Nucleic Acids Research, Vol. 20, No. 12 2933–2940

Self-destruction and tolerance in resistance of mammalian cells to alkylation damage

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Received April 14, 1992; Revised and Accepted May 21, 1992

INTRODUCTION

Clinical resistance to chemotherapeutic drugs is a common phenomenon. It can represent a general defensive strategy against many structurally unrelated compounds or it may be specific for particular agents. In this article, we consider resistance, both constitutive and acquired, to agents that methylate DNA. The phenomenon of induced resistance to methylating agents raises important questions about how cells are able to modify their normal metabolic processes to avoid the lethal consequences of DNA damage.

O^6-methylguanine (O^6-meGua) is one of the major products of DNA methylation by methylnitrosamines and methylnitrosamides. It has been clear for some time that O^6-meGua is important in mutation and cell transformation by methylating agents. Only more recently has it become generally accepted that O^6-meGua in DNA can also kill cells. The DNA repair enzyme that specifically demethylates this base, O^6-meGua-DNA methyltransferase (MGMT), is normally essential to the cellular defence against methylating agents. Thus, transformed human cell lines that have switched off expression of MGMT (Mex^- or Mer^-) are hypersensitive to various genotoxic properties of O^6-meGua in DNA but can be complemented by expression of either bacterial (Reviewed in 1) or human (2, 3) MGMT. There is an additional, less well defined, defence mechanism that protects against the cytotoxicity of O^6-meGua in DNA. This process, which cells acquire following exposure to methylating agents, can also confer resistance to some of the chromosomal damage induced by O^6-meGua but apparently not to the associated mutagenesis. Since this phenotype of 'methylation tolerance', allows increased survival and unchanged mutation induction, it has serious implications for cellular transformation.

In this review, we will concentrate on recent work on the regulation of MGMT expression and attempt to provide a description and working hypothesis for the phenomenon of methylation tolerance.

SELF-DESTRUCTION

Mapping and expression of O^6-meGua-DNA methyltransferase (MGMT)

The most recent comprehensive review of the function and suicidal properties of mammalian MGMTs (1) immediately predated the isolation and characterization of cDNA clones for the human MGMT. Three groups, each using a different experimental approach, reported the isolation of full length MGMT cDNA clones that exhibited a gratifying similarity (4–6). The cDNA was used to map the human MGMT gene to chromosome 10 (5) and subsequently to the tip of the long arm (7,8). More recently, MGMT cDNAs have been cloned from hamster (9); rat (10–12); mouse (13–15) and a genomic clone isolated from yeast (16). All MGMT proteins from both prokaryotic and eukaryotic sources share an identical self-destructive reaction mechanism in which they transfer the methyl group of O^6-meGua to one of their own cysteine residues. The automethylated MGMT is an inactive product of this reaction and active protein is not regenerated. All MGMT proteins contain a perfectly conserved PCHRV pentapeptide at the active site in which the cysteine residue serves as the methyl group acceptor. The overall homology among the bacterial and mammalian methyltransferases is high, particularly around this acceptor site which is located in the C-terminal region of the protein.

Several recent findings partly clarify the regulation of mammalian MGMTs and in particular the molecular events that underlie the Mex^- phenotype. Tano *et al* (4) showed that loss of MGMT activity in Mex^- cell lines could be a consequence of either a deleted gene or apparent transcriptional silencing. Analysis of a large number of Mex^+ and Mex^- lines (17) has confirmed that the MGMT gene is normally retained without significant rearrangement in Mex^- lines and is most probably transcriptionally silent. Since expression of human MGMT cDNAs in *E. coli* produces normal-length, fully-functional protein (5,18), post-translational modification of human MGMT is apparently not required for its activity.

The high frequency at which Mex^- lines arise and the occasional instability of the phenotype in culture (19–22) suggests that the silencing might be epigenetic. MGMT expression has been reactivated in a mouse cell line by treatment with 5-azacytidine (23) although several attempts to reactivate the human MGMT by similar treatment have been unsuccessful (23, 24). Recently some light has been shed on this apparent paradox by the interesting observation (25,26) that MGMT expression is associated with CpG hypermethylation in the MGMT gene of several human tumor cell lines. Loss of MGMT expression in Mex^- lines is associated with a lower level of 5-meCyt in MGMT sequences. 5-azacytidine treatment of Mex^+ cells induces the expected reduction in the level of cytosine methylation in the MGMT gene with a consequent loss of MGMT

expression (25). Thus, in these cases hypermethylation of the MGMT gene and its expression appear to be causally related and the connection between MGMT expression and cytosine methylation is the inverse of that usually observed. Hypomethylation of CpG sequences in the silent MGMT gene has also been demonstrated in a number of Mex^- human lymphoblastoid lines transformed *in vitro* with Epstein Barr virus (S. Cairns-Smith, P. K.; Cancer Res. Submitted for publication). In contrast, the MGMT gene in a cell lines that undergoes reversible phenotypic switching from Mex^- to Mex^+ (22) is hypermethylated in non-expressing cells, and a switch to the expressing phenotype is accompanied by demethylation of MGMT sequences. The variable relationship between cytosine methylation and expression of the MGMT gene in different cell lines clearly requires further investigation.

The recent identification and sequence determination of the promoter region of the human MGMT gene (27) provides a step towards understanding the complex control of its expression. The promoter region as a whole is highly GC rich and contains the clustered CpG sequences that are characteristic of promoters of housekeeping genes. The unmethylated promoter fragment is functional in murine NIH3T3 cells and seems to be able to function in either Mex^+ or Mex^- human lines (B. Kaina; cited in 27). Thus, the apparent dependence on transcription from a methylated copy in certain Mex^+ lines may not be directly related to MGMT promoter regulation. Fine structure mapping to determine the methylation pattern of the MGMT promoter in Mex^+ and Mex^- cells may resolve this question. An interesting aspect of MGMT gene regulation has been provided by attempts to map the human gene by assaying MGMT activity in hamster/human hybrid cells. Strauss (19) reviewed data from S. Mitra's laboratory indicating that enzyme expression could not be correlated with the consistent presence of any single human chromosome. Using a similar approach, Zunino *et al* (28) were later able to assign the MGMT gene to human chromosome 10. However, they found that MGMT expression was absent if chromosome 10 was accompanied by human chromosomes 3 and/or 14. These authors raised the intriguing possibility that genes encoding factors suppressing the expression or activity of MGMT are present on these chromosomes. The relationship between such putative factors and the methylation state of the MGMT gene might be an interesting area to explore.

