Development and Validation of a New, Sensitive Immunochemical Assay for O6-Methylguanine in DNA and Its Application in a Population Study

Panagiotis Georgiadis1, Stella Kaila1, Paraskevi Makedonopoulou1, Eleni Fthenou2, Leda Chatzi2, Vasiliki Pletsas1, and Soterios A. Kyrtopoulos1

Abstract

Background: Investigations of the presence of the precarcinogenic DNA adduct O6-methylguanine (O6-meG) in humans and its association with exposure or cancer risk have been hindered by the absence of analytic methods of adequate sensitivity and throughput. We report the development, validation, and application of an ELISA-type assay for O6-meG appropriate for large-scale population studies.

Methods: In the new analytic method, restriction enzymes are used to digest DNA to fragments of size expected to contain no more than one O6-meG residue. Anti-adduct antisera are used to transfer O6-meG–containing fragments to a solid surface, where they are detected using anti-ssDNA antisera, the high ratio of normal nucleotides to adducts providing a strong signal enhancement.

Results: An assay with a limit of detection of 1.5 adducts/10^9 nucleotides using 10 μg of DNA, a dynamic range of approximately two orders of magnitude and satisfactory precision and accuracy characteristics was established and validated. Analysis of samples from 120 subjects from the Rhea mother–child cohort in Crete led to the detection of O6-meG in 70% of maternal and 50% of cord blood buffy coat samples at mean levels of 0.65 and 0.38 adducts/10^8 nucleotides, respectively.

Conclusions: The frequent observation of O6-meG in human DNA is compatible with dietary compounds (e.g. N-nitroso compounds or their precursors), or endogenous processes being responsible for the formation of this adduct.

Impact: The new assay opens the way for large-scale population studies of O6-meG as a biomarker of exposure or risk. The approach used in this assay can, in principle, be extended to any DNA adduct for which suitable antisera are available.

Introduction

A number of studies have reported that low levels of aberrant methylation of DNA are frequently observed in human populations. The methylated DNA bases that have most commonly been found in such studies are N7-methylguanine (N7-meG) and O6-methylguanine (O6-meG), detected in DNA isolated from blood lymphocytes or leukocytes, gastrointestinal tissues, lung, urinary bladder, and other human tissues (1–6). These aberrant bases are believed to represent DNA damage caused by methylating agents of dietary, environmental, or endogenous origin. The presence of O6-meG in human DNA is of particular significance because this base is well known to be cytotoxic and strongly mutagenic (7–10), causing G→A mutations via a direct miscoding mechanism, and seems to be of major importance in the development of tumors in experimental animals treated with methylating carcinogens (11–12). The observation (13–15) of a high frequency of G→A mutations in the K-ras or p53 genes of human colon and lung tumors lacking O6-meG–DNA methyltransferase (MGMT), an enzyme that specifically repairs O6-meG in DNA (16), implies a possible etiologic involvement of this DNA lesion in the carcinogenic mechanism, underlining the need for the study of its presence in human tissues.

Experimental studies have shown that O6-meG is formed in DNA with particularly high efficiency by SN2-type methylating agents (17), best represented by the N-nitroso compounds (NOC), a group of chemicals to which there is widespread human exposure and which include many powerful experimental carcinogens (18). Therefore, the levels of this DNA adduct in humans...
may quantitatively reflect exposure to NOCs or other methylating carcinogens and possibly serve as a biomarker of cancer risk. The potential of O6-meG to serve as a biomarker of exposure to NOCs is of particular interest, given the difficulty of estimating human exposure to dietary or endogenously formed NOCs.

