Detection of DNA Alkylphosphotriesters by $^{32}$P Postlabeling: Evidence for the Nonrandom Manifestation of Phosphotriester Lesions in Vivo

Yves Guichard, George D. D. Jones, and Peter B. Farmer

Medical Research Council Toxicology Unit, Centre for Mechanisms of Human Toxicity [Y. G., P. B. F.] and Department of Chemistry [G. D. D. J.], University of Leicester, Leicester LE1 9HN, United Kingdom

ABSTRACT

Many genotoxic carcinogens react with the sugar-phosphate backbone in DNA to form phosphotriester (PTE) adducts. These lesions are relatively abundant and persistent for some alkylating carcinogens and may therefore serve as useful biomarkers with which to assess genotoxic exposure and potential mutagenic risk. In the present study, we have developed a $^{32}$P postlabeling method that permits analysis of total methyl and/or ethyl PTE in DNA at the femtomole level. The technique is based on the inability of all known nucleolytic enzymes to cleave the internucleotide PTE bond. Consequently, complete digestion of alkylated DNA with these nucleases in the presence of an alkaline phosphatase yields PTE-dinucleoside phosphates. These species are then converted to the corresponding dinucleoside phosphates ($^{32}$pdNPs) by treatment with alkali to permit subsequent $^{32}$P labeling. The resulting labeled dinucleotides ($^{32}$pdNPs) are then analyzed by PAGE. Validation of this method has been carried out using a polydeoxythymidylic acid oligonucleotide containing a site-specific methyl PTE. The method has been applied to the in vitro analysis of calf thymus (CT) DNA treated with dimethylsulfate (DMS) or diethylsulfate (DES) and to the analysis of liver DNA from mice treated in vivo with nitrosodiethylamine. In each case, autoradiograms of the polyacrylamide gels showed the anticipated five bands representing the sixteen labeled dinucleotides, with proportional increases observed as the concentrations of DMS or DES used in the in vitro treatment of CT DNA were increased. The identity and frequency of the nucleosides located 5’ to the PTE lesions were obtained by nuclease P1 digestion of the gel-isolated $^{32}$pdNPs and by analysis of the released labeled mononucleotides, $^{32}$pdN, by high-performance liquid chromatography with radioactivity detection. Results obtained from CT DNA treated with DMS or DES showed that the frequency of the four detected nucleotides reflected the normal nucleoside content of CT DNA, indicating the random formation of methyl and ethyl PTE adducts in the in vitro modified DNA. However, studies using liver DNA from three strains of mice treated in vivo with nitrosodiethylamine indicated that the frequency of the thymidine and the 2′-deoxyguanosine 5′ to the ethyl PTE was significantly different from the corresponding normal nucleoside content. These results are indicative of (a) the nonrandom formation of ethyl PTE in vivo and/or (b) base sequence-specific ethyl PTE repair.

INTRODUCTION

An initial key stage in genotoxic chemical carcinogenesis is the interaction of the chemical carcinogen with DNA. Consequently, numerous studies have been undertaken to characterize the products of the reactions of chemical carcinogens with DNA and to elucidate their biological significance (1). Because the carcinogenic and mutagenic effects of alkylating agents are generally considered to be the result of DNA base alkylation (2), the majority of studies of the interaction of chemical carcinogens with DNA have focused on the formation of adducts with the DNA bases. However, in addition to reacting with DNA base moieties, certain carcinogens react with the oxygen of the internucleotide phosphodiester linkages to form PTE3 adducts (3). A wide range of alkylating carcinogens can form PTE adducts in DNA, including dialkylsulfates, alkyl methanesulfonates, and N-alkylnitrosoureas (4). In addition, cyclophosphamide and cyanoethylene oxide have also been shown to react with the sugar-phosphate backbone in DNA (5, 6).

The role of PTE adducts in carcinogenesis is unknown. Mutation resulting from PTE formation has not been fully studied, but such adducts may influence cellular function by altering the binding of proteins to DNA (7). For certain alkylating carcinogens, the resulting PTE adducts are chemically stable in DNA, and their relative abundance has been reported in various studies (8, 9). The proportion of total alkylation products manifest as PTE adducts in salmon sperm DNA treated with ethyl nitrosourea is 55.3%, whereas for the less carcinogenic agent ethylmethanesulfonate, PTE adducts constitute 12.0% of total alkylation products (9). Furthermore, the extent of PTE formation tends to parallel that of other oxygen alkylation products such as on the O6-alkylated adduct of guanine or the O4-alkylated adduct of thymine, which are known to be highly mutagenic. O6-Ethylguanine represents 9% of DNA alkylation products for ethyl nitrosourea and 2% of DNA alkylation products for ethylnitrosourea (9). Thus, PTEs might serve as valid markers for known mutagenic lesions in DNA.