Inducibility

The ability to greatly increase its level of active methyltransferase is an important facet of the adaptive response to alkylation damage employed by bacteria and some lower eukaryotes (29,30). Mammalian cells do not mount an adaptive response but some do display a DNA damage-dependent induction of MGMT activity. The effect is perhaps best documented in rats in which prior chronic feeding with alkylating agents enhances the ability of the liver to remove O^6-meGua from its DNA (31). Unlike the highly specific induction of the adaptive response to alkylating agents in *E. coli*, many unrelated DNA damaging agents are able to induce increased repair in liver cells. However, the enhanced ability to remove O^6-meGua is always the result of an increase in MGMT activity. The ability to respond to inducing treatments is retained by some hepatic cells in culture. Thus, there is a 2–5-fold increase in MGMT activity after pretreatment of cultured H4 rat hepatoma cells with agents as diverse as γ-rays and 2-methyl-9-hydroxy ellipticine (32). No increases were observed in human or mouse fibroblasts or CHO cells (33). These observations have recently been confirmed and extended (34,35) to show that the increased enzyme activity in rat (but not a human) hepatoma lines is correlated with an increased steady-state level of MGMT mRNA, indicating that the response might be controlled at the level of MGMT transcription. Significantly, hamsters (which are extremely sensitive to tumor induction by dimethylnitrosamine) are unable to replace the hepatic MGMT molecules depleted during repair whereas rapid recovery (induction) to beyond pretreatment levels is initiated within 24hr in rat liver (36). More recently, it has been shown that induction of MGMT activity in rat liver by treatment with nitrosamines is also associated with a higher steady-state level of MGMT mRNA, although the levels of other, unrelated mRNAs are also increased (J. Hall, Personal communicaton).

The observation that the induction of MGMT in mammalian cells is so far confined to rat hepatocytes implies that the process might be a liver-specific response of rodents. The lack of specificity in the inducing signal urges caution, however. There are numerous damage-inducible transcripts in mammalian cells for which there is so far no apparent function: an example is DNA polymerase β which is 'induced' in CHO cells by MNNG treatment (37) although the increased mRNA levels do not seem to be correlated with increased DNA polymerase activity. Indeed, unless the rate-limiting step for DNA repair is polymerization, increased DNA polymerase β alone would be of little value. The single step reaction mechanism of the MGMT protein, however, confers the unique advantage that any increase in its intracellular level will always bring about a proportional increase in the ability to repair O^6-meGua. Since numerous non-repair related functions are also induced by DNA damage to rat liver, the possibility remains that activation of transcription factors (38) results in increased transcription of many genes. Only those enzymes whose activity levels are controlled by the availability of mRNA might exhibit induction at the protein level.

O^6-meGua and mutation induction

The determination of mutational spectra has defined two areas of contemporary interest in the role of MGMT in mutation avoidance. The first of these is the issue of sequence- and DNA strand-specificity of induced mutation. The second concerns the role of MGMT in protection against spontaneous mutation.

O^6-meGua in DNA introduces G to A transitions. The distribution of these mutations is not random and certain DNA sequences are mutational hot-spots (39–41). Most significantly, the second G in the sequence 5′-GpG-3′ is mutated at high frequency. This is best exemplified by the c-Ha-*Ras* gene in MNU-induced rat mammary tumors where activating G to A transitions occur exclusively in the second G of the GGA of codon 12 (42).

To explain the targeted activation, the relative contributions of differential reactivity towards MNU and MNNG, differential repair of O^6-meGua and effects on DNA replication have all been separately considered. Some of the mutational bias can be ascribed to preferential methylation at the central position of the codon since MNU exhibits sequence selectivity (39, 43). An additional component is the effect of flanking sequence on misreplication of the methylated base (44,45). Non-uniform repair of O^6-meGua is also considered to be a likely contributor to mutational bias (46, 47). The sequence context of a central O^6-meGua in codon 12 of H-*ras* makes it the poorest among several related substrates for a bacterial MGMT *in vitro*. Further, antibodies raised against O^6-meGua were least able to recognize

the methylated base when it was in this sequence (48). Thus, O^6-meGua in particular sequence contexts in DNA is apparently poorly accessible for DNA-protein interactions-including those which involve its own repair enzyme. However, comparison of mutational spectra in Mex^+ and MGMT depleted human cells indicated that the presence of MGMT conferred protection against mutation induction but did not alter the types or distribution of MNNG-induced mutations (49). This suggests that *in vivo*, sequence dependent variations in the rate of repair by MGMT is not a major factor in determining the distribution of O^6-meGua-induced mutations.

Certain types of DNA damage are preferentially removed from the transcribed strand of transcriptionally active genes in mammalian cells (50). A consequence of this repair bias would be relative enrichment of mutations in the non-transcribed strand and this is indeed observed for hamster and human cells (51,52). Reed and Hutchinson (53) suggested that there might be a repair bias towards the transcribed strand for O^6-meGua. After MNNG treatment of mammalian cells G to A transitions are overwhelmingly found on the non-transcribed strand (49). The same strand bias occurs after MNNG treatment of Mex^- and Mex^+ cells, however, indicating that the strand distribution of MNNG-induced mutations is not due to preferential repair by MGMT of the transcribed strand. A similar conclusion has been reached by Palombo *et al* (54) who used a shuttle vector carrying the *E. coli gpt* gene under the control of an inducible promoter. When the vector is present in human cell lines treated with MNU, there is again a non-random distribution of G to A transitions that is highly biased towards the non-transcribed strand of the *gpt* gene, but the strand-specific distribution is unchanged by its transcriptional state. Thus, the bias in MNU-induced mutation is not a consequence of either preferential MGMT repair or of transcriptional activity. Interestingly, a major determinant of the bias is apparently the codon sequences themselves and in particular, the probability that an O^6-meGua-derived mutation will give rise to a selectable mutation (55).

