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MUTAGENIC SPECIFICITY OF ALKYLATED AND OXIDIZED DNA BASES AS DETERMINED BY SITE-SPECIFIC MUTAGENESIS

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Summary. - *This work demonstrates the use of the tools of site-specific mutagenesis to study the mutagenic activity of two DNA adducts, O⁶-methylguanine and* cis-*thymine glycol. The former adduct is one of the methylated bases formed by carcinogenic and mutagenic alkylating agents. It was built into the single-stranded genome of bacteriophage M13 and replicated in* Escherichia coli (E. coli). *The mutation frequency of O⁶-methylguanine was 0.4% in physiologically normal cells. In cells in which the repair systems for O⁶-methylguanine were compromised by challenge with an alkylating agent, the mutation frequency rose to approximately 20%. DNA sequencing revealed that O⁶-methylguanine induced exclusively G→A transitions, which was most consistent with it pairing with thymine during DNA synthesis. The mutagenic effects also were investigated of* cis-*thymine glycol isomers, which are major, stable products of ionizing radiation and oxidative damage to DNA. By techniques similar to those employed for the study of O⁶-methylguanine mutagenesis, a single thymine glycol was situated in an M13 phage genome. The genome was replicated in* E. coli *that were physiologically normal, induced for SOS functions, or deficient in the* nth *gene product and, in all cases, the mutagenic processing of thymine glycol* in vivo *yielded mutant progeny phage at a frequency of 0.3-0.4%. All mutations occurred at the site that originally contained thymine glycol, and all were demonstrated by DNA sequencing to have resulted from targeted T→C transitions. These data suggest that thymine glycol pairs with guanine during replication.*

Riassunto (Applicazione della mutagenesi sito-specifica per lo studio della specificità mutagena di basi alchilate e ossidate). - *Questo studio mostra l'uso della mutagenesi sito-specifica per lo studio dell'attività mutagena di due addotti al DNA, O⁶-metilguanina e* cis-*timina glicole. Il primo addotto è una delle basi metilate formate dagli agenti alchilanti cancerogeni e mutageni. E' stato costruito nel genoma a singolo filamento del batteriofago M13 e replicato in* Escherichia coli (E. coli). *La frequenza di mutazione della O⁶-metilguanina è risultata dello 0,4% in cellule in condizioni fisiologiche. La frequenza di mutazione era circa del 20% in cellule in cui i sistemi di riparo per la O⁶-metilguanina erano stati compromessi da un pretrattamento con un agente alchilante. L'analisi della sequenza del DNA ha mostrato che la O⁶-metilguanina induceva esclusivamente transizioni del tipo G→A, in accordo con l'accoppiamento della base modificata con la timina durante la sintesi del DNA. E' stato anche condotto uno studio degli effetti mutageni degli isomeri del* cis-*timina glicole, che sono i prodotti stabili indotti sul DNA in maggiore quantità dalle radiazioni ionizzanti e dal danno ossidativo. Con tecniche simili a quelle usate nello studio della mutagenesi della O⁶-metilguanina, un singolo glicole della timina è stato costruito nel genoma del fago M13. Il genoma è stato replicato in cellule di* E. coli *che erano fisiologicamente normali, indotte per le funzioni di SOS, o deficienti nel prodotto del gene* nth *e, in tutti i casi, il processamento mutageno del glicole della timina* in vivo *ha dato luogo ad una progenie fagica mutante alla frequenza della 0,3-0,4%. Tutte le mutazioni erano localizzate nel sito che conteneva originariamente il glicole della timina e l'analisi della sequenza metteva in evidenza transizioni T→C. Questi dati suggeriscono che il glicole della timina si accoppi con la guanina durante la replicazione.*

Introduction

Most genotoxins act by binding to DNA nucleotides, forming covalent products that are referred to as adducts. Misreplication or misrepair of DNA adducts can cause mutations, which can contribute to or represent the sole basis for initiation of genetic disease [1]. Because of the close mechanistic relationship of DNA adducts to the origin of disease states, adduct measurement in body fluids and accessible tissue samples offers a means to

assess exposure to environmental chemicals, drugs, and radiation [2]. The practical utility of DNA adducts as biomonitors would be enhanced if it were possible to focus upon the adducts most closely related mechanistically to mutation or other genotoxic endpoints. Knowledge of the biological importance of individual DNA adducts has been constrained, however, because the reaction of most activated carcinogens and mutagens with DNA nearly always results in a wide range of DNA adducts. The large number of adducts is a consequence of the multiplicity of pathways that convert chemicals to reactive intermediates, and to the complexity of DNA as the target for modification.

