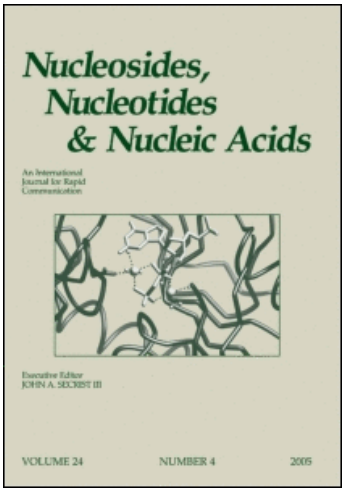

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Repair Kinetics of Abasic Sites in Mammalian Cells Selectively Monitored by the Aldehyde Reactive Probe (ARP)

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REPAIR KINETICS OF ABASIC SITES IN MAMMALIAN CELLS SELECTIVELY MONITORED BY THE ALDEHYDE REACTIVE PROBE (ARP)‡

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ABSTRACT: Human methylpurine N-glycosylase (MPG) activity was investigated by monitoring abasic (AP) sites resulting from removal of alkylated bases. The amount of AP sites in MMS-treated HeLa cells transiently increased at 3 h, then gradually decreased to 40% at 24 h. The presence of adenine, an inhibitor of AP endonucleases, in the repair incubation of MMS-treated cells induced moderate accumulation of AP sites, suggesting inhibition of the activities of MPG as well as AP endonucleases by adenine metabolites.

A monofunctional alkylating agent, methylmethanesulfonate (MMS) has been widely used to investigate the base excision repair mechanism, because the treatment of mammalian cells by this reagent results in the production of two major damages, 7-methylguanine (7mG) and 3-methyladenine (3mA), almost exclusively[1]. 7mG and 3mA constitute approximately 80 % and 10 % of total base damages, respectively. These N-alkylated bases are removed from DNA by methylpurine N-glycosylases (MPG)[2,3]. The half lives of 3mA and 7mG in living cells have been estimated as about 3 h and 3 days, respectively, while their chemical half lives without enzymatic catalysis are about 1 day and 6 days, respectively[4]. Recently, eukaryotic MPG (or alkyl-N-purine glycosylase) genes have been cloned separately in several laboratories[5-10]. Using the recombinant protein, it has been shown that mammalian MPG recognizes 1-N^6-ethenoadenine[11-13] and

‡This paper is dedicated for the late Professor Tsujiaki Hata.

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hypoxanthine[14] in addition to methylated purines. Roy et al. have shown that the N-terminal region of the protein is essential for the substrate recognition based on the data with mouse MPG[15] and a chimeric protein between mouse and human MPG[16]. While the three dimensional structure of *E. coli* Alk A protein, a major prokaryotic MPG, provided valuable insight into the structure-function relationship of this type of enzymes[17,18], the *in vivo* function of the mammalian homologs remains to be clarified.

One of the possible approaches to uncover the *in vivo* function of human MPG is measuring the intracellular level of AP sites resulting from removal of alkylated bases by this enzyme. The level of AP sites in alkylated DNA is affected by MPG that is a major methylpurine glycosylase in human cells and converts alkylated bases into AP sites and AP endonucleases that initiate the subsequent repair processing of AP sites. In this study, intracellular levels of AP sites in MMS-treated HeLa cells were selectively monitored in the presence and absence of adenine, a potent inhibitor of mammalian AP endonucleases[19], by using the optimized aldehyde reactive probe (ARP) assay (see also FIG. 1)[20,21]. Furthermore, the effects of adenine and its metabolites, NAD^+ and adenosine diphosphoribose (ADPR), on the activity of MPG were studied *in vitro* to obtain the biochemical basis of the observed *in vivo* effects of adenine.

MATERIALS AND METHODS

Preparation of DNA substrates.