Despite the recognized significance of the presence of O6-meG in human DNA, investigations of its occurrence in human populations have been relatively limited (17). In the first study to report the presence of this adduct in human DNA, Foiles et al. (19) used an HPLC/radioimmunoassay method to detect it in 5 of 20 samples of human placenta at levels in the range 0.8 to 32 adducts/10^8 nucleotides. Subsequently other investigators found the adduct in DNA from human white blood cells, 0.1–0.9 adducts/10^8 nucleotides, and liver, 2.2–13 adducts/10^8 nucleotides (20, 21). In a medium-size study, O6-meG was detected using the "competitive repair assay" in 21 of 407 samples of blood leukocyte DNA, collected from individuals from 17 regions worldwide, at levels ranging from 1.65 (limit of detection) to 5.9 adducts/10^8 nucleotides (22). In this study, O6-meG was found more frequently in subjects from regions with higher consumption of nitrate-treated foods and incidence of gastric cancer. Finally, an investigation in nonsmoking, asymptomatic women, using a more sensitive variation of the same assay, revealed the presence of O6-meG in maternal and cord blood leukocyte DNA in approximately 80% of 35 mother–baby pairs (0.35–4.2 adducts/10^8 nucleotides; ref. 23).

Systematic, population-based studies of the presence of O6-meG in human DNA and its association with specific dietary or environmental exposures would be of great value in the evaluation of human exposure to NOCs, dietary or endogenously formed, and might help to elucidate the possible role of this group of chemicals in human carcinogenesis. Such studies have so far been hampered by the lack of analytic methods of sufficient sensitivity and throughput to permit their use with relatively large cohorts. We wish to report here the development and validation of a new ELISA-type assay for O6-meG that provides substantially improved sensitivity and throughput and is suitable for large-scale molecular epidemiology studies. The method is based on the use of restriction enzymes to digest DNA to fragments up to 4,000 base pairs (bp), which are expected to contain only 1 or no O6-meG residues. Antisera raised against the adduct are subsequently used to remove adduct-containing fragments from the bulk of the DNA and transfer them to a solid surface where they are detected using anti-ssDNA antisera. The high ratio of nucleotides to adducts in each fragment provides strong signal enhancement and therefore high sensitivity. After extensive validation, the method was used for the investigation of the presence of O6-meG in maternal and cord blood leukocyte DNA obtained from a mother–child cohort in Greece.

Materials and Methods

N-Methyl-N-nitrosourea (MNU), guanine, adenine, N7-meg, O6-meG, and MGMT were obtained from Sigma Chemical Co. A high-binding, 96-well, microtiter plates for ELISA were from Greiner Labortechnik. Antisera were mainly obtained from CHEMICON Inc., whereas specific reagents for ELISA such as l-block (casein) and CDP-Star with Emerald II were obtained from Tropix. Rabbit anti-O6-meG antisera were raised against an O6-meG–BSA conjugate and their IgG fraction was semipurified using a commercially available protein A purification kit (Sigma Chemical Co.). Restriction enzymes were obtained from New England Biolabs Inc. All other biochemicals were from Sigma Chemical Co. unless otherwise stated.

Purification of DNA

The purification of DNA from cells, buffy coats, and tissues was carried out with QIAGEN Blood & Cell Culture DNA mini and midi purification kits, using the manufacturer’s procedure with minor modifications. DNA purified with this method had an average length of 35,000 to 50,000 bp.

Cell culture

A HeLa Tet-On cell line (MGMT deficient:Mer−, MMR proficient; Clontech Tet-On Gene Expression Systems and Cell Lines) was used. To minimize the possible presence of endogenously formed O6-meG in control DNA used in the immunoassay, the control DNA was isolated from the cells in which expression of the human MGMT gene could be induced to very high levels (upto 50 fmol protein/μg DNA) by culturing the cells in the presence of doxycycline (V. Pletsa et al., unpublished data). The same cell line, without any transformation, was used for adduct detection after exposure to MNU. Both native and transformed HeLa cell lines (no MGMT and inducible MGMT) were grown in DMEM (Gibco; containing glucose 4.5 g/L, L-glutamine, and pyruvate) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) at 37°C in a humidified incubator with 5% CO2.

Animal studies

Pairs of Sprague–Dawley rats (200–250 g), fed normal rat chow and water ad libitum, were injected intraperitoneally (i.p.) with N-nitrosodimethylamine (NDMA) dissolved in physiologic saline at doses of 0.01 to 1 mg/kg and sacrificed after 6 hours. DNA was isolated from rat liver and analyzed for O6-meG.

