Detection of O\(^6\)-Methyldeoxyguanosine in Human Placental DNA\(^1,2\)

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ABSTRACT

A monoclonal antibody specific for \(O^6\)-methyldeoxyguanosine (\(O^6\)-MedGua) was developed. When used in a competitive enzyme-linked immunosorbent assay, 50% inhibition of binding was achieved with 0.5\(\mu\)mol \(O^6\)-MedGua. When the competitive enzyme-linked immunosorbent assay was coupled with high-performance liquid chromatography, 2\(\mu\)g of DNA could be analyzed giving a lower limit of detection of 0.5\(\mu\)mol \(O^6\)-MedGua/mol deoxyguanosine. This assay was used to test for \(O^6\)-MedGua in DNA from placental tissues and three of 10 from nonsmoking women with detectable concentrations of \(O^6\)-MedGuo. Concentrations ranged from 0.6 to 1.6\(\mu\)mol \(O^6\)-MedGua/mol deoxyguanosine. Activity of \(O^6\)-alkylguanine DNA alkyltransferase was also measured. There was no apparent relationship between \(O^6\)-alkylguanine DNA alkyltransferase activity and \(O^6\)-MedGua concentrations in the 20 subjects, nor did mean \(O^6\)-alkylguanine DNA alkyltransferase activity differ between the two groups. Although no apparent relationship between smoking history and \(O^6\)-MedGua concentration was found in this preliminary study, this is the first report of a structurally identified DNA adduct in human placenta.

INTRODUCTION

We have previously reported the development of an immunos assay for \(O^6\)-MedGua which we used to demonstrate that the tobacco-specific nitrosamine NNK was a DNA-methylating agent (1). We have since improved the immunoassay by developing a monoclonal antibody with greater affinity for \(O^6\)-MedGua. The increased level of sensitivity gained using this antibody has allowed us to assay human tissue for the presence of \(O^6\)-MedGua. We chose to assay placenta because it is more readily available in large quantities than lung tissue and because it has previously been shown that smoking-related DNA adducts are formed in placenta (2, 3).

The presence of \(O^6\)-MedGua in human tissue has been reported previously in esophageal DNA of patients from Linxian county (Peoples Republic of China) with cancer of the esophagus (4). Higher concentrations of \(O^6\)-MedGua were found in the Chinese cancer patients than in a control population from Europe. Linxian county has a higher rate of esophageal and gastric cancer than other areas of China. A number of studies have found a higher level of environmental exposure to nitrosamines and their precursors in this region of China compared to other regions of the country. The bulk of the exposure seems to come from moldy foods and pickled vegetables (reviewed in References 5 and 6). These data suggest an association between environmental nitrosamine exposure, formation of \(O^6\)-MedGua and development of cancer. In this study we sought to determine whether a relationship between exposure to NNK through smoking and concentrations of \(O^6\)-MedGua in placenta DNA could be established.

MATERIALS AND METHODS

Chemicals. \(O^6\)-MedGua and 7-methyl-2'-deoxyguanosine were synthesized as described by Farmer et al. (7). 2'-Deoxycytidine, 2'-deoxyadenosine, thymidine, 2'-deoxycytidine, and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). \(O^6\)-Methylguanosine was obtained from VEGA Biotechnologies Inc. (Tucson, AZ). The \(O^6\)-MeGuo-BSA conjugate was prepared by the method of Erlanger and Beiser (8). [\(\text{H}\)]Nitrosomethylurea was obtained from NEN Research Products (Wilmington, DE).