The field of site-specific mutagenesis developed in response to the need for systems that can rank the relative biological importance of DNA adducts. The technology of this field utilizes a combination of organic synthesis, biochemical, and recombinant DNA methodologies to situate adducts individually into preselected sites in the genomes of viruses and plasmids [1]. The site-specifically modified vectors thus produced are replicated within cells, where the ability of the DNA adduct to induce mutations *in vivo* can be assessed. The frequency of mutations is scored and the qualitative basis for the mutant phenotype is determined by DNA sequencing. These data allow direct evaluation of the qualitative and quantitative biological importance of individual DNA adducts and can serve as a basis for selection of the adducts most suited for application as biomarkers in environmental monitoring. In this manuscript, we describe the use of the tools of site-specific mutagenesis to define the mutagenic activity of two DNA adducts, O^6-methylguanine (O^6-MeGua) and *cis*-thymine glycol (t').

Site-specific mutagenesis studies using O^6-methylguanine

The DNA adduct chosen for our initial studies was O^6-MeGua, which is one of the methylated bases formed by carcinogenic N-nitroso compounds and other alkylating agents. The persistence of the O^6-alkylguanines correlates well with the mutagenic and carcinogenic effects of alkylating agents [3-4]. The molecular rationale for the mutagenic effects of this adduct derives from the fixation by O^6-alkylation of guanine of the enol tautomer of the base, in which it has been predicted to base-pair with thymine during replication.

Mutation frequency of O^6-methylguanine in single-stranded DNA in vivo

The initial step for preparing a viral genome containing O^6-MeGua was the synthesis of a tetradeoxynucleotide, d{5'-Tpm^{6}GpCpA-3'} (m^{6}G = O^6-methyldeoxyguanosine). The modified tetranucleotide was synthesized by the phosphotriester method [5] and inserted by using recombinant DNA techniques into a 4-base gap positioned at the sole PstI site in the duplex genome of *Escherichia coli (E. coli)* virus M13mp8 [6]. The product of this reaction is referred to herein as O^6-MeGua-M13mp8 (Fig. 1). The method of construction of the modified duplex provided a means for removing the DNA strand opposite the adduct (note the nick in Fig. 1), and hence it was possible in our genetic studies to evaluate the fate of the adduct in either single-stranded (ss) or double-stranded (ds) genomes. It was desirable to have the option to remove the complementary strand because the major repair protein for O^6-MeGua is less active on ss DNA than on ds substrates. With either ss or ds O^6-MeGua-M13mp8, the method of construction resulted in formation of equal quantities of genomes in which the sole adduct was at position 6255 in the (+) strand and at position 6256 in the (-) strand.

Mutagenesis of O^6-MeGua in ss DNA was studied by denaturing ds O^6-MeGua-M13mp8 in alkali and introducing the ss product into *E. coli* MM294A cells [7]. A mixture of wild-type and mutant phage was produced, which was used to produce the ds replicative form (RF) DNA; the wild-type phage were derived either from repaired O^6-MeGua-M13mp8 or from replication events in which the lesion did not lead to mutation. The method used to differentiate mutant and wild-type phage was based on the supposition that mutations were formed in the PstI recognition site because this was the site originally occupied by O^6-MeGua. Mutations affecting this site rendered the RF DNA insensitive to cleavage by PstI, permitting isolation of a pure mutant phage population for DNA sequencing and for calculation of mutation frequencies. Because the adduct was present in *either* of the complementary strands of the M13mp8 genome (see Fig. 1), mutations could arise at genome position 6256 (when the adduct was in the (-) strand) or at position 6255 (when it was in the (+) strand). DNA sequencing of the mutants revealed that O^6-MeGua induced exclusively G→A transitions [7]. The same results were obtained independently by Hill-Perkins *et al.* [8] and Bhanot and Ray [9].

