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Fluorescence Postlabeling Assay of DNA Damage Induced by *N*-Methyl-*N*-nitrosourea[1]

Rama Jain and Minoti Sharma[2]

Department of Biophysics, Roswell Park Cancer Institute, Buffalo, New York 14263

ABSTRACT

We have used a new technique to assay DNA adduct by combining enzymatic digestion of DNA to nucleotides and fluorescence postlabeling. The assay relies on the selectivity of nucleotide chromatography by high-performance liquid chromatography and the sensitivity of fluorescence detection. This report describes the fluorescence postlabeling assay of N^7-(N7-methyldGuo) and O^6-methyl-2′-deoxyguanosine (O^6-methyldGuo) in calf thymus DNA exposed to *N*-methyl-*N*-nitrosourea. Using a conventional fluorescence detector, fluorescence postlabeling assay detected 1 modified nucleotide/10^6 normal nucleotides in 100 μg DNA. Laser induced fluorescence detection offers a linear response of the chromatographic signal from 10^{-13}-10^{-16} mol fluorescent nucleotides (correlation coefficient, 0.998) improving the detection limit of the assay to 1 modified nucleotide/10^8 normal nucleotides in 10 μg DNA. Fluorescence postlabeling analysis of the authentic markers shows that both N7- and O^6-methyldGuo can be assayed with similar detection sensitivity. However, the inherent nature of the instability of N7-methyldGuo, even under physiological conditions, makes its quantitation difficult by any postlabeling technique. O^6-MethyldGuo, on the other hand, can be detected with sufficient selectivity and sensitivity by a fluorescence postlabeling assay. Quantitative efficiency of enzymatic excision and chemical postlabeling of O6-methyldGuo were validated by fluorescence postlabeling analysis of synthetic model DNA. Fluorescence postlabeling assay complements ^{32}P-postlabeling assay for O^6-methyldGuo without requiring the handling and disposal of radiolabel. Fluorescence postlabeling assay has potential, therefore, to monitor the level of O^6-alkyldGuo in human DNA exposed to both environmental and chemotherapeutic alkylating agents.

INTRODUCTION

Chemical agents like MNU,[3] dimethyl sulfate, and methylmethane sulfonate are thought to exert their carcinogenic or mutagenic effects by methylating the nucleic acids in the target tissues (1, 2). As a result, a broad spectrum of methylated adducts are formed in DNA. The presence of these alkylated adducts, in particular O^6-methyl- and N^7-methylguanines, appears to be related to DNA damage which ultimately can lead to tumor formation (3). 7-Methylguanine has been reported to occur in higher level than O^6-methylguanine (4). N7-MethyldGuo may serve as a surrogate for the promutagenic marker O^6-methyldGuo (5). O^6-MethyldGuo is considered to play a role in mutagenic effects of methylating agents (6). Methylation of O^6-guanine is also an important step in the cytotoxic effects of different antineoplastic alkylating agents such as methyltriazines and nitrosoureas (7, 8).

Numerous methods have been devoted to examine the distribution of alkylated deoxyguanosine adducts in general (9) and to assay N7-methyldGuo (4, 10–12) and O^6-methyldGuo (11, 13, 14) in particular. The combined HPLC (15, 16) or immunoaffinity purification (17) with ^{32}P-postlabeling assay, initially developed by Randerath *et al.* (18), have been the most sensitive assay reported thus far to detect the methylated dGuo using small (100 μg) DNA samples. However, all the ultrasensitive methods reported, thus far, involve the use of radioactive labeling. We explored the application of fluorescence postlabeling assay to detect these methylated DNA adducts. Fluorescence postlabeling assay advocates a novel approach combining the basic idea of postlabeling the enzymatically digested DNA with a fluorophore and fluorescence detection (19). This report describes fluorescence postlabeling assay of N^7-methyl-dGMP and O^6-methyl-dGMP in calf thymus DNA exposed to MNU.

MATERIALS AND METHODS

Chemicals. The standard deoxynucleotides, 1-methylimidazole, 1-(3,3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 5-dimethylaminonaphthalene 1-sulfonyl chloride (dansyl chloride), nitrosomethylurea, and calf thymus DNA were purchased from Sigma. 1,8-Diazabicyclo[5.4.0]undec-7-ene, ethylenediamine, triisopropylbenzenesulfonyl chloride, anhydrous methylamine, anhydrous pyridine, and 2-cyanoethyl phosphate (barium salt dihydrate) were obtained from Aldrich Chemical Company. Dimethyl sulfate was bought from Fluka. Protected mononucleotide phosphodiester and protected mononucleoside were purchased from Gallard-Schesinger Biochemicals.