Standard O6-meG–containing DNA

DNA standards containing different levels of O6-meG were prepared with DNA from HeLa cells. One milligram of purified DNA was dissolved in 0.1 mol/L of Tris-HCl, pH 7.4, and treated overnight at 37°C with different...
amounts of MNU that had been dissolved in DMSO shortly before use and diluted in complete medium to the required concentrations. The DNA was then precipitated with cold ethanol, washed, dried, and redissolved in distilled water prior to the determination of its O6-meG content by HPLC. For this determination, the DNA was subjected to acid depurination (0.1 mol/L of HCl, 70°C, 30 minutes) and the supernatant was analyzed on a Particil SCX column by using as elution buffer 20 mmol/L of ammonium formate, pH 4, and flow 2 mL/min. Detection was based on UV (254 nm) and fluorescence (excitation 286 and emission 366 nm) and comparison of the eluted bases with a standard mixture of purines that were quantified by their UV absorption. The extinction coefficient used for O6-meG was ε = 9,800 (ε = 280; pH 7).

For the determination of the specific activity of MGMT (used to selectively and quantitatively remove O6-meG from DNA), standard [3H]-methylated DNA was used. Commercial calf thymus DNA was methylated with [3H]-N-methyl-N-nitrosourea (17 Ci/mmol of MNU; Amersham Biochemicals), purified by repeated ethanol precipitations, and analyzed as described earlier. The eluting O4-[3H]-meG peak was detected by liquid scintillation counting and quantified on the basis of the specific activity of MNU. Prior to its use as a substrate to determine the specific activity of a human MGMT preparation, this DNA was depleted of its N-methylpurine content by heating to 80°C for 16 hours in neutral buffer followed by extensive dialysis. For MGMT activity quantitation, this substrate was incubated overnight with purified human MGMT, followed by acid precipitation, exhaustive washing of the precipitate, and radioassaying as previously described (24).

**Determination of O6-meG in DNA by ELISA**

The conduct of the newly developed assay is schematically described in Figure 1. A 96-well microtiter plate was coated with anti-rabbit IgG by the addition of 100 µL of antiseraum (10 µg/mL) in coating buffer (Na2CO3 0.015 mol/L, NaHCO3 0.035 mol/L; pH 9.6) and overnight incubation at 4°C. After washing 3 times with water, nonspecific binding sites were blocked with 310 µL of casein (0.25% in PBS/0.5% Tween) for 1.5 hours at 37°C. After washing 2 cycles of PBST. In the meantime, duplicate samples of DNA (unknown or standard, 1 µg dissolved in 55 µL of buffer) were exhaustively digested with MspI (5 × 2 units/µg DNA) by overnight reaction in microcentrifuge tubes at 37°C, using the supplier’s buffering conditions. The digested DNA was then made single-stranded by heating at 95°C for 10 minutes, snap frozen in liquid nitrogen, and placed on ice. Subsequently, an equal volume of rabbit anti-O6-meG antiserum, previously purified with protein A (diluted 3,750-fold in 2 x PBS, 1% Tween, 0.5% casein), was added and allowed to react with adduct-containing DNA fragments at 37°C for 1.5 hours in a shaking water bath. After this incubation, 100 µL of each DNA-antiserum mixture was transferred to a well of the microplate prepared as described earlier and incubated for 1.5 hours at room temperature to allow binding of the antibody-bound DNA complexes, followed by washing with 5 cycles of PBST. Two sequential steps then followed: (a) addition of semipurified monoclonal anti-ssDNA mouse antibody diluted 100-fold in PBST, 0.25% casein, and (b) addition of goat anti-mouse IgG conjugated with alkaline phosphatase and diluted also in PBST, 0.25% casein. Each step was followed by washing with 5 cycles of PBST. Finally, 2 extra washing cycles with Tris buffer (20 mmol/L Tris–1 mmol/L MgCl2, pH 9.5) were carried out. A chemiluminescence signal was finally generated by the addition of CDP-Star substrate containing Emerald II enhancer (100 µL) and incubation at room temperature for 30 minutes. Chemiluminescence was measured using a Safire II Microplate Luminometer (TECAN) at 542 nm.

