Antibodies against 7-Methyldeoxyguanosine: Its Detection in Rat Peripheral Blood Lymphocyte DNA and Potential Applications to Molecular Epidemiology

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ABSTRACT

Polyclonal antibodies have been raised against the imidazole ring-open form of 7-methyldeoxyguanosine (7-mdGua). A combined high performance liquid chromatography/immunoassay method has been developed using these antibodies which provides a specific and sensitive way to quantitate 7-mdGua in DNA. Following enzyme hydrolysis and chromatographic purification of 7-mdGua, the adduct is quantitatively converted to the ring-open form and can be measured at levels as low as 0.05 pmol by immunoassay. With 1 mg of DNA a level below 1 adduct per 10⁷ normal deoxyxynucleosides can be measured. Using DNA modified by radiolabeled carcinogens, a good correlation between 7-mdGua levels, as measured by immunoassay or radioactivity, was obtained. In rats treated with dimethylnitrosamine (0.4 and 1.0 mg/kg), both 7-mdGua and O⁶-methyldeoxyguanosine were detected in peripheral blood lymphocyte DNA. In addition the levels of both adducts at time points up to 48 h posttreatment were very similar to those seen in liver DNA from the same animals. The measurement of 7-mdGua, quantitatively the major methylation adduct, in small cell samples such as lymphocytes has great potential in determining the exposure of humans to environmental methylating agents such as nitrosamines.

INTRODUCTION

Alkylating agents, including N-nitroso compounds, interact with DNA to yield a variety of reaction products. The predominant adduct (70 to 90%) following reaction of monofunctional methylaing agents with DNA is at the nucleophilic position 7 of guanine yielding 7-mdGua (see Ref. 1). While this adduct does not appear to be directly mutagenic (2, 3), the minor alkyl adducts O⁶-mdGua and O⁴-mThd have been shown to lead to point mutations in DNA following replication (for reviews see Refs. 1 and 4).

Human environmental exposure to N-nitroso compounds is well documented (5), while the role of these compounds in tumor induction in humans is the subject of extensive research (6). One major difficulty in establishing a causal relationship between exposure to an environmental carcinogen and development of a tumor at a specific site has been the lack of sufficiently sensitive techniques to allow low level exposure to be assessed. Recently a number of antibodies recognizing alkylating and other carcinogen DNA adducts have been developed (7, 8). We have previously developed radioimmunoassays for the minor, promutagenic alkyl adducts O⁶-mdGua and O⁴-mThd (9, 10) to facilitate measurement of human exposure at an individual level. Using this approach we detected O⁶-mdGua in esophageal and stomach mucosa DNA from populations at high risk of cancer at these two sites and in geographical areas where environmental exposure to nitrosamines is likely (11). O⁶-mdGua was present in low levels (1 adduct/10⁷ to 10⁸ normal deoxyxynucleosides), probably reflecting the efficient repair of this adduct by O⁶-alkylguanine DNA alkyltransferase in human tissues (12). Consequently these adduct levels were likely to represent a small fraction of those initially induced, in turn limiting the sensitivity of this approach.

As described above, 7-mdGua is more abundant than O⁶-mdGua upon alklylation of DNA and also may persist in cellular DNA for an extended period due to its slow repair (13). However, the quartemization of the ring nitrogen resulting from alklylation at position 7 destabilizes the glycosyl bond of 7-mdGua leading to two other possible consequences: (a) depurination, yielding apurinic/apyrimidinic sites (14), the latter of which are potentially mutagenic (15); in vitro DNA at 37°C and pH 7.0, 7-mdGua is lost relatively slowly by this chemical depurination with a half-life of about 150 h (14); (b) alkali induced cleavage of the imidazole ring yielding the ring-open form of the adduct 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine (7-mdGua irø) (16-18). Ring fission has been reported to chemically stabilize the adduct in DNA (19, 20) although the presence of this form in mammalian DNA following in vivo exposure to methylating agents is unclear (21-23). In addition to spontaneous chemical depurination, DNA glycosylases capable of relatively slow repair of 7-mdGua have been detected in in vitro cellular protein extracts from bacteria (18, 24), rat liver (25, 26), hamster liver (25), and human lymphoblasts (27).

