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Review

The role of O^6-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis

Daniel B. Yarosh

Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Building 37, Room 5D17, 9000 Rockville Pike, Bethesda, MD 20205 (U.S.A.)

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1. Introduction

The challenge of molecular biology is to explain complex biological processes with biochemical principles. No problem is greater than understanding chemical carcinogenesis from the initial reaction of carcinogens with target cells to malignant transformation. Nevertheless, substantial progress has been made in following the path from DNA adducts produced by simple alkylating agents to tumors in rats and mice. These findings of course have important implications for cancer in humans. The attention of many researchers has focused on the DNA lesion O^6-methylguanine (O^6-MeGua) and its persistence in cells. This review will summarize the evidence for the importance of O^6-MeGua, the repair processes which determine the persistance of this methylated base in DNA, and the data on the biological consequences of O^6-MeGua in DNA.

2. Historical perspective

(A) The importance of O^6-MeGua

The most frequent DNA-base modification produced by alkylating agents such as alkyl alkanesulphonates and alkylnitrosoureas is 7-alkylGua (for review see Singer, 1979). This lesion was initially thought to be responsible for alkylation-induced mutagenesis and carcinogenesis. However, a poor correlation was generally recognized between the degree of methylation or ethylation of the 7 position of guanine in DNA and the mutagenicity of alkyl alkanesulphonates in phage test systems (Loveless and Hampton, 1969) or the carcinogenicity of these drugs in rats (Swann and Magee, 1968, 1971). Loveless (1969) produced O^6-methyldeoxyguanosine (O^6-MedG) by treatment of deoxyguanosine with methylnitrosourea (MNU) and showed that the more mutagenic ethyl methanesulphonate (EMS) produced O^6-alkyldG while the non-mutagenic methyl methanesulphonate (MMS) did not. He suggested that alkylation of the O^6-position of guanine would disrupt base paring with cytosine and that this would provide a more satisfactory explanation of the mutagenic and carcinogenic effects of alkylating agents. O^6-MeGua was subsequently identified in the DNA of *E. coli* (Lawley and Orr, 1970) and mouse cells (Lawley and Thatcher, 1970) treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and shown to cause mistakes in transcription by RNA polymerase (Gerchman and Ludlum, 1973).

A much stronger correlation was then observed between the persistence of O^6-alkylGua in animal tissue after treatment with alkylating carcinogens and the appearance of tumors. The liver carcinogen MNU was shown to produce much more O^6-MeGua in rat-liver DNA than the non-carcinogen MMS (O'Connor et al., 1973). Rat tissues in which alkylating agents produced tumors were shown to repair little or no O^6-alkylGua while other organs which initially had a similar or higher level of alkylation repaired O^6-alkylGua much faster and did not develop tumors (Goth

and Rajewsky, 1974; Kleihues and Margison, 1974; Nicoll et al., 1975). The mechanism of this loss of O^6-alkylGua was mysterious. While extracts prepared from various rat tissues removed ^{3}H-O^6-MeGua from alkylated DNA in vitro (Pegg, 1978; Pegg and Hui, 1978) no ethanol-soluble radioactivity was detected, indicating that glycosylases or endonucleases were not likely to be involved in the repair.

(B) The adaptive response in bacteria

The repair of alkylation damage in *E. coli* DNA became of great interest with the discovery of the adaptive response (Samson and Cairns, 1977). In attempts to measure the mutation rate of cells continuously exposed to low levels of MNNG, *E. coli* populations were found to cease accumulating mutants after the first 30 min of treatment. This resistance to MNNG induced mutagenesis decayed after removal of the MNNG or was prevented by addition of chloramphenicol during treatment (see also Sklar, 1978; Sklar and Strauss, 1980). Accompanying the resistance to mutagenesis was an increase in survival of cells exposed to large doses of MNNG. These results were interpreted to reflect the induction of a new DNA repair system distinct from *rec–lex* SOS repair because increased survival was accomplished without increased mutagenesis, in contrast to SOS repair which coordinately produces increased mutagenesis and survival. The resistance after MNNG adaptation was only to alkylating agents and not to UV, in contrast to SOS repair which increases survival after UV treatment.

Jeggo and colleagues firmly established the difference between SOS repair and the adaptive response. Mutants defective in SOS repair were shown to be competent for adaptation (Jeggo et al., 1977), and Ada mutants, mapped at 47 min on the *E. coli* map, were isolated which lacked the adaptive response (Jeggo, 1979; Sedgwick, 1982). However, among the mutants there was poor correlation between their deficiency in adaptation to alkylation-induced killing and mutagenesis. In addition, *polA* and *dam 3* mutants could adapt to mutagenesis but not cell killing (Jeggo et al., 1978). This suggested that the mechanism of resistance to mutagenesis was distinct from the resistance to cell death.

The molecular basis of the adaptive response was soon illuminated. Lawley and Orr (1970) had first reported repair of O^6-MeGua in *E. coli* DNA. Adapted *E. coli* repaired O^6-MeGua in DNA more efficiently than unadapted cells, but the repair capacity was limited and mutations only began to accumulate in adapted cultures when repair of O^6-MeGua was saturated (Schendel and Robins, 1978). In fact in adapted wild-type AB1157 strains there appeared to be about 3000 molecules of the *ada* gene product per cell, if each molecule repaired a single O^6-MeGua base (Robins and Cairns, 1979). The mechanism by which O^6-MeGua was repaired by the *ada* gene product was as mysterious as was its repair by rat-tissue extracts. While extracts from adapted wild type strains could repair O^6-MeGua in DNA treated with ^{3}H-MNU, and extracts from adapted Ada mutants could not, ^{3}H-O^6-MeGua was not detected as a free base, indicating there was no excision by glycosylases or endonucleases (Karran et al., 1979). The solution to this puzzle was made possible by isolation of mutants which constitutively expressed the adaptive response (Sedgwick and Robins, 1980). Using partially purified extracts from one of these mutants, Olsson and Lindahl (1980) demonstrated that the methyl group was transferred from O^6-MeGua in DNA to a cysteine residue of an acceptor protein, producing an *S*-methyl-L-cysteine (*S*-MeCys) residue. A guanine base was restored by this repair (Foote et al., 1980).