In Escherichia coli, methyl PTE can be removed by the native form of the O6-methylguanine-DNA methyltransferase (Ada protein; Ref. 10). This repair mechanism is inducible (11) and specific for the S-isomer of methyl PTE (10, 12, 13). A related mechanism has also been reported in the eukaryote Aspergillus nidulans (14), but a mechanism for repair of alkyl PTE in higher eukaryotic systems has not yet been demonstrated. Studies using rodents (15–17) or human fibroblast cell lines (18) exposed to alkylating agents have demonstrated the long biological half-lives of methyl PTE and ethyl PTE, which exceed those of other known DNA alkylation products. Thus, PTE lesions may serve as ideal biomarkers with which to assess cumulative genotoxic exposure.

One of the reasons that so little attention has been paid to the detection of carcinogen-induced PTEs in DNA has been the lack of suitable analytical methods. Consequently, the extent of PTE formation by most carcinogens is largely unknown. Early efforts to quantify total PTE in DNA involved analysis of the alkali-induced strand break at the sites of PTE lesions (3, 19, 20), but this is a relatively nonspecific approach presumably confounded by the presence of other alkali-labile lesions in the DNA. Recently, a different approach exploiting the transalkylation of PTE to a stronger nucleophile has been reported in attempts to identify the PTE-forming species (21), but this method has not been applied to in vivo studies.

Received 5/24/99; accepted 1/4/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a Medical Research Council Realizing Our Potential Awards grant (to P. B. F.).
2 To whom requests for reprints should be addressed, at Medical Research Council Toxicology Unit, Hodgkin Building, University of Leicester, P. O. Box 138, Lancaster Road, Leicester LE1 9HN, United Kingdom. Phone: 44-116-252-5619; Fax: 44-116-252-5616; E-mail: pb11@le.ac.uk.

3 The abbreviations used are: PTE, phosphotriester; dNpN, 2′-deoxynucleoside 3′-phosphate-2′-deoxynucleosides (dinucleoside phosphates); dNpP(R), 2′-deoxynucleoside 3′-alkylphosphate; dTP(Me)2Te, 2′-thymidine 3′(methylphospho)2′-thymidine; CT, calf thymus; DMS, dimethylsulfate; DES, diethylsulfate; NDEA, nitrosodimethylamine; T4 PNK, T4 polyuridylic kinase; SVDPD, snake venom phosphodiesterase; NPI, nuclease P1; HPLC, high-performance liquid chromatography; CMHT, Centre for Mechanisms of Human Toxicity, poly(dT)$_{16}$, polydeoxythymidylic acid oligonucleotide; DNA-P, DNA-phosphates.
with DMS or DES and to the analysis of liver DNA from mice treated in vivo with NDEA.

MATERIALS AND METHODS

Chemicals. DES, CT DNA (type 1), 2'-deoxynucleosides, and 2'-deoxyxynucleoside 5'-monophosphates were purchased from Sigma Chemical Co. Ltd. (Poole, United Kingdom). DMS was from Fisher Scientific Ltd. (Loughborough, United Kingdom). Poly(dT)16 was purchased from Pharmacia Biotech (St. Albans, United Kingdom). [γ-32P]ATP (Redivue; >3000 Ci/mmol) was from Amersham International (Little Chalfont, United Kingdom). All other reagents and solvents of ultra pure grade were purchased from either National Diagnostics (Hull, United Kingdom) or Life Technologies, Inc. (Paisley, United Kingdom). The dNpdNs, dTp(Me)dT, and methyl PTE-containing oligonucleotide [(dT)3dTp(Me)(dT)7] were prepared by the Protein and Nucleic Acid Chemistry Laboratory (CMHT, University of Leicester, Leicester, United Kingdom). Liver DNA from SWR, BALB/c, and C57BL/6J mice treated in vivo with a single i.p. dose of NDEA (90 mg/kg) was provided by Drs. R. Singh and V. Oreto (Medical Research Council Toxicology Unit).

Enzymes and Buffers. DNase I (type II from bovine pancreas) and SVPD (type IV from Crostalus atrax) were purchased from Sigma Chemical Co. Ltd. NP1 was purchased from Pharmacia Biotech. Shrimp alkaline phosphatase, T4 PK, and kinase buffer were purchased from Amersham International.