It is possible that the more stable and abundantly formed adduct 7-mdGua will be present in higher levels than O⁶-mdGua in DNA from people exposed to environmental alkylating agents. This may increase assay sensitivity allowing measurements to be made on smaller quantities of DNA including samples from, for example, lymphocytes, oral mucosa, and biopsy material. Antibodies against 7-mdGua have been described (before see "Discussion"). We report here the development and characterization of high affinity antibodies against 7-mdGua irø and an ELISA/HPLC methodology which allows quantitation of 7-mdGua in lymphocyte and liver DNA isolated from rats exposed in vivo to DMN. This approach has great potential in determining the exposure of humans to environmental alkylating agents such as nitrosamines.

MATERIALS AND METHODS

Chemicals. Unlabeled DMN and dimethyl sulfate were from Merck-Schuchardt, Munich, Federal Republic of Germany, while [¹⁴C]DMN (8 mCi/mmol) and [³H]MNU (29 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL. Fatty acid free BSA, Limulus polyphemus hemolymph HC, 7-mdGua, and 7-methyl-2'-deoxyguanosine 5'-triphosphate were from Sigma, St. Louis, MO. Freund's adjuvant was purchased from Difco Laboratories, Detroit, MI. Alkaline phosphatase and snake venom phosphodiesterase were obtained from Worthington. Polyethylene glycol 400 was from Fluka. Methyltransferase was from Boehringer, Mannheim, Federal Republic of Germany. Oligonucleotides were from Dynatech, Plochingen, Federal Republic of Germany, and dimethyl sulfoxide and 3,3',5,5'-tetramethylbenzidine were from Ald-
DETECTION OF 7-mdGua BY HPLC/ELISA

Richard Chemical Co., Gillingham, Dorset, United Kingdom. Alkalization of DNA by \( [\text{H}]\text{JMN2} \) was as described previously (28). PBS was prepared in the laboratory as follows: 140 mM NaCl; 2.7 mM KCl; 1.5 mM KH\(_2\)PO\(_4\); 10 mM Na\(_2\)HPO\(_4\); 2H\(_2\)O.

Marker Compounds. 7-mdGua was prepared either by dimethyl sulfate methylation of 2'-deoxyguanosine or 2'-deoxyguanosine 5'-monophosphate according to the method of Lawley (29) or by enzyme hydrolysis of 7-mdGua 5'-triphosphate. Using the former method the 7-mdGua was separated on a Sephadex G-10 column eluting with water. This 7-mdGua peak was further purified on Aminex A7 and reverse phase chromatography using conditions described under “Enzyme Hydrolysis/HPLC Separation.” A sample of 7-mdGua prepared in this way was hydrolyzed with 0.1 N HCl to remove the sugar molecule. The product of this hydrolysis was shown to cochromatograph as expected with authentic 7-mG (Sigma) on UltraspHERE ODS (5 \( \mu \)m; 4.6-mm x 12.5-cm column equipped with a 4.6-mm x 4.5-cm guard column) with a flow of 1 ml/min using a methanol (solvent A) water heptafluorobutyric acid (0.1%, v/v) (solvent B) gradient. Gradient as follows: 5 min 3% solvent A-97% solvent B; 15 min 80% solvent A-20% solvent B (Waters gradient curve 8); 5 min 80% solvent A-20% solvent B. 7-mG eluted at 14 min under these conditions. The 7-mdGua iro was then prepared by treating the purified 7-mdGua with 0.2 M NaOH (4 h, room temp) and two peaks were observed on reverse phase HPLC (Fig. 2). The concentration of the 7-mdGua iro stock solution was determined spectrophotometrically and dilutions were made for use as standard inhibitors in ELISA. The extinction coefficient of 7-mdGua iro was determined as 15,260 at \( \lambda_{max} \) 270 nm with a solution in 0.25 M phosphate buffer, pH 8.4.