Enzymes. Bovine pancrease DNase 1, micrococcal nuclease, and nuclease P_1 were obtained from Sigma. *Crotalus atrox* phosphodiesterase 1 and calf spleen phosphodiesterase were purchased from Pharmacia and Boehringer Mannheim, respectively.

Preparation of 3′- and 5′-Monophosphates of N^7-Methyl-2′-deoxyguanosine. To obtain 7-methylated guanine derivatives, 2′-deoxyguanosine 3′- and 5′-monophosphates (10 mg/ml each) were reacted separately with dimethyl sulfate (100 mM) in 0.5 M sodium phosphate buffer, pH 6.5, at 24°C for 1 h following a reported procedure (20). The methylated derivatives were purified by C_{18} reversed phase HPLC (5 μm, 10 mm x 25 cm) under isocratic conditions with 0.1 M ammonium acetate, and the isolated products were desalted on the same system using a 30-min linear gradient of 0–50% methanol in water. The identity of N^7-methyldeoxyguanosine monophosphates was established by depurination to N^7-methylguanine which coeluted in HPLC with authentic marker.

Preparation of 5′-Monophosphate of O^6-Methyl-2′-deoxyguanosine. The scheme for the preparation of O^6-methyl-dGMP is shown in Fig. 1. Completely protected derivative O^6-methyldGuo (II) was prepared from completely protected dGuo (I) by a procedure developed by Jones *et al.* (21); facile sulfonylation of position 6 of guanine by triisopropylbenzenesulfonyl chloride, followed by its displacement with anhydrous triethylamine, generated an unstable 6-trimethylamino compound which, in turn, was converted to the O^6-methyl derivative (II) with a quantitative yield. After detritylation by 10% benzenesulfonic acid, the 5′-hydroxy derivative (III) was purified by column chromatography in silica using 0–3% methanol in dichloromethane. A portion of the purified product (III) was phosphorylated with β-cyanoethyl phosphate to IV while the rest was converted to the dinucleoside monophosphate d(CpO^6-mGuo) by phosphotriester approach in solution phase by procedures described previously (19). The products IV and V were isolated by C_{18} reversed phase HPLC (5 μm, 10 mm x 25 cm) using a linear gradient of 0–20% acetonitrile in 0.1 M ammonium acetate. The isolated products were desalted on the same system using a linear gradient of 0–100% methanol in water. Bacterial alkaline phosphatase treatment of IV provided a product chromatographically identical with O^6-methyldGuo standard. The products IV and V were also characterized by NMR.

^{1}H NMR (D_2O) measurements were made on a Bruker WP200 spectrometer.

The chemical shift values (∂) of the dinucleoside monophosphate (V) in ppm with reference to sodium 3-trimethylsilylpropionate-2,2,3,3,-d_4 were 1.68–1.72 (m, 1H, CH-2″,), 2.29–2.30 (m, 1H, CH-2″), 2.52–2.62 (m, 1H, GH-2^{11}),

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: MNU, *N*-methyl-*N*-nitrosourea; dGuo, deoxyguanosine; N7-methyldGuo, N^7-methyl-2′-deoxyguanosine; O^6-methyldGuo, O^6-methyl-2′-deoxyguanosine; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; s, singlet; d, doublet; t, triplet; m, multiplet.