The adaptive response in *E. coli*, therefore, consists in part of the induction of O^6-methylguanine-DNA methyltransferase (O^6-MT) which transfers the methyl group from O^6-MeGua in DNA to a protein acceptor. In addition to the methyltransferase, the adaptive response involves the induction of a DNA glycosylase which catalyzes the release of *N*-methylated purines (Karran et al., 1982a; Evensen and Seeberg, 1982). This glycosylase, *N*-3 methyladenine-DNA glycosylase II, is the product of the *alk* gene (Yamamoto et al., 1978) and its induction is controlled by the *ada* gene (Karran et al., 1982a; Evensen and Seeberg, 1982). The sensitivity of adapted Alk$^-$ mutants to alkylation induced cell killing (Evensen and Seeberg, 1982) resolves the paradox that not all Ada mutants show equal loss of adaptation to muta-

genesis and killing. By this interpretation, induction of *N*-3 methyladenine-DNA glycosylase II protects against lethal lesions and induction of O^6-MT protects agains mutagenic lesions. Polymerase I is required for repair of the apurinic sites produced by the glycosylase.

An adaptive response similar to *E. coli* has been detected in *B. subtilis* (Morohoshi and Munkata, 1983; Hadden et al., 1983) and *M. luteus* (Ather et al., 1984), but no adaptation was observed in *S. typhimurium, H. influenzae* (Ather et al., 1984), or in *S. cerevisiae* (Hadden et al., 1983).

(C) The adaptive response in mammals

DNA-repair activities analogous to *E. coli* O^6-MT were soon identified in mouse liver (Bogden et al., 1981), rat liver (Renard and Verly, 1980; Mehta et al., 1981), human liver (Pegg et al., 1982), and these activities were present in human cells in culture (Waldstein et al., 1982a; Myrnes et al., 1982; Foote et al., 1983; Yarosh et al., 1983a, b). Evidence for an adaptive response in mammalian cells which paralleled the *E. coli* response was more ambiguous. Chronic administration of dimethylnitrosamine (DMN) to rats increased the capacity of the liver to repair O^6-MeGua (Montesano et al., 1979). The increase in O^6-MeGua repair capacity and in O^6-MT was produced in the liver by partial hepatectomy or non-alkylating hepatotoxins (Buckley et al., 1979; Pegg et al., 1981; Pegg and Perry, 1981) and in the kidney by unilateral nephrectomy (Pegg and Wiest, 1983). This spectrum of inducing treatments differs from those inducing the adaptive response in *E. coli*, where only DNA alkylation is effective. In addition the increase in O^6-MT protein is much less in rats than in *E. coli* (Pegg et al., 1981; Renard and Verly, 1983).

Results with rats were not easily extended to other rodent species. Treatment of mice, hamsters or gerbils with alkylating or non-alkylating carcinogens or with partial hepatectomy failed to increase O^6-MeGua repair or O^6-MT activity (O'Connor et al., 1982; Bamborschke et al., 1983; Lindamood et al., 1984).

The first evidence of an 'adaptive'-like response in mammalian cells in culture was reported by Samson and Schwartz (1980) who found that pretreatment of Chinese hamster ovary (CHO) cells and SV40-transformed human skin fibroblasts with low doses of MNNG increased their resistances to alkylation induced cell killing and sister-chromatid exchanges (SCEs). CHO cells were found to have increased resistance to MNNG-induced mutation after adaptation (Samson and Schwartz, 1983; Schwartz and Samson, 1983). However, CHO cells failed to remove O^6-MeGua from DNA (Goth-Goldstein, 1980) and lacked either constitutive or inducible O^6-MT activity (Foote and Mitra, 1984) and therefore the mechanism for this response in CHO cells may differ from that in *E. coli*. In another laboratory normal human fibroblasts did not show increased resistance to alkylation-induced cytotoxicity after pretreatment with MNNG (Karran et al., 1982b). An 'adaptive'-like response also has been reported for another line of Chinese hamster cells, V79 (Kaina, 1982). Cells pretreated with MNNG acquired resistance to the mutagenic effects of MNNG, MNU, to a lesser extent EMS, but not all to MMS, ENU, mitomycin C or UV (Kaina, 1983a, b). Pretreatment with MNNG also reduced methylation, but not ethylation, induced chromosome alterations and sister-chromatid exchanges (Kaina, 1983a, b). Because the doses and agents used for maximum resistance to mutagenesis and clastogenesis differed, the underlying mechanisms for these two endpoints were likely different (Kaina, 1983a, b). There was no evidence that this response was due to changes in the level of O^6-MT, especially in light of the absence of repair of O^6-MeGua in V79 cells (Warren et al., 1979). Others have failed to observe the acquired resistance to either cell killing, mutagenesis or to increased repair of methylpurines in V79 cells (Durant et al., 1981; Fox et al., 1982).

Recently, rat hepatoma cells pretreated with alkylating agents have been reported to show increased resistance to alkylation induced cell killing and mutagenesis and an increased O^6-MeGua repair capacity (Laval and Laval, 1984) similar to the response shown by rat liver cells in vivo. Rat-kidney cells in culture did not show an increase in O^6-MT levels after pretreatment (Foote and Mitra, 1984).