Animals. Male BD IV rats (150–200 g), bred in the animal house of the International Agency for Research on Cancer, were used in all experiments. The animals received rat chow and water ad libitum. Groups of three rats were treated p.o. with 0.4 mg/kg [\(^{14}\)C]DMN or groups of eight rats were treated with 1.0 mg/kg unlabeled DMN in 0.16 ml sterile NaCl solution. Six, 24, or 48 h after treatment, rats were anesthetized with ether, and blood was obtained by cardiac puncture. Routinely, 5–6 ml of blood were obtained per rat. Lymphocytes were isolated by lymphocyte separation medium (Flow Laboratories, Puteaux, France), using the method described by the suppliers. Cell pellets were stored at -80°C prior to DNA extraction. Rat livers were removed and washed for 30 min with Tris buffer (0.4 ml/min) until all material was then eluted with 0.1 M sodium acetate-0.5 M NaCl buffer (pH 7.0) at 1 ml/min. The column was then washed with 30 ml with Tris buffer (0.4 ml/min) until all material absorbing at a wavelength of 280 nm had been eluted. Adsorbed material was then eluted with 0.1 M sodium acetate-0.5 M NaCl (pH 4.5) until the absorbance at 280 nm had returned to baseline (total volume, approximately 20 ml). The eluate was dialyzed extensively against a 1:10 dilution of PBS. The dialysate was concentrated to a volume equivalent to the serum volume applied to the column, using polyethylene glycol 6000 (Sigma). This resulted in a recovery of 70% of antibody activity as assayed by ELISA. Antibody was stored at -20°C, at a dilution ready for use in ELISA.

Enzyme Hydrolysis/HPLC Separation. DNA was hydrolyzed and chromatographed on Aminex A7 cation exchange resin as described previously and \( \delta^4 \)-mdGua was isolated and assayed by radioimmunoassay (11). In the experiments described in this paper the DNA adducts 7-mdGua iro and 7-mdGua were found to elute as a single peak after 8 min (Fig. 1) while the ribonucleosides 7-mGua and 7-mGua iro and the ring-open and closed forms of the free base 7-mG, if present, would elute at 8, 13–16, 23, and 56 min, respectively. It was observed that the enzymatic hydrolysis conditions used, involving overnight incubation at 37°C in Tris buffer, pH 8, caused approximately 60% of 7-mdGua, but not 7-mdGua iro, to ring-open. In addition, further ring-opening can occur during passage through the Aminex column (pH 8; 50°C). Due to the separation of 7-mdGua and 7-mdGua iro from 7-mdGua iro in this chromatographic step the possible contamination from RNA could be avoided. However, it should be noted that on a more recent Aminex A7 column the 7-mdGua iro has been found to elute later than 7-mdGua and partially chromatograph with 7-mdGua iro. We have been unable to explain this observation but as a result it is important to stress (a) the need to characterize specifically each column with authentic marker compounds to ensure that the correct fractions are taken for immunoray and (b) the priority of eliminating RNA prior to chromatography. We have given an additional treatment of the purified DNA with RNase A and RNase T\(_1\) prior to chromatography to further avoid this problem.

Fractions 5–8 (7–11 min) from the Aminex A7 column were pooled, air dried, dissolved in 0.2 M NaOH (pH 10.0), and incubated at 37°C for 4 h to ensure a homogeneous imidazole ring-opened product (33). The fractions were neutralized with 0.2 M HCl and air dried and a second HPLC separation was performed on a Supelcosil LC-18 reverse phase column (25 x 0.4 cm) eluting isocratically with 10 mM NH\(_4\)HCO\(_3\) (pH 7.0) at 1 ml/min. Two rotamers of 7-mdGua iro elute at 8 and 10 min (Fig. 2). These regions (fractions 7–17) were pooled, air dried, and dissolved in PBS for ELISA. Fractions 18–28 were similarly treated as an internal control column blank.

ELISA. The assay procedure was a modification of an ELISA used in our laboratory for other carcinogens (34). An ELISA was chosen to avoid the difficulty of preparing a stable, high specific activity tracer of 7-mdGua iro which would be required for radioimmunoassay. Dynatech 96-well plates were coated with 4 ng of 7-mdGua iro-BSA conjugate in PBS and dried overnight. This conjugate had a nucleoside-protein ratio of approximately 15:1. The standard inhibitor solutions were prepared as described above. Primary antisera were used at a dilution of 1:50,000 prior to purification and 1:35,000 after purification. A 90-min preincubation in ELISA with 100 \( \mu \)l/well of 10% fetal calf serum in PBS was performed to reduce nonspecific binding. All other assay conditions were as described previously (34).