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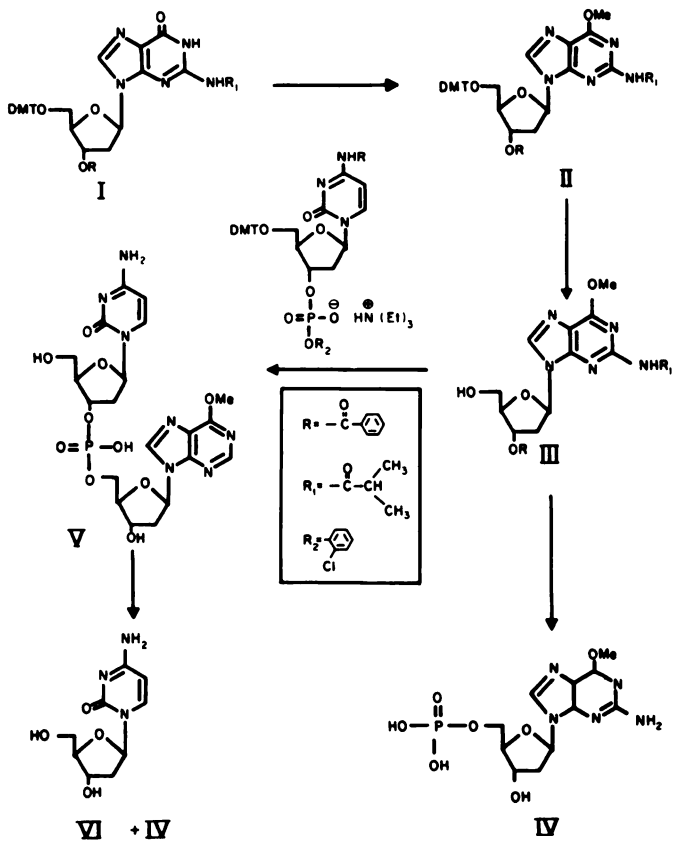

Fig. 1. Scheme for the chemical synthesis of the 5′-monophosphate derivative of O⁶-methyldGuo and the dinucleoside monophosphate d(CpO⁶-mG).

GH-2[11]), 2.81–2.94 (m, 1H, GH-2′), 3.62–3.65 (m, 2H, CH-5′, 5), 4.04 (s, 3H, O-CH_3), 4.07 (m, 3H, CH-4′, GH-5′, 5), 4.2 (m, 1H, GH-4′), 4.6 (s, 1H, CH-3′), 6.05–6.09 (d, 1H, CH-5), 6.25 (t, 1H, CH-1′), 6.30 (t, 1H, GH-1′), 7.72–7.76 (d, 1H, CH-6), 8.15 (s, 1H, GH-8). The identity of the dinucleoside monophosphate was also determined by nuclease P_1 digestion (see Fig. 1). Cochromatography of the digest with standard deoxycytidine (VI) and 5′-monophosphate of O⁶-methyldGuo (IV) further confirmed the structure of V.

Methylation of DNA. Calf thymus DNA (1 mg) was treated with nitrosomethylurea (2.5 mg) in 66 mM Tris buffer (pH 8) for 1 h at 37°C and precipitated with 2 volumes of ethanol and NaCl (1/50, v/v) following the reported procedure (4).

HPLC Separation of Normal and Methylated Nucleotides. HPLC was performed using a Beckman Altex pump and a variable wavelength detector with an Altex spectrophotometer flow cell. Nucleotides were separated on a Radial-Pak LC cartridge 8MBC$_{18}$ (10 μm, 8 mm x 10 cm) using a 30-min linear gradient of 0–20% acetonitrile in 0.01 M ammonium acetate, pH 6. The profiles were detected at 254 nm. The postlabeled nucleotides were analyzed on a Microsorb C_{18} column (5 μm, 4.6 mm x 25 cm) using a Shimadzu fluorescence detector with excitation and emission wavelengths set at 340 and 520 nm, respectively. The elution was performed under isocratic conditions in 20% acetonitrile in 0.1 M ammonium acetate for N7-methyldGuo. A 30-min gradient of 20–100% acetonitrile in 0.1 M ammonium acetate was used to assay O⁶-methyldGuo.

Fluorescence Postlabeling Assay of DNA Adducts. DNA (50–100 μg) was digested to nucleoside 3′- or 5′- monophosphates by published procedures (18, 19, 22). The entire digestion mixture was fractionated by HPLC under the conditions used to separate the standard, normal, and modified nucleotides (Fig. 2). Fractions corresponding to the retention times of the methylated nucleotides were collected, prior to labeling, to enrich the modified nucleotides from the normal nucleotides. The collected fractions were then lyophilized and labeled with dansyl chloride following procedures described previously (19). Typically the lyophilized residue was treated with 10 μl distilled deionized water, 20 μl ethylenediamine cocktail, pH 6, and reacted for 6 h at room temperature. The pH of the mixture was adjusted to 9.5 by adding 30 μl sodium carbonate-bicarbonate buffer (1 M, pH 10) before reacting with 25 μl dansyl chloride (1 g/10 ml acetone; Pierce Hypovial). The reaction mixture was stirred in the dark for 1 h at room temperature and filtered on a centrifuge (2000 × *g*) using an Ultrafree-MC unit with a Durapore membrane, 0.22 μm. The filtrate was stored at −22°C until ready for HPLC analysis.