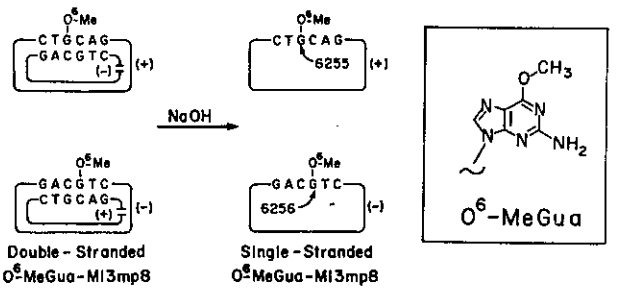


Fig. 1. - Single- and double-stranded M13mp8 genomes containing O^6-methylguanine.

The exact mutation frequencies of O6-MeGua in the (-) and (+) strands are MF_1 and MF_2, respectively:

$$MF_1 = \frac{M^-}{(M^- + WT^-)} \quad (1)$$

$$MF_2 = \frac{M^+}{(M^+ + WT^+)} \quad (2)$$

where M and WT are the numbers of mutant and wild-type phage produced from the adduct-containing DNA strand indicated by the superscript. It was possible to distinguish mutants originating from the adduct in the (+) and (-) strands by the color of their plaque on an indicator agar. However, as elaborated in Loechler *et al.* [7], it was impossible to distinguish wild-type phage derived from the (+) and (-) strands (WT^+ and WT^- in the above equations), because they were phenotypically identical.

Therefore, instead of calculating MF_1 and MF_2 (the true mutation frequencies of the lesion in each strand) we calculated the quantities MF^- and MF^+, which represent the *fraction* of the progeny phage population with mutations originating from the adducted (-) or (+) strands, respectively:

$$MF^- = \frac{M^-}{(M^- + WT^-) + (M^+ + WT^+)} \quad (3)$$

$$MF^+ = \frac{M^+}{(M^- + WT^-) + (M^+ + WT^+)} \quad (4)$$

Equations 3 and 4 were used to calculate the data in Table 1.

As shown in Table 1, the mutation frequencies of the adduct in the (+) and (-) strands of ss M13mp8 (MF^+ and MF^-, respectively) were determined to be 0.36 and 0.08%, respectively. The sum of the values for the mutation frequencies in the individual strands is defined as the *total mutation frequency* of O6-MeGua, MF^t, which was determined to be approximately 0.4%. That is, 0.4% of the phage issued from host cells transfected with an equal mixture of the adducted genomes shown in Fig. 1 had mutations at either of the two possibly adducted sites.

Role of DNA repair in protecting cells against O6-methylguanine mutagenesis

Others have investigated the *in vitro* miscoding characteristics of O6-MeGua in experiments that typically have involved random incorporation of O6-substituted guanines into DNA or RNA polymers, which then were copied with polymerases [10-12]. Subsequently, the replication products were analyzed for the presence of noncomplementary nucleotides. The results of such experiments demonstrated that DNA and RNA polymerases misreplicate O6-alkylguanines approximately one-third of the time. Although less work has been done to estimate the mutation frequency *in vivo*, the few data that are available indicate a similar [13] or slightly higher [14] mutation frequency, as compared to that observed *in vitro*.

The mutation frequency we observed *in vivo* was several orders of magnitude less than that determined or predicted by the experiments cited above. The most likely reason for this apparent discrepancy is the fact that in our studies a *single* adduct was built into the genome, and this single lesion probably was removed quickly in *E. coli* by repair proteins associated with the adaptive response (specifically, the O6-MeGua DNA-methyltransferase (MT)). MT acts by transferring the methyl group from O6-MeGua in DNA to itself [15]; the alkylated repair protein is not believed to turn over, and thus it is irreversibly inactivated in the process of dealkylating the genome. We exploited the suicide-inactivation mechanism of the MT to diminish the intracellular capacity to repair O6-MeGua. Two minutes before O6-MeGua-M13mp8 was introduced into the host cells, the cells were treated with N-methyl-N'-nitro-N-nitrosoguanidine