RESULTS

Chromatographic Purification of 7-mdGua iro. Considerable characterization of the elution profile of 7-methylguanine, -guanosine, and -deoxyguanosine in imidazole ring-open or closed forms is required (see “Materials and Methods” for details). These results are summarized in Fig. 1. In an enzyme hydrolyzed DNA sample a number of these products could be detected and separated by reversed phase HPLC.
DETECTION OF 7-mdGua BY HPLC/ELISA

7-medG 7-medG i.r.o. dT dC dA dG O6-medG

Elution time (min)

10 20 30 40 50 60

pmol of inhibitor required for 50% inhibition in ELISA

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pmol of inhibitor required for 50% inhibition in ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-mdGua i.r.o.</td>
<td>0.625</td>
</tr>
<tr>
<td>7-mdGua</td>
<td>2.8</td>
</tr>
<tr>
<td>7-ethyl-dGua i.r.o.</td>
<td>3.0 x 10^-3</td>
</tr>
<tr>
<td>7-mdGua</td>
<td>1.25 x 10^-3</td>
</tr>
<tr>
<td>7-mdG</td>
<td>6.5 x 10^-3</td>
</tr>
<tr>
<td>O6-medGua</td>
<td>2.5 x 10^-3</td>
</tr>
<tr>
<td>O6-mThd</td>
<td>&gt;5.0 x 10^-3</td>
</tr>
<tr>
<td>3-Methyladenine</td>
<td>&gt;1.0 x 10^-3</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>5.5 x 10^-3</td>
</tr>
<tr>
<td>Thymidine</td>
<td>6.0 x 10^-4</td>
</tr>
<tr>
<td>Deoxycytosine*</td>
<td>&gt;5.0 x 10^-4</td>
</tr>
<tr>
<td>Deoxyadenosine*</td>
<td>&gt;5.0 x 10^-4</td>
</tr>
</tbody>
</table>

* Maximum amount of inhibitor used was not sufficient to give 50% inhibition in ELISA.

Fig. 1. Schema of the chromatographic elution profile of 7-mdGua (7-medG), 7-mdGua i.r.o., thymidine (dT), deoxycytosine (dC), deoxyadenosine (dA), and O6-mdGua (O6-medG) on Aminex A7. 7-mdGua, 7-mdGua i.r.o., 7-mG elute at 8, 13 to 16, 23, and 56 min, respectively. For further details see “Materials and Methods.”

Fig. 2. Reverse phase chromatographic separation of 7-mdGua i.r.o. Peaks eluting at 8 and 10 min represent the two rotameric forms, F1 and F2, of 7-mdGua i.r.o. (inset). The peak at 4 min represents the breakthrough peak. Fractions collected after HPLC (0.5 ml/fraction) were analyzed by ELISA. Recovery, as calculated by inhibition measurements, was 94-102%.

Hypothesized to be present. Initially it is necessary to ensure that no naturally occurring 7-mdGua from RNA is present to interfere in ELISA. Ribonucleosides eluted just prior to and separate from the corresponding deoxyribonucleoside on Aminex A7. Thus, RNA contamination of DNA preparations could be conveniently monitored. In addition, this can be measured by a sensitive HPLC assay for uridine (35). In the experiments reported here we also had a chromatographic separation of RNA and DNA derived 7-methyl adducts (see “Materials and Methods”) and this further reduced the possibility of RNA derived 7-mdGua being included in fractions taken for ELISA. It should also be pointed out that the antiserum we used had a 4.5-fold higher specificity for 7-mdGua i.r.o. than 7-mdGua (Table 1) and that this may be further improved by monoclonal antibodies which we have produced to this adduct.

The DNA hydrolysis conditions used did not lead to appreciable loss of 7-mdGua by depurination. This was shown by hydrolyzing two aliquots of the same DNA sample from a rat treated with [14C]DMN by (a) standard enzyme hydrolysis, followed by Aminex A7 chromatography, or (b) acid hydrolysis (0.1 N HCl, 70°C, 30 min) followed by chromatography on Partisil 10SCX (25 x 0.4 cm). In this way the quantity of 7-mdGua present after enzyme hydrolysis was shown to be equivalent to the 7-mG liberated by acid hydrolysis (Fig. 4).