Partially depurinated DNA for the standard of ARP assay was prepared by acid-heat treatment as follows[22]. Calf thymus DNA (Worthington Biochem.) was treated with 100 μg/mL RNAase A (Type II, Sigma) at 37 °C for 1 h, and extracted with phenol-chloroform. The purified DNA (500 μg/mL) was dialyzed against AP buffer (10 mM sodium citrate, 0.1 M NaCl, pH 5.0), heated at 70 °C for 0-50 min, and then precipitated with ethanol. For the preparation of the methylated DNA substrate for MPG, purified calf thymus DNA was treated with 0-120 mM methylmethanesulfonate (MMS, Sigma) in NTE buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl) at 37 °C for 1 h. To prepare DNA containing 1-N^6-ethenoadenine (εA), purified calf thymus DNA was treated with 0.5 M chloroacetaldehyde (CAA) in 0.1 M sodium cacodylate buffer (pH 7.0) at 37 °C for 12 h[11]. Modified DNAs were precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at the concentration of 1 mg/mL.

MMS treatment of HeLa cells and repair incubation.

HeLa RC355 cells[23] was cultivated in Eagle's MEM medium supplemented with 10% fetal bovine serum (Hyclone) in 100 mm dishes. Log-phase culture was washed with

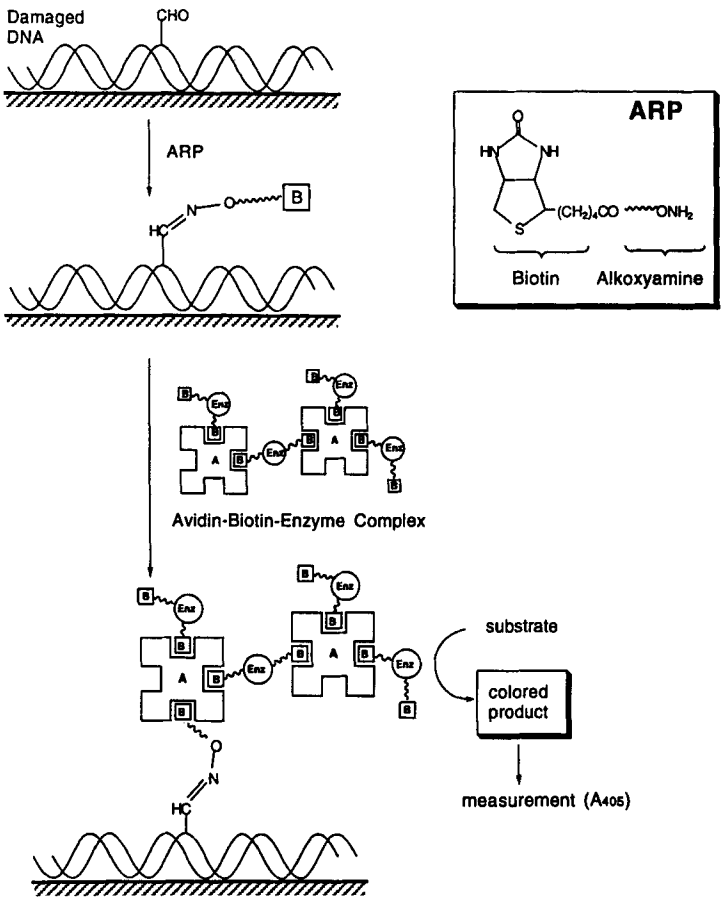

FIG. 1 Schematic diagram of ARP assay. The aldehyde group in AP sites is tagged by the aldehyde reactive probe (ARP), then the amount of the probe attached to DNA is quantitated using an avidin-biotin-enzyme amplification system.

PBS(-) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), and treated with MMS in PBS [PBS(-) supplemented with 0.9 mM $CaCl_2$ and 0.49 mM $MgCl_2$] at 37 °C for 1 h. The cells were washed with PBS(-) and then incubated in 10 mL of the fresh medium at 37 °C for up to 24 h. The medium was discarded by aspiration and the cells were washed by PBS(-). To test the cellular repair capacity of methylated DNA damages, the cells were harvested immediately after the MMS treatment or after various incubation periods, and DNA was extracted as described below.

DNA extraction from MMS-treated cells.

DNA was extracted from MMS-treated RC355 cells by the neutral guanidine thiocyanate phenol-chloroform (GPC) method[24]. Briefly, the cells were lysed in solution D

(4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine sodium salt, 0.1 M 2-mercaptoethanol) and extracted with equal volume of TE-saturated phenol (pH 7.5) and 0.1 volume of 3 M sodium acetate (pH 5.2). 0.5 volume of chloroform was added and the mixture was centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was collected and DNA was precipitated with 2-propanol.