Overexpression of the constitutive Ogt methyltransferase in *E. coli* protects against spontaneous mutation (56) and the simultaneous absence of both the Ada and Ogt methyltransferases confers a mutator phenotype on non-dividing *E. coli* (57). Both sets of observations imply the existence of an environmental or endogenous source of an alkylating agent that generates O^6-meGua (or the minor lesion O^4-meThy, which is repaired by the same enzyme) residues in cellular DNA. O^6-meGua does not destroy the ability of DNA to act as a template for either transcription or replication and it is likely to induce identical copying errors by both RNA and DNA polymerases. Advantageous errors in mRNA that would allow a particular cell to divide might be mirrored by identical base substitution mutations during subsequent DNA replication, thus fixing as a mutation the phenotypic change that resulted from base misinsertion during transcription. O^6-meGua, therefore represents an attractive candidate for generation of advantageous spontaneous mutations under non-growing conditions.

Among mammalian cells, the spontaneous mutation spectra in the APRT gene of CHO cells that differ in their expression of MGMT are almost identical in both frequency and distribution. The exceptions are a significant reduction in the frequency of G to A transitions and an increase in G to T transversions (Aquilina *et al.*, Cancer Research; Submitted). A reasonable explanation for the former observation would be that non-enzymatic DNA methylation makes a significant contribution to spontaneous mutation in these cells. In general, however, the variations in spontaneous mutation spectra among mammalian cells are much greater than those in induced spectra (58) and there is no obvious unifying process underlying the origins of most spontaneous mutations in mammalian cells.

TOLERANCE

Phenotypic description

Tolerance to methylating agents can be simply defined as resistance to the cytotoxicity of O^6-meGua in DNA that is not accompanied by increased repair of the methylated base.

Although Baker *et al* (59) made the important observation that the cytotoxic and mutagenic effects of alkylating agents towards HeLa cells were separable phenomena, the first suggestion of methylation tolerance was by Goldmacher *et al* (60) who isolated an MNNG-resistant variant of the human lymphoblastoid cell line TK6. The variant was similar to the parental line in that it did not express MGMT and could remove only 3-methyladenine from its DNA. It sustained the same initial level of DNA methylation which indicated that its tolerance of methylation damage was not due to the multidrug resistant (MDR) phenotype in which protection is conferred *via* exclusion of drug from the cell. The resistant line exhibited a slightly increased MNNG-induced mutation frequency but a more striking 40-fold increase in spontaneous mutation rate.

In a study of MNNG-resistant variants of (Mex^-) HeLaMR cells, Goth-Goldstein (61) found no difference in initial level of damage or rates of removal of methylated bases between parental and resistant lines. By isolating resistant variants from a number of initially clonal HeLaMR populations, she could rule out selection of a pre-existing resistant sub-population and suggested that an epigenetic mechanism might explain the high frequency at which tolerant lines arise. A second study of HeLa cell variants demonstrated the absence of correlation between the persistence of O^6-meGua in DNA and cellular sensitivity to killing (62). The tolerant phenotype is not confined to human cell lines. Goth-Goldstein and Hughes (63) isolated a CHO cell line that exhibited resistance to killing by MNNG and MNU, was cross-resistant to streptozotocin and (slightly) to MMS but displayed unaltered sensitivity to ENU, ENNG, UV-light and X-rays. Interestingly, the tolerant cells were somewhat hypersensitive to chloroethylnitrosourea. The levels of the methylpurine-DNA glycosylase activity in cell-free extracts were unaltered in the tolerant cell line which remained MGMT-deficient.

In the above examples, tolerant cells were isolated following exposure of (Mex^-) cells to a single high concentration of MNNG. An alternative approach has been to induce resistance by stepwise increasing concentrations of methylating agent. This approach was used to isolate resistant variants of (Mex^-) HeLa S3 cells that were cross-resistant to MNU and only marginally to MMS and DMS (24). The enhanced resistance was not accompanied by detectable MGMT activity in cell-free extracts. Aquilina *et al* (64) applied selection with increasing concentrations of MNNG to a $MGMT^+$ CHO cell line (65). Cells initially acquired a moderate (two-fold) increase in resistance as a result of a two-fold increase in MGMT. Subsequent increments in resistance were not associated with increased repair of O^6-meGua or any other methylpurine and the absence of cross-resistance to other classes of DNA damaging agents indicated that high level MNNG resistance was due to tolerance. This was the first demonstration that tolerance could be induced in MGMT-

expressing cells. Subsequently a similar protocol has been used successfully to isolate tolerant variants of a Mex^+ HeLa line (66, G. Aquilina, M. B.; unpublished). Tolerant cells selected by this type of protocol are generally much more resistant than those isolated in a single step and they illustrate that tolerance does not seem to be an all or none phenomenon but rather appears to be of an incremental nature.

Proof that tolerance was specific for O^6-meGua among methylated bases, was provided by Green *et al* (67) who examined the properties of MNU-resistant Mex^- MRC5 human fibroblasts that had been transfected with the *E. coli ada* O^6-meGua-DNA methyltransferase gene. They demonstrated that while expression of the Ada protein conferred resistance to MNU on the parental MRC5 cells, no increased protection resulted from methyltransferase expression in cells previously selected for tolerance to MNU. The data best fitted a model in which tolerance and the Ada protein acted on the same lethal lesion. These tolerant cells were also slightly hypersensitive to a chloroethylating agent. A surprising property of the tolerant MRC5 cells was their cross-resistance to low concentrations of 6-thioguanine (6-TG), a base analog that is normally cytotoxic *via* incorporation into DNA. This observation was extended by Aquilina *et al* (68, 69) who demonstrated that resistance in tolerant CHO lines was due neither to a loss of HPRT activity nor to the exclusion of 6-TG from DNA. On the contrary, tolerant cells were distinguished from the parental line in their ability to carry out multiple rounds of replication of DNA highly substituted with 6-TG. The demonstration that variant lines isolated as $HPRT^+$ / 6-TG-resistant exhibited a cross-resistance to MNU confirmed that tolerance extended to both O^6-meGua and 6-TG in DNA. Other base analogs tested; 2-aminopurine, 2,6-diaminopurine, 5-azacytidine, 5-bromodeoxyuridine, 8-azaguanine and 8-azaadenine were equally cytotoxic to normal and tolerant cells (67,68).