Fusion. Immunization of mice and the fusion protocol for the production of monoclonal antibodies were performed essentially as described previously (9). Briefly, female C57B6J mice were immunized with 100\(\mu\)g of \(O^6\)-MeGuo-BSA conjugate in 0.1\(\mu\)l of saline emulsified with an equal volume of Freund’s complete adjuvant (GIBCO, Grand Island, NY), given in a split dose, i.p. and s.c. A second injection was given 2 weeks later in incomplete Freund’s adjuvant. Four weeks after the second injection individual mice were boosted with 100\(\mu\)g of conjugate in saline given i.p. on Days 1, 2, 3, and 4. On Day 5 a mouse was sacrificed and the spleen removed for fusion. The fusion partner SP2/O (10) was maintained in RPMI 1640 medium (Whittaker M.A. Bioproducts, Walkersville, MD) supplemented with 1\(\text{mM}\) sodium pyruvate, 2\(\text{mM}\) L-glutamine, 25\(\text{µg/ml}\) gentamicin and 15% fetal bovine serum (GIBCO). A spleen cell suspension was made by forcing the spleen through a fine mesh stainless steel screen followed by centrifugation. Red cells were lysed by resuspending the cell pellet in 5\(\text{mL}\) of cold tris-ammonium chloride (nine parts 0.83% NH\(_4\)Cl, one part 170 cold tris-ammonium chloride) and incubating for 5\(\text{min}\) on ice. The cells were washed, resuspended in 10\(\text{mL}\) of Hank’s balanced salt solution and counted. The spleen cells were mixed with the SP2/O cells at a ratio of 5:1 in a 17 \(\times\) 100-mm polypropylene test tube (no. 2006; Falcon, Oxnard, CA) and centrifuged. One\(\text{mL}\) of the fusing solution [50% Hank’s balanced salt solution, 45% polyethylene glycol (Kodak, Rochester, NY; M\(_4\), 1450] and 5% dimethyl sulfoxide] was added to the pellet and the pellet was broken into small clumps by gentle agitation and incubated for 5\(\text{min}\) at 37\(\text{°C}\). The fused cells were centrifuged and the supernatant discarded. The cells were washed and resuspended in 50\(\text{mL}\) of complete RPMI 1640 medium supplemented with hypoxanthine (0.1\(\text{mM}\), aminopterin (0.4\(\text{µg/ml}\) and thymidine (16\(\text{µg}\)). The cells were cultured in 96-well tissue culture plates at 150\(\text{µl/well}\) and incubated at 37\(\text{°C}\) and 5%\(\text{CO}_2\) in air. On Day 6 the cultures were fed with 50\(\text{µL}\) of complete RPMI 1640 medium supplemented with hypoxanthine and thymidine. By Day 14 the colonies were sufficiently large to be screened for antibody production by ELISA.

DNA Isolation. Specimens of maternal blood were obtained at about the 7th month of pregnancy. Placenta were obtained at delivery from healthy volunteers as part of an ongoing study described elsewhere (11). Placental specimens were frozen at −70\(\text{°C}\) within about 30 min of delivery, stored at −80\(\text{°C}\), and shipped on dry ice. Questionnaires were administered to the mother a few days following delivery. Specimens from 10 subjects who reported smoking cigarettes throughout pregnancy and 10 who reported never smoking cigarettes were chosen for this study. These specimens were also used in a previous study meas-

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The abbreviations used are: \(O^6\)-MedGua, \(O^6\)-methyldeoxyguanosine; BSA, bovine serum albumin; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay: AAT, \(O^6\)-alkylguanine DNA alkyltransferase; SCE, sister chromatid exchange.

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using aromatic adducts by the $^{32}$P-postlabeling assays (2, 11). They were matched approximately for time of storage at −80°C. Individual placenta samples weighing between 40 and 65 g were thawed and excess blood drained and collected. The placentas were cut into small chunks, placed in isotonic saline sodium citrate buffer and homogenized. The homogenate was centrifuged at 10,000 x g for 15 min and the pellet was resuspended in 1 M NaCl with 0.1% sodium dodecyl sulfate. A portion of the supernatant was frozen for later testing of AAT activity. The resuspended pellet was then extracted twice with a half volume of chloroform:isooamyl alcohol (5:1 v/v) and the DNA precipitated with an equal volume of cold ethanol. The DNA was redissolved in 0.1 M sodium acetate buffer pH 7.0 and treated with ribonuclease A (Sigma) for 30 min at room temperature. Two volumes of 2 M NaCl were then added and the DNA extracted with chloroform:isooamyl alcohol. The DNA was then precipitated with an equal volume of cold ethanol, washed with acetone, dried, and stored at −20°C. The recovery of DNA ranged from 10 to 100 mg.