ARP Assay.

The ARP assay for detection of AP sites in DNA was performed as reported with slight modifications[20,21]. In ARP assay, aldehyde groups in AP sites are specifically tagged and quantified by the biotin-conjugated prove as illustrated in FIG. 1. Aminoplates (Sumitomo Bakelite, #MS-3696F) or 0.1% protamine sulfate-coated plates (Corning, #25880-96)[25] were used for immobilizing DNA instead of UV-treated plates. The aminoplates were pretreated with $NaBH_4$ to reduce background signals. 200 μL of DNA solution (10 μg/mL) was added into each well of the aminoplate and the plate was incubated at 37 °C overnight. After the plate was washed with TPBS [PBS(-) containing 1% Tween 20], AP sites were modified with 5 mM ARP at 37 °C for 1 h followed by ABC solution (avidin-biotinylated horseradish peroxidase complex, Vector Laboratories) at room temperature for 30 min. Then, the plate was washed with TPBS and the substrate for horseradish peroxidase (1 mg/mL ABTS, 51 mM Na_2HPO_4, 24 mM citric acid) was added to each well. After 2 h, the absorbance at 405 nm was taken. The number of AP sites were determined by using heat-depurinated DNA containing defined numbers of AP sites as a standard. In an alternative method, DNA (10-200 μg/mL) was first treated with 5 mM ARP in solution and then diluted DNA (0.2-1.0 μg/mL) was added to the protamine-coated plate. The plate was incubated at 37 °C for 1 h and subjected to ARP assay as described above.

Quantitation of DNA bound to aminoplates.

Since the binding efficiency of DNA to the aminoplate has not been determined previously, it was measured using [^{3}H]-labeled DNA. Radioactive DNA were prepared by cultivating RC355 cells in a growth medium containing [^{3}H]-thymidine (550 kBq, 370 MBq/mL) at 37 °C overnight. [^{3}H]-labeled DNA was extracted by the GPC method and precipitated with ethanol. The precipitate was dissolved in TE buffer and the radioactivity was determined by a liquid scintillation counter (9,650 cpm/μg DNA). DNA was diluted to 0-20 μg/mL and 200 μL of the solution was added to each well of the aminoplate. The plate was incubated at 37 °C overnight and washed with TPBS to remove unbound radioactivity. The wells were cut out from the plate and measured for the remaining radioactivity to determine the amount of bound DNA. As shown in FIG. 2, the amount of

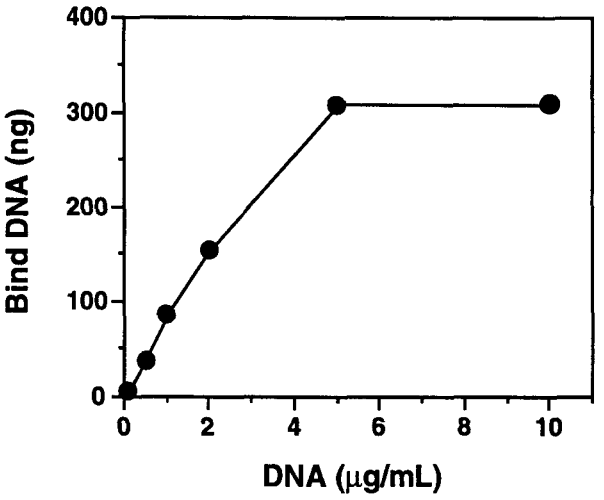

FIG. 2 Binding efficiency of DNA to aminoplates. [^{3}H]-labeled DNA (9,650 cpm/μg DNA) was prepared as described in Materials and Methods. Solutions (200 μL) containing the indicated concentrations of [^{3}H]-labeled DNA were added to the wells of aminoplates. The plates were incubated at 37 °C overnight. The radioactivity bound to each well was measured by a scintillation counter. The amount of DNA bound to the well was calculated using the specific activity of [^{3}H]-labeled DNA.

DNA bound to the plate increased with the DNA concentration and saturated at 5 μg/mL. Therefore, DNA was plated at the concentration of 10 μg/mL for ARP assays.