In all cases where it has been measured, the methylating agent-induced mutation frequency in tolerant human and hamster cells is closely similar to that of their respective parental lines (61,62,24, G. Aquilina and M.B; unpublished) indicating that tolerance is confined to cytotoxicity of O^6-meGua in DNA. The data for sister chromatid exchange (SCE) induction are more contradictory. No difference was observed in MNNG-induced SCE between resistant and parental cells in two studies (62,63) while protection against MNU-or 6-TG-induced SCE was apparent in tolerant CHO cells selected by MNNG and 6-TG respectively (69). It should borne in mind, however, that the cells that have acquired resistance to SCE induction are apparently the ones that exhibit the more extreme degrees of tolerance, and this discrepancy perhaps reflects the sensitivity of the assay. Alternatively, resistance to the SCE-inducing effect of O^6-meGua (and 6-TG) in DNA may require a further modification in phenotype after acquisition of the initial level of MNNG or MNU resistance.

In summary, tolerance to the cytotoxic but not the mutagenic effects of methylating agents is a widespread phenomenon. The degree of tolerance is correlated with the S_N1 character of the agent and all available evidence indicates that resistance is a consequence of a specific acquired ability to circumvent the cytotoxic effects of O^6-meGua in DNA. The resistance does not seem to extend to DNA damage induced by ethylating agents, UV-light, large chemical adducts or gamma rays. The only other modified DNA base that is known to be subject to this type of tolerance is 6-thioguanine (6-TG) which exerts its effect *via* its incorporation into DNA. O^6-meGua and 6-TG have closely similar molecular volumes and they also share the property of being unable to form a stable base pair with either of the normal pyrimidines (70). The cross-resistance of tolerant cells to 6-TG and O^6-meGua, together with their unaltered sensitivity to DNA ethylation indicates that an important feature of the tolerated base is its similarity to normal guanine.

Tolerance and DNA replication

To understand how cells become resistant to the lethal effects of a particular DNA damage, it is useful to have some idea of how that damage kills cells. Tolerance to UV damage is generally conceived as a process by which cells overcome blocks to replication fork progression. Mammalian cells are thought to tolerate replicon-blocking UV photoproducts *via* gap formation and *de novo* synthesis in the daughter DNA strand (71). While the deficiencies of the experimental approach that led to this model have been pointed out (72), it is generally agreed that UV photoproducts do block DNA synthesis and that this blockage is related to their lethal effect. UV photoproducts and the methylated base 3-methyladenine, which is a potentially lethal lesion in *E.coli* (73) are blocks to purified DNA polymerases in a simple *in vitro* template/primer system (74,75). Other potentially lethal modified bases such as those produced by cisPt or AAAF and abasic sites all share this ability to arrest DNA replication. In contrast, O^6-meGua does not efficiently terminate DNA chain growth *in vitro* (75) although it does slow replication fork progression (45,76). (It should be noted, however, that the replication blocking ability of this base may be dependent on sequence context (77)). The lethal effect of O^6-meGua in DNA thus may be a consequence of a property other than its ability to arrest replication.

Measurements of DNA synthesis in intact cells confirm that the effect of O^6-meGua in DNA is unusual. Chemical or physical DNA damage usually results in immediate inhibition of DNA synthesis. Despite its lethal effect, O^6-meGua in DNA is not a direct block to DNA replication. Plant and Roberts (78) and later others (79,80) showed that MNU or MNNG treatment of Mex^- cells produces a unique dose-dependent inhibition of DNA synthesis that is apparent only in the second S phase following treatment. No effect is observed in the first S phase. These data imply that O^6-meGua·C base pairs can be replicated quite well and only when the replicated O^6-meGua is itself replicated in the daughter cell is there any impairment of DNA synthesis. Consistent with this interpretation is the finding that MNU/MNNG treated cells are able to divide once before ceasing to proliferate (60,80) and that the appearance of MNNG-induced SCE requires two S phases (81). That the second S phase inhibition of replication is related to cytotoxicity is indicated by the observation that 'dead' cells, as defined by membrane integrity, sorted by cell sorting are predominantly arrested in the S phase some 24 hours or more after treatment with MNNG (80). Cell death involves DNA degradation but preliminary experiments (P.K., P. Macpherson; unpublished) indicate that this process is not associated with the nucleosomal 'ladder' characteristic of apoptosis. The intuitive expectation that methylation tolerant cells will not undergo MNU-induced inhibition of replication in the second S phase has recently been confirmed experimentally in HeLa cells (P.K., P. Branch; unpublished), indicating a connection between the delayed DNA synthesis inhibition and the lethality of MNU. The tolerant HeLa cells remain unchanged in their sensitivity to the ENU-induced

Table 1. DNA O^6-Alkylguanine at D_{37} doses to human and Chinese hamster cells

Cell Line	Agent	D_{37} (μM)	O^6-AlkylGua/ Cell	Reference
HeLa	MNU	10	3200	108
HeLa	MNNG	0.05	1800	108
HeLa	MNU	6.0	1000	61
TK6	MNNG	0.04	500	60
Fibroblast	MNNG	0.15	440	110
Human Tumor	MNNG	0.15	280	110
CHO	MNU	60	3400	82
CHO	ENU	3000	45000	82
Fibroblast	ENU	2500	52000	110, 111
V79	ENU	2300	25000	112, 113

DNA synthesis inhibition that occurs immediately following treatment.

It appears then, that the mechanism of cell killing by O^6-meGua in DNA, although involving an impaired DNA replication, differs fundamentally from that associated with UV and UV-mimetic agents. There are some indications that it is the cell's attempts to process O^6-meGua in DNA that results in its death. Firstly, DNA-O^6-meGua is a very efficient cytotoxic lesion. At a D_{37} dose for UV light in an excision-defective xeroderma pigmentosum cell, there are $\geq 5.10^4$ photoproducts, or on average approximately 1 per replicon (72). Table 1 shows the calculated cellular load of O^6-meGua at the D_{37} of Mex^- human and CHO cells. The values are scattered but the average is at least an order of magnitude lower than for UV photoproducts. Thus, significant cytotoxicity results from relatively few O^6-meGua lesions. Secondly, at equitoxic doses of ENU and MNU in hamster cells which are unable to remove either O^6-meGua or O^6-ethylguanine (O^6-etGua) to any significant extent, there is a much higher level of O^6-etGua than O^6-meGua in DNA (Table 1). It seems likely that the closer similarity of O^6-meGua to unsubstituted guanine allows it to be recognized by a normal cellular function and this recognition is related to its efficient cytotoxicity. In agreement with this notion, MNU/MNNG tolerance is recessive in cell-cell hybrids (G. Aquilina, M. B.; unpublished data) suggesting that it is acquired as a consequence of the loss of a normal function rather than the gain of a new one.