Alkylation of CT DNA. CT DNA (1 mg/ml, ~3 mm DNA-P) was methylated or ethylated by treatment with DMS or DES, respectively, (1–10 μM) in sodium phosphate buffer (0.5 M, pH 6.0) at room temperature for 4 h (8) and then precipitated by the addition of 0.1 volume of sodium acetate (3 M) and 0.8 volume of 2-propanol. The DNA pellet was washed twice with 70% ethanol, evaporated to dryness using a DNA centrifugal evaporator (DNA 110; Savant, Holbrook, United Kingdom), and the residue was dissolved in an appropriate volume of water (10–50 μl) in 30 μl in 32P end labeling by

Digestion. DNA, the methyl PTE-containing oligonucleotide, and the methyl PTE dinucleoside phosphate were all "digested" using a modification of the procedures described by Weinfeld et al. (30) and Saris et al. (26). Samples of DNA (10 μg), methyl PTE-containing oligonucleotide (32 pmol), or methyl PTE dinucleoside phosphate (8 pmol) were incubated overnight at 37°C with DNase I (0.4 unit), SVPD (0.02 unit), NP1 (0.5 unit), and shrimp alkaline phosphatase (0.4 unit) in 30 μl of digestion buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 4 mM MgCl2, and 2 mM ZnCl2]. The enzymes were then precipitated by the addition of 3 volumes of ice-cold ethanol and removed by centrifugation at 4°C (17,000 × g, 15 min). The supernatant was evaporated, and the resulting residues were dissolved in water (100 μl), heated at 100°C for 10 min to inactivate residual nuclease and phosphatase activity, and then stored at −20°C.

Alkaline Hydrolysis of PTE. Hydrolysis with ammonia of the digested PTE-containing DNA, oligonucleotide, or dinucleoside phosphate was carried out by using the procedure described by Saris et al. (26). An equal volume of aqueous ammonia (25%) was added to an aliquot (50 μl) of digest solution, and the reaction was incubated in a tightly capped vial at 70°C for 24 h. The reaction mixture was evaporated to dryness using a DNA centrifugal evaporator, and the residue was dissolved in an appropriate volume of water (10–50 μl) and then stored at −20°C.

32P Labeling. Each phosphorylation reaction mixture (10 μl) contained the supplied kinase buffer (used 1×), 5 μl of the digested solution (untreated or treated with alkali) or the PTE-containing oligonucleotide or known amounts of the dinucleoside phosphate standards, 5 μCi of [γ-32P]ATP (~1 pmol), T4 PNK (7.5 units), and 100 fmol of poly(dT)16 when used as a labeling reference. The samples were incubated at 37°C for 1 h. When poly(dT)16 was used to consume excess [γ-32P]ATP, the reaction was incubated for an additional 30 min with 40 pmol of poly(dT)16 plus T4 PNK (4 units). At the end of the reaction, an equal volume of formamide loading buffer (90% formamide, 0.02% bromphenol blue, and 0.02% xylene cyanol in 100 mM Tris, 90 mM boric acid, and 1 mM EDTA; Ref. 31) was added, and 5 μl of the mixture were analyzed by PAGE.

PAGE of the 32P-Labeled Compounds. Samples were loaded onto a 20% polyacrylamide/7 M urea gel. The gel electrophoresis equipment used consisted of a power supply (EPS 3500 XL; Pharmacia Biotech) together with a Model S2 Sequencing Gel Electrophoresis System unit using 33.5 (width) × 41.5-cm (length) glass plates, 0.8-mm spacers, and a 32-tooth comb (Life Technologies, Inc.). Standard conditions, electrophoresis was carried out at 1100 V until

PTE-containing DNA

D N a s e I , S V P D , N P 1 , S A P

dNp(R)dN + n[dN]

Alkaline hydrolysis

dNpdN + n[dN]

1) [γ32P]ATP, T4 PNK
2) Polyacrylamide gel electrophoresis

32PdNpdN

1) NP1
2) 32P-HPLC

32PdN

Fig. 1. 32P postlabeling strategy.
the bromophenol blue marker migrated 11–12 cm (3–4 h). Radiolabeled products were visualized by autoradiography on Kodak X-OMAT K film (30 × 34 cm) or by storage PhosphorImager analysis (model 42SE; Molecular Dynamics, Sunnyvale, CA; using ImageQuant software version 3.2). The level of dNpdN (fmol) in a sample was determined as follows: (fmol of dNpdN used in the standard or mixture of standards) × (integrated volume for $^{32}$pdNpdN obtained from the samples)/ (integrated volume of $^{32}$pdNpdN obtained from the standard or mixture of standards). When poly(dT)$_{16}$ was used as a labeling reference, the obtained value was multiplied by the following correcting factor: [integrated volume for labeled poly(dT)$_{16}$ obtained from the standard(s)]/ [integrated volume of labeled poly(dT)$_{16}$ obtained from the samples].