RESULTS

Known mixtures of standard and adducted nucleotides were separated by HPLC. Fig. 2*a* shows the resolution of 3′-monophosphates of standard deoxynucleoside and N7-methyldGuo. Fig. 2*b* shows the resolution of 5′-monophosphates of standard deoxynucleoside and O⁶-methyldGuo.

The extent of digestion of methylated DNA was determined by HPLC fractionation of the digest. The digestion efficiency, calculated from the integrated area of excised dAmp peak and the response factor of the standard dAMP, was found to be about 90% of control DNA. The digestion of control DNA was quantitative by both 3′- and 5′-digestive enzymes. The labeling efficiency of the methylated nucleotides was determined, as described previously (19), by HPLC analysis of the reaction mixture both before and after labeling. The labeling yield was quantitative for both the modified and the normal nucleotides.

Fig. 3 shows the fluorescence postlabeling assay of 5′-monophosphate of O⁶-methyldGuo. Fig. 3, *b* and *c*, represents analyses of MNU treated calf thymus DNA and DNA model d(CpO⁶-mG), respectively. Both the profiles detected a peak corresponding to the retention time of the labeled O⁶-methyldGuo 5′-monophosphate peak at 18 min. The

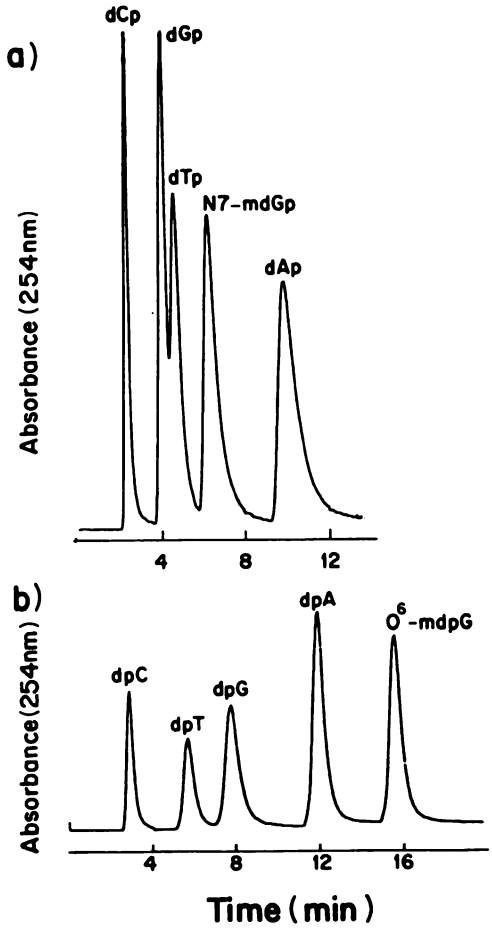

Fig. 2. HPLC elutions profiles of methyl-dGMP and standard nucleotides detected by absorbance at 254 nm. *a*, 3′-monophosphate of N7-methyldGuo and standard deoxynucleosides; *b*, 5′-monophosphate of O⁶-methyldGuo and standard deoxynucleosides.

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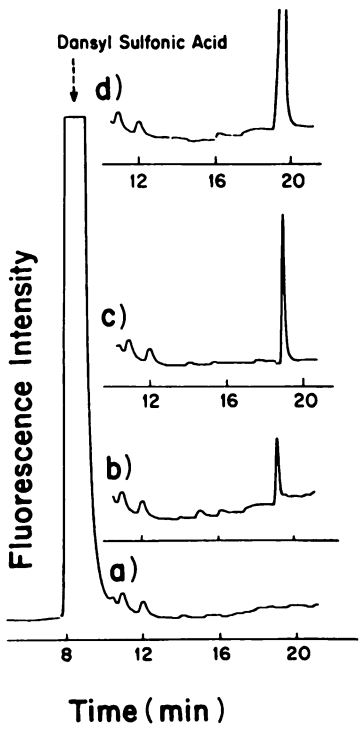

Fig. 3. Fluorescence postlabeling assay of 5′-monophosphate O⁶-methyldGuo in (*a*) calf thymus DNA, (*b*) MNU treated calf thymus DNA, (*c*) dinucleoside monophosphate d(CpO⁶-mG), and (*d*) MNU treated calf thymus DNA coeluted with O⁶-mdpG.

identity of the peak was confirmed further by cochromatography of the postlabeled DNA digest with dansylated O^6-methyl-dGMP (Fig. 3*d*). Fluorescence postlabeling assay of control DNA did not detect such a peak (Fig. 3*a*). The large peak at 10 min shown in Fig. 3*a*, also present in all the profiles, is due to dansyl sulfonic acid generated from hydrolysis of excess dansyl chloride.