The sensitivity of DNA replication to ionizing radiation indicates two targets for inhibition (83). One target corresponds to the size of an average replicon. The second is $\geq 10^9$ Da in size and is thought to represent a cluster of replicons requiring coordinate initiation. A single 'hit' in this large domain has profound effects on DNA replication. The relatively low number of these targets—around 10^3 per cell—is in the same range as the low numbers of O^6-meGua lesions required for cytotoxicity, and suggests that the cytotoxic effect of O^6-meGua may be related to an ability to block replicon initiation rather than elongation. In this regard it is perhaps significant that the effect of the other tolerated base analog, 6-TG, on SV40 replication *in vivo* is an impairment of replication initiation (84). Recently, low level γ-radiation of human cells has been shown to inhibit the replication of small autonomously replicating plasmids at doses too low to result in damage to the plasmids themselves (85). This effect has been ascribed to a '*trans*-acting' factor, presumed to be activated or synthesized in response to genomic DNA strand breakage and whose activity outlives the inducing DNA damage signal. An alternative suggestion has been made that the mechanism acts effectively in *cis* because of the close association of replicating episomal and chromosomal DNA at the nuclear matrix (86). Whatever the explanation for its mechanism, there clearly exists a means of amplifying the DNA synthesis inhibitory effects of relatively minor amounts of DNA damage.

Models of methylation tolerance

It has been suggested that tolerance may result from an enhanced ability to repair methylation lesions in DNA other than O^6-meGua (24,62,87). While the apparent recessive nature of the tolerant phenotype would indicate otherwise, and the circumstantial evidence linking O^6-meGua to cytotoxicity is strong, a definite answer to this question awaits a molecular characterization of tolerance. One model for tolerance invokes the loss of a DNA mismatch repair system. An apparent connection between the lethality of O^6-meGua and mismatch correction was inferred from the abrogation of hypersensitivity to MNNG-induced killing in an *E. coli dam* mutant strains by the introduction of a second mutation that inactivated the post-replication mismatch correction system (88,89). The absence of strand-discrimination signals for mismatch correction in *dam* mutants was presumed to be an important contributor to O^6-meGua cytotoxicity. Goldmacher *et al* (60) adapted this model to human cells to explain the properties of their resistant (tolerant) lymphoblastoid cell lines. An important feature of these particular lines was their apparent mutator phenotype. This property is expected if tolerance results from the loss of a mismatch correction function. The spontaneous mutation rates of tolerant cells have not perhaps received the attention they merit.

An outline of the mismatch correction model (88,60) together with recent supporting evidence (90,66) is as follows:

When a replication fork encounters O^6-meGua it is likely to be slowed but, with some dependence on sequence context (44), will usually incorporate and extend a TMP residue (while there is apparently no thermodynamically 'good' complementary base for O^6-meGua but O^6-meGua·T introduces less structural distortion of DNA (91)). Subsequently, O^6-meGua·C and O^6-meGua·T pairs are recognized by the mismatch correction system which initiates excision of the perceived incorrect pyrimidine. Repair synthesis, which occurs in the strand opposite O^6-meGua, is doomed to failure owing to the inability to find a good complementary match for the methylated base. The attempted correction at O^6-meGua results in a 'repair patch' in the strand opposite the methylated base which itself remains in DNA. Tolerance would arise when the mismatch repair system no longer initiates this abortive correction. Day *et al* (90) have pointed out that the model is consistent with their observations of elevated MNNG-induced DNA repair synthesis and the persistence of MNNG-induced strand breaks in Mex^- cell lines. The effects on DNA replication may be explained by the strand breaks persisting into the subsequent cell cycle and inhibiting replication initiation.

The suggestion of persistent strand breaks is not new. DNA discontinuities associated with O^6-meGua were proposed 20 years ago by Plant and Roberts (78) to explain the delayed replication inhibition by MNU and MNNG. Direct experimental evidence of a role for O^6-meGua-associated strand breakage in the cytotoxicity of methylating agents has been obtained by DNA alkaline elution (92). Both hamster and Mex^- human cells respond to alkylating agents by immediately introducing a large number of alkali-sensitive sites or nicks into their DNA. In

hamster cells, a large proportion of these lesions have the properties of abasic (AP) sites (93) that apparently arise at oxygen alkylated bases. Their rate of appearance is too rapid to be accounted for by the known rates of chemical or enzymatic loss of the N-alkylated bases and their disappearance suggests some kind of active repair event. An involvement in cell killing is inferred from the observation that the nicks (or abasic sites) persist in a sensitive Mex$^-$ HeLa line (92). Methoxyamine (MX), a compound that reacts with abasic sites to render them insensitive to cleavage by mammalian AP endonuclease(s) (94, 95), protects cells against the cytotoxicity of MNU (96) suggesting that chain breakage at rapidly-formed abasic sites is linked to cell killing. As might be expected, the protective effect of MX is not seen in tolerant CHO cells (P. Fortini; unpublished). Although indirect, the evidence indicates that AP sites associated with sites of oxygen alkylated DNA bases are involved in killing and that altered processing of these lesions by tolerant cells is the basis of their resistance.

Biochemical approaches

Attempts are underway to reconcile the *in vitro* biochemical properties of O^6-meGua with the biological effects of methylating agents and the particular nature of methylation tolerance. The effects of O^6-meGua on the interaction of SV40 T antigen with its target DNA sequence in the SV40 origin of replication have been investigated and the data serve to underline the importance of DNA sequence context in the biological effects of this methylated base. In some sequences, a single O^6-meGua is sufficient to abolish T antigen interaction with one of its binding sites whereas multiple substitution of the second T antigen binding site is without effect (97). The presence of O^6-meGua or 6-thioguanine in DNA can also inhibit recognition by restriction endonucleases and the former has been shown to alter the efficiency of the cytosine 5-methyltransferase in a sequence-dependent fashion (98–100). Human cells contain at least two distinct proteins that recognize and bind to single base pair mismatches in DNA (101,102). One is specific for G·T while the other recognizes several purine·pyrimidine and pyrimidine·pyrimidine mispairs. While their roles are unknown, it is possible that they are involved in mismatch correction. Neither activity is able to recognize O^6-meGua-containing base pairs (103) and both are present in apparently normal levels in extracts of a number of tolerant cell lines. Human cell extracts that are able to cleave oligonucleotides containing a single G·T mismatch (104) do not nick O^6-meGua·T or O^6-meGua·C base pairs at the same position (S. Griffin, P.K.; unpublished).

