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IMIDAZOLE OPEN RING 7-METHYLGUANINE : AN INHIBITOR OF DNA SYNTHESIS

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Guanine methylated at the N7 position (me^7G) is susceptible to cleavage of the imidazole ring yielding : 2,6-diamino-4-hydroxy-5N-methyl-formamidopyrimidine (rom^7G).

DNA synthesis catalysed by E.coli DNA polymerase I, using as templates poly(dGC) containing either me^7G or rom^7G, show that rom^7G blocks DNA chain elongation. It implies a potential killing effect. Furthermore rom^7G does not induce mispairing with either dAMP or dTMP. me^7G does not affect DNA synthesis. The results suggest that, beside AP-sites, rom^7G is a potential killing lesion in cells treated by alkylating agents.

The treatment of cells by alkylating agents such as alkylsulfates, alkyl methanesulfonate or nitrosoureas results in mutagenesis and killing. The main targets of such compounds on DNA are the N^7 of guanine, the N^3 of adenine and the O^6 of guanine (1), although all the nitrogens and oxygens of the bases can be alkylated (2). So far the mutagenic properties of this class of compounds have been attributed to the alkyl O^6 guanine (3). In the case of the nitrosoureas used in cancer chemotherapy, it has been postulated that the lethal action is due to depurination and/or formation of cross links (4).

The N^7 position of guanine is by far the main target of alkylating agents and such alkylation persists for generations in cellular DNA (5). It is generally admitted that this lesion is not harmful to the cell, although it is removed enzymatically with low efficiency (6, 7, 8). Guanine, methylated at the N^7 position (me^7G), is susceptible to depurination yielding apurinic/apyrimidinic sites (AP-sites) and to alkali cleavage of the imidazole ring yielding the ring-open form (rom^7G) : 2,6-diamino-4-hydroxy-5-N-

ABBREVIATIONS : me^7 G : 7-methylguanine
rom^7 G : 2,6-diamino-4-hydroxy-5N-methyl-formamidopyrimidine.
DMS : dimethyl sulfate

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methylformamidopyrimidine (9,10). This reaction occurs slowly *in vitro* at neutral pH and it has not been excluded that it can occur in *Micrococcus luteus* crude extracts (6). Nevertheless, the opening of the ring may be of importance because of the large amount of me^{7}G generated by the action of methylating agents on DNA. Upon X-irradiation, the imidazole open ring form of purine is the main lesion identified in DNA (11). In this paper, we show the effect of the presence of rom^{7}G in the poly(dGC) as a template for *E.coli* DNA polymerase I.

MATERIALS AND METHODS

Preparation of poly(dGC) templates containing me^{7}G, rom^{7}G and AP-sites.

Poly (dGC) was alkylated by dimethyl sulfate as described for DNA (6) and modified as follows. The polynucleotide - P.L. Biochemical product - was dissolved in 100 µl of 0.3 M Na cacodylate-perchloric acid buffer (pH 7.5) to a final concentration of 2 mM dMNP residues. It was alkylated with 5 mCi of [^{3}H] DMS (New England Nuclear, 4.7 mCi/mmole). The reaction mixture was incubated for 1 hour at 37°C, then dialysed at 4°C against several changes of buffer containing 10 mM Tris-HCl (pH 8.0) 20 mM KCl, and 0.1 mM Na_2 EDTA until all ethanol-soluble materials had been removed. Under these conditions about 0.5 % of the guanine residues are methylated. Alkylation of poly(dGC) by dimethyl sulfate (Aldrich Milwaukee, WI), diluted if necessary in 5 µl of benzen, was performed under the same conditions.

In order to induce imidazole ring opening of me^{7}G , the alkylated poly(dGC) (DMS-poly(dGC) was treated at 25°C for increasing time length either in 0.2 N NaOH or in 50 mM Na_2 H PO_4 -NaOH (pH 11.4) (10, 12).

