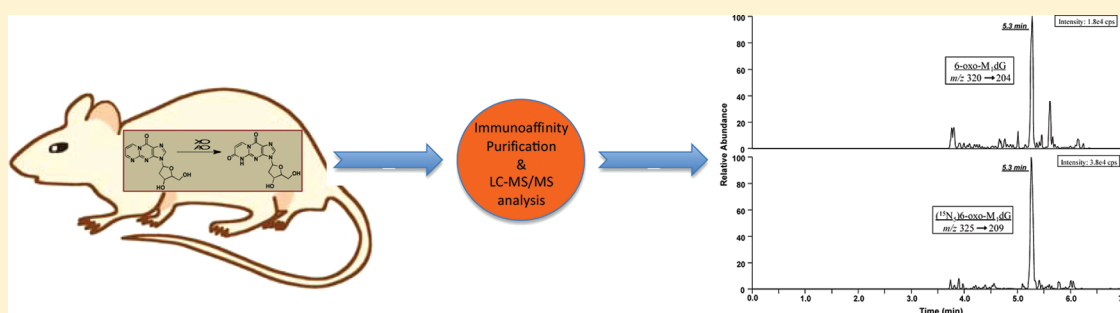


Selection of Monoclonal Antibodies Against 6-oxo-M₁dG and Their Use in an LC-MS/MS Assay for the Presence of 6-oxo-M₁dG in Vivo

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S Supporting Information



ABSTRACT: Oxidative stress triggers DNA and lipid peroxidation, leading to the formation of electrophiles that react with DNA to form adducts. A product of this pathway, (3-(2'-deoxy- β -D-erythro-pentofuranosyl)-pyrimido[1,2- α]purine-10(3H)-one), or M₁dG, is mutagenic in bacterial and mammalian cells and is repaired by the nucleotide excision repair pathway. In vivo, M₁dG is oxidized to a primary metabolite, (3-(2'-deoxy- β -D-erythro-pentofuranosyl)-pyrimido[1,2- α]purine-6,10(3H,5H)-dione), or 6-oxo-M₁dG, which is excreted in urine, bile, and feces. We have developed a specific monoclonal antibody against 6-oxo-M₁dG and have incorporated this antibody into a procedure for the immunoaffinity isolation of 6-oxo-M₁dG from biological matrices. The purified analyte is quantified by LC-MS/MS using a stable isotope-labeled analogue (¹⁵N₅]-6-oxo-M₁dG) as an internal standard. Healthy male Sprague–Dawley rats excreted 6-oxo-M₁dG at a rate of 350–1893 fmol/kg·d in feces. This is the first report of the presence of the major metabolite of M₁dG in rodents without exogenous introduction of M₁dG.

INTRODUCTION

M₁dG (3-(2'-deoxy- β -D-erythro-pentofuranosyl)-pyrimido[1,2- α]purine-10(3H)-one) is one of many endogenous exocyclic lesions derived from the reaction of DNA with bifunctional electrophiles generated by lipid, protein, or DNA peroxidation. Being an endogenous constituent of human and rodent genomes,^{1–3} M₁dG is mutagenic in bacterial and mammalian cells^{4,5} and leads to mispairing when replicated in vitro by multiple different DNA polymerases.⁶ M₁dG is repaired by nucleotide excision repair.⁴

We have previously developed a monoclonal antibody against M₁dG⁷ and incorporated the antibody into analytical schemes using immunoaffinity purification of M₁dG followed by mass spectrometric detection.^{3,8} These studies indicated that adult humans excrete M₁dG at a rate of 12 fmol/kg·d in urine.⁸ Subsequent investigations into the metabolism and elimination of M₁dG revealed that administration of M₁dG to rodents at doses from 8 mg/kg to 6 pg/kg leads to its rapid disappearance from plasma due to oxidation and formation of the single, stable metabolite, 6-oxo-M₁dG (3-(2'-deoxy- β -D-erythro-pentofuranosyl)-pyrimido[1,2- α]purine-6,10(3H,5H)-dione).^{9–11} In both humans and rats, xanthine oxidase appears to be

responsible for this oxidation, while aldehyde oxidase also plays a role in M₁dG metabolism in humans.^{10,11} Because 6-oxo-M₁dG is the sole metabolite of M₁dG in rats, it appears to be an ideal surrogate for M₁dG as an in vivo biomarker for oxidative stress.