Fig. 4 shows fluorescence postlabeling assay of 3′-monophosphate of N7-methyldGuo. MNU-treated DNA detected two peaks at 16 and 22 min (Fig. 4*b*). The peak at 16 min was identified as 3′-monophosphate N7-methyldGuo by cochromatography with the dansylated, authentic marker. The peak at 22 min, also found in the control DNA in a lesser extent, was not identified. The retention time of the dansylated 3′-monophosphate of ring-opened 7-methyldGuo did not correspond to the peak at 22 min seen in the profile 4b (results not shown).

The profiles shown in Fig. 3*b* and Fig. 4*b* are from analysis of 3.8 and 5 μg of methylated DNA, respectively.

Fig. 5 shows the typical response of the chromatographic signal (integrated peak area) detected by a laser induced fluorescence detector as a function of a dansylated nucleotide. The relationship is linear from 10^{-13} to 10^{-16} mol (correlation coefficient, 0.998, each point being the average of three independent determinations). Using a conventional detector, the relationship was linear from 10^{-10} to 10^{-13} mol (23).

DISCUSSION

Fluorescence postlabeling assay combines the selectivity of nucleotide chromatography by HPLC with the sensitivity of fluorescence detection.

Fig. 2 shows that both the methylated nucleotides could be resolved from the corresponding normal nucleotides by HPLC. The 5′-monophosphate of N7-methyldGuo did not separate well from the standard 5′-mononucleotides under the elution condition used to obtain the profiles shown in Fig. 2 (results not shown). The eluent used was a weak buffer (0.01 M ammonium acetate, pH 6) so that the isolated nucleotides can be concentrated and salt removed by lyophilization. The 3′-monophosphate of O⁶-methyldGuo, on the other hand, resolves as well as the 5′-monophosphate of O⁶-methyldGuo by HPLC from the corresponding standard nucleotides (16). As a result, both N^7-methyl and O^6-methyl derivatives of 3′-dGMP can be enriched from the normal nucleotides in a single run, in less than 20 min, by HPLC fractionation of a 3′-digest of methylated DNA. In immunoaffinity/^{32}P-postlabeling technique, 30 min incubation were required for maximum binding of the antigen (O⁶-methyldGuo) with the affinity gel which was followed by drainage and washing with 10 ml each of water, buffer, and eluent. The scheme adopted in the combined HPLC, ^{32}P-postlabeling, and immunoprecipitation method (16) is laborious. In addition to HPLC fractionation of digested DNA before labeling, the scheme also requires HPLC fractionation of postlabeled nucleotides from [^{32}P]ATP prior to their detection by immunoprecipitation using monoclonal antibody. Besides, such a method also involves, for specificity purpose, proper screening of appropriate monoclonal antibody for each adduct of different chemical structure.

Using a conventional fluorescence detector, the detection limit (10^{-13} mol) allows the estimation of 1 methylated nucleotide/10^6 dGp from 100 μg DNA. Combined HPLC/^{32}P-postlabeling has reported the detection of 1 methylated nucleotide/10^7 dGuo in 100 μg DNA (4,

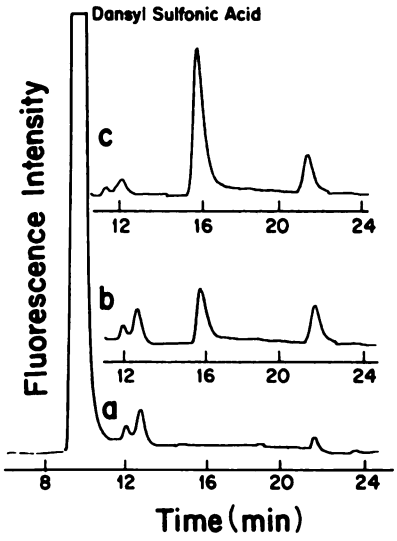

Fig. 4. Fluorescence postlabeling of 3′-monophosphate N7-mdGuo in (*a*) calf thymus DNA, (*b*) MNU treated calf thymus DNA, and (*c*) MNU treated calf thymus DNA coeluted with N7-mdGp.