**Analysis of the 5'-labeled Nucleotides in the $^{32}$pdNpdN Species.** The radioactive fragments of gel containing the $^{32}$pdNpdN species were excised from the gel and then soaked overnight at room temperature in 1 ml of water. After removing the water, the gel fragments were rinsed with an additional 0.5 ml of water, and the washes were combined. Before NP1 digestion, the combined washes were desalted by lowering the pH to 4 (by adding an appropriate volume of 50 mM HCl solution) and then applying the mixture to a Water-Sep-Pak C$_{18}$ light cartridge previously conditioned in a 200 nm ammonium formate buffer (pH 4). The cartridge was washed with 10 ml of the same buffer, and $^{32}$pdNpdN species eluted with 0.8 ml of 50% v/v methanol/water mixture. The obtained fraction was taken to dryness and resuspended in 50 µl of NP1 buffer (10 mM sodium acetate and 1 mM ZnCl$_2$). After adding NP1 (0.6 unit), the reaction mixture was incubated for 1 h at 37°C and subjected to HPLC with all four 5'-mononucleotides as UV markers. HPLC was performed using a Varian Star 9012 pump system (Varian United Kingdom Ltd., Surrey, United Kingdom) connected with a U-V-Vis detector (Varian Star 9050) and a radioactivity detector ($\beta$-ram; LabLogic, Sheffield, United Kingdom). The data were analyzed by Laura software (LabLogic). Absorbance of the eluate was monitored at 254 nm. Analyses were performed at room temperature using a reverse-phase C$_{18}$ column (apex, 5 µm; 3 × 100 mm; HPLC Technology Co. Ltd., Macclesfield, United Kingdom). The HPLC gradient conditions were as follows: solvent A, 50 mM ammonium acetate (pH 4.5); solvent B, methanol/water (80:20, v/v); gradient: 0 min, 0% B; 5 min, 5% B; 20 min, 20% B; and 25 min, 0% B at a flow rate of 1 ml/min.

**Results**

**Analysis of a Methyl PTE-containing Oligonucleotide.** To determine whether a PTE DNA lesion could be detected as its corresponding dNpdN species by the postlabeling protocol outlined in Fig. 1, a methyl PTE dinucleoside phosphate [dTp(Me)dT] and an oligonucleotide containing a single methyl PTE [d(T)$_3$dTp(Me)(dT)$_7$] were examined. The protocols for the $^{32}$P labeling stage and separation of the resulting labeled compounds by PAGE were adapted from protocols described by Weinfeld et al. (30, 32) for the analysis of oxidative and radiogenic DNA damage. The autoradiogram in Fig. 2 shows the labeled compounds obtained after various treatments of (dT)$_3$dTp(Me)(dT)$_7$ and dTp(Me)dT. Each sample contained a limiting amount of poly(dT)$_{16}$ as a labeling reference. In parallel, the dinucleoside phosphate dTpdT was labeled under identical conditions. Alkali hydrolysis of the enzyme-digested samples was carried out using aqueous ammonia according the protocol of Saris et al. (26). This protocol was judged appropriate because labeling of the digested and alkali-treated methyl PTE-containing oligonucleotide and the digested alkali-treated PTE-dinucleoside phosphate yielded the anticipated labeled dinucleotide ($^{32}$pdTpdT). This species was also detectable in a small amount in samples that have been labeled without the alkali treatment. This is presumably the result of some minor hydrolysis of the bonds of the phosphate esters during the heating of the digest sample to inactivate residual nuclease and phosphatase activity (see “Materials and Methods”).

**Levels of generated dTpdT species in each sample, as determined by $^{32}$P labeling and storage phosphor image analysis, were derived from comparison with a standard sample of authentic dTpdT that was labeled in parallel (see “Materials and Methods”).** After DNA digestion and alkali hydrolysis, 63% of the dTpdT(Me)dT species were detected as dTpdT species, whereas 58% of methyl PTE present in the methyl PTE-containing oligonucleotide could be detected as the corresponding dTpdT species. Consequently, the overall yield of the method was judged to be ~60%.