Fractions 5-8 (7 to 11 min) from Aminex A7 containing any remaining 7-mdGua and 7-mdGua i.r.o. were subjected to alkali treatment to ensure complete imidazole ring-opening and this material was subjected to a second purification by reverse phase chromatography prior to ELISA. Under these chromatographic conditions two peaks of the same magnitude can be resolved eluting at approximately 8 and 10 min (Fig. 2). The rechromatography of one of these peaks under the same conditions again produced two peaks and this is consistent with the presence of two rotamers as shown by Chetsanga et al. (20) and Kadlubar et al. (22). Both peaks were equally recognized by the purified polyclonal antiserum used in ELISA (Fig. 2) and assay of each fraction, followed by quantitation by ELISA, showed a 94-102% recovery of a known amount of authentic 7-mdGua i.r.o. This result is to be expected because a mixture of the two rotamers was used for coupling to protein for immunogen preparation.

An additional validation of the chromatographic separation was performed by using an enzyme hydrolyzed liver DNA sample from a [14C]DMN treated rat and chromatographing on Aminex A7 alone or on both Aminex A7 and the reverse phase column. 7-mdGua was quantitated by radioactivity in the relevant fractions and it was shown that the amount of radioactivity present between 7 and 11 min on Aminex A7 corresponded to the quantity eluting in peaks at 8 and 10 min on the reverse phase column (Fig. 4).

Antibody Specificity. Specificity of antibodies was examined by comparing the relative amounts of normal or modified deoxy- and ribonucleosides as well as free base required to produce 50% inhibition of antibody-antigen binding in ELISA. As reported in Table 1, the antiserum primarily recognizes
adducts with a 7-methyl modification, with the imidazole opening being an important determinant. The presence of a sugar moiety is also recognized with, in addition, a slightly increased reactivity with deoxynucleoside compared to ribonucleoside. Deoxyribonucleosides modified at other sites are weakly effective inhibitors as are unmodified deoxyribonucleosides.

Antibody Sensitivity. Titers of primary antisera with 7-mGua iro conjugated to BSA or HC were almost identical. The work described in this paper used exclusively the anti-7-mGua iro-HC antibodies to allow the use of the more readily soluble 7-mGua iro-BSA as solid phase antigen in ELISA. This combination allowed us to minimize binding of the antigen to epitopes on the protein. Nevertheless, in the crude antiserum preparation, antibodies strongly reacting with BSA were evident, resulting in a low sensitivity of ELISA (50% inhibition, 140 pmol) and a flat curve with a maximum inhibition of approximately 70% (Fig. 3). Purification of the antiserum by affinity chromatography (see “Materials and Methods”) resulted in a greater than 250-fold increase in sensitivity with a 50% inhibition value of 0.63 pmol. Using 7-mGua iro-BSA coupled to CNBr activated Sepharose 4B we found that conditions required to elute the specifically retained antibodies caused their inactivation. Consequently an affinity matrix was prepared using instead deoxyguanosine-BSA coupled to the gel, the rationale being that sufficient affinity of the antiserum for the parent nucleoside would allow both retention on the column and easy elution. Using purified antibody the lower limit of detection in ELISA was set at 20% inhibition, i.e., 0.05 pmol/ELISA well or 2 pmol/ml sample.

Assay Validation. Two experiments were performed to compare 7-mdGua as quantitated by ELISA or by radioactivity, from DNA treated in vitro or in vivo with radiolabeled methylating agents. Calf thymus DNA, alkylated in vitro by [3H]-MNU, was enzymatically digested and an aliquot was analyzed by Aminex A7 chromatography. An aliquot of the fractions from the 7-mdGua (iro) peak were counted for radioactivity while the remainder was dried, reconstituted in PBS, and assayed by ELISA. The levels of modification were 2.18 and 2.05 pmol 7-mdGua/μmol dGua by the two methods, respectively. Similar experiments were performed on liver DNA from rats treated with DMN. The dose of 0.4 mg/kg involved treatment with [14C]DMN (8 mCi/mmol); numbers in parentheses, determination of 7-mdGua by radiocounts following separation of enzymically digested DNA by Aminex chromatography (see also Fig. 4). Each data point for liver represents the mean ± SE of at least three estimations involving individual rats. For lymphocytes, DNA was extracted from cells pooled from 3 (0.4 mg/kg DMN) or 8 (1.0 mg/kg DMN) rats.