The removal of me^{7}G by cleavage of the glycosydic bond was obtained by heating DMS-poly(dGC) at 100°C for 30 min., in 5mM KPO_4 buffer pH 7.2

Chromatographic analysis of methylated products in poly(dGC)

Base analysis was performed after hydrolysis of treated poly(dGC) in 0.1 M HCl for 17 hours at 37°C. Authentic samples of me^{7}G (Sigma) and rom^{7}G (a gift form Dr. Lindahl) were added as reference molecules. The bases were separated by thin-layer chromatography on Eastman-Kodak plates, as previously described (6). After migration, the markers were localized by ultraviolet absorption. Strips of 0.5 cm were cut transversally, further cut into small fragments and the radioactivity was measured by liquid scintillation with a counting efficiency of about 30 %.

Alternatively me^{7}G and rom^{7}G were also quantified using high performance liquid chromatography as already described (13), and a C_{18} µbondapak (Waters Milford MA). The mobile phase was ammonium phosphate buffer 50 mM (pH 4.0) containing 5 % methanol. The order of elution was rom^{7}G : 6 ml and me^{7}G:15 ml. The amount of the different compounds was measured using liquid scintillation and optical density determination.

Replication of poly(dGC) templates

The polymerisation mixture (100 µl) contained 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 50 µM [^{3}H] dGTP (30 dpm per pmole), 50 µM [^{3}H] dCTP (30 dpm per pmole), 50 µM poly (dGC) and 0.2 unit of *E.coli* polymerase I (EC 2.7.7.7)

(Boeringer, Mannheim, Germany). The mixture was incubated for 45 min. at 37°C. It was then spotted on GF/C filters (Whatman). The filters were washed three times at 0°C with a solution containing 5 % trichloracetic acid (w/v) and 2 % Na_4 pyrophosphate (w/v), once with 5 % trichloracetic acid, soaked in ethanol, and dried. The radioactivity bound to the filter was measured by liquid scintillation. The replication fidelity assay (100 µl) was as described above with addition of 25 µM ($[^{32}P]\alpha$ dATP (2000 cpm/pmole) or 25 µM $[^{32}P]\alpha$ -dTTP (2000 cpm/pmole). The labelled triphosphates were purchased from NEN and had a specific activity >600 mCi/mmole. The purety of triphosphates was monitored by PEI cellulose chromatography (14). The blanck values without either polymerase or template were substracted, they never exceeded in the reported experiments 0.05 pmole of any triphosphate added.

RESULTS AND DISCUSSION

The activity of E.coli DNA polymerase I was measured using as templates poly(dGC) containing different lesions, namely me^7G, open ring me^7G (rom^7G), and apurinic sites (AP-sites). The template was prepared by incubating poly(dGC) with DMS (DMS-poly(dGC). The analysis of the alkylated template shows that all the radioactivity cochromatographed with me^7G (Fig. 1A). Under the conditions described in Materials and Methods, methylated pyrimidines migrate with oligonucleotides. Therefore 3-methylcytosine accounts for less than 1 % of the total methylated bases in DMS-poly(dGC). This result was confirmed independently by Bio-gel-P_2 chromatography (6). When DMS-poly(dGC) is treated under alkaline conditions, me^7G is converted into the opened ring form (rom^7G). A short treatment yields a template containing both lesions (Fig. 1B), while a 15 hours treatment results in a quantitative conversion of me^7G into rom^7G (Fig. 1C).

As shown in Figure 2, pretreatment of the template by DMS concentrations lower than 20 mM, which yield about 10 % of me^7G (see insert Figure 2 and reference 15), does not impair DNA synthesis. In contrast, when me^7G is converted into rom^7G, we observe a marked inhibition of DNA synthesis. If the me^7G are excised by depurination, yielding AP-sites, the same severe inhibition is observed, as expected (16, 17). The kinetics of inhibition of DNA polymerase by AP-sites or by rom^7G are the same, showing that the inhibiting events derive from the same lesion, namely me^7G.