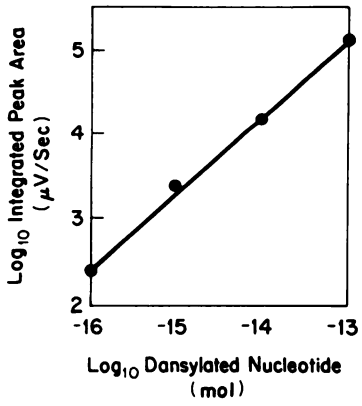

Fig. 5. A typical relationship of chromatographic signal and amount of fluorescence nucleotide by HPLC using laser induced fluorescence detection (correlation coefficient, 0.998).

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15). Cooper *et al.*, by combining immunoaffinity column with ^{32}P-postlabeling, reported that with their method when applied to human DNA samples, O⁶-methyldGuo can easily be detected at a level of three or four modifications in 10^7 normal nucleotides. Assuming a detection limit of 50 dpm above background, it was estimated that the assay should be able to detect as little as one modified guanine in 10^8 normal guanines in 100 μg DNA (17). Recently, another group from Germany has reported the same detection limit of O⁶-methyldGuo, using a 100-μg size DNA sample, by a combination of HPLC prefractionation, ^{32}P-postlabeling, and immunoprecipitation (16). As shown in Fig. 5, a laser induced fluorescence detector enhances the detection limit of fluorescence postlabeling assay by three orders of magnitude ($10^{-13} \rightarrow 10^{-16}$ mol). The laser induced detector was developed by the cell design of Yeung *et al.* (24). Using the same argument, such a detection limit should easily allow the detection of 1 methylated nucleotide/10^8 dGp in a 10-μg DNA sample. Thus, this newly developed technique has detection sensitivity comparable to that of radiolabeling assay for DNA modification.

The N7-methyldGuo adduct can spontaneously depurinate or undergo ring fission due to an unstable quaternary structure (10, 25). Ring opened N7-methylguanosine nucleotide has been reported to be resistant to nuclease P_1 and good substrate for polynucleotide kinase (20). Such an observation suggests that perhaps treatment of methylated DNA with 70% ethanol containing 50 mM NaOH for 10 min at room temperature for complete conversion of N7-methyldGp to ring opened product would be more reliable for quantitative analysis of N7-methyldGp. However, C_{13} NMR analysis shows that imidazole ring opening of N7-methyldGp takes place at physiological pH and that the ring opened product is very mobile. The molecule exists in different conformations depending on the solvent and in water is capable of existing in different kinds of aggregates (26).

Fluorescence postlabeling assay of O⁶-methyldGp does not suffer from any background interference as shown in the profile from control DNA in Fig. 3*a*. The technique was validated by quantitative excision of O⁶-methyldGp from the dinucleotide monophosphate d(CpO⁶-mG) by nuclease P_1 digestion and fluorescence postlabeling analysis of the digest by HPLC (Fig. 3*c*). The same overall procedures were used to assay MNU-treated DNA. Due to the inherent nature of instability, reliable quantitation of N7-methyldGp seems to be difficult by any postlabeling technique. On the other hand, the above result shows that O⁶-methyldGp can be assayed with sufficient specificity and sensitivity by fluorescence postlabeling assay.

O⁶-methyldGp was detected at higher level in normal esophageal mucosa isolated from individual in Linxin, China, with a high risk for esophageal cancer as compared to individuals from European countries with lower incidence of esophageal cancer (27). *O*⁶-Alkylguanines are an important class of DNA lesions produced by mutagenic monofunctional alkylating agents, as well as by bifunctional chloroethylnitrosoureas used as cancer chemotherapeutic agents (28, 29). Fluorescence postlabeling assay complements the ultasensitive assays for O⁶-methyldGp (15–17) and yet has a distinct advantage over the reported procedures because the assay does not involve the use of radioactive label. Therefore, fluorescence postlabeling assay for *O*⁶-alkyldeoxyguanine has potential not only for biomonitoring human exposure to environmental alkylating agents but also to correlate the therapeutic index with the efficacy of those drugs that induce *O*⁶-alkylguanine lesions. Such a correlation study would help to design improved treatment protocol for the management of neoplastic disease.

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