Using an *in vitro* system, we have recently shown that replication of an SV40 origin-containing plasmid by extracts of HeLa(Mex$^-$) is slowed by the presence of a low number (about 1) of O^6-meGua residues introduced into the plasmid by prior MNU treatment. Replication by extracts of tolerant HeLa(Mex$^-$) cells is unimpaired. The inhibitory effects of O^6-meGua are associated with O^6-meGua-dependent repair synthesis (SV40 T antigen-independent DNA synthesis) that is not carried out by extracts of tolerant cells (S. Ceccotti, P.K., M.B.; unpublihed). These preliminary data demonstrate that O^6-meGua·C base pairs in DNA slow the replication fork and stimulate apparent repair synthesis. Further, the ability to carry out this 'repair' is most likely associated with the cytotoxicity of the methylated base. By situating O^6-meGua at specific sites in DNA, the size and location of the 'repair patch' can be evaluated. In addition, the effect of the modified base on replication of the leading and lagging strand can be separately determined along with any differences in the ability of cell extracts to process and replicate DNA containing O^6-meGua·C or O^6-meGua·T mispairs.

About 10 years ago, the phenomenon of tolerance was summarized thus: '.........the investigation of tolerance of DNA base damage in replicating mammalian cells is fraught with experimental difficulties and interpretive complexities' (72). We have no doubt that this is still true. However, those particular attempts to understand the process of tolerance to UV damage in mammalian cells were ultimately frustrated by the limitations of the analytical techniques. We are now more fortunate in having powerful *in vitro* assays which allow biochemical characterization of pathways that were previously only approachable as phenomena. The use of assays for specific DNA-protein interactions (105), DNA excision repair (106) and DNA replication (107, 108) should, in the near future, help unravel the molecular changes that underlie this particular pathway of acquired drug resistance.

ACKNOWLEGEMENTS

We thank our numerous colleagues for their views on the manuscript and in particular Jenny Dogliotti and Shaun Griffin for discussions and Professor S. Fortini for molecular volume estimations. We are also indebted to Drs. Janet Hall and Geoffrey Margison for communicating their unpublished data.

REFERENCES

1. Pegg, A. E. (1990) Cancer Res., **50**, 6119–6129.
2. Kaina, B., Fritz, G., Mitra, S. and Coquerelle, T. (1991) Carcinogenesis, **12**, 1857–1867.
3. Wu, Z., Chan, C. L., Eastman, A. and Bresnick, E. (1991) Molecular Carcinogenesis, **4**, 482–488.
4. Tano, K., Shiota, S., Collier, J., Foote, R. S. and Mitra, S. (1990) Proc. Natl. Acad. Sci. USA, **87**, 686–690.
5. Rydberg, B., Spurr, N. and Karran, P. (1990) J. Biol. Chem., **265**, 9563–9569.
6. Hayakawa, H., Koike, G. and Sekiguchi, M. (1990) J. Mol. Biol., **213**, 739–747.
7. Gardner, E., Rydberg, B., Karran, P. and Ponder, B. A. J. (1991) Genomics, **11**, 475–476.
8. Natarajan, A. T., Vermeulen, S., Darroudi, F., Valentine, M. B., Brent, T. P., Mitra, S. and Tano, K. (1992) Mutagenesis, **7**, 83–85.
9. Rafferty, J. A., Elder, R. H., Watson, A. J., Cawkwell, L., Potter, P. M. and Margison, G. P. (1992) Nucleic Acids Res., **20**, 1891–1895.
10. Potter, P. M., Rafferty, J. A., Cawkwell, L., Wilkinson, M. C., Cooper, D. P., O'Connor, P. J. and Margison, G. P. (1991) Carcinogenesis, **12**, 727–733.
11. Sakumi, K., Shiraishi, A., Hayakawa, H. and Sekiguchi, M. (1991) Nucleic Acids Res., **19**, 5597–5601.
12. Rahden-Staron, I. and Laval, F. (1991) Biochem. Biophys. Res. Commun., **177**, 597–602.
13. Shiraishi, A., Sakumi, K., Nakatsu, Y., Hatakawa, H. and Sekiguchi, M. (1992) Carcinogenesis, **13**, 289–296.
14. Shiota, S., von Wronski, M. A., Tano, K., Bigner, D. D., Brent, T. P. and Mitra, S. (1992) Biochemistry, **31**, 1897–1903.
15. Santibanez-Koref, M., Elder, R. H., Fan, C.-Y., Cawkwell, L., McKie, J. H., Douglas, K. T., Margison, G. P. and Rafferty, J. A. (1992) Mol. Carcinogenesis, **5**, 161–169.
16. Xiao, W., Derfler, B., Chen, J. and Samson, L. (1991) EMBO J., **10**, 2179–2186.
17. Pieper, R. O., Futscher, B. W., Dong, Q., Ellis, T. M. and Erickson, L. C. (1990) Cancer Commun., **2**, 13–20.
18. von Wronski, M. A., Shiota, S., Tano, K., Mitra, S., Bigner, D. D. and Brent, T. P. (1991) J. Biol. Chem., **266**, 1064–1070.
19. Strauss, B. S. (1990) Mutat. Res., **233**, 139–150.

