.6 ± 2.1% (standard deviation) for 25 determinations from each cell line. In dose-response experiments, P388 cells produced approximately twice as much strand breakage per unit alkylation as L5178YAI1 cells and it was possible to detect strand breaks in P388 at doses of MMS that produced no observable strand breaks in L5178YAI1 cells. Quantitative descriptions of these observations were produced as empirical equations by linear regression of the data (see legend to Fig. 5).

Fig. 4. The radioactivity profile of methylpurines from cells treated with equitoxic doses of MMS. P388 (○) and L5178YAI1 (●) cells were treated with \(^{14}\text{C}\)MMS at 40 μg/ml (0.131 μCi/μg) and 180 μg/ml (0.033 μCi/μg), respectively, for 1 h at 37°C. DNA was extracted and purified using phenol/cresol, and purines were released in 0.1 M-HCl at 70°C for 30 min. This solution was analysed by HPLC and fractions were collected for scintillation counting that represented 1 min of flow.
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Evidence of excision repair

Since alkylation and the removal of alkylation products were found to be similar in these cell lines, it was unlikely that strand breaks reflected chemically altered sites in DNA that were spontaneously labile in the alkaline conditions of analysis. To examine whether DNA breaks were enzyme-mediated the experiment was repeated at reduced temperature. It was found that alkylation of DNA, after exposure to 260 μg/ml MMS at 11.5 °C for 1 h was similar to that at 37 °C (250 ± 42 pg MMS equivalents/μg DNA) but that the incidence of strand breaks was at least eight times less (0 % relative loss in L5178YAII, 10 % in P388). These observations suggested that strand breaks arose as a result of enzymic action.

Since the cells differed in sensitivity to the toxic effects of MMS, the strands breaks may have been the result of non-specific endonuclease activity related to cell killing. To investigate this possibility an experiment was designed to follow the time-course over which strand breaks were lost. When similar numbers of strand breaks were produced in both cell lines they were found to be removed at the same rate (see Fig. 6). From this it could be deduced that the appearance and loss of strand breaks reflected repair activity, and that in this situation ligation is not rate-limiting in the sensitive cell line.
If strand breaks are the results of excision repair then ligation should be a late enzyme-mediated step following DNA repair synthesis. To investigate the involvement of late steps in the excision repair pathway two experiments were performed. The first examined the temperature dependence of strand-break removal and the second the effects of inhibiting DNA polymerase activity using araC and HU. The results are shown in Figs 7 and 8. Removal of strand breaks is clearly optimal at 37°C, consistent with an enzyme-mediated process. The involvement of one or more DNA polymerases in the MMS-induced repair process is shown by the high level of strand breaks accumulated in the presence of the inhibitors. Also, it can be seen in Fig. 8 that whilst alkylation of DNA is similar in both cell lines, at equal doses of MMS, more strand breaks appear in P388 over the short time-course and persist longer than in L5178YAII cells. However, in the presence of polymerase inhibitors (HU and araC) this did not occur and similar levels of strand breaks were observed in both cell lines.
Fig. 7. The temperature dependence of strand break removal in lymphoma cells. P388 (●) and LS178YAII (○) cells were radiolabelled with thymidine (0.2 $\mu$Ci/ml($^{3}$H) or 0.02 $\mu$Ci/ml($^{14}$C)) and exposed to MMS (as in Fig. 6) for 1 h at 37 °C. After exposure, cultures were incubated for 1 h at temperatures between 0 °C and 43 °C and assayed by alkaline elution. The points show individual experiments.

Fig. 8
From this series of observations it was possible to conclude that strand break production and removal is likely to represent the operation of a repair process and that, at equal doses of MMS, P388 cells produce a greater number of long-lived strand breaks than L5178YAI1 cells.

**Correlations**

Since the dose of MMS reflected the same degree of alkylation in both cell lines it was possible to rearrange the empirical equations shown in Figs 1 and 5 such that dose was eliminated. By doing this the correlation between strand breaks and cell survival could be defined. This is plotted in Fig. 9. It can be seen that the gradient of the exponential kill phase of each curve is very similar for both cell lines, which indicates that long-lived strand breaks may be common toxic lesions. The shoulder in the survival curve seen in L5178YAI1 cells may therefore reflect the presence of strand breaks that are relatively short-lived due to the rapid completion of the repair processes, whilst at higher doses of MMS long-lived strand breaks may be generated, resulting in a similar effect on cell survival to that produced in P388 cells at all doses studied.
DISCUSSION

An investigation of both gross alkylation and alkylated purines after MMS treatment of these cell lines indicated that there were no observable differences in the distribution, amount or removal of the alkylation products studied.

In vitro, MMS is known to produce a number of alkylation products in DNA, the major product being 7-methylguanine and the minor products consisting of 3-methyladenine, O-methylguanine, and small amounts of phosphotriesters (Beranek et al. 1980). In the present study no O-methylguanine was detected. It is a very minor product in vitro, and may have been present in vivo at levels beyond the resolution of the technique used in this study.