When the template for DNA polymerase I is poly(dC) containing me^3cytosine untreated of treated under alkaline conditions, the pattern of replication

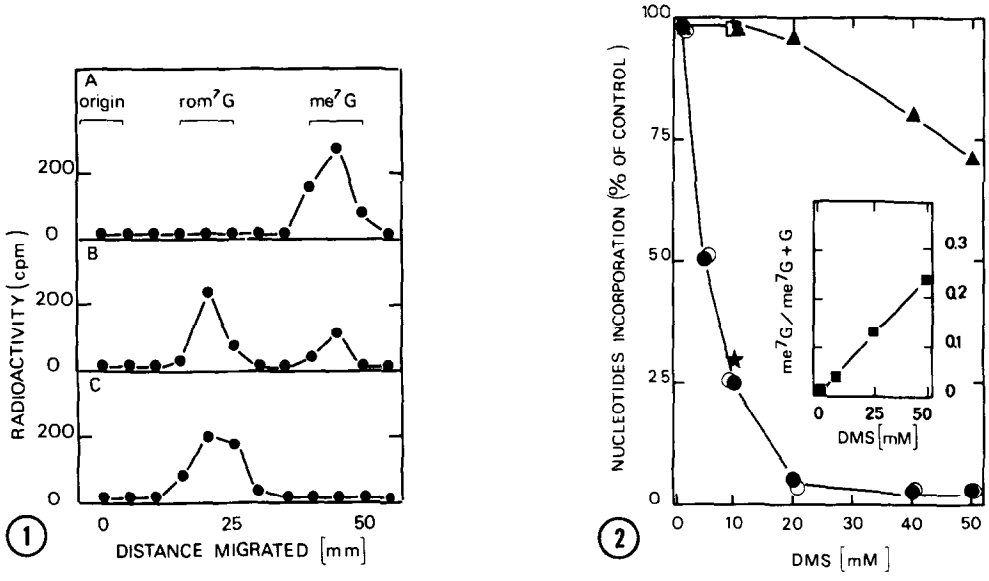

Figure 1 : Thin-layer chromatographic analysis of methylated products in poly(dGC) untreated or treated under alkaline conditions

Poly(dGC) was alkylated by [^{3}H]dimethyl sulfate and further treated or not under alkaline conditions for increasing lenght of time. The methylated poly (dGC) was hydrolysed and the products were chromatographied on cellulose plates.
(A) DMS-poly(dGC) untreated control ;
(B) DMS-poly(dGC) incubated with 0.2 M NaOH for 2 Hours ;
(C) DMS-poly(dGC) incubated with 0.2 M NaOH for 15 Hours.

Figure 2 : Activity of E.coli DNA polylmerase I using as template poly(dGC) alkylated by increasing amounts of dimethyl sulfate and either untreated or further treated at neutral or alkaline pH.

Poly(dGC) was either alkylated with increasing amounts of DMS, dialysed, and treated with 0.2 N NaOH for 15 Hours, or treated with NaOH and alkylated. The depurination of DMS-poly(dGC) was obtained by incubating the methylated polynucleotide in 5 mM K-phosphate buffer (pH 7.2) for 30 min. at 100°C. The amount of me^{7}G in DMS-treated poly(dGC) was determined by HPLC and was reported in the INSERT. The template used to measure DNA synthesis was poly(dGC) either alkylated with increasing amounts of DMS (▲—▲), or treated under alkaline conditions prior to alkylation (□), or after alkylation (●—●).

The alkylated poly(dGC) was heat depurinated (○—○) or treated alkaline conditions and heat-treated (★).

remains unchanged (data not shown). These results rule out the possible role of me^{3}cytosine derivative as an inhibitor of DNA polymerase. We have also checked the stability of the glycosidic bond of me^{7}G during alkaline treatment. The result shows that less than 1 % of the alkylated bases are released as ethanol-soluble products after a 24 hours treatment at alkaline pH.