ELISA. DNA was dissolved in 50 mM Tris (pH 7.2) and the concentration determined by the fluorescence method of Lefterica and Paigen (12). The O-methylguanosine content of DNA was determined by its percentage by weight, 22.8% (1). A 2-mg sample was then enzyme hydrolyzed with deoxyribonuclease I, snake venom phosphodiesterase, and alkaline phosphatase as previously described (13). The ELISA was performed essentially as described previously (1). Briefly, polystyrene ELISA plates were coated with 5 ng O'-MedGuo-BSA conjugate. The plates were washed and 50 μl of reconstituted HPLC fraction or standard were added followed by 50 μl of O6A4 monoclonal antibody. The mixtures were incubated for 1 h.

When screening fusions for clones producing O6-MedGuo specific antibody, 100 μl of tissue culture supernatant was added to each well. The plates were washed and 100 μl of alkaline phosphatase labeled goat anti-mouse antibody (KPL, Gaithersburg, MD) was added and incubated for 1 h. The plate was then washed and 100 μl of p-nitrophenyl phosphate (Sigma), 1 mg/ml in 1 M diethanolamine buffer (pH 9.8), was added and the absorbance at 405 nm was read after 30 min using a Bio-Tek EL308 ELISA reader (Bio-Tek, Burlington, VT). The extent of inhibition of binding of O6A4 antibody was converted to the concentration of O6-MedGuo by use of a log-logit plot generated using synthetic O6-MedGuo standard.

HPLC. The enzyme hydrolysates of DNA were analyzed by two HPLC systems. System A used two Waters µBondapak C8 3.9-mm x 30-cm reversed-phase columns (Millipore Corp., Bedford, MA). The solvent system was a linear gradient of 0–100% methanol in 20 mM potassium phosphate (pH 5.6) at 1 ml/min in 60 min. System B used a Whatman Partisil M9 ODS-3 9.4-mm x 50-cm reversed-phase column (Whatman Inc., Clifton, NJ). The solvent system was a linear gradient of 0–35% methanol in H2O at 4 ml/min in 50 min. The retention time of O6-MedGuo in these two systems was determined by injecting synthetic standard. Six 1-min fractions were collected in the region where O6-MedGuo had been determined to elute. Fractions were lyophylized and reconstituted in 300 μl of phosphate buffered saline for analysis by ELISA.

Assay for AAT. The activity of AAT in cytosolic protein fractions of placenta was tested using the method of Pegg et al. (14). One mg of 3H-methylated DNA, obtained by reaction with N[3H]methyl-N-nitrosourea and containing 1.4 pmol of O6-MedGuo, was mixed with 5 μg of protein in a final volume of 3 ml of assay buffer [65 mM Tris (pH 8.3), 1.25 mM dithiothreitol, and 0.35 mM EDTA]. The mixture was incubated for 30 min at 37°C and the reaction stopped by the addition of 50 μl of 6 N HCl followed by heating at 70°C for 1 h. The sample was then centrifuged to 1 ml and passed through an Amicon Centrifree tube to remove excess protein. The concentration of O6-methylguanine in the hydrolysate was measured using an HPLC equipped with a FlowOne Beta radioactive flow detector (Radiomatic Inst., Tampa, FL). Two Whatman 10 SCX strong cation exchange columns connected in series were used. The program consists of a linear gradient of 0–32% buffer B in buffer A in 40 min at 1 ml/min [A = 20 mM ammonium formate, 6% MeOH (pH 4.0); B = 300 mM ammonium formate (pH 4.0), 8% MeOH].