The rates of removal of purine alkylation products in vivo indicated that the process was probably enzyme-mediated (Lawley & Brookes, 1963; Margison, Capps, O'Connor & Craig, 1973) and also consistent with a repair mechanism that was equally efficient at removing alkylation products in both cell lines. It is therefore concluded that alkylation products or the rate of their removal did not constitute the major toxic lesion in these cells, and therefore were not responsible for the differential sensitivity observed.

Strand breaks have previously been shown to accumulate in human cells treated with MMS (Scudiero & Strauss, 1974; Karran, Higgins & Strauss, 1977). This has been postulated to be the basis of the efficient chemotherapeutic activity of this compound (Strauss et al. 1979). In the present study P388 cells were found to accumulate approximately twice as many strand breaks per unit alkylation in comparison to L5178YAI cells. The results shown in Figs 6–8 suggest that these breaks are the product of an excision-repair system rather than the spontaneous breakdown of DNA at cell death or the breakdown of chemically modified sites in DNA during analysis by alkaline elution.

In the repair of DNA in mammalian cells a steady state is thought to exist between the production of strand breaks by incision and their removal by ligation. This dynamic equilibrium is at its most impressive during the repair of u.v. damage in DNA when few strand breaks can be observed to accumulate (Erixon & Ahnstrom, 1979). However, the equilibrium can be shifted by the addition of polymerase inhibitors such as HU and araC. In the presence of these inhibitors large numbers of strand breaks accumulate. This property has been effectively used to detect and quantitate enzymic incision (Erixon & Ahnstrom, 1979; Collins & Johnson, 1979; Collins, Downes & Johnson, 1980; Snyder, Carrier & Regan, 1981; Squires, Johnson & Collins, 1982). The dynamic equilibrium is shifted such that long-lived strand breaks are generated. High levels of long-lived strand breaks have also been observed in the absence of inhibitors in cells defective in the late stages of excision repair, such as those found in the immunodeficient individual 46B.B, described by Squires & Johnson (1983) and Teo et al. (1983). The longevity of strand breaks produced by excision repair therefore seems to depend on the state of the dynamic equilibrium, which may be shifted by dysfunction of the late steps in excision repair. This can be caused directly, by mutations in the enzymes themselves; or indirectly brought about by the use
of inhibitors or mutations in other parts of DNA metabolism such as the ADP-ribosyl transferase system (Creissen & Shall, 1982).

The present studies with L5178YAI cells suggest that the mutation responsible for resistance in these cells lies in the late steps of excision repair, since after equal doses of MMS in the presence of inhibitors (araC and HU) both L5178YAI and P388 cells accumulate long-lived strand breaks to a similar degree, whilst in the absence of inhibitors fewer long-lived strand breaks accumulate in L5178YAI cells. These breaks have also been shown to be correlated with cell killing in both cell lines and therefore are likely to be the major toxic lesions. Data that seem to contradict these inferences can be found in Fig. 6, where equal levels of strand breaks in the cell lines are shown to be removed with similar kinetics. On close examination of this experiment it is apparent that highly toxic and different doses of MMS have been used, which generate less than 1% and 10% survival in P388 and L5178YAI cells, respectively. Both of those survival levels lie on the logarithmic linear portion of the correlation between survival and strand breaks (see Fig. 9). It can therefore be deduced that at these levels of survival both cell lines accumulate long-lived strand breaks. The data in Fig. 6 therefore suggest that once long-lived strand breaks are produced in these cell lines they are removed with similar kinetics. This information is consistent with the previous conclusions, in that it is the differential generation of long-lived strand breaks at equal levels of alkylation that is responsible for the difference in sensitivity to MMS observed in these cell lines. Similar conclusions have been reached to explain hypersensitivity in Chinese hamster ovary (CHO) cell lines. In this case the hypersensitive line was shown to accumulate more long-lived strand breaks than the less-sensitive normal cell line (Thompson et al. 1982). This CHO cell line was also hypersensitive to a wide range of agents including u.v. light and alkylating agents, and therefore the lesion was postulated to occur in ligase function. By analogy it is likely that the mutation in L5178YAI cells also occurs in ligase function. In support of this no differences in repair synthesis were observed after u.v., X-rays or alkylation, when these cells were compared with other lymphoma cell lines including P388 (Fox & Fox, 1973b,c,d).

The precise nature of the mutation cannot be deduced from the information available at present but it is possible that it could be in the ADP-ribosyl transferase system. 3-Aminobenzamide is known to inhibit specifically ADP-ribosyl transferase, and it is likely that ADP-ribosylation of ligase is required for its activity (Teo et al. 1983; Creissen & Shall, 1982). It is therefore possible that studies with 3-aminobenzamide would reveal that the resistance shown by L5178YAI could be modulated to the same level as that seen in P388 cells.

In conclusion, the present work supports the suggestion that long-lived strand breaks are a major factor responsible for cell killing after alkylation, and that the mutation that confers resistance on L5178YAI cells occurs in the late steps of excision repair and may be associated with the ADP-ribosyl transferase system.

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REFERENCES


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