For the quantitation of O6-meG in unknown DNA samples, a standard curve consisting of DNA standards with known O6-meG content was constructed with each microplate well. These standards were prepared by mixing HeLa DNA, methylated with MNU, and accurately analyzed by HPLC as described earlier (O6-meG content: 1 adduct/13,000 nucleotides), with unmodified HeLa DNA extracted from MGMT-overexpressing cells as described earlier.

**Human study**

Samples of Buffy coat were collected in the context of the NewGeneris project from subjects participating in the
Rhea mother-child cohort, Crete (25), which is investigating the association between maternal exposures during pregnancy and birth outcomes and childhood disease. Ethical clearance for the study was provided by the University of Crete Ethics Committee and informed consent was obtained from all participating subjects. Venous blood from the mother and from the umbilical cord were collected at delivery in heparinized Vacutainer tubes and processed for buffy coat preparation within 1 hour. The buffy coats were stored at –80°C until transfer to the assay laboratory. In the present report, data are presented for part (120 mother-child pairs) of the cohort under study.

**Results**

A new sandwich ELISA method for the determination of O6-meG in DNA, which provides high sensitivity and throughput appropriate for use in large-scale human studies, has been developed. The principle of the method (Fig. 1) is based on the restriction digestion of the DNA to be analyzed to fragments of such size that for DNA with damage levels corresponding to general environmental exposure, practically all resulting fragments contain 1 or no adduct residues. The adduct-containing DNA fragments thus generated are captured on a microwell plate surface using anti-adduct antibodies whereas the remaining DNA fragments are removed and may, in principle, be used for the determination of a different adduct. The O6-meG captured on the solid surface, along with the DNA fragments in which it is located, is subsequently quantified indirectly by the reaction of the normal nucleotides of the DNA fragments with anti-ssDNA antibodies. The large ratio of normal nucleotides to adducts provides a strong signal enhancement that results in high sensitivity. Using this assay, a single operator can readily analyze 60 samples per week, a number that may be substantially increased with the automation of some of steps of the assay.

In setting up the assay, optimal conditions were identified by systematically varying the following experimental parameters: incubation temperature of different stages (4°C, room temperature, 37°C), incubation times (1 hour, overnight), antiserum dilutions, restriction enzymes. In relation to the antiserum dilutions, dilutions yielding maximum signal-to-noise ratio were identified for the goat anti-rabbit IgG used for coating the plates and the rabbit anti-O6-meG. For the mouse anti-DNA and the goat anti-mouse IgG–AP conjugate employed in the last 2 steps of the assay, the signal-to-noise ratio continued to increase with decreasing antiserum dilutions, and for this reason, final dilutions were adopted on considerations of material availability and cost (results not shown).

As regard the selection of restriction enzymes, it was anticipated that optimally sized DNA fragments would, on one hand, have to be sufficiently small for the adduct-antibody complex to be efficiently transferred from the aqueous phase to the solid surface and, on the other hand, carry a large enough number of anti-DNA antibody-binding epitopes to provide the required signal enhancement. After preliminary tests with multiple restriction enzymes, 4 enzymes (MspI, Hinfl, AluI, and ScrI) were selected for systematic testing on the basis of their being consistent with the following criteria: (a) yielding complete digestion of DNA to fragments of size giving adequate assay sensitivity, (b) being relatively insensitive to CpG methylation, (c) not requiring extreme conditions (e.g., high temperature), and (d) being relatively inexpensive (in view of the large amounts of enzyme required per digestion). In silico digestion of a randomly selected part of the human genome (210,000 bp), using the NEB-cutter 2.0 software, indicated that the above-mentioned enzymes have 374, 655, 1,140, and 1,709 restriction sites, respectively. As shown in Figure 2A, optimal signal using 5 μg of DNA was obtained with MspI and Hinfl, the 2 enzymes producing longer fragments, whereas the use of enzymes giving shorter (e.g., AluI and ScrI) fragments gave remarkably lower sensitivities. MspI was finally adopted for regular use because its action is not affected at all by CpG methylation sites, unlike Hinfl that is blocked by some combinations of overlapping methylated CpG sites.