In addition to the evidence of Samson and Schwarz for an 'adaptive'-like response in human cells, a specific claim has been made that pretreatment of human tumor and HeLa cells with low

doses of MNNG increased by 3-fold the level of O^6-MT activity, and that this increase was blocked by the addition of cycloheximide (Waldstein et al., 1982a, b). Other laboratories have not observed this response using the same human tumor cells strains (Yarosh et al., 1984) or HeLa cell line (Myrnes et al., 1982; Foote and Mitra, 1984) or in human lymphoblastoid cells (Sklar et al., 1981) or in normal human fibroblasts (Karran et al., 1982b).

3. Enzyme properties

(A) Substrate

O^6-MT removes alkyl groups from the O^6 position of guanine, but ethyl groups were repaired much more slowly than methyl groups (Sedgwick and Lindahl, 1982; Pegg et al., 1983). In *E. coli*, as the size of the alkyl group increased the importance of nucleotide excision repair increased and the participation of the adaptive response in survival and mutagenesis decreased (Warren and Lawley, 1980; Todd and Schendel, 1983). In human cells, O^6-MT activity was correlated with greater resistance to cytotoxicity produced by methylating agents than ethylating agents (Scudiero et al., 1984a). O^6-MT also removes substituted ethyl groups, such as chloroethyl and hydroxyethyl moieties, from the *O*-6 position of guanine. The cytotoxicity of the chloroethylnitrosoureas has been proposed to be caused by interstrand crosslinks in DNA resulting from the slow conversion of O^6-2-chloroethylguanine to a crosslink with the base pairing partner cytosine (Kohn, 1977; Tong et al., 1982, 1983). O^6-MT activity in human tumor cells provided resistance to cross-link formation and cell killing by these drugs (Erickson et al., 1980). The purified *E. coli* O^6-MT repaired O^6-hydroxyethylguanine and prevented crosslink formation by bis-chloroethylnitrosourea (Robins et al., 1983). The partially purified rat O^6-MT repaired O^6-hydroxyethylguanine but more slowly than it demethylated O^6-EtGua (Pegg et al., 1984). In human lymphoblast extracts, O^6-MT activity co-purified with an activity which suppressed cross-link formation by chloroethylnitrosoureas, and their similar heat stabilities and susceptibilities to inhibitors suggests that in fact O^6-MT prevented crosslink formation (Brent, 1984).

Other methylated oxygens in DNA are repaired by the bacterial O^6-MT. O^4-methylthymine (O^4-MeThy) was demethylated, but more slowly than O^6-MeGua (McCarthy et al., 1983, 1984; Ahmmed and Laval, 1984). Although other DNA-base oxygens can be methylated, the distinguishing feature of O^6-MeGua and O^4-MeThy is that the methyl group protrudes into the major groove of DNA. Methylphosphotriesters are also repaired by the uncleaved 37000 mol. wt. form of the *E. coli* O^6-MT (McCarthy et al., 1983; Teo et al., 1984).

The mammalian O^6-MT does not share the same range of substrates as the *E. coli* O^6-MT. O^6-MT partially purified from rat liver did not repair O^4-MeThy or methylphosphotriesters (Pegg et al., 1983; Dolan et al., 1984). Human liver fractions or human tumor cells in culture repaired O^6-MeGua in DNA very efficiently, but only slightly demethylated methylphosphotriesters and did not remove O^4-MeThy at all (Yarosh, unpublished results). In human fibroblasts ethylphosphotriesters were not removed, O^4-ethylthymine (O^4-EtThy) had a half-life of 4–5 days, but O^6-EtGua was removed with a half-life of less than 20 h (Bodell et al., 1979). C57/BL mice treated with MNU or ENU removed very little alkylphosphotriesters from the DNA of many organs including liver (Shooter and Slade, 1977) although O^6-MT was present in C57/BL primary tissue and cells in culture (Yagi et al., 1984) and has been partially purified from the liver of this strain of mouse (Bogden et al., 1981). In rat liver O^4-alkylThy was removed very slowly, with a half-life of 4 days or longer, and alkylphosphotriesters were essentially not repaired, while O^6-alkylGua had a half-life of 13 h or less (O'Connor et al., 1973; Shooter and Slade, 1977; Shooter et al., 1977; Scherer et al., 1980; Singer et al., 1981; Swenberg et al., 1984). The difference in repair among the bases in rat DNA led to the remarkable situation in which O^6-EtGua, which was formed 3–4 times more frequently than O^4-EtThy by diethylnitrosamine (DEN), was found at 2% of O^4-EtThy levels in rats continuously exposed to DEN for 10 weeks (Swenberg et al., 1984). Thus the mammalian O^6-MT appears to act almost exclusively on small alkyl groups at the *O*-6 position of guanine, and only very inefficiently on alkylphosphotriesters or O^4-alkylThy.

The bacterial (Lindahl et al., 1982), the rat (Pegg et al., 1983; Hora et al., 1983) and the human O^6-MT (Harris et al., 1983) had greater activity on double-stranded DNA than denatured or single-stranded DNA. This preference for double-stranded DNA may explain the observation that alkylating agents which produce O^6-MeGua cause multiple mutations at the replication forks of the bacterial chromosome (Cerda-Olmedo et al., 1968; Guerola et al., 1971; Hince and Neal, 1977; Sklar and Strauss, 1980) because these regions have higher concentrations of single-stranded DNA preceding the replication fork and thus O^6-MeGua is less likely to be removed before the DNA is replicated (Sklar and Strauss, 1980; Lindahl et al., 1982). The concentration of O^6-MeGua per DNA base in double-stranded DNA also influences the rate of methyl transfer by O^6-MT. The time required for complete repair by rat liver O^6-MT increased 15-fold when the frequency of O^6-MeGua per Gua base in DNA was decreased 250-fold (Scicchitano and Pegg, 1982). This suggests that the enzyme acts progressively, although the energy source for this translocation is unknown. From such a model we would predict bi-phasic repair kinetics, with a rapid initial phase when O^6-MeGua is relatively dense and a slower phase when repair had diluted its concentration. Bi-phasic kinetics have in fact been reported for human fibroblasts (Medcalf and Lawley, 1981; Shiloh and Becker, 1981), human lymphoma cells (Sklar et al., 1981) and rat hepatoma cells (Laval and Laval, 1984). Bacterial and human O^6-MT also repaired O^6-MeGua produced in tRNA by MNU treatment (P. Karran, personal communication).