20. Arita, I., Tatsumi, K., Tachibana, A., Toyoda, M. and Takebe, H. (1988) Mutat. Res., **208**, 167–172.
21. Arita, I., Fujimori, A., Takebe, H. and Tatsumi, K. (1990) Carcinognesis, **11**, 1733–1738.
22. Karran, P., Stephenson, C., Macpherson, P., Cairns-Smith, S. and Priestley, A. (1990) Cancer Res., **50**, 1532–1537.
23. Mitani, H., Yagi, T., Leiler, C. Y. M. and Takebe, H. (1989) Carcinogenesis, **10**, 1879–1882.
24. Ishida, R. and Takahashi, T. (1987) Carcinogenesis, **8**, 1109–1113.
25. Pieper, R. O., Costello, J. F., Kroes, R. A., Futscher, B. W., Marathi, U. and Erickson, L. C. (1991) Cancer Communications, **3**, 341–353.
26. Wang, Y., Kato, T., Ayaki, H., Ishizaki, K., Tano, K., Mitra, S. and Ikenaga, M. (1992) Mutat. Res., **273**, 221–230.
27. Harris, L. C., Potter, P. M., Tano, K., Shiota, S., Mitra, S. and Brent, T. P. (1991) Nucleic Acids Res., **19**, 6163–6167.
28. Zunino, A., Arena, G., Rossi, O., Archidiacono, N., Rocchi, M., Romeo, G. and Abbondandolo, A. (1991) Mutagenesis, **6**, 395–397.
29. Lindahl, T., Sedgwick, B. and Sekiguchi, M. (1988) Ann. Rev. Biochem., **57**, 133–157.
30. Baker, S. M., Margison, G. P. and Strike, P. (1992) Nucleic Acids Res., **20**, 645–651.
31. Montesano, R., Bresil, H., Planche-Martel, G., Margison, G. P. and Pegg., A. E. (1980) Cancer Res., **40**, 452–458.
32. Leferbvre, P. and Laval, F. (1986) Cancer Res., **46**, 5701–5705.
33. Laval, F. (1990) Mutat. Res., **233**, 211–218.
34. Fritz, G., Tano, K., Mitra, S. and Kaina, B. (1991) Mol. Cell. Biol., **11**, 4660–4668.
35. Fukuhara, M., Hayakawa, H., Sakumi, K. and Sekiguchi, M. (1992) Jpn. J. Cancer Res., **83**, 72–77.
36. Hall, J., Bresil, H., Serres, M., Planche-Martel, G., Wild, C. P. and Montesano, R. (1990) Cancer Res., **50**, 5426–5430.
37. Fornace, A. J., Zmudzka, B., Hollander, M. C. and Wilson, S. H. (1989) Mol. Cell. Biol., **9**, 851–853.
38. Stein, B., Rahmsdorf, H. J., Steffen, A., Litfin, M. and Herrlich, P. (1989) Mol. Cell Biol, **9**, 5169–5181.
39. Richardson, F. C., Boucheron, J. A., Skopek, T. R. and Swenberg, J. A. (1989) J. Biol. Chem., **264**, 838–841.
40. DuBridge, R. B., Tang, P., Hsia, H. C., Lelong, P. M., Miller, J. H. and Calos, M. P. (1987) Mol. Cell. Biol., **7**, 379–387.
41. Horsfall, M. J., Gordon, A. J., Burns, P. A., Zielenska, M., van der Vliet, G. M. and Glickman, B. W. (1990) Environ. Mol. Mutagen, **15**, 107–122.
42. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanka, D. and Barbacid, M. (1985) Nature, **315**, 382–385.
43. Sendowski, K. and Rajewski, M. F. (1991) Mutat. Res., **250**, 153–160.
44. Singer, B., Chavez, F., Goodman, M. F., Essigmann, J. M. and Dosanjh, M. K. (1989) Proc. Natl. Acad. Sci. USA, **86**, 8271–8274.
45. Dosanjh, M. K., Galeros, G., Goodman, M. F. and Singer, B. (1991) Biochemistry, **30**, 11595–11599.
46. Topal, M. D., Eadie, J. S. and Conrad, M. (1986) J. Biol. Chem.., **261**, 9879–9885.
47. Dolan, M. E., Oplinger, M. and Pegg, A. E. (1988) Carcinogenesis, **9**, 2139–2143.
48. Georgiadis, P., Smith, C. A. and Swann, P. (1991) Cancer Res., **51**, 5843–5850.
49. Lukash, L. L., Boldt, J., Pegg, A. E., Dolan, M. E., Maher, V. M. and McCormick, J. J. (1991) Mutat. Res., **250**, 397–409.
50. Mellon, I., Bohr, V. A., Smith, C. A. and Hanawalt, P. C. (1986) Proc. Natl. Acad. Sci. USA, **83**, 8878–8882.
51. Vrieling, H., Venema, J., van Rooijen, M-L., van Hoffen, A., Menichini, P., Zdzienicka, M. Z., Simons, J. W. I. M., Mullenders, L. H. F. and van Zeeland, A. A. (1991) Nucleic Acids Res., **19**, 2411–2415.
52. McGregor, W. G., Chen, R. H., Lukash, L., Maher, V. M. and McCormick, J. J. (1991) Mol. Cell. Biol., **11**, 1927–1934.
53. Reed, J. and Hutchinson, F. (1987) Mol. Gen. Genet., **208**, 446–449.
54. Palombo, F., Kohfeldt, E., Calcagnile, A., Nels, P. and Dogliotti, E. (1992) J. Mol. Biol., **223**, 587–594.
55. Palombo, F., Bignami, M. and Dogliotti, E. (1992) Nucleic Acids Res., **20**, 1349–1354.
56. Takano, K., Nakamura, T. and Sekiguchi, M. (1991) Mutat. Res., **254**, 37–44.
57. Rebeck, G. W. and Samson, L. (1991) J. Bacteriol., **173**, 2068–2076.
58. Zhang, L. H., Vrieling, H., van Zeeland, A. A. and Jenssen, D. (1992) J. Mol. Biol., **223**, 627–635.
59. Baker, R. M., Vanvoorhis, W. C. and Spencer, L. A. (1979) Proc. Natl. Acad. Sci. USA, **76**, 5249–5253.
60. Goldmacher, V. S., Cuzick, R. A. and Thilly, W. G. (1986) J. Biol. Chem., **261**, 12462–12471.
61. Goth-Goldstein, R. (1987) Carcinogenesis, **8**, 1449–1453.
62. Samson, L. and Linn, S. (1987) Carcinogenesis, **8**, 227–230.
63. Goth-Goldstein, R. and Hughes, M. (1987) Mutat. Res., **177**, 267–276.