Table 1. - *Mutation frequency (%) of O6-methylguanine in single-stranded O6-MeGua-M13mp8*

MNNG Challenge *(a)* µg/ml	O6-MeGua-M13mp8		M13mp8	
	MF^- *(b)*	MF^+ *(b)*	MF^t *(b)*	MF *(c)*
0	0.08	0.36	0.4	≤ 0.03
17	1.3	4.1	5.4	≤ 0.11
33	3.6	4.7	8.3	≤ 0.20
50	4.1	13.7	17.8	≤ 0.16

(a) Host cells were challenged with these concentrations of MNNG 2 min prior to DNA uptake; *(b)* MF^+, MF^- and MF^t are % of progeny phage with mutations in the (+) and (-) strands, and the total of the (+) and (-) strands, respectively; *(c)* Mutation frequency (upper limit) of unmodified M13mp8.

(MNNG). This treatment produced O^6-MeGua residues, as well as other adducts, in the host chromosome, and repair of these lesions depleted the endogenous reserves of the MT and thus diminished the ability of cells to repair the single adduct in O^6-MeGua-M13mp8.

Table 1 presents the results of an experiment in which the mutation frequency of O^6-MeGua was examined in a series of cell populations that had been pretreated with increasing doses of MNNG; *i.e.*, treatments that generated a range of reduced MT activities within the host cells. As expected the mutant fraction derived from O^6-MeGua-M13mp8 increased with the level of MNNG treatment. As a control to the above experiment, *E. coli* cells challenged identically with MNNG were transformed with ss wild-type M13mp8 DNA. Mutation of this DNA to PstI insensitivity was insignificant.

At the highest level of MNNG challenge (50 μg/ml), MF[1] had increased to almost 50 times the comparable value in unchallenged cells. The mutation frequency of this sample (approximately 20%) does not necessarily represent the inherent mutation efficiency of O^6-MeGua, because mutagenesis was still increasing at this level of MNNG challenge (Table 1); rather, this value represents the lower limit of the *in vivo* mutation frequency of this lesion. Interestingly, at its present value this mutation frequency is at the lower end of the range measured for mutagenesis of O^6-alkylguanines *in vitro* and it is within a factor of two or three of the level predicted by indirect *in vivo* measurements [13, 14].

Replication errors induced by *cis*-thymine glycol

Ionizing radiation causes a wide variety of DNA lesions, including base modifications, strand breaks, cross-links and abasic sites [16, 17]. The hydroxyl radical is the major reactive species responsible for many DNA damages in aerated irradiated aqueous solutions [18]. This radical is highly reactive toward thymine bases, generating *cis*-5,6-dihydroxy-5,6-dihydrothymine, or *cis*-thymine glycol (t') (Fig. 2) as one of the major stable products [19]. Oxidation, either through hydroxyl radical attack or by other mechanisms also gives rise to this thymine modification [20]. Several *in vitro* studies have shown that t' inhibits DNA synthesis in most sequence contexts [21-24]. We recently have extended the study of t' genetic toxicology by investigating its effects on replication *in vivo*.

Construction of a bacteriophage M13 genome containing a single cis*-thymine glycol at a unique site*

The hexanucleotide, d{GCTAGC}, was oxidized under a variety of reaction conditions, and the oxidation products were analyzed by reversed phase HPLC. Previous

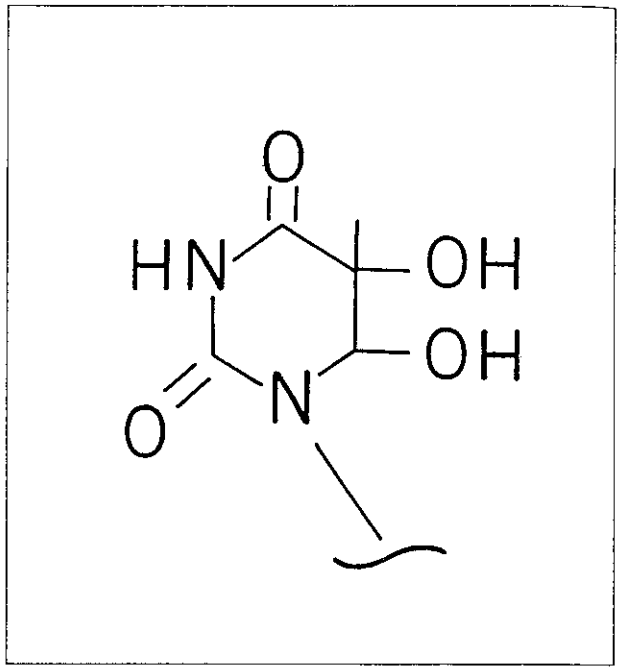


Fig. 2. - Thymine glycol.