**Influence of total DNA quantity**

The sensitivity of the assay, expressed in terms of the lowest adduct frequency that can be detected, depends on the maximum amount of DNA that can be added per well without substantially altering the adduct-specific signal. Figure 2B shows that no significant alteration of the signal generated by 0.5 and 1 fmol O6-meG occurred in the presence of a total amount of DNA in the range 1 to 20 μg.

**Assay sensitivity, precision, and accuracy**

As already mentioned, a standard curve, using DNA of known O6-meG content, was constructed with each microtiter plate and used to calculate the adduct levels in unknown samples analyzed on the same plate, thus minimizing between-assay variation. The standard curve was constructed by mixing human DNA from HeLa cells methylated at a level of 1 O6-meG/13,000 normal nucleotides (thus ensuring that no MspI digestion fragments would contain more than 1 adduct) with O6-meG-free DNA from MGMT-overexpressing HeLa cells. As shown in Figure 3A and B, the chemiluminescence signal obtained was linearly related (r² > 0.98) to the amount of the amount of O6-meG in DNA added at both high (0.5–5 fmol) and low (0.05–0.5 fmol) adduct ranges. The limit of detection of the assay was estimated at 50 attomole O6-meG, whereas the coefficient of variation (CV) was 2.5% and 5.5% for the high and low adduct ranges, respectively. When using 10 μg of DNA per well, the limit of sensitivity corresponds to 1.5 adducts/10⁷ nucleotides.

A number of experiments were conducted to further evaluate the assay’s accuracy and precision: (a) Samples of methylated DNA prepared using [3H]-MNU and analyzed by HFLC as described in Materials and Methods.
Methods were also analyzed using the newly developed ELISA. As shown in Table 1, closely similar values were obtained by the 2 methods. Furthermore, analysis of DNA from HeLa cells treated with MNU concentrations ranging from 0.005 to 1 mM yielded a linear dose–response curve, showing the dynamic range of the assay (Fig. 4A). (b) Pairs of Sprague–Dawley rats were treated with 10 to 1,000 μg/kg of NDMA for 6 hours and the O6-meG levels in liver DNA were measured by the new assay on 2 consecutive days by 2 different researchers. As shown in Figure 4B, a linear dose–response curve was observed and the combined day-to-day and operator-to-operator CV was 8.7%. Additional data on the day-to-day variation of the assay are given in the following text.

**Assay specificity**

To check that the signal obtained in the assay reflects specifically and quantitatively O6-meG present in DNA, standard O6-meG–containing DNA was incubated overnight with MGMT of known specific activity (see Materials and Methods), using amounts expected to remove 33% or 100% of the adduct residues. As shown in Figure 4C, this treatment resulted in the reduction of the slope of the dose–response curve by 33% in one case and complete loss of signal in the other.

**O6-meG in mother and cord blood DNA**

Following the above-mentioned extensive validation, the new assay was employed in the analysis of 120 pairs of maternal and cord blood DNA samples collected from the Rhea mother–child cohort (25). Using 10 μg of DNA per well, 84 (70%) maternal and 68 (57%) cord blood DNA samples contained detectable levels of O6-meG. Adduct levels in maternal DNA had a mean value of 0.65 adducts/10⁸ nucleotides (taking the content of samples with nondetectable adducts as 50% of the limit of detection) and ranged up to 3.64 adducts/10⁸ nucleotides (Fig. 5A). Adduct levels in cord blood had a mean value of 0.38 adducts/10⁸ nucleotides and ranged up to 2.35 adducts/10⁸ nucleotides. There was a weak but significant correlation between adduct levels in maternal and cord blood DNA ($r = 0.28; P < 0.001$; Fig. 5B), with the levels in maternal samples being significantly higher ($P < 0.001$; 2-tailed Wilcoxon test).