(B) Size and stability

The O^6-MT purified from *E. coli* was 18000 mol. wt. (Demple et al., 1982) but the product of the cloned *ada* gene was a 37000 mol. wt. polypeptide (Sedgwick, 1983). Antibodies prepared against the 18-kd protein recognized the 37-kd protein, synthetic oligonucleotides prepared from the amino acid sequence of the 18-kd protein hybridized to the cloned *ada* gene, both proteins repaired DNA identically, and in fact the 18-kd protein was derived from the 37-kd protein by rapid cleavage upon cell lysis (Teo et al., 1984). The 37-kd O^6-MT may be the native form of the enzyme and the 18-kd protein an artifact of purification.

The partially purified rat O^6-MT has been measured to be between 18.5 and 20 kd (Pegg et al., 1983; Hora et al., 1983). The O^6-MT in crude extracts of mouse cells was 22 kd (Yagi et al., 1983) and the human O^6-MT from cells in culture and tissue has been reported to be between 21 kd and 24 kd (Myrnes et al., 1982; Harris et al., 1983; Yarosh et al., 1984a). No evidence has been reported of cleavage of a mammalian O^6-MT protein.

The bacterial and mammalian O^6-MT may differ in heat stability. The purified *E. coli* O^6-MT was very heat-resistant, with a half-life of 45 min at 95° (Demple et al., 1982). Partially purified human O^6-MT from placenta (Yarosh et al., 1984a) or lymphocytes (Brent, 1984) has been reported to rapidly lose activity at 45°, while another laboratory has reported that O^6-MT partially purified from human lymphoid cells retained 54% of its activity after 10 min at 95° (Harris et al., 1983). This difference may be resolved when the human O^6-MT is available in pure form. The bacterial O^6-MT was less active but still functional at 5° or less (Foote et al., 1980; Lindahl et al., 1982) while the mammalian O^6-MT was virtually inactive at these temperatures (Bogden et al., 1981; Harris et al., 1983).

(C) Suicide kinetics

The unique feature of the DNA-repair reaction of O^6-MT is the suicidal attack of the protein on the methyl group of O^6-MeGua. The reaction is extremely rapid and reaches a plateau, despite the presence of excess O^6-MeGua, when all the O^6-MT molecules have reacted. In *E. coli*, the half-life of the reaction at 37° was 15 min or less in cells (Schendel and Robins, 1978; Robins and Cairns, 1979), less than 10 min in crude extracts (Foote et al., 1980), and less than 1 min using the purified protein (Lindahl et al., 1982). In human cells or cell extracts at 37° the half-life was about 10 min or less (Sklar et al., 1981; Yarosh et al., 1983; Foote et al., 1983; Harris et al., 1983). The reaction was equally as rapid with the rodent O^6-MT (Bogden et al., 1981; Scicchitano and Pegg, 1982). Once the O^6-MT in a cell has reacted, only RNA

and protein synthesis restored the original levels of O^6-MT (Robins and Cairns, 1979; Warren and Lawley, 1980; Waldstein et al., 1982b; Yarosh et al., 1984a), demonstrating that the O^6-MT is not rejuvenated after its reaction with a methyl group, and remains as a dead end complex.

Transfer of the methyl group produced an *S*-MeCys residue in both bacterial and mammalian O^6-MT (Olsson and Lindahl, 1980; Bogden et al., 1981; Mehta et al., 1981; Pegg et al., 1982). Loss of O^6-MeGua from DNA was coupled with a stoichiometric formation of *S*-MeCys (Lindahl et al., 1982; Bogden et al., 1981; Pegg et al., 1982; Craddock et al., 1982; Waldstein et al., 1983), indicating that this is the exclusive mechanism for demethylation of O^6-MeGua. Purification of the *E. coli* O^6-MT has demonstrated that the transferring and accepting functions reside on the same protein and that a single O^6-MT molecule can act only once (Demple et al., 1982; Lindahl et al., 1982). The transferring and accepting functions of either the rat (Hora et al., 1983) or human (Harris et al., 1983) O^6-MT were performed by the same size protein. In a reaction in which the enzyme was limiting, dilution of the human O^6-MT diminished the methylated protein reaction product in direct proportion to the dilution factor, as predicted if a single molecule was required for repair, and not as a square of the dilution factor, as predicted if two molecules must interact for repair (Yarosh, unpublished results). These results suggest that the mammalian O^6-MT also acts as both the transferring and accepting protein. Rat-liver chromatin proteins containing O^6-MT were reacted with ^{3}H-O^6-EtGua-DNA and digested with trypsin, yielding two different labeled peptides (Renard et al., 1983). This implies that there may be two acceptor sites per O^6-MT molecule in rat liver, but such an important difference from the bacterial protein should be confirmed using purified rat O^6-MT.