reports on the oxidation of thymine indicated that mild treatment with $KMnO_4$ generates 5-hydroxy-5-methylbarbituric acid and t' as the major products, whereas OsO_4 forms the latter predominantly [22, 25]. Oxidation of d{GCTAGC} by permanganate (1.4 mM $KMnO_4$, 37 °C, 5 min) at pH 8.6 provided several products eluting before the unmodified hexanucleotide. The major product was shown to be d{GCt'AGC} by release of t' (as well as the expected amounts of guanine, cytosine, and adenine) deoxynucleosides after enzymatic hydrolysis, reduction of t' deoxynucleoside to thymine by HI, and by its reaction with a monoclonal antibody that shows high specificity for t' (unpublished results). In parallel, a separate portion of d{GCTAGC} was oxidized with OsO_4 (2% OsO_4, 37 °C, 10 min) at pH 8.6. The major product was chromatographically identical with the peak of d{GCt'AGC} synthesized by permanganate oxidation.

A six-base insertion mutant of M13mp19, which contained a d{GCTAGC} sequence in the center of the unique SmaI site of the polylinker interruption of the *lacZα* fragment, was created by a procedure previously described [26]. This genome is denoted M13mp19-NheI, because disruption of the SmaI site generated a new, unique NheI site (GCTAGC) in the phage genome. This insertion clone produced colorless plaques on *Su*⁻ strains of *E. coli* because of the in-frame *amber* codon (underlined above) in the *lacZ* gene. Mutations affecting the *amber* codon generated blue plaques in the presence of β-galactosidase indicator dyes.

Denaturation, followed by renaturation of SmaI-linearized M13mp19 RF DNA with the ss genome (+ strand) of M13mp19-NheI produced a heteroduplex with

a six-base gap in the (-) strand. Following 5'-phosphorylation with polynucleotide kinase, the t'-containing hexamer, d{GCt'AGC}, was ligated into the gap in the presence of T4 DNA ligase (efficiency ~ 30%). This generated a singly adducted genome with the adduct uniquely located at position 6275. A slightly altered protocol [6] allowed us to introduce t' into, in equal amounts, the (+) or the (-) strands of the phage DNA (at positions 6274 or 6275, respectively; e.g., see Fig. 1). By this protocol, a nick was situated in each duplex in the DNA strand opposite the one containing the adduct; the presence of the nick made it possible to remove the complementary strand by denaturation (with alkali, heat or formamide) resulting in singly adducted ss genomes. Evidence that ligation occurred at the NheI site, and that ligation occurred on both sides of the gap, was obtained (unpublished results obtained as in Basu *et al.* [26]).

Effects of cis-*thymine glycol on viral DNA replication in* E. coli

Transformation of several strains of *E. coli* with ss and ds M13 genomes containing t' was performed. No significant reduction in the yield of progeny phage was noted in strains MM294A (wild type), BW415 (*Δnth*; this is the gene responsible for endonuclease III, a t' repair protein), BW419 (overproduces endonuclease III), DE1018 (*recA*430) and DE667 (constitutively expresses SOS functions). The results of these experiments suggest either facile bypass or rapid repair of the t' base.

The possible mutagenic processing of t' during replication was investigated by using a mutant selection scheme similar to that described above for O^6-MeGua; however, NheI was the selective enzyme rather than PstI. In all genetic environments listed above, t' was not detectably mutagenic in *double-stranded* DNA. The lower limit of detection of the mutation assay under the conditions employed was approximately 0.01%.