**$^{32}$P Labeling of All of the Possible Dinucleoside Phosphates.** The autoradiogram in Fig. 3A shows the labeled compounds obtained on $^{32}$P labeling of all possible dinucleoside phosphates expected after digestion and alkali hydrolysis of alkylated DNA. Sixteen authentic dinucleoside phosphate standards were analyzed individually and in an equimolar mixture. For these experiments, residual [$^{32}$P]ATP used in the reaction was consumed using a large excess of poly(dT)$_{16}$ (see “Materials and Methods”). Results obtained from the equimolar mixture of standards indicated that under the gel electrophoresis conditions used, the $^{32}$pdNpdN species are grouped into five prominent bands, bands 1–5. Their relative intensity, as measured by storage phosphor image analysis, was approximately 1:4:6:4:1. Bands 1 and 5 contain only the $^{32}$PdGpdG and $^{32}$PdCpdC species, respectively; bands 2, 3, and 4 consist of labeled dinucleotides containing the base sequences [GA, GT, AG, TG], [CG, GC, AT, TA, TT, AA], and [TC, CT, AC, CA], respectively. For the individual dinucleoside phosphates, the molar quantity of $^{32}$pdNpdN species in each band was determined by multiplying the fraction of the total activity of the lane found in each band by the molar quantity of [$^{32}$P]ATP used in the reaction. The results indicated a similar labeling efficiency for each dinucleoside monophosphate of 50 ± 15%.

The quantitative aspects of the method were further investigated by $^{32}$P-labeling various amounts of the 16 dNpdN standards in an equimolar mixture, using a fixed amount of poly(dT)$_{16}$ as a labeling reference. Fig. 3B shows the ratio of the 16 $^{32}$pdNpdN species labeled poly(dT)$_{16}$ observed when 0.39–100 fmol of the equimolar mixture of dNpdN standards were labeled in the presence of a limiting amount of poly(dT)$_{16}$. At levels of the dNpdN standards lower than 3.12 fmol...
DETECTION OF PHOSPHOTRIESTERS IN DNA

bands, bands 1–5. Poly(dT) 16 was used to consume excess fmol, indicating that the corresponding 32 pdNpdN species were grouped into five prominent possible dNpdN standards, individually (2.5 fmol of each) and in an equimolar mixture (40 fmol). Levels of PTE adducts in DNA samples were calculated by taking into account the amount of digested and alkali-treated DNA used for analysis and the overall yield of the method (=60%). The observed levels vary from 0.9–17.5 methyl PTE/10^4 DNA-P in the 0.1–10 mM DMS-modified samples and from

(<0.2 fmol/dNpdN), the measurements obtained were indistinguishable from those of control samples containing just water. This background appears because of compounds present in the commercial preparations of T4 PNK or [γ-32P]ATP. These contaminants, once labeled, comigrate with the 32 pdNpdN species on gel electrophoresis. From these experiments, we judged the detection limit of this assay to be ~5 fmol for the detection of the 16 dinucleoside phosphates.

Analysis of in Vitro Alkylated Modified DNA. The conditions established above were applied to the detection of PTE DNA adducts formed by reacting an alkylating agent with DNA in vitro.

In the first experiment, CT DNA was incubated with 10 mM DMS. A control sample was an equal concentration of CT DNA in the same buffer without DES. Before the labeling step, DNA digests were either nontreated or treated with alkali. Residual [γ-32P]ATP was consumed by an excess of poly(dT)\textsubscript{16}. In parallel, an equimolar mixture of dNpdN standards was labeled under identical conditions. Compared with control DNA digests, autoradiograms obtained from DES-treated DNA digests revealed the presence of five prominent bands corresponding to 32 pdNpdN species (Fig. 4). These bands were far more intense in the alkali-treated digest, indicating that the detected dinucleoside phosphates arise from PTE-dinucleoside phosphates generated by digestion of DES-treated DNA. By comparison with the mixture of standards labeled under identical conditions (see “Materials and Methods”), the level of dinucleoside phosphates was calculated as ~200 fmol. The detectable bands in the nonalkali-treated digest may be due to a partial PTE hydrolysis during sample preparation (see above). The detected activity in the alkali-treated control samples is at the detection limit of the assay.