Table 2 Methylation levels in liver and lymphocyte DNA from rats treated with DMN (0.4 or 1.0 mg/kg)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>7-mdGua μmol adduct/µmol DNA</th>
<th>7-mdGua μmol adduct/µmol dGua</th>
<th>86-mdGua μmol adduct/µmol DNA</th>
<th>86-mdGua μmol adduct/µmol dGua</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>6 h</td>
<td>145 ± 23 (138 ± 22)</td>
<td>125 ± 22 (119 ± 22)</td>
<td>27 ± 6 (26 ± 5)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>6 h</td>
<td>125 ± 22 (120 ± 22)</td>
<td>127 ± 22 (123 ± 22)</td>
<td>27 ± 6 (24 ± 5)</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>263 ± 22 (259 ± 22)</td>
<td>322 ± 22 (317 ± 22)</td>
<td>12 ± 6 (11 ± 5)</td>
</tr>
</tbody>
</table>

* ND, not done.

DISCUSSION

In this paper two significant results are reported: (a) a sensitive immunological method for quantitating the major DNA adduct 7-mdGua has been developed and validated; (b) it has been shown that peripheral blood cell DNA can be modified upon p.o. administration of a methylating agent (DMN) requiring metabolic activation. The detection and quantitation of 7-mdGua are particularly valuable for assessing human exposure to environmental methylating agents because this adduct (a) represents a major fraction of total methylation and (b) may accumulate upon repeated exposure due to the relative stability of the adduct. This adduct may thus be a suitable indicator of exposure to environmental methylating agents over an extended period of time, which represents a valuable tool in increasing the sensitivity and specificity of epidemiological studies.

Previous workers have raised antibodies against 7-mGua,
mainly with a view to purifying tRNA or mRNA molecules containing this modified nucleoside (30, 33, 37, 38). When these antibodies have been applied in immunoassays to quantitate 7-mdGua the sensitivity has been limited, around 100 pmol or more having been required to give 50% inhibition of antibody-antigen binding (33, 37, 38). We used the imidazole ring-opened form of 7-mdGua as an immunogen because (a) the 7-mdGua iro molecule is much more stable than the ring-closed form and a quantitative conversion of ring-closed to ring-open form can be obtained. Having this stable adduct is of importance for preparation of immunogens and during analysis following enzyme hydrolysis and chromatographic separation, and (b) we hypothesized that this molecule, a highly modified pyrimidine, may elicit a stronger immune response than a simple methyl adduct at position 7 of guanosine. It should be noted that when the Erlanger and Beiser (31) coupling procedure is used for 7-mdGua, a major proportion of the conjugate is in the ring-open form due to the high pH used at room temperature during the coupling procedure (33).7

In summary the assay system we have used is (a) to isolate 7-mdGua and any 7-mdGua iro occurring in vivo by chromatographic separation after enzyme hydrolysis, (b) to quantitatively convert this to 100% 7-mdGua iro (c) to purify this material with a second chromatography step, and (d) to assay using specific antibodies. This methodology was validated by comparing data obtained by ELISA with that obtained by 14C- or 3H-methyl-labeled DNA in the same samples. There was a good agreement between the two methods (Fig. 4). This approach has permitted the quantitation of low levels of 7-mdGua with the avoidance of possible contamination with normally occurring 7-mGua. Although it has been suggested that 7-mdGua iro may occur in vivo, in rat tissues (21, 22), quantitation of both the ring-open and -closed forms is obtained using these antibodies and HPLC separation. The combination of two chromatographic steps and the immunoassay, besides resulting in a high specificity, results also in a sensitivity which allows as little as 0.05 pmol 7-mdGua iro to be quantitated in one assay. In practice, to assay in quadruplicate on two different days requires around 0.4 pmol of adduct. This figure represents the limit of detection independent of the amount of DNA available. Fluorescence measurements of 7-mG allows routine quantitation down to 50 pmol (39) while nuclear magnetic resonance has been reported to allow as little as 1 pmol 7-mdGua to be quantitated using marker compounds (40). As yet this latter method, involving relatively expensive equipment, takes a long time to perform and has not been validated at these low levels using biological samples. The use of one of these methods to confirm ELISA data would be a particularly powerful approach.