(D) Regulation of enzyme levels

Unadapted *E. coli* contained very low levels of O^6-MT, approximately 13–60 molecules per cell (Mitra et al., 1982), and unadapted *B. subtilis* had about 240 molecules per cell (Hadden et al., 1983; Morohoshi and Munkata, 1983). Normal human fibroblasts contained 40 000–80 000 molecules per cell (Metcalf and Lawley, 1981), human lymphoid cells had 10 000–25 000 per cell (Harris et al., 1983) and human tumor cells had between 30 000 and 100 000 per cell (Yarosh et al., 1983a; Foote et al., 1983; Foote and Mitra, 1984; Myrnes et al., 1982; Waldstein et al., 1982a). Rat cells had about one-fifth the basal level of O^6-MT as human cells (Grafstrom et al., 1984) with between 21 000 and 28 000 molecules per cell in rat kidney cells in culture (Foote and Mitra, 1984). In both rats and humans, the highest levels were found in liver, followed by organs of the digestive tract such as stomach, colon and intestine, while brain tissue had approximately one-tenth the level of liver (Goth and Rajewsky, 1974; Singer et al., 1981; Myrnes et al., 1983; Krokan et al., 1983; Grafstrom et al., 1984; Kyrtopoulos et al., 1984). The higher levels of O^6-MT in organs which might be exposed first to dietary alkylating carcinogens, and the correlation between rat-liver cell differentiation and O^6-MT levels (Hesse et al., 1984) is suggestive of genetic regulation of O^6-MT levels during differentiation. Although it is tempting to speculate that O^6-MT levels are related to DNA mass, in cases where DNA content is comparable, such as *E. coli* and *B. subtilis* or human and rat liver or liver and brain, differences in O^6-MT levels indicate this is not the case.

Rapid production of O^6-MeGua in DNA and rapid demethylation by O^6-MT resulted in depletion of cellular levels of the protein because of its stoichiometric and not catalytic activity (Schendel and Robins, 1978; Robins and Cairns, 1979; Waldstein et al., 1982b; Zlotogorski and Erickson, 1983; Yarosh et al., 1984a). Restoration of the original level required RNA and protein synthesis as noted before, and the net rate of O^6-MT production was about 6000 molecules per hour in adapted *E. coli* (Robins and Cairns, 1979) and between 750 and 4500 molecules per hour in human tumor cells (Yarosh et al., 1984a; Foote and Mitra, 1984). In human tumor cells, at least, the basal level of O^6-MT may be determined by proteolytic degradation and turnover of the repair protein (Yarosh et al., 1984a).

In *E. coli* depletion of the O^6-MT pool leads to the adaptive response, including increased levels of O^6-MT and protection against alkylation induced mutagenesis. The maximal adaptive response was obtained after a dose of MNNG which was just

sufficient to produce enough O^6-MeGua to exhaust the constitutive level of O^6-MT (Lindahl et al., 1983). Adaptation is produced by many methylating agents such as MNNG and MMS, which produce different proportions of methylpurines in DNA, but they induced the adaptive response only at doses which produced equivalent levels of O^6-MeGua (P. Karran, personal communication). Ethylating agents efficiently produce O^6-EtGua but were poor inducers of the adaptive response (Jeggo, 1979; Warren and Lawley, 1980) and in DNA O^6-EtGua was not as good a substrate for O^6-MT as O^6-MeGua (Sedgwick and Lindahl, 1982). The evidence so far suggests the adaptive response is induced in response to O^6-alkylguanine, not other alkylpurines, and the signal may be the alkylated form of O^6-MT.

The *ada* gene of *E. coli* codes for O^6-MT (Teo et al., 1984) but it also regulates adaption to mutagenesis and cell killing (Jeggo, 1979). A plasmid carrying the cloned *ada* gene conveyed MNNG resistance to Ada$^-$ mutants and resulted in high constitutive levels of O^6-MT and *N*-3 methyladenine-DNA glycosylase II, which were induced to even higher levels (Sedgwick, 1983), suggesting that the *ada* gene product positively regulates its own synthesis and that of the glycosylase. Many of the Ada$^-$ mutants isolated so far appear to lack both the repair and regulatory functions of the induced O^6-MT protein. They contained a normal basal level of O^6-MT activity (Mitra et al., 1982). Upon adaption they produced a 37-kd protein which cross-reacted with the anti-O^6-MT antibody (Teo et al., 1984) but no O^6-MT activity was detected (Jeggo, 1979; Karran et al., 1979; Mitra et al., 1982) and the *alk* gene product was not induced (Evensen and Seeberg, 1982). Another class of Ada mutants (Adc) overproduced, without adaption, O^6-MT indistinguishable from wild-type but did not overproduce the glycosylase, and 'reverted' to the Ada$^-$ phenotype (Sedgwick and Robins, 1980; Mitra et al., 1982; Demple et al., 1982; Karran et al., 1982a).

A model for regulation of the adaptive response is that following O^6-MeGua formation in DNA *ada* gene expression is increased by a positive signal, perhaps the methylated form of O^6-MT. In addition the *ada* gene product induces expression of the *alk* gene and perhaps other genes. The *aid* B gene, whose function is unknown, is under the control of the *ada* gene (Volkert and Nguyen, 1984). The amount of *ada* gene product begins to rise 4–5 min after MNNG treatment, reaches the induced levels after 20 min and remains there for 2–3 h (Teo et al., 1984), in agreement with a report that chloramphenicol enhanced MNNG induced mutations only if it was added by 20 min after MNNG addition (Sklar and Strauss, 1980). The adaptive response decays by 4 h (Samson and Cairns, 1977), presumably because O^6-MeGua has been repaired and the inducing signal has been diluted by cell division.