Partial purification of truncated-human MPG.

E. coli MV1932 cells (*tag*$^-$*alkA*$^-$)[26] harboring plasmid pDG23 was grown and disrupted as described previously[27,28]. The plasmid pDG23 containing human MPG cDNA has been characterized[5]. In the MPG cDNA, N-terminal 31 amino acids were truncated, but the expressed protein retained the activity and substrate specificity essentially similar to those of intact MPG (Kubo et al., unpublished data). Cells were disrupted by sonication and cell debris were removed by centrifugation (38,000 *g*). After nucleic acids were precipitated by polyethylene imine and centrifugation, ammonium sulfate (55% saturation) was added to the supernatant to precipitate proteins. The precipitated proteins were redissolved in MPG storage buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10% glycerol), and an aliquot (3 mL) was dialyzed against buffer A (100 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). The dialysate was applied onto an Econo-pac heparin cartridge (5 mL, Bio-Rad) preequilibrated with the same buffer. The heparin cartridge was washed with buffer A (20 mL) and eluted with a linear

gradient of NaCl (0-1 M, 50 mL) in the same buffer. Fractions containing the MPG activity (0.2-0.35 M NaCl, 8 mL) were pooled and dialyzed against buffer B (20 mM HEPES-KOH, pH 8.0, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 M NaCl). The dialysate was then loaded onto a POROS HS column (1 mL, Perseptive) preequilibrated with buffer B. After washing with buffer B (10 mL), the column was eluted with a linear gradient of NaCl (0-1 M, 25 mL) in buffer B. Truncated human MPG was eluted at 0.4 M NaCl.

Glycosylase assay.

The N-glycosylase activity of MPG for alkylated purines was assayed by measuring AP sites formed after removal of these damages. Partially purified MPG (16 μg/mL) was incubated with MMS- or CAA-treated DNA (100 μg/mL) in 20-50 μL of MPG assay buffer (35 mM HEPES-KOH, pH 7.5, 0.5 mM EDTA, 0.5 mM DTT) at 37 °C for 1 h (MMS) or 12 h (CAA). The reaction mixture was extracted with phenol-chloroform and DNA was precipitated with ethanol. To determine the effect of adenine and its metabolites on the MPG activity, incubation was carried out in the presence of 1 mM of each compound. The number of abasic sites in MPG-treated DNA was measured by ARP assay as described above.

RESULTS AND DISCUSSION

Repair kinetics of methylpurines in RC355 cells.

In mammalian cells, the major DNA base damages produced by methylmethanesulfonate (MMS) are 3-methyladenine (3mA, ca. 10%) and 7-methylguanine (7mG, ca. 80%)[1]. These methylated bases are removed mainly by 3-methyladenine DNA glycosylase (MPG) leaving AP sites in DNA[2,3]. Subsequently, AP sites are processed by AP endonucleases that primarily generate 3'-OH and 5'-deoxyribose phosphate (dRp) termini[29-31]. Then, the 5'-dRp moiety is removed by deoxyribophosphodiesterase (dRpase)[32] or Pol ß[33]. In this study, ARP assay was used to monitor the cellular base excision repair process covering from excision of the damaged bases to removal of the 5'-dRp moiety. FIG. 3 shows the change in the amount of AP sites in MMS-treated RC355 cells during the repair incubation. The background level of AP sites in untreated cells was estimated to be less than 1 AP site per 10^5 nucleotides and did not affect the ARP assay. Quantitation of total methylated purines using a heat treatment of isolated DNA revealed that only 10 % of the methylated purines were converted to AP sites immediately after the MMS treatment (data not shown). In the MMS-treated cells, the level of AP sites transiently increased at 3 h, then gradually decreased with incubation time. The dilution effect of damaged DNA by intact one due to cell growth in the repair incubation period was negligible since the MMS doses used here virtually inhibited cell proliferation. This was