To confirm a dose-response relationship between alkylating agent treatment and PTE-derived dinucleoside phosphates, CT DNA was incubated with various concentrations (0–10 mM) of DMS or DES in vitro. Alkali-treated digests were labeled in the presence of a limiting amount of poly(dT)\textsubscript{16} as a labeling reference. Fig. 5A shows the resulting autoradiogram of the polyacrylamide gel and demonstrates the appearance, with increasing doses of DMS and DES, of the five characteristic bands indicating the formation of methyl-PTE and ethyl-PTE, respectively. Levels of dinucleoside phosphates in each sample were determined by comparison with the mixture of standards labeled under identical conditions (see “Materials and Methods”). Fig. 5B shows a clear dose response for the detected dinucleoside phosphates as the concentration of the alkylating agent used in the in vitro treatment was increased. The levels of PTE adducts in DNA samples were calculated by taking into account the amount of digested and alkali-treated DNA used for analysis and the overall yield of the method (~60%). The observed levels vary from 0.9–17.5 methyl PTE/10^4 DNA-P in the 0.1–10 mM DMS-modified samples and from
In this study, we have shown that PTE DNA adducts induced by three different alkylating agents can be detected by a novel 32P postlabeling protocol. The assay is based on the observation that PTE lesions prevent the hydrolysis by nucleases of the internucleotide PTE bond (22, 23). Hence, enzymatic digestion of alkylated DNA with the nucleases DNase I, SVPD, and NP1, plus a phosphatase, generates PTE-dinucleoside phosphates. However, these compounds are not substrates for phosphorylation by T4 PNK (26); consequently, they have to be converted into either the corresponding dinucleotide phosphates or 3'-phosphate alkylated mononucleotides. This 32P postlabeling strategy for detection of PTE DNA adducts has been previously applied in in vitro studies (26, 27), using TLC or TLC/HPLC to separate the PTE-derived labeled species. Saris et al. (26) found that the dinucleoside phosphates represent 80–100% of the labeled compounds obtained after alkali hydrolysis and labeling. Using this strategy, we exploited PAGE to partially separate and analyze the complex mixture of labeled dinucleotides produced. This methodology is similar to that described by Weinfield et al. (30, 32) for the detection of oxidative and radiogenic DNA damages. The 32P postlabeling assay

0.4–9.2 ethyl PTE/10⁶ DNA-P in the 0.5–10 mM DES-modified samples.

Analysis of PTE DNA Adducts Induced by NDEA in Vivo. To investigate whether this 32P postlabeling method was capable of detecting PTE DNA adducts formed in vivo, we analyzed samples of liver DNA from three strains of mice that were either nontreated (control) or treated with a single i.p. dose of NDEA (90 mg/kg). Compared with the control samples, autoradiograms resulting from the NDEA-treated samples show the five alkali-induced bands, indicating the presence of ethyl PTE adducts (Fig. 6). The levels of ethyl PTE adducts in liver DNA from the three strains of treated mice were calculated as 25.3 ± 4.1 PTE/10⁶ DNA-P.

Analysis of the Nucleosides 5' to the PTE DNA Lesion. The gel resolution of the 16 32pdNpdN species was not sufficient to permit analysis of individual PTE-derived dinucleoside phosphates. However, the identity and frequency of the labeled nucleotides, 32pdN, located 5' to the PTE lesions were obtained by NP1 digestion of the gel-isolated 32pdNpdN species and analysis by reverse-phase HPLC with radioactivity detection. Typical HPLC chromatograms for CT DNA treated in vitro with 10 mM DES and for liver DNA from BALB/c mouse treated in vivo with NDEA are shown in Fig. 7. In both cases, the major labeled eluted compounds correspond to one of the expected four normal 5'-mononucleotides in DNA. The relative amounts of each radioactively labeled nucleotide were obtained from the observed area peak ratios. The variation of this assay, based on the repeated analysis of an equimolar mixture of the dinucleoside phosphate standards (which were labeled, gel-isolated, and NP1-digested), was found to be ≈10% (data not shown). Table 1 shows the frequency of the nucleosides 5' to the PTE lesions obtained from in vitro and in vivo studies compared with the normal nucleoside content as determined by HPLC analysis of the digested DNA samples (see “Materials and Methods”). Results obtained from CT DNA treated in vitro with DMS or DES show that the frequency of the four nucleosides 5' to the PTE adducts reflected the normal nucleoside content. Results obtained for liver DNA from mice treated in vivo with NDEA are different. The frequency of the thymidine 5' to the PTE adducts was significantly higher than the normal thymidine content, whereas the frequency of 2'-deoxyguanosine 5' to the PTE adducts was significantly less than the normal 2'-deoxyguanosine content.

DISCUSSION

In this study, we have shown that PTE DNA adducts induced by three different alkylating agents can be detected by a novel 32P postlabeling protocol. The assay is based on the observation that PTE lesions prevent the hydrolysis by nucleases of the internucleotide PTE bond (22, 23). Hence, enzymatic digestion of alkylated DNA with the nucleases DNase I, SVPD, and NP1, plus a phosphatase, generates PTE-dinucleoside phosphates. However, these compounds are not...
that we have developed has been validated using an oligonucleotide containing a site-specific methyl PTE and permits the simultaneous analysis of several samples. The method was shown to be suitable for the detection of PTE DNA adducts in CT DNA treated in vitro with DMS or DES and in liver DNA from mice treated in vivo with NDEA. In every case, the resulting autoradiograms clearly show the five bands representing the 16 possible dinucleotide species.