To further validate the precision of the new assay when measuring very low adduct levels of environmental relevance, 16 randomly selected maternal or cord blood DNA samples were analyzed for a second time within a period of 2 months. As shown in Figure 6, the repeated measurements were in good agreement, considering that the trace quantities of O6-meG were measured ($r = 0.89, CV = 20\%$).

**Discussion**

Several methods have been developed in the past for the measurement of O6-meG in DNA. Previously reported immunoassays for this DNA adduct (26–29) had a limit of detection of a few femtomoles or more, and the lowest adduct frequency they could detect was of the order of $10^{-7}$ or more. A significantly more sensitive enzymatic assay, based on the repair of O6-meG by MGMT (23, 24), while proving useful in the context of studies with patients on chemotherapy with high doses of methylating drugs, failed to detect the adduct in blood leukocyte DNA of most subjects with general environmental exposure, or required large amounts of DNA (150 μg). Low sensitivity, and an associated requirement for prohibitively large amounts of DNA, also characterizes other reported analytic methods for O6-meG based on...
mass spectrometry (30, 31). Current trends in molecular epidemiology, especially the need to exploit material stored in biorepositories constructed in the context of large population cohorts, make imperative the development of assays that combine high sensitivity and high throughput. While $^{32}$P-postlabeling, thanks to its high sensitivity, has made possible the study in this context of bulky, hydrophobic DNA adducts, it cannot be usefully employed for the study of O6-meG. The assay reported here constitutes a significant advance in this direction by combining a sensitivity that approaches that of $^{32}$P-postlabeling with the convenience and throughput capability of ELISAs.

Imunoassays have been extensively used for the detection of other adducts (e.g., BPDE–DNA adducts; ref. 32). Most of these assays were based on the competition of unknown or standard DNA with plate-immobilized, heavily modified DNA for the binding of specific anti-adduct antibodies and have certain disadvantages: (a) the standard curve is sigmoid, complicating adduct quantitation, (b) the antibodies recognize the adducts in heavily modified DNA with higher affinity than in less modified DNA, and (c) a substantial amount of DNA is required to achieve high sensitivity. Previously reported sandwich ELISAs for DNA adducts aimed at the detection of the adduct-bound anti-adduct IgG molecules, relying on signal amplification at subsequent steps to achieve high sensitivity. In the presently reported assay, the adduct–anti-adduct IgG complexes are initially selectively moved from solution to a solid surface and subsequently detected and quantified by reaction of their normal nucleotide chains with anti-ssDNA antisera. Because the binding site for each anti-DNA antibody extends over approximately 60 nucleotides, a DNA fragment of 1,000 to 4,000 bp (typically produced by MspI), can bind tens of anti-ssDNA IgG molecules, thus providing a substantial initial signal augmentation that is further amplified in subsequent steps by standard immunochromatographic procedures.

Critical for correct adduct quantification by the above-mentioned approach is the initial restriction digestion of the DNA samples to fragments that contain no more than a single adduct residue. After testing various restriction enzymes, MspI was selected for routine use in the assay as giving maximum sensitivity, which reflects the production of fragments that optimally combine, on one hand, efficient transfer from solution to the solid phase and, on the other, the presence of a large enough number of anti-ssDNA binding epitopes. It is worth noting that, following the capture of the O6-meG–containing fragments on

![Figure 3](image_url). Standard curve for O6-meG in DNA; range: 0–5 fmol (A) and 0–0.5 fmol (B). Ten micrograms of DNA were added per well and reactions were carried out in triplicate.