64. Aquilina, G., Frosina, G., Zijno, A., DiMuccio, A., Dogliotti, E., Abbondandolo, A. and Bignami, M. (1988) Carcinogenesis, **9**, 1217–2122.
65. Ding, R., Ghosh, K., Eastman, A. and Bresnick, E. (1985) Mol. Cell. Biol., **5**, 3293–3296.
66. Roberts, J. J. and Basham, C. (1990) Mutat. Res., **233**, 253–263.
67. Green, M. H. L., Lowe, J. E., Petit-Frere, C., Karran, P., Hall, J. and Kataoka, H. (1989) Carcinogenesis, **10**, 893–898.
68. Aquilina, G., Zijno, A., Moscufo, N., Dogliotti, E. and Bignami, M. (1989) Carcinogenesis, **10**, 1219–1223.
69. Aquilina, G., Giammarioli, A. M., Zijno, A., DiMuccio, A., Dogliotti, E. and Bignami, M. (1990) Cancer Res., **50**, 4248–4253.
70. Rappaport, H. P. (1988) Nucleic Acids Res., **16**, 7253–7267.
71. Lehmann, A. R. and Karran, P. (1981) Int. J. Cytol., **72**, 101–146.
72. Friedberg, E. C. (1985) DNA Repair. W. H. Freeman and Company, New York.
73. Evensen, G. and Seeberg, E. (1982) Nature, **296**, 773–775.
74. Moore, P. and Strauss, B. (1979) Nature, **278**, 664–666.
75. Larson, K., Sahm, J., Shenkar, R. and Strauss, B. (1985) Mutat. Res., **150**, 77–84.
76. Snow, E. T., Foote, R. S. and MItra, S. (1984) J. Biol. Chem., **259**, 8095–8100.
77. Voigt, J.M. and Topal, M.D. (1992) Proc. Amer. Assoc. Cancer Res. **33**, 171 (Abstract)
78. Plant, J. E. and Roberts, J. J. (1971) Chem.-Biol. Interactions, **3**, 337–342.
79. Schwartz, J. L. (1989) Mutat. Res., **216**, 111–118.
80. Black, K. A., McFarland, R. D., Grisham, J. W. and Smith, G. J. (1989) Am. J. Pathol., **134**, 53–61.
81. Kaina, B. and Aurich, O. (1985) Mutat. Res., **149**, 451–461.
82. Bignami, M., Dogliotti, E., Aquilina, G., Zijno, A., Wild, C. P. and Montesano, R. (1989) Carcinogenesis, **10**, 1329–1332.
83. Painter, R. B. and Young, B. R. (1975) Radiat. Res., **64**, 648–656.
84. Maybaum, J., Bannison, A. N., Roethel, W. M., Ajmera, S., Iwaniec, L. M., Terbush, D. R. and Kroll, J. J. (1987) Mol. Pharmacol., **32**, 606–614.
85. Lamb, J. R., Petit-Frere, C., Broughton, B. C. and Lehmann, A. R. and Green, M. H. L. (1989) Int. J. Radiat. Biol., **56**, 125–130.
86. Cleaver, J. E., Rose, R. and Mitchell, D. L. (1990) Radiat. Res., **124**, 294–299.
87. Dunn, W. C., Tano, K., Horesovsky, G. J., Preston, R. J. and Mitra, S. (1991) Carcinogenesis, **12**, 83–89.
88. Karran, P. and Marinus, M. G. (1982) Nature, **296**, 868–869.
89. Eadie, J. S., Conrad, M., Toorchen, D. and Topal, M. D. (1984) Nature, **308**, 201–203.
90. Day, R. S., Babich, M. A., Yarosh, D. B. and Scudiero, D. A. (1987) J. Cell Sci. Suppl., **6**, 333–353.
91. Gaffney, B. L. and Jones, R. A. (1989) Biochemistry, **28**, 5881–5889.
92. Kalamegham, R., Warmels-Rodenhiser, S., MacDonald, H. and Ebisuzaki, K. (1988) Carcinogenesis, **9**, 1749–1753.
93. Fortini, P., Bignami, M. and Dogliotti, E. (1990) Mutat. Res., **236**, 129–137.
94. Sanderson, B. J. S., Chang, C., Grollman, A. P. and Henner, W. D. (1989) Biochemistry, **28**, 3894–3901.
95. Rosa, S., Fortini, P., Karran, P., Bignami, M. and Dogliotti, E. (1991) Nucleic Acids Res., **19**, 5569–5574.
96. Fortini, P., Rosa, S., Zijno, A., Calcagnile, A., Bignami, M. and Dogliotti, E. (1992) Carcinogenesis, **13**, 87–93.
97. Bignami, M., Karran, P. and Lane, D. (1991) Biochemistry, **30**, 2857–2863.
98. Tan, N. W. and Li, B. F. L. (1990) Biochemistry, **29**, 924–9240.
99. Smith, S. S., Kan, J. L. C., Baker, D. J., Kaplan, B. E. and Dembek, P. (1991) J. Mol. Biol., **217**, 39–51.
100. Hepburn, P. A., Margison, G. P. and Tisdale, M. J. (1991) J. Biol. Chem., **266**, 7985–7987.
101. Jiricny, J., Hughes, M., Corman, N. and Rudkin, B. B. (1988) Proc. Natl. Acad. Sci. USA, **85**, 8860–8864.
102. Stephenson, C. and Karran, P. (1989) J. Biol. Chem., **264**, 21177–21182.
103. Karran, P. and Stephenson, C. (1990) Mutat. Res., **236**, 269–275.
104. Wiebauer, K. and Jiricny, J. (1989) Nature, **339**, 234–236.
105. Fried, M. and Crothers, D. M. (1981) Nucleic Acids Res., **9**, 6505–6525.
106. Wood, R. D., Robins, P. and Lindahl, T. (1988) Cell, **53**, 97–107.

107. Stillman, B. W. and Gluzman, Y. (1985) Mol. Cell. Biol., **5**, 2051–2060.
108. Li, J. J. and Kelly, T. J. (1985) Mol. Cell. Biol., **5**, 1238–1246.
109. Roberts, J. J., Pascoe, J. M., Plant, J. E., Sturrock, J. E. and Crathorn, A. R. (1971) Chem.-Biol. Interactions, **3**, 29–47.
110. Teo, I. A. and Arlett, C. F. (1982) Carcinogenesis, **3**, 33–37.
111. Teo, I. A., Lehmann, A. R., Müller, R., and Rajewski, M. F. (1983) Carcinogenesis, **4**, 1075–1077.
112. Natarajan, A. T., Simons, J. W. I. M., Vogel, E. W. and van Zeeland, A. A. (1984) Mutat. Res., **128**, 31–40.
113. van Zeeland, A. A., Mohn, G. R., Neuhäuser-Klaus, A. and Ehling, U. H. (1985) Environmental Health Perspectives, **62**, 165–169.