Cotinine and SCE Analysis. The average serum cotinine value was obtained as described by Haley et al. (15). Sister chromatid exchanges were measured using 100 μM BUdR in cultures for eight subjects included in this study using methods described by Lundgren et al. (16).

RESULTS

Several fusions were performed. In the one yielding the cell line used in this study, one mouse was immunized as described in "Materials and Methods," sacrificed, and its spleen cells fused with SP2/O. Two weeks after fusion, 231 of 288 wells contained one or more viable colonies. When tested by ELISA, 30 of the 231 colonies were found to bind to the O6-methylguaninosa-BSA conjugate. Of these 30, the binding of two could be inhibited with O6-MedGuo. These two colonies were cloned by limiting dilution in hanging drop tissue culture. The surviving clone was designated O6A4.

The isotype of O6A4 antibody was determined using isotype-specific antibodies in an ELISA and found to be an IgG, with k light chains. Further studies were performed using O6A4 ascites fluid diluted 1:20,000. The specificity of the O6A4 antibody was determined in a competitive ELISA assay using O6-MedGuo and related compounds as inhibitors. The results of these studies are shown in Table 1 and Fig. 1. O6-MedGuo and O6-methylguanine were the strongest inhibitors requiring as little as 0.51 pmol to achieve 50% inhibition of binding; 7-methyldeoxyguanosine was 2,000-fold less reactive. Only two DNA bases, deoxycytidine and deoxyadenosine, had measurable reactivity, and were at least 10,000-fold less reactive than O6-MedGuo. Using this antibody and assuming 15% inhibition as the minimum reliable level of detection, the ELISA could detect as little as 120 fmol of O6-MedGuo. The sensitivity of the method described above is limited by the amount of DNA present.

Table 1 Specificity of O6A4 monoclonal antibody for O6-MedGuo

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pmol F</th>
</tr>
</thead>
<tbody>
<tr>
<td>O6-MedGuo</td>
<td>0.51</td>
</tr>
<tr>
<td>O-MeGua</td>
<td>0.81</td>
</tr>
<tr>
<td>7-MedGuo</td>
<td>1.060</td>
</tr>
<tr>
<td>Deoxycytosine</td>
<td>5.850</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>148,000</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>&gt;187,000</td>
</tr>
<tr>
<td>Thymidine</td>
<td>&gt;100,000</td>
</tr>
</tbody>
</table>

* pmol of inhibitor needed for 50% inhibition of binding in the ELISA.

† 7-MedGuo, 7-methyldeoxyguanosine.

Fig. 1. Inhibition curves for O6A4 in the competitive ELISA. Plots were generated from least squares fit of log-logit transformed data. □, O6-MedGuo; ○, O6-methylguanine; ●, 7-methyldeoxyguanosine; ▲, deoxycytosine; △, deoxyadenosine.
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hydrolysate which can be placed in a single ELISA plate well. Assuming 100 μg of DNA per well as the maximum the lowest detectable concentration of O⁶-MedGuo is 1.8 μmol/mol deoxyguanosine. In order to analyze larger amounts of DNA, the DNA hydrolysate was separated by HPLC. Fig. 2 shows the results of an analysis of 2 mg of placenta DNA. Six 1-min fractions starting at 29 min were collected and analyzed by ELISA. Maximal inhibition was seen at 31 min which corresponds to the elution time of synthetic O⁶-MedGuo standard. Comparable results were obtained using HPLC system B with the adduct appearing at 43 min (data not shown). Using this system with 2 mg of DNA, and diluting the HPLC fractions to a volume sufficient for six ELISA plate wells, the detection limit was 0.5 μmol O⁶-MedGuo/mol deoxyguanosine.