<table>
<thead>
<tr>
<th>Mean O6-meG value measured by HPLC</th>
<th>O6-meG measured by ELISA (same-day triplicates)</th>
<th>Mean (SD) ELISA value</th>
<th>Mean ELISA value as % of HPLC value</th>
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<tbody>
<tr>
<td>Mean O6-meG value measured by HPLC</td>
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<td>Mean (SD) ELISA value</td>
<td>Mean ELISA value as % of HPLC value</td>
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<td>26.1</td>
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<td>87.4</td>
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<td>35.5</td>
<td>30.3, 35.5, 37.2</td>
<td>34.3 (3.6)</td>
<td>96.6</td>
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the surface of the microwells, the remaining DNA is removed and, in principle, may be used in a similar manner for the analysis of additional adducts.

Using the fully optimized assay, it is possible to detect as little as 50 attomole O6-meG, which, for 10 μg DNA per well, corresponds to 1.5 adducts/10⁹ nucleotides. This sensitivity can be increased further by using more DNA per well, as it was found that at least up to 20 μg of DNA may be used without adversely affecting assay performance. Sensitivity can also be further increased, if necessary, by using higher concentrations of mouse anti-ssDNA antiserum as well as of the goat anti-mouse IgG–alkaline phosphatase conjugate. The dynamic range of the assay covers more than 2 orders of magnitude, as indicated by the linear dose–response curves observed with DNA samples obtained after the treatment of rats or cells in culture with different doses of MNU or NDMA, respectively (Fig. 3A and B). The adduct levels measured in these samples are in good agreement with those previously reported by other studies (24, 33). The accuracy of the assay was confirmed by direct comparison of adduct levels measured in the same samples by ELISA and by HPLC using [³H]-O6-meG–DNA, which indicated that adduct levels measured by the ELISA were 87% to 96% of the levels measured by HPLC.
those measured by HPLC. The intra-assay CV, typically below 5%, and the interassay and operator-to-operator CV of 8.8% are excellent and comparable with those of other sensitive methods for adduct detection (32).

The chemical specificity of immunoassays is limited by the specificity of the antisera employed. The anti-adduct antiserum used in the current assay has been raised against O6-meG coupled to a carrier protein and its specificity has been previously shown to be primarily for O6-meG and, to a small degree, other types of O6-alkylguanine adducts (34). The specificity of the currently reported assay for O6-meG was further confirmed by using the MGMT protein to specifically remove known amounts of O6-meG from standard DNA, something that resulted in the expected quantitative loss of signal (Fig. 3C).

The new assay is currently being employed in the NewGeneris project, which involves the analysis of samples from more than 1,000 mother–child pairs. Here, we report the outcome of measurements of O6-meG in DNA from buffy coats of 120 pairs of maternal and cord blood samples from this study. The donors were nonsmoking, pregnant women living on the island of Crete who suffered general environmental exposure. Using 10 μg of DNA per well, measurable levels of adducts were found in 70% of the maternal and 57% of the cord samples at levels (of the order of 1 adduct/10^8 nucleotides) comparable with those previously reported by us using an analytic method that required much larger amounts of DNA (23). A weak but statistically significant correlation between maternal and cord blood adducts was observed, with levels in cord blood being, on average, 42% lower than those in maternal blood. The relatively weak correlation may reflect involvement of an unstable ultimate methylating species or varying maternal and fetal metabolic capacities. However, the presence of this correlation implies that transplacental transfer contributes to fetal exposure to methylating agents. The source of the observed adducts is not known; however, their frequent occurrence is compatible with their resultant exposure to dietary or endogenously formed methylating agents, including NOCs. Further evaluation of the dietary and other determinants of the adduct levels in this population is ongoing.

In conclusion, we have developed and validated a highly sensitive assay for the detection of O6-meG suitable for large-scale epidemiologic studies. The approach used in the development of the currently reported assay can, in principle, be used for the development of assays for any DNA adduct for which antibodies are available and, in our hands, has so far led to the successful development of sensitive assays for O6-meG (present report) and PAH–DNA adducts (unpublished). In addition, using this assay, we have shown the presence of O6-meG, a precarcinogenic adduct, in maternal and cord leukocyte DNA in a large proportion of women with general environmental and dietary exposure, indicating that DNA methylation constitutes a significant genotoxic burden for the general population.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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