4. Biological consequences

(A) E. coli

The Ada mutants have been essential to understanding the effects of O^6-MT in cells. These mutants fail to induce not only the *ada* gene product but also the *alk* gene product, complicating the analysis of the O^6-MT defect. Ada mutants have increased mutagenesis and reduced survival compared to wild-type after MNNG treatment (Jeggo, 1979), but it is the absence of the *N*-3 methyladenine-DNA glycosylase II of the *alk* gene which is likely to produce the MNNG hypersensitivity (Karran et al., 1982a; Eversen and Seeberg, 1982). This is supported by the observation that a plasmid carrying the *ada* gene produced high levels of O^6-MT but did not increase the MNNG resistance of an Alk$^-$ mutant (Sedgwick, 1983). Confirmation must await the identification of a mutant defective in O^6-MT and competent in the *alk* gene product, but O^6-MeGua in DNA is not likely to be lethal in *E. coli*.

The majority of mutations induced by MNNG in *E. coli* were G–C to A–T transitions (Coulondre and Miller, 1977) and they only began to accumulate in a population when O^6-MeGua accumulated in DNA (Schendel and Robins, 1978), suggesting that O^6-MeGua is mutagenic by mispairing with thymine. In vitro O^6-MeGua in DNA was mispaired with thymine more frequently than it was paired with cytosine by *E. coli* polymerase I (Abbott and Shaffhill, 1979; Snow et al., 1984). O^4-MeThy in DNA, which is also a substrate for O^6-MT, caused mispairing with guanine (Abbott and Saffhill, 1977; Singer et al., 1978) and may

also lead to transition mutations.

The O^6-MeGua: Thy pairing is not 'normal' in that the presence of O^6-MeGua in tempates inhibited DNA replication (Snow et al., 1984). The mispairing is removed by the mismatch correction system of *E. coli*, and mutants which are defective in this system were hypersensitive to MNNG (Karran and Marinus, 1982). The mismatch system did not prevent mutagenesis by O^6-MeGua (Sklar and Strauss, 1980; Karran and Marinus, 1982), presumably because the pairing O^6-MeGua: Cyt is also recognized as a mismatch. It was O^6-MT activity which prevented mutation resulting from mismatched based and abolished the hypersensitivity to MNNG of mismatch repair mutants (Karran and Marinus, 1982; Eadie et al., 1984).

(B) Mammalian cells

The analysis of the effect of O^6-MT in mammalian cells is aided by the existence of cell strains which have little or no O^6-MT activity. CHO and V79 cells derived from hamsters and 34I cells derived from a mouse mammary carcinoma failed to repair O^6-MeGua and lacked 06MT activity (Warren et al., 1979; Goth and Rajewsky, 1974; Yagi et al., 1984). A group of human tumor cell strains failed to reactivate MNNG-treated adenovirus 5 (MNNG-Ad5) and this phenotype is designated Mer$^-$ (Day and Ziolkowski, 1977; Day et al., 1980a, b). All 16 Mer$^-$ strains tested had little or no O^6-MT activity while all 15 Mer$^+$ strains tested contained activity (Yarosh et al., 1983a, b; Foote et al., 1983; Yarosh, unpublished results). A similar phenotype of some Epstein–Barr virus-transformed human lymphoblastoid cell lines is designated Mex$^-$ and is defined by cells which failed to remove O^6-MeGua from their DNA, and in addition these cells lacked O^6-MT activity (Sklar and Strauss, 1981; Harris et al., 1983). Transformation by other DNA and RNA viruses, such as SV40, Rous sarcoma virus and adenovirus in addition to Epstein–Barr virus produced Mer$^-$ cells with high frequency (Day et al., 1980a; Heddle and Arlett, 1980; Yarosh et al., 1983a, b; Sklar and Strauss, 1983). An SV40-transformed mouse strain also lacked O^6-MT activity (Yagi et al., 1984). Mer$^-$ variants have been detected in the HeLa cell line (Baker et al., 1979) and Mer$^+$ cells have arisen as variants of a Mer$^-$ human melanoma cell line (Hayward and Parsons, 1984). All normal human fibroblast strains tested were Mer$^+$ (Day, personal communication) with the exception of fetal fibroblast strain GM0011 from the Institute for Medical Research, Camden, NJ, which failed to reactivate MNNG-Ad 5 and was deficient in O^6-MT activity (Middlestat, Day and Yarosh, unpublished results).

Another class of human tumor cells, designated Mer$^+$ Rem$^-$, has been identified by their increased sensitivity to MNNG cytotoxicity compared to Mer$^+$ Rem$^+$ cells (Scudiero et al., 198b). Although these cells were not deficient in basal levels of O^6-MT (Yarosh et al., 1983a), they were slower than Mer$^+$ Rem$^+$ cells in regenerating activity after depletion by MNNG treatment (Day and Ziolkowski, 1981; Scudiero et al., 1984b).

In Ad 5, unlike *E. coli*, the evidence indicates that O^6-MeGua in DNA is a lethal lesion. MNNG-Ad 5 infecting Mer$^-$ cells had a correlation of 2.3 O^6-MeGua per Ad 5 genome per lethal hit (Day et al., 1984). No other lesion is as likely a candidate because Mer$^-$ cells repaired *N*-7 methylguanine and *N*-3 methyladenine as efficiently as did Mer$^+$ cells (Day et al., 1980a, 1984) and other alkylated bases occurred less frequently than 1 per Ad 5 genome per lethal hit. In addition the finding that every Mer$^-$ strain tested was defective in O^6-MT activity implied that unrepaired O^6-MeGua inactivated Ad 5.