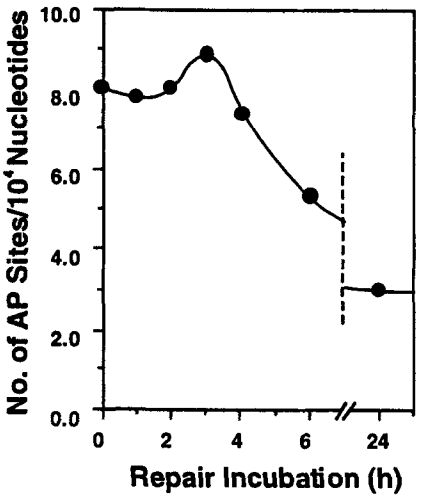

FIG. 3 Repair kinetics of AP sites in MMS-treated RC355 cells. Log-phase RC355 cells were treated with MMS (11.8 mM) in PBS at 37 °C for 1 h. Immediately after the treatment, the cells were washed and incubated in a fresh medium. At the time indicated, the cells were harvested and DNA was extracted. Then AP sites in DNA were assayed by ARP method. The number of AP sites were standardized relative to that observed without the repair incubation.

also supported by the fact that recovery of DNA from cells was essentially independent of the incubation time. Although the level of AP sites gradually decreased with incubation time, it still remained about 40% of that observed immediately after the MMS treatment even after 24 h of the incubation. These AP sites are likely originated from the major methylated base, 7mG, that is a considerably stable damage with a half life of 3 days in cells[4].Interestingly, a transient increase in the level of AP sites was apparent after 3 h of the repair incubation. This increase was not an experimental artifact and reproducibly observed in four repeated experiments. It is conceivable that in cells, the release of methylpurines by MPG is coupled with the subsequent processing of resulted AP sites by AP endonucleases since AP sites are mutagenic and lethal for cells[34-38]. Therefore, one might expects that AP sites are removed from DNA at a constant rate. However, the transient increase in the AP sites suggests that rapid cellular repair takes place to eliminate certain damage(s). Probably 3mA, which is more toxic than 7mG[3], may be quickly removed from DNA. This result also appears to be consistent with the observation by Bodell and Banerjee that repair DNA synthesis increased several hours after MMS-treatment of cells[39].

Effect of adenine on the repair of AP sites in RC355 cells.

In order to estimate the rate of base excision process in mammalian cells, repair incubation was performed in the presence of adenine, an inhibitor of human AP endonucleases[19]. RC355 cells were treated by different concentrations of MMS, then incubated with or without 5 mM adenine for 24 h. AP sites in extracted DNA were quantified by ARP assay. The amounts of AP sites immediately after the MMS treatment and after 24 h of the repair incubation without or with adenine are shown in FIG. 4. In the absence of adenine, the levels of AP sites at 24 h were reduced to about 50 % of those observed immediately after the MMS treatment. The addition of adenine induced moderate accumulation of AP sites. The levels of the accumulated AP sites were at most 2-fold higher than those observed without adenine. These results were rather unexpected since AP sites derived from persisting 7mG by the MPG action should have been accumulated when the activity of cellular AP endonucleases was inhibited by adenine. As mentioned in the previous section, only 10 % of the methylated purines were converted to AP sites immediately after the MMS treatment. Thus, the remaining 90 % of the initially produced methylprurines should be subjected to repair by MPG during the repair incubation, thereby significantly increasing the number of AP sites over the initial level. The moderate effects of adenine on the accumulation of AP sites can be explained if added adenine or its metabolites affect not only the activity of cellular AP endonucleases responsible for the conversion of AP sites but also that of MPG responsible for the formation of AP sites. Thus the effects of adenine and its metabolites on the activity of MPG were investigated *in vitro* (see below).

Effect of adenine and its metabolites on MPG activity *in vitro*.

To test whether or not adenine-related compounds inhibited the MPG activity, human MPG was partially purified and incubated *in vitro* with MMS- and chloroacetaldehyde (CAA)-treated calf thymus DNA in the presence and absence of 1 mM adenine metabolites (FIG. 5).