One limitation of our previous studies of alkylated bases in human tissues from Linxian County, People's Republic of China (11), was the quantity of DNA required to measure the minor adduct O4'-mdGua. Other workers who have since confirmed these observations of O-alkylated adducts in human tissues (41, 42) have similarly used several mg of DNA. In addition, the analysis of surgical samples does not allow random selection of the sample to be analyzed, limits the number of individuals which can be assayed, and restricts epidemiological studies to retrospective analyses. Determining the presence of DNA alkylation adducts in peripheral blood cell DNA could overcome some of these difficulties.

The detection of 7-mdGua in rat peripheral blood cell DNA following in vivo systemic treatment with DMN (see Table 2) is consistent with previous findings on the metabolism of this carcinogen and its adverse biological effects. Umbenhauer and Pegg (43) have shown that freshly isolated liver cells are capable of metabolizing DMN and that the intermediate (nitrosohydroxymethylmethylamine), with a half-life of 10 s (44), is able to alkylate extracellular DNA. Kuroki et al. (45) detected the mutagenic effect of various nitrosamines in V79 Chinese hamster cells only when a microsomal activation system was present, and chromosomal aberrations were induced in lymphocytes of rats treated with 30 mg/kg DMN (46). Incubation of intact human erythrocytes with rat liver microsomes and DMN resulted in the methylation of erythrocyte proteins (47). All these observations indicate that DMN is presumably metabolized in the liver and the active intermediate is sufficiently stable to interact with blood cell components. In fact, Osterman-Golkar et al. (48) reported a level of alklylation (as measured by the amounts of 3-methylhistidine) approximately the same in hemoglobin and liver proteins, and this approach has been proposed to monitor exposure to alkylating agents such as DMN by measuring alkylated derivatives of various amino acids (e.g., cysteine, histidine) of hemoglobin (see Ref. 49). However, in the case of nitrosamines such as DMN, the level of detection has been found to be low, since the existence of a high background level of dimethylcysteine limits the detection of this adduct in hemoglobin at levels below 12.5 mg/kg of DMN in rats, in spite of the high sensitivity of the analytical method of detection (50). Similarly measurement of 7-mG in human urine is limited by the high levels of this molecule (approximately 10 mg/24 h) present as a normal component of urine (51). Recently, Carmella and Hecht (52) described the formation of 4-hydroxy-1-(3-pyridyl)-1-butanoate hemoglobin adducts in rats treated with [3H]-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanoate and N'-nitrosornicotine, nitrosamines present in tobacco smoke.

In summary, the methodology presented in this paper now permits the quantitation of 7-mdGua, in addition to O4'-mdGua and O4'-mThd, using a single DNA sample with three specific antibodies. From 20 ml of blood one may obtain around 200 µg DNA from lymphocytes, or 0.2 µmol of dGua. With a detection limit of 0.4 pmol 7-mdGua, a level of 2 µmol 7-mdGua per mol dGua could be detected. In order to produce this level of alkylation in rat liver approximately 5.2 µg/kg DMN (data derived from Ref. 36) are required which, based on a dose/m² comparison (rat, 0.05 m²; human, 1.5 m²) translates to 0.45 µg/kg DMN in humans. This assumes no accumulation of adduct upon repeated exposure. However, Pegg and Hui (36) have shown that upon low daily doses of DMN (1 µg/kg for liver DNA analyses; 10 µg/kg for kidney) the level of 7-mG accumulated during the duration of the experiment (20 days for liver, 50 days for kidney) suggesting that the 7-mG adduct is sufficiently stable to accumulate and give an indication of past exposure to a methylating agent.

Furthermore, we have obtained preliminary data of methylation of lymphocyte DNA in cancer patients treated with the chemotherapeutic agent MNU which is further evidence of the validity of this approach.a

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REFERENCES

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