The absence of O^6-MT is involved in human cellular sensitivity to MNNG. Virtually all Mer$^-$ and Mex$^-$ cells had increased cytotoxicity after MNNG treatment compared to Mer$^+$ or Mex$^+$ cells (Baker et al., 1979; Day et al., 1980a, b; Shiloh and Becker, 1981; Sklar and Strauss, 1981, 1983; Scudiero et al., 1984b). The hypersensitivity was not as great in some of the virally transformed strains and the HeLa variant A6 as in the tumor derived strains. This may indicate that O^6-MeGua is not a lethal lesion in human cells or that mechanism other than O^6-MT can prevent the lethal effects of O^6-MeGua. Mer$^-$ strains were also hypersensitive to cell killing induced by DNA cross-linking agents which produced O^6-alkylGua intermediates (Erickson et al., 1980; Scudiero et al., 1984a).

In contrast, O^6-MT activity is not well corre-

lated with cellular resistance to DNA alkylation in rodent cells. Compared to strains which possessed O^6-MT activity two mouse strains which lacked activity were more sensitive to MNNG-produced cytotoxicity while mouse strain 34I and hamster strain CHO which lacked activity were no more sensitive (Yagi et al., 1984; Yarosh, unpublished results). O^6-MT may still protect against lethality because pretreatment of rat hepatoma cells with MNNG coordinately increased O^6-MeGua repair capacity and cellular resistance to MNNG (Laval and Laval, 1984).

Attempts have been made to dissect the Mer^-, Mex^-, and Rem^- phenotypes by genetic analysis. Fusion of two Mer^- strains or mixing of extracts from two Mer^- strains failed to produce O^6-MT activity, suggesting that the Mer^- strains tested shared the same defect (Yarosh et al., 1983a, 1984b). Fusion of Mex^- lines with a Mex^+ line produced hybrids with MNNG resistance intermediate between those of the parents (Ayres et al., 1982). Fusion of a Mer^- strain with each of three Mer^+ Rem^- strains produced hybrids with more, equivalent and less MNNG resistance than the Mer^+ Rem^- parent (Yarosh et al., 1984b). In all these hybrids there was a rough correlation between O^6-MT activity or O^6-MeGua repair capacity and MNNG resistance, although exceptions were noted. The genetic regulation of O^6-MT appears to be more complex than the dominance or recessiveness of a single allele. A Mer^- strain was treated with DNA from a Mer^+ cell and several clones were selected which had increased resistance to MNNG cytotoxicity, but only one had the full Mer^+ phenotype, including O^6-MT activity (Yarosh et al., 1984c). This is the clearest case of dissociation between O^6-MT activity and MNNG resistance. These results, together with experiments in which mixing Mer^- extracts with Mer^+ extracts failed to inhibit O^6-MT activity (Yarosh et al., 1983a; Foote et al., 1983), suggest that the Mer^- defect does not actively destroy O^6-MT. CHO variants hypersensitive to MNNG produced cytotoxicity were treated with normal human DNA and clones were isolated with increased resistance to MNNG, but no evidence was presented that these clones contained O^6-MT activity (Waldren et al., 1983).

The absence of O^6-MT is correlated with other biological endpoints. MNNG induced much higher levels of sister-chromatid exchanges (SCEs) in Mer^- cells than Mer^+ cells (Day et al., 1980a). However in rodent cells only 2 of 4 strains lacking O^6-MT had MNNG-induced SCE levels higher than strains with O^6-MT (Yagi et al., 1984) and lesions other than O^6-MeGua have been more strongly associated with SCEs in CHO cells (Heflich et al., 1982). MNNG induced greater DNA-repair replication in Mer^- cells than in Mer^+ cells (Day et al., 1980b) suggesting that cells may respond to persistent lesions with abortive DNA synthesis.

In forward mutational assays with human fibroblasts or rodent cell lines O^6-MeGua was more strongly correlated with mutagenesis induced by many alkylating agents than other lesions studied (Newbold et al., 1980; Suter et al., 1980; Medcalf and Wade, 1983), although in reversion assays this was not the case (Suter et al., 1980; Fox and Brennand, 1980). In a rat mammary tumor induced by a single dose of MNU, the H-*ras*-1 oncogene was activated by a G–C to A–T transition, exactly as would be predicted if O^6-MeGua was responsible for the mutation and neoplastic transformation (Sukumar et al., 1983). Mer^- cells had a higher frequencies of MNNG-induced mutations than Mer^+ cells, suggesting that O^6-MT protects against alkylation mutagenesis (Baker et al., 1979).

Mer^- strains have been identified in 19 of 93 human tumor strains tested, and in at least two cases they were derived from tumors of patients whose normal fibroblasts were Mer^+ (Day and Ziolkowski, 1979; Day et al., 1980a, b; Scudiero et al., 1984b). This suggests the possibility that spontaneously arising Mer^- cells were transformed by alkylation induced somatic mutation and that these Mer^- tumors might be treated with alkylating antineoplastic agents which selectively kill Mer^- cells. Support for these hypotheses comes from direct measurements of O^6-MT activity in human tissues and tumors. In Fig. 1 data has been assembled from published reports and our previously unpublished results on O^6-MT levels in human tumor strains, tumor samples and normal tissue. Each symbol represents the average of measurements of individual cell strains, tumor biopsies or tissue samples. O^6-MT activity has been plotted