The activity of MPG was determined by ARP assay. While adenine showed no appreciable effect on the MPG activity to remove methylated purines, its metabolites, NAD^+ and ADPR, suppressed the activity significantly (FIG. 5A). NAD^+ and ADPR also inhibited the activity on εA in CAA-treated calf thymus DNA. It has been shown that both NAD^+ and ADPR are potent inhibitor of human AP endonucleases; NAD^+ abolishes the endonuclease activity at 1 mM and ADPR inhibits it more than 80% at 0.1 mM[19]. In addition, adenine, hypoxanthine, adenosine and AMP have been also shown to exert the inhibitory effects. As the observed inhibitory effects of NAD^+ and ADPR on MPG are moderate even at 1 mM, it

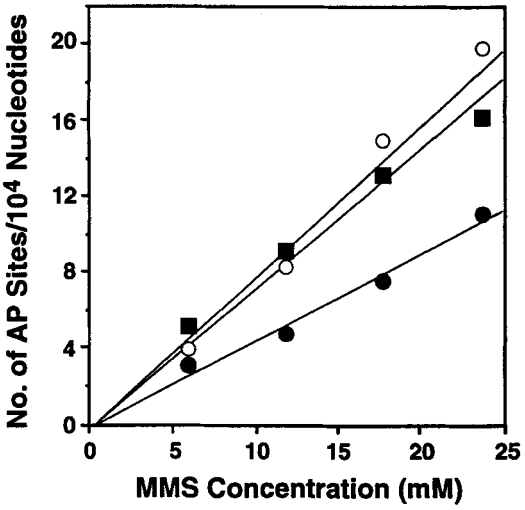

FIG. 4 Effects of adenine on the repair of AP sites in MMS-treated RC355 cells. Log-phase RC355 cells were treated with various concentrations of MMS in PBS at 37 °C for 1 h. Immediately after the treatment, the cells were washed and incubated in a fresh medium for 24 h in presence and absence of adenine. DNA was extracted immediately after the MMS treatment (○)or after the repair incubation with (■)or without (●)5 mM adenine. The number of AP sites in DNA was determined by ARP assay using protamine plates.

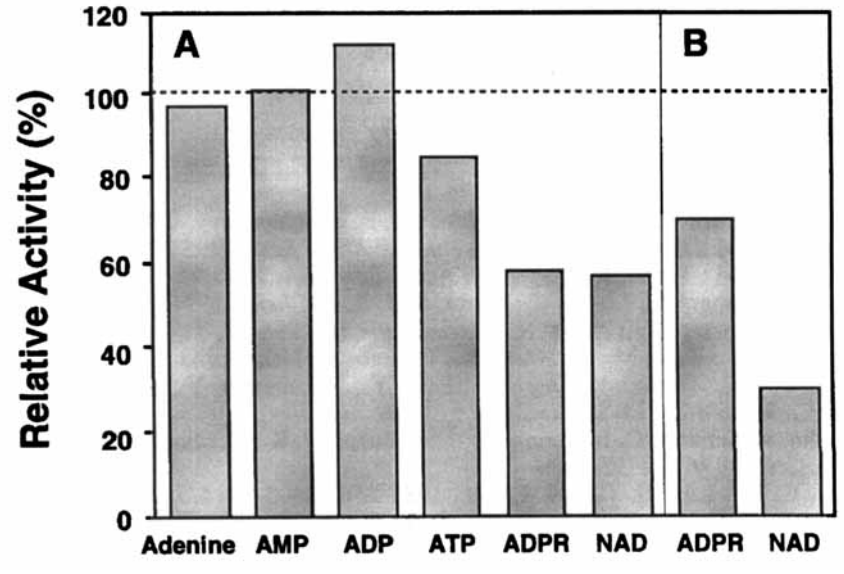

FIG. 5 Effects of adenine metabolites on MPG activity. Partially purified human MPG was incubated with calf thymus DNA containing methylpurines (A) or ethenoadenine (B) in the presence or absence of the adenine derivatives (1 mM). The number of AP sites in DNA resulting from removal of the alkylated purines by MPG was determined by ARP assay. The MPG activities were standardized relative to that without adenine derivatives.

is possible that removal of the methylated purines in cells is not affected so severely by the adenine-treatment as compared with the AP endonuclease activity. Presumably, the intimate balance of these inhibitory effects on MPG and AP endonucleases resulted in the moderate increase in the AP sites in the adenine-treated cells as compared with non-treated cells (FIG. 4). The present results also suggest that common inhibitors of the enzymes acting in the base excision repair process may be useful for understanding the questions as to how DNA lesions are processed in cells.

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