Study subjects included 13 whites and 7 blacks; two of the blacks were smokers. The age of the subjects was 30.3 ± 5.5 for the nonsmokers and 24.5 ± 5.0 for the smokers. The 10 smokers reported using between four and 30 cigarettes per day during the third trimester of pregnancy, with an average of 18 cigarettes per day.

Using the method described above, detectable concentrations of O⁶-MedGuo were found in the placental DNA of five of the 20 women (Table 2). Positive results were confirmed by two methods. First, when a DNA sample was found positive using one HPLC system a second 2-mg sample was tested using the second HPLC system. If this sample was also positive a portion of the positive fractions were reanalyzed using the first HPLC system. All five of the positive samples in Table 2 were positive in all three tests. There was no apparent association between O⁶-MedGuo concentration or AAT activity and smoking. Two of the 10 smokers and three of the 10 nonsmokers had detectable concentrations of O⁶-MedGuo. This is also the first positive identification of a DNA adduct with known structure in human placenta. O⁶-MedGuo was not detected in a previous study of blacks. Four of the seven blacks (57%) but only one of 13 whites (8%) had detectable concentrations of O⁶-MedGuo. This difference proved sensitivity is especially important in human studies where exposure is less intense than that achieved in animal studies with a commensurate lower level of adduct formation. using a nonparametric statistical test [the Kendall Tau β correlation coefficient and its associated test for statistical significance (18)] there was evidence of an inverse association between concentrations of O⁶-MedGuo, but not AAT, and caffeine consumption during pregnancy (Tau β = -0.40, P = 0.03). There was no association between concentrations of O⁶-MedGuo or AAT activity and maternal age, consumption of charcoal broiled fish or alcohol during pregnancy, birth weight or birth length of the infant, or the interval between birth of the infant and freezing the placenta (in each case the P value associated with Tau β was >0.10). There was also no association between concentrations of O⁶-MedGuo or AAT activity and adduct 1 determined by ³²P-postlabeling as described by Eversen et al. (18) or frequencies of sister chromatid exchanges in peripheral blood lymphocytes from cord blood or maternal blood. Although the number of specimens for which both procedures were conducted was only eight, a direct association was suggested between AAT activity and sister chromatid exchange activity in cord blood (Tau β = 0.57, P = 0.05).

Table 2 O⁶-MedGuo concentration and AAT activity in placenta

<table>
<thead>
<tr>
<th>Race</th>
<th>Race</th>
<th>O⁶-MedGuo μmol</th>
<th>AAT*</th>
<th>Cotinine ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>Black</td>
<td>1.6 ± 0.7</td>
<td>72</td>
<td>7</td>
</tr>
<tr>
<td>Black</td>
<td>1.2 ± 1.0</td>
<td>98</td>
<td>0</td>
<td>9.5</td>
</tr>
<tr>
<td>Black</td>
<td>0.6 ± 0.1</td>
<td>83</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Black</td>
<td>&lt;0.5</td>
<td>61</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Black</td>
<td>&lt;0.5</td>
<td>75</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>134</td>
<td>1</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>128</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>132</td>
<td>0</td>
<td>16.9</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>63</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>94</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>Smokers</td>
<td>Black</td>
<td>0.6 ± 0.2</td>
<td>55</td>
<td>109</td>
</tr>
<tr>
<td>Black</td>
<td>&lt;0.5</td>
<td>39</td>
<td>302</td>
<td>7.7</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>30</td>
<td>314</td>
<td>8.0</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>66</td>
<td>380</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>1.6 ± 0.2</td>
<td>147</td>
<td>114</td>
<td>21.0</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>96</td>
<td>51</td>
<td>9.0</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>ND</td>
<td>56</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>98</td>
<td>323</td>
<td>9.8</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>95</td>
<td>124</td>
<td>NT</td>
</tr>
<tr>
<td>White</td>
<td>&lt;0.5</td>
<td>108</td>
<td>245</td>
<td>NT</td>
</tr>
</tbody>
</table>

a) Mean and standard deviation of triplicate determinations of O⁶-MedGuo, μmol/mol deoxyguanosine.

b) fmol O⁶-MedGuo removed in 30 min/mg protein at 37°C.
c) Maternal serum cotinine, ng/ml.
d) ND, not detected; NT, not tested.