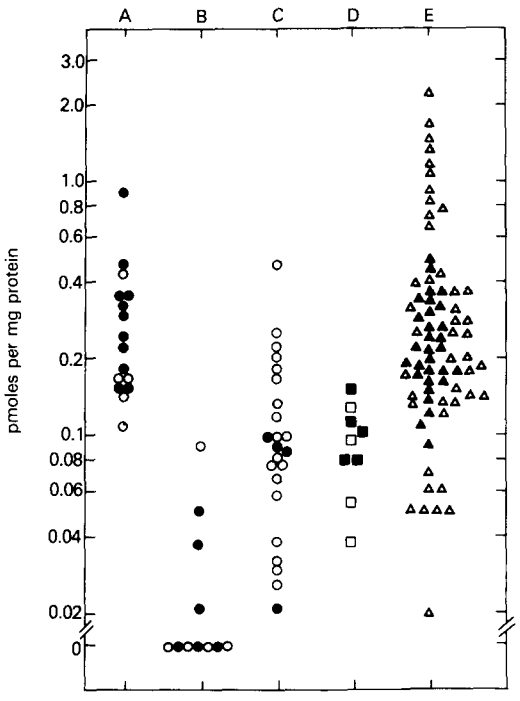

Fig. 1. O^6-MT activity in human tumor strains, brain tumor samples, normal brain and non-brain tissue. Activity is expressed as pmoles O^6-MT per mg protein of crude extract. Data compiled from references described in text. (A) Mer^+ tumor strains; (B) Mer^- tumor strains; (C) brain tumor and metastases samples; (D) normal brain tissue samples; (E) normal non-brain tissue samples. ○, brain tumor strains or samples; ●, non-brain tumor strains or samples; ■, fetal brain tissue; □, adult brain tissue; ▲, fetal non-brain tissue; △, adult non-brain tissue.

as pmoles O^6-MT per mg protein in crude extract on a logarithmic scale along the abscissa in order to display better the entire range of measurements.

In column A the range of O^6-MT activity for Mer^+ strains derived from human tumors is shown, and in column B the range for Mer^- strains is shown (Yarosh et al., 1983a, b, unpublished results). The open circles represent data for brain tumor-derived strains and closed circles are data for non-brain tumor strains. In column C the range of O^6-MT activity in human brain tumor samples (open circles) and samples from metastases (closed circles) is shown (Wiestler et al., 1984). The range of values for the brain tumor samples approximates the combined range of the Mer^+ and Mer^- values, although no tumor biopsies lacked O^6-MT activity entirely, perhaps because very large amounts of sample extract were used to increase sensitivity of the assay. The values for O^6-MT activity in tumor strains and brain tumors can be compared with the range of values for activity in normal brain tissue (column D) or non-brain tissue (column E) (Pegg et al., 1982; Myrnes et al., 1982, 1983; Krokan et al., 1983; Wiestler et al., 1984; Yarosh, unpublished results). Open symbols represent data for adult tissue and closed symbols are for fetal tissue. Although the range of values for normal brain samples in column D falls below the range of Mer^+ tumor cell strains (including brain tumor strains) in column A, there appears to be some brain tumors in column C with even lower levels of O^6-MT than normal brain tissue. Among the non-brain tissues shown in column E are liver, stomach, kidney, small intestine, colon, lung and placenta. The preponderance had O^6-MT activity above 0.1 pmoles per mg protein, in the range of Mer^+ tumor cell strains, and the highest values, above 1.0 pmoles per mg protein, were from adult liver.

These data indicate that the range of O^6-MT activity measured in tumor strains reflects the range of activities in normal tissue and in tumor biopsies. Although many tumors may not lack O^6-MT activity entirely, some have significantly less activity than normal tissue. In a survey of 24 tumors in which non-malignant tissue at the tumor site was also obtained, 3 tumors had O^6-MT activity lower than in the normal tissue by between 2- and 7-fold (Myrnes et al., 1984). The same laboratory has reported that of another 4 patients in which tumor and non-tumor tissues were obtained from the same organ, one tumor had less than half the O^6-MT activity of the normal tissue (Myrnes et al., 1983). 2 of 6 patients with gastric malignancies had lower O^6-MT levels in the tumor tissue than in surrounding normal mucosa (Kyrtopoulos et al., 1984).

More studies of this type are needed.

Conclusions

O^6-MeGua is demethylated by a remarkable and unique repair mechanism in many bacterial and mammalian cells, implying a form of evolutionary conservation and/or strong selection for

this method of DNA repair. Although the basic features of the repair reaction are the same in *E. coli* and humans, there are several important differences, especially in substrate specificity and inducibility, which warrant careful extrapolation from one system to the others.

O^6-MT appears to repair premutagenic lesions in all organisms studied but its importance in survival after DNA alkylation is greatest to Ad 5 infecting human cells and of little consequence to *E. coli*. Strong theoretical arguments and some circumstantial evidence link O^6-MT activity to the prevention of oncogenic transformation.

O^6-MT activity may provide a dose threshold below which DNA alkylation does not produce all of its potential biological effects. However, the absence of significant inducible activity in human cells, the reduced efficiency of O^6-MT as the density of O^6-MeGua in DNA decreases, and the low affinity for single-stranded DNA all suggest that this low dose threshold may provide little protection to the public health.

Important questions remain unanswered. The signal for induction of the adaptive response in bacteria has not been conclusively identified, and the mechanism by which this signal stimulates transcription of the *ada* gene is a crucial subject for understanding gene regulation. Other genes and functions yet to be identified may be under *ada* gene control. Much less is known about O^6-MT regulation in mammalian cells, where the primary question is whether there is any significant, reproducible adaptive response in cells other than those of the rat.

The enzymology of O^6-MT has made much progress but the evidence of protein cleavage in bacterial cells raises the question of whether there is controlled processing of O^6-MT. Processing of O^6-MT has not been ruled out in mammalian cells and critical objectives should be purification of this protein and the production of antibodies against it.

Finally, the molecular basis of the Mer^-/Mex^- defect has yet to be fully explored, particularly the production of O^6-MT defects by viral transformation. The hypersensitivity of Mer^-/Mex^- human tumor cells to alkylating agents offers the possiblity of developing selective chemotherapy protocols using drugs which produce O^6-alkylGua and crosslinks. An understanding of the regulation of O^6-MT in human cells may permit its activation in normal cells and inhibition in tumor cells for the prevention and treatment of cancer.

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