For the digestion stage, we followed the protocol described by Weinfeld et al. (30) and Saris et al. (26), which used NP1 in addition to SVPD for the digestion of DNA to nucleosides (33). The two exonuclease enzymes have different specificities. SVPD interacts with the bases 3’ to a phosphodiester linkage, and a missing base or certain modified bases at this position inhibit cleavage of the phosphodiester bond. On the other hand, NP1-mediated phosphodiester cleavage is inhibited by a missing base or certain modified bases immediately 5’ to the phosphodiester linkage (34). Results obtained in our laboratory have shown that the use of these two enzymes simultaneously for the digestion of alkylated DNA allows for the sole detection of PTE-derived dinucleoside phosphates, with other possible lesions such as alkylated bases and apurinic/apyrimidic sites being shown not to interfere (data not shown). The specificity of the method was confirmed by the fact that the band intensity of the dinucleoside phosphates corresponding to PTE adducts were strongly amplified in the alkali-treated samples. Our method, which is designed for the analysis of PTE-derived labeled dinucleotides, could be applicable to the detection of any carcinogen-induced PTE as long as the PTE-dinucleoside phosphates obtained after digestion can be hydrolyzed to yield the corresponding dinucleoside phosphates.

Quantitation of total PTE in DNA was estimated from the level of PTE-derived dinucleoside phosphates detected. At this time, the sensitivity of the method is limited by interfering compounds present in the labeling reaction. The minimum detectable amount of dNdpN species as an equimolar mixture was ≈5 fmol, allowing the detection of ≈2–3 PTE/10^6 DNA-P when 0.5 μg of DNA is used. The yield of the method was determined to be ≈60%, using a site-specific methyl PTE oligonucleotide. However, despite a similar labeling efficiency for the 16 dinucleoside phosphates, it is uncertain whether the result obtained for the detection of a single PTE lesion contained in a single homo-oligonucleotide can be extrapolated to the circumstance of alkylated DNA because of the potential for sequence-dependent differences in the yields of products on alkali hydrolysis (35). Thus, levels of PTE adducts determined in this study represent a semiquantitative approximation. However, the results obtained in vitro showed that levels of methyl PTE in DNA treated with DMS are, on average, twice that of the levels of ethyl PTE in DNA treated with DES. This is in agreement with the previous observation that methylation agents are more reactive than ethylating agents to form PTE in DNA (36). Furthermore, our values obtained from liver DNA of mice treated with NDEA in vivo are in the same range (10–30 ethyl-PTE/10^6 DNA-P) as those obtained from liver DNA of rats given NDEA in drinking water (where the total level of PTE was measured by alkali-induced strand break analysis; Ref. 3). In addition, our estimations of ethyl-PTE are, on average, twice the levels of N-7 ethylguanine and O^6 ethylguanine DNA adducts that have been determined by immunoslot-blot analysis of the same samples. The latter observation is in broad agreement with results obtained from a study of rats treated with ethylnitrosourea, where the total level of ethyl PTE [extrapolated from d(Tethyl)PdT detected by HPLC] were found to be three times higher than the level of N-7 ethylguanine in liver DNA (16). The levels of any PTE adducts in liver DNA of non-treated mice were below the detection limit of our assay; analogously, no base adducts were detected by immunoslot-blot analysis of the same samples.

An obvious drawback of this postlabeling method is that for every type of PTE generated, up to 16 labeled compounds can be produced, and these are not fully separated by PAGE. On the other hand, advantage can be taken of this situation through the use of NP1 to obtain the identity and frequency of the nucleosides located 5’ to the alkylated phosphates. The outcome of this type of analysis carried out on CT DNA modified with DMS or DES indicates that the frequency of the four nucleosides 5’ to PTE reflects the normal base content of CT DNA, suggesting that the reaction of the alkylating agents with internucleotidic phosphodiester groups within a double-strand DNA is random in vitro. However, analysis of liver DNA from three strains of mice treated in vivo with NDEA indicates that the frequency of the thymidine and the 2’-deoxyguanosine 5’ to the ethyl PTE is significantly different from the corresponding normal nucleoside content. Because DES and NDEA give the same PTE products in DNA, the difference in the results in vitro and in vivo suggests (a) the nonrandom formation of ethyl PTE in vivo; and/or (b) sequence-specific ethyl PTE repair. With regard to (a), this could arise from sequence-dependent differences in the chemical reactivity of DES and metabolized NDEA toward phosphate groups in DNA. However, with regard to (b), despite the fact that PTE DNA repair has not yet been reported in the eukaryotic system, the results obtained in vivo could suggest the intervention of a sequence-specific ethyl PTE repair mechanism. Overall, the results obtained in this study are in agree-

\[\text{Frequency of the nucleosides 5’ to the PTE lesions (%)}\]