All studies of M₁dG metabolism *in vivo* have been conducted with exogenously administered deoxynucleoside. Thus, it is not known if 6-oxo-M₁dG is actually present in unadulterated intact animals. In order to address this question, we developed a highly specific monoclonal antibody (mAb) against 6-oxo-M₁dG and covalently linked the antibody to sepharose beads. The resultant antibody-sepharose matrix (the gel) was used for the immunoaffinity purification of 6-oxo-M₁dG from urine and feces followed by LC-MS/MS quantification against the stable isotope-labeled internal standard, [¹⁵N₅]-6-oxo-M₁dG. Utilizing this method, we report for the first time that 6-oxo-M₁dG is endogenously produced in rodents and is excreted in urine and feces.

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MATERIALS AND METHODS

Na₂CO₃, NaHCO₃, NaCl, water, acetonitrile, mariculture keyhole limpet hemocyanin (mckLH), HCl, acetic acid, and methanol were obtained from Thermo Fisher Scientific (Waltham, MA, USA). ABTS (diammonium salt), thimerosal, KH₂PO₄, Na₂HPO₄, KCl, 30% H₂O₂, Tween 20, bovine serum albumin (BSA), dimethylformamide (DMF), dimethylsulfoxide (DMSO), K₂CO₃, sodium periodate, sodium methoxide, NaBH₄, hypoxanthine, xanthosine, xanthine, ethyl *cis*-3-iodoacrylate, ethyl *cis*-3-bromoacrylate aminopterin, ethanolamine, cyanogen bromide-activated sepharose beads, diethylene glycol, 2'-deoxyguanosine, sodium hydroxide, formic acid, sodium azide, glycine, and citric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat antimouse IgG (H + L) [0.8 mg/mL] and IgG (Fc) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Phosphate buffered saline (PBS) was purchased from Invitrogen (Carlsbad, CA, USA). Chemicals were used as received from these commercial sources unless specified. Solvents were HPLC-grade purity or higher.

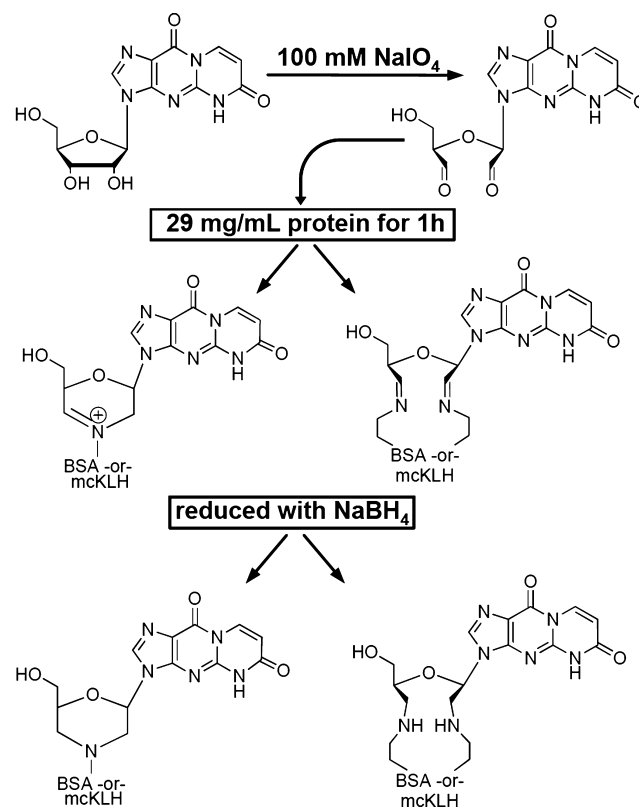
Synthesis of 6-oxo-M₁Guo. The synthesis of 6-oxo-M₁Guo was based on a previously described synthetic scheme¹¹ with some modifications. Guanosine was dissolved in a minimum amount of a DMF/DMSO mixture (1:1, v/v). K₂CO₃ (1.5 equiv) was added to the solution and the reaction mixture was held at 60–65 °C for 18 h. Ethyl *cis*-3-iodoacrylate (0.25 equiv per h for 5 h) was added to the reaction mixture. Solvents were evaporated using a high-vacuum rotary evaporator. The residue was dissolved into a minimum amount of methanol, and sodium methoxide (1.5 equiv) was added (dropwise in a 0.5 M solution). 6-oxo-M₁Guo was purified on Biotage SP1 flash chromatography system (Biotage, Uppsala, Sweden) using the following gradient; 1% to 5% acetonitrile in 30 column volumes followed by a 5% to 15% gradient in 10 column volumes. Separation was achieved on a 12 + M C18 column, and the purified compound showed a single sharp peak by reverse-phase HPLC. ¹H NMR (600 MHz, DMSO-*d*₆): δ 8.65 (d, 1H, H8, *J* = 7.8 Hz), 8.29 (s, 1H, H2), 6.24 (d, 1H, H7, *J* = 8.4 Hz), 5.82 (d, 1H, H1'), 5.48 (d, 1H, H2'-OH), 5.20 (d, 1H, H3'-OH), 5.03 (d, 1H, H5'-OH), 4.49 (d, 1H, H2'), 4.15 (d, 1H, H3'), 3.91 (d, 1H, H4'), 3.63–3.55 (m, 2H, H5'). The ¹H NMR may be seen in Figure A of the Supporting Information.