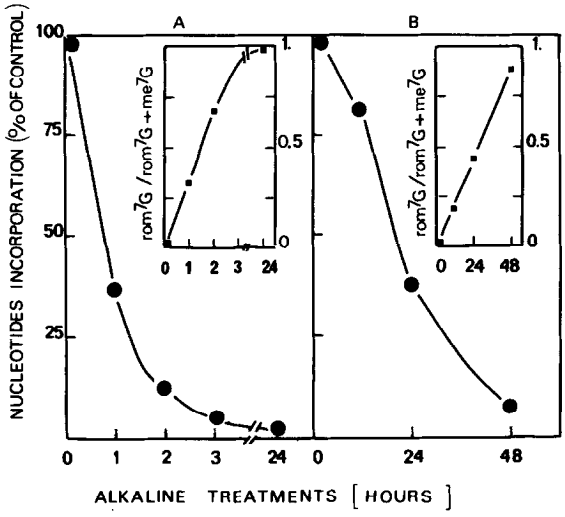

Figure 3 : Kinetics of inhibition of E.coli DNA polymerase I when an open ring form me^{7}G is introduced in the template by either of two different chemical treatments.

The template were obtained by alkylation of poly(dGC) with 20 mM DMS, followed by treatments at alkaline pH under two different conditions.
(A) E.coli polymerase I activity using as template DNA-poly(dGC) treated with NaOH for increasing lengths of time.
Insert : Kinetics of the appearance of rom^{7}G in DMS-poly(dGC) under the same conditions.
(B) E.coli polymerase I activity using as template DMS-poly(dGC) treated under mild alkaline pH for increasing lengths of time. Insert : kinetics of the appearance of rom^{7}G in DMS-poly(dGC) under the same conditions.

In order to ascertain the role of rom^{7}G as a block of DNA replication, this compound was generated in alkylated poly(dGC) using two different conditions (0.2 M NaOH or Na_2HPO_4 at pH 11.4). The results presented in Fig.3 show that under the two conditions used to convert me^{7}G to rom^{7}G the residual DNA synthesis is related to the amount of rom^{7}G generated. It should be emphazised that the inhibition is directly proportional to the amount of rom^{7}G generated regardlesss of the conditions used to catalyse the conversion that is, either mild alkaline conditions for a long time or strong conditions for a short time. These results suggest that the open ring form of me^{7}G blocks DNA replication. We have also investigated whether this arrest of elongation is accompanied by a misincorporation. The experiment shows that it is not the case, as the ratios dAMP/dCMP and dTMP/dCMP are not significantly different from the ratios observed in untreated poly(dGC) (Table I). This indicates that, given the sensitivity of the method under our experimental conditions,

TABLE I : Fidelity of DNA synthesis catalysed by E.coli polymerase I using as template poly(dGC) containing either me^7 G or rom^7G.

Poly (dGC) was alkylated with 10 mM DMS, and incubated for 15 hours with 0.2 N NaOH at 25°C. All analyses were performed three times, on separate alkylated preparations.

Poly (dGC) treatment	0	DMS	NaOH	DMS + NaOH
Major DNA lesion	0	me^7G	0	rom^7G
$[^3H]$ dCMP inc. (pmoles)	546	534	781	150
$[^{32}P]$ dAMP inc. (pmoles)	0.09	0.12	0.09	< 0.05
dAMP/dCMP x 10^4	1.6	2.2	1.2	< 3.3
$[^3H]$ dCMP inc. (pmoles)	596	522	715	185
$[^{32}P]$ dTMP inc. (pmoles)	< 0.05	< 0.05	< 0.05	< 0.05
dTMP/dCMP x 10^4	< 0.8	< 1.0	< 0.7	< 2.7

there is no mispairing of a dAMP or a dTMP with a rom^7G residue, only a block of DNA replication.

Our results show that the open ring form of me^7G is powerful block of DNA chain elongation. Its properties are comparable as those of AP-sites and both act as potentially killing lesions. The open ring form of purine does not appear to have miscoding properties *in vitro*, which suggest that it is not directly involved in direct mutagenesis as compared to O^6me Guanine (3, 18). It appears that me^7G, a harmless lesion introduced in great amounts by alkylation, could give rise to a harmful one either by depurination or through an opening of the imidazole ring. It should be recalled that the open ring forms of purines are the main lesions introduced by X-rays in DNA (11) and could be a cause of cell death. To overcome these deleterous effects (19), the cells have evolved specific constitutive DNA repair systems : AP-endonucleases, insertases and formamidopyrimidine DNA-glycosylases (for review see 20, 21).

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