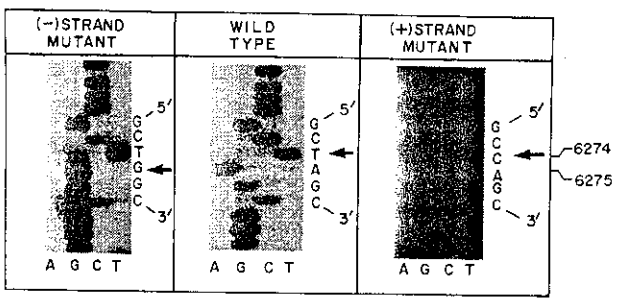
In contrast, t' displayed significant mutagenic activity in *single-stranded* DNA. In all strains tested, the mutagenic efficiency of the lesion was ~ 0.3-0.4%. Some of these data are presented in Table 2. In four separate experiments the mutation frequency of t' generated by the $KMnO_4$ procedure in ss DNA varied from 0.2-0.6%. A parallel experiment was done with genomes prepared with hexanucleotide generated by OsO_4 to determine whether the type or amount of mutagenesis was dependent upon the route of modified oligonucleotide preparation. As shown in Table 2, the mutation frequency was the same, regardless of the method of oxidation. Interestingly, the mutagenic efficiency of t' was not dependent upon, nor was it increased by, the induction of SOS functions (MM294A cells + UV [50 J/m^2]). Additionally, t' was mutagenic in strain DE1018, which is impaired in inducing SOS functions because of a mutation in RecA that makes it defective in cleaving LexA.

In all genetic backgrounds evaluated, DNA sequencing revealed that the exclusive mutation of t' in ss DNA was a T→C transition (Fig. 3). The transition occurred either at position 6274 or 6275; we assume that the mutations at these positions arose from misreplication of the adduct in the (+) and (-) strands, respectively. The nature of the transition induced in this experiment suggests that t' paired with guanine during replication. A quantitative strand bias was observed, in that the mutants arising from the (+) strand were approximately

Table 2. - *Mutation frequency of* cis-*thymine glycol (in ss DNA)*

GCt'AGC derived from:	**Cells**	**Mutation frequency (%)**
OsO_4 oxidation	MM294A	0.4
$KMnO_4$ oxidation	MM294A	0.2
"	"	0.2
"	"	0.3
"	"	0.6
"	MM294A + UV (SOS-induced cells)	0.2
"	DE1018 (*RecA*430)	0.2

Ratio of mutants [(+) strand : (-) strand] = 2.8 : 1



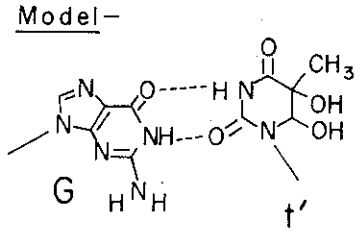


Fig. 3. - DNA sequence of the mutants induced by *cis*-thymine glycol. Autoradiograms showing the sequences of the mutants derived from mutagenic processing of t' in the (-) and (+) strand of M13 are shown in the left and right panel, respectively. The sequence of wild type M13mp19-NheI is shown in the middle panel. At the bottom is a base pair hypothesized to occur during t' mutagenesis.

three times more abundant than those arising from the (-) strand (Table 2). This strand bias in favor of t'-induced mutants arising from the (+) strand is similar to that observed for O^6-MeGua.

Perspective on the practical value of site-specific mutagenesis

An assumption toxicologists often are forced to make is that dose-response relationships are linear from the high doses at which it is convenient to make biological measurements down to the much lower doses at which normal human exposure to chemicals or radiation occurs. High doses are used in conventional genotoxicity studies because the genetic target in which a measurement is to be made is usually small, and a high overall level of modification of the cellular genome ensures that enough damage will fall within the target to generate a measurable response. Unfortunately, high doses often result in much higher levels of genome modification than would be encountered normally, and DNA repair and other geno-protective systems can saturate, giving rise to a non-linear dose-response curve. One of the attractive but often overlooked features of making genetic measurements using site-specifically modified genomes is that they enable the effects of DNA damage to be measured in cells that have a damage dose as little as one adduct per cell. Hence, genetic measurements made using the probes described in this review are likely to reflect accurately the ways that a given organism might respond in its natural environment, at the doses of chemicals and radiation routinely encountered.

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