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Frequency of the nucleosides 5’ to the PTE lesions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT DNA^a</td>
<td>da dG dC T</td>
</tr>
<tr>
<td>DMS-treated</td>
<td>28.6 22.5 24.2 24.7</td>
</tr>
<tr>
<td>DES-treated^b</td>
<td>29.6 29.2 21.7 23.4 22.6 22.5 26.1 24.9</td>
</tr>
<tr>
<td>Normal base content^c</td>
<td>28.3 ± 0.5 23.2 ± 0.8 23.9 ± 1.0 24.6 ± 1.2</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
</tr>
<tr>
<td>Liver from mice^d</td>
<td></td>
</tr>
<tr>
<td>NDEA-treated^e</td>
<td>25.6 ± 1.0 15.3 ± 1.6 23.3 ± 0.4 35.8 ± 0.7</td>
</tr>
<tr>
<td>Normal base content^f</td>
<td>27.2 ± 0.5 22.9 ± 0.4 23.6 ± 0.9 26.3 ± 1.0</td>
</tr>
</tbody>
</table>

^a CT-DNA (3 μmol DNA-P) was incubated in vitro with 10 μmol DMS or DES.
^b Data from two separate analyses.
^c Frequency of the four normal mononucleosides determined by HPLC analysis of the digested DNA samples (HPLC conditions are described in “Materials and Methods”). Values represent the mean ± SD obtained from three analyses.
^d Three strains of mice were treated in vivo with an i.p. dose of NDEA (90 mg/kg).
^e Values represent the mean ± SD obtained from the analysis of one or two animals of each strain.
^f Significantly different from the corresponding normal base content at P < 0.05 by the Student’s t test.

\[\text{Liver from mice}^d\]

\[\text{NDEA-treated}^e\]

\[\text{Normal base content}^f\]

\[\text{SD obtained from three analyses.}\]

\[\text{Data from two separate analyses.}\]
general acceptance that adduct distribution in the genome is not random (2, 37). In many cases, because of the structural organization of the genome, base-adduct formation has preferential sites of alkylation in DNA, some of which have been shown to be sequence dependent (38). Furthermore, lesion repair has been shown to be base sequence dependent, varying in extent between individual nucleotide sites (39–41), as well as dependent on the genomic localization of the lesion and the functional status of the gene (42–44).

To date, there is no record of a compound that reacts with the phosphates of nucleic acids that is not a carcinogen. This observation prompts the question as to whether the level of such lesions can be related to cancer incidence. Because our assay for PTE involves the determination of the dinucleoside phosphates derived from PTE adducts (after digestion and alkali hydrolysis), it should have a wide applicability. One of the ultimate aims is to apply the assay to the analysis of human DNA from populations exposed to suspected environmental carcinogens. Further methodological development will focus on analysis of the carcinogen-containing species that are also released during the alkali hydrolysis. Our assay should permit a study of the role of the DNA sequence in the formation and removal of PTE. This type of information will be helpful in understanding the mechanisms of PTE adduct formation and their biological significance in vivo.

ACKNOWLEDGMENTS

We thank Dr. K. Lilley (Protein And Nucleic Acid Chemistry Laboratory, CMHT, University of Leicester) for technical assistance. We also thank Dr. K. Lilley (Protein And Nucleic Acid Chemistry Laboratory, CMHT, University of Leicester) for technical assistance.

REFERENCES

16. de Jonge, L., Menkveld, G. J., Debeij, R. J., and Yates, A. D. Formation and stability of alkylation products of nucleic acids and purines (including imida-zone ring-opened 7-alkylguanosine) and alkylphosphotriesters in liver DNA of adult-rats treated with ethylhydroxamate or dimethylhydroxanne. Carcinogenesis (Lond.), 7: 393–403, 1986.
Detection of DNA Alkylphosphotriesters by $^{32}$P Postlabeling: Evidence for the Nonrandom Manifestation of Phosphotriester Lesions in Vivo

Yves Guichard, George D. D. Jones and Peter B. Farmer


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/5/1276

Cited articles
This article cites 40 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/5/1276.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/5/1276.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/60/5/1276.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.