Synthesis of 6-oxo-M₁dG and Stably Labeled Analogue. 6-oxo-M₁dG and its stable isotope-labeled analogue, [¹⁵N₅]-6-oxo-M₁dG, were synthesized as previously described.¹² Briefly, anhydrous dG (1.6 g, 6 mmol) and anhydrous K₂CO₃ (0.93 g, 6.75 mmol) were dissolved in anhydrous DMF (18 mL). One milliliter of a 0.55 M solution of ethyl *cis*-3-bromoacrylate in anhydrous DMF was added to the reaction every 15 min for 4 h. The reaction was stirred for an additional 2 h then cooled and filtered. The filtrate was evaporated under high vacuum and the residue was dissolved in water (4 mL) and 5% acetic acid (4 mL). The product was purified by reversed-phase (C18) medium pressure liquid chromatography with a Biotage SP1 apparatus (Biotage) using a prepacked 25 × 75 mm Biotage FLASH 25 + S cartridge (KP-C18-HS) and a FLASH 25 C18 samplelet for sample loading. A pure final product, 6-oxo-M₁dG (0.92 g, 50.6%), was obtained. [¹⁵N₅]-6-oxo-M₁dG was synthesized by substituting [¹⁵N₃]-dG for dG (Cambridge Isotope Laboratories, Andover, MA, USA) and employing the scheme described above. The ¹H NMR of 6-oxo-M₁dG and its stably labeled analogue matched the spectrum previously reported for this compound.¹¹ The purified products both showed only one peak by reverse-phase HPLC. Mass spectrometric analysis of 6-oxo-M₁dG showed an [M + H]⁺ peak at *m/z* 320, while analysis of [¹⁵N₅]-6-oxo-M₁dG showed an [M + H]⁺ peak at *m/z* 325. The *m/z* = 325 corresponds to the 6-oxo-M₁dG value plus 5 additional mass units from the 5 ¹⁵N atoms incorporated into the [¹⁵N₃]-dG starting material. A *m/z* 320 peak was not observed for [¹⁵N₅]-6-oxo-M₁dG, indicating that no unlabeled 6-oxo-M₁dG was present in the internal standard.

Conjugation of 6-oxo-M₁Guo to BSA and mckLH. 6-oxo-M₁Guo (12 mg) was dissolved in 750 μL of 100 mM aqueous sodium periodate. Protein (20 mg of BSA or mckLH) was reconstituted with 700 μL of PBS preadjusted to a pH of 9.5 with 5% K₂CO₃. The 6-oxo-

M₁Guo solution and the protein were combined and agitated. After 1 h, 45 μL of a 1 M diethylene glycol solution was added to the mixture (to quench excess oxidizing agent) followed by 700 μL of 0.45 M NaBH₄ (aqueous). After another 12 h, the pH of the mixture was adjusted to ~7.0 with 1.0 M formic acid. The mixture was kept at this pH for 1 h. Then, the pH was increased to 8.5 by the careful addition of 1 M aqueous ammonium hydroxide solution. This mixture was dialyzed against PBS buffer twice for 24 h. The sample was lyophilized and stored at 4 °C. Scheme 1 shows the conjugation reaction of 6-oxo-

Scheme 1. Conjugation Reaction between 6-oxo-M₁Guo and the Carrier Protein (BSA or mckLH)



M₁Guo with a lysine residue of the carrier protein. Figure B of the Supporting Information depicts the scheme for preparation of the conjugated protein.