Fig. 2. HPLC separation of deoxyribonucleosides obtained by enzyme hydrolysis of 2 mg of placenta DNA using system A. dC, deoxycytosine; dG, deoxyguanosine; dA, deoxyadenosine. One-min fractions were collected from 29–35 min and assayed for O⁶-MedGuo. Solid bars, percentage of inhibition in the ELISA.

DISCUSSION

The development of the O6A4 monoclonal antibody has allowed us to improve the sensitivity of our assay 5-fold over our previously published assay which used rabbit antisera. Improved sensitivity is especially important in human studies where exposure is less intense than that achieved in animal studies with a commensurate lower level of adduct formation. This report is only the second to show the presence of O⁶-MedGuo in human DNA and the first to demonstrate its presence in human placenta. This is also the first positive identification of a DNA adduct with known structure in human placenta. O⁶-MedGuo was not detected in a previous study of 10 human placental specimens (several of which were included in this study), which used an analytic technique 10-fold less sensitive than that used here (18). DNA adducts have been centrations of O⁶-MedGuo (Table 2). The frequency of detectable concentrations of O⁶-MedGuo was higher among blacks. Four of the seven blacks (57%) but only one of 13 whites (8%) had detectable concentrations of O⁶-MedGuo. This difference was statistically significant by the Fisher's exact test (P = 0.03). Using a nonparametric statistical test [the Kendall Tau β correlation coefficient and its associated test for statistical significance (18)] there was evidence of an inverse association between concentrations of O⁶-MedGuo, but not AAT, and caffeine consumption during pregnancy (Tau β = -0.40, P = 0.03). There was no association between concentrations of O⁶-MedGuo or AAT activity and maternal age, consumption of charcoal broiled fish or alcohol during pregnancy, birth weight or birth length of the infant, or the interval between birth of the infant and freezing the placenta (in each case the P value associated with Tau β was >0.10). There was also no association between concentrations of O⁶-MedGuo or AAT activity and adduct 1 determined by ³²P-postlabeling as described by Eversen et al. (18) or frequencies of sister chromatid exchanges in peripheral blood lymphocytes from cord blood or maternal blood. Although the number of specimens for which both procedures were conducted was only eight, a direct association was suggested between AAT activity and sister chromatid exchange activity in cord blood (Tau β = 0.57, P = 0.05).
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demonstrated in human placenta previously by both 32P-postlabeling and immunoassay but their identification was not clear (2, 3). The thin-layer chromatographic patterns of placenta DNA adducts found by 32P-postlabeling did not correspond to those of known adducts including benzo(a)pyrene-7,8-diol-9,10-epoxide-DNA adducts. The antibodies used in the immunoassay of benzo(a)pyrene diol epoxide-DNA adducts in human placenta are known to cross-react with other hydrocarbon adducts such as those from chrysene-1,2-diol-3,4-epoxide (2). Thus, while the evidence for the presence of hydrocarbon adducts as a class in human placenta is strong, their identities remain unclear. We believe the evidence presented in this study argues strongly for the presence of O'-MedGuo in human placenta. The strength of the evidence is based on two factors, namely the specificity of the antibody and the retention time on HPLC. The combination of these two factors in the same analytical method make it unlikely that our results are due to artifact.

Within the limitations of the study (small sample size and sensitivity of the assay) we were unable to demonstrate a relationship between smoking and O'-MedGuo formation. There are however a number of other environmental sources of methylating agents which could lead to the formation of O'-MedGuo. The intake of nitrosamines in the diet has been estimated to be 1.1 µg/day in Germany and 2.3 µg/day in Japan (19). The major sources of nitrosamines identified in these studies were beer and broiled fish. Nitrosamines capable of causing DNA methylation have also been found in hair care products at concentrations ranging from 8 to 1180 ppb (20, 21). Various household cleaning agents contain methylating nitrosamines at concentrations ranging from 45 to 660 µg/kg (22). Nitrosamines are also formed endogenously from the reaction of nitrosatlatable amines and amides with nitrosating agents, in particular nitrates. In western Europe and the United States, the estimated consumption of nitrate is 90 mg/day and of nitrite 4 mg/day (19). The major source of these compounds is vegetables. N-Nitrosodimethylamine and N-nitrosoproline excreted in human urine at levels of 38 ng and 3.3 µg per day, respectively (23). Clearly there are ample sources of methylating agents other than NNK which could lead to the formation of O'-MedGuo in human placenta. Our study was not specifically designed to assess total human exposures to methylating agents, so that the contribution of each of these sources to O'-MedGuo concentrations could not be directly investigated. Although the number of specimens included in our study was small, two interesting associations were observed which may warrant further investigation; a higher frequency of detectable O'-MedGuo adducts among blacks compared with whites and a positive association between AAT activity and frequencies of SCE in lymphocytes.

O'-MedGuo has been detected (0.03–0.25 µmol/mol deoxyguanosine) in esophageal and gastric DNA of patients with esophageal cancer from the Linxian region of China (4). The concentrations of O'-MedGuo were higher in the Chinese population than in a control population from western Europe, but lower than those found in the present study. This may reflect differences in metabolic activity between these tissues. Our study is in agreement with the China study in finding that AAT activity was not responsible for differences in concentrations of O'-MedGuo.

Our observation of a difference in O'-MedGuo concentrations among blacks and whites may be due to a number of factors including genetics and differences in environmental exposure to nitrosamines. A larger study will be needed to confirm this observation and to determine the basis for the difference.

A variety of factors were found to influence the extent of formation of O'-MedGuo in tissues of rats treated with NNK (1, 24, 25). The most important of these factors was tissue type. O'-MedGuo was found only in tissues where NNK is known to cause tumors, i.e., liver, lung, and nasal mucosa. The type of exposure was also important. With acute exposure, concentrations of O'-MedGuo peak within 24 h in liver and nasal mucosa. With continued chronic exposure O'-MedGuo concentrations decline to approximately half their peak concentrations in liver and nasal mucosa. In lung, however, O'-MedGuo concentrations increase to a plateau over a period of 2 weeks. The decline in O'-MedGuo concentrations seen with chronic exposure is presumed to be the result of AAT activity (24). In lung, O'-MedGuo formation was found to differ according to cell type (25). Methylation was highest in Clara cells. In this study NNK may have caused higher methylation in specific cell types which was missed because of dilution. Differing levels of AAT activity in individual cell types within the placenta may also result in concentrations of O'-MedGuo in specific cell types which may have been missed. Although NNK is known to cross the placental barrier in mice, its binding to placenta DNA relative to that of other tissues has not been investigated (26). As discussed above O'-MedGuo could be formed by a number of environmental exposures other than tobacco smoke. The results of this study suggest that this lack of specificity in O'-MedGuo formation may mask O'-MedGuo formation due to exposure to tobacco smoke, at least in placenta. For this reason O'-MedGuo formation is not an ideal dosimeter of exposure to tobacco smoke. One of our objectives in future studies is to develop immunoassays specific for nonmethyl adducts derived from NNK. Adducts of this type, which would presumably be derived from nicotine-related compounds, could be specific for tobacco exposure and if formed in detectable amounts could serve as excellent dosimeters of exposure to tobacco products. Preliminary studies indicate that such hemoglobin and DNA adducts are formed in rats exposed to NNK (27, 28).

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Detection of $O^6$-Methyldeoxyguanosine in Human Placental DNA

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