Immunization and Hybridoma Preparation. Four BALB/cJ mice and four A/J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were injected subcutaneously with 50 μg of 6-oxo-M₁Guo-KLH and Freund's complete adjuvant (primary boost). Four wk after the initial immunization, the mice were boosted subcutaneously (first boost) with the same dose of conjugate, but with the substitution of incomplete adjuvant (which was also used in subsequent boosts). Two wk after the first boost, the mice were tail bled and antibody titers were assessed by direct and competitive ELISA as described below. A second boost was administered 4 wk subsequent to the first, and after 2 wk, sera were again extracted and subjected to ELISA evaluation. A third boost was administered 25 wk after boost two, and the sera were collected and screened approximately 2 wk later. On the basis of the cumulative ELISA data, a single BALB/cJ mouse (BALB/cJ R) showing the most selective and concentrated anti-6-oxo-M₁dG titer was chosen for a fourth and final boost given intraperitoneally and lacking adjuvant to prepare it for splenocyte extraction. Four days after the final boost, the mouse was sacrificed by cervical dislocation and the spleen harvested.

Splenocytes were isolated and subjected to polyethylene glycol-mediated fusion with both Sp/20 and NS1 murine myeloma cells (obtained from the University of Virginia and the University of

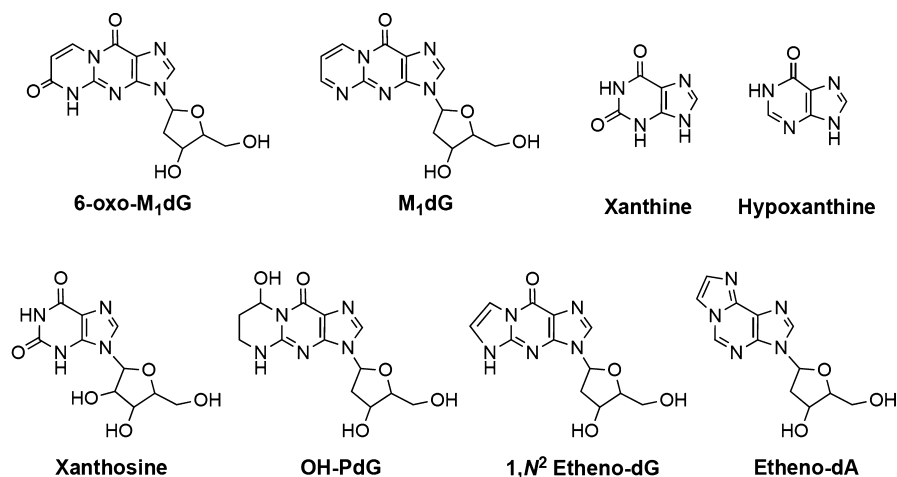


Figure 1. 6-oxo-M₁dG, M₁dG, and structural analogues that were used as competitive antigens during ELISA analysis of murine sera, hybridoma supernatant, and purified antibodies.

Nebraska Medical University School of Dentistry, respectively) and allowed to recover for 24 h in liquid culture. The products of the fusion were evenly distributed into 24 96-well plates, with 12 plates corresponding to fusions of Sp/20 cells and splenocytes and 12 plates corresponding to fusions of NS1 cells and splenocytes. Cells were grown in Iscove's Modified Dulbecco's Medium with 20% Fetal Bovine Serum (Invitrogen). Hybridomas were selected by growing in the presence of aminopterin (5×10^{-8} M, Sigma) and HT supplement (1:100 dilution, Invitrogen) for 14 days with media and aminopterin replenishment every 3 days.

Supernatant was removed and screened for antigen-specific antibodies by ELISA, with 6-oxo-M₁dGuo-BSA conjugate used as the antigen (described below). Positive hybridoma supernatants were rescreened in the presence of xanthine and 6-oxo-M₁dG. Xanthine was employed as a competitor to identify antibodies not specific to 6-oxo-M₁dG. Clones exhibiting anti-6-oxo-M₁dG activity and specificity in addition to positive growth and productivity were selected and plated on 24-well plates. Of these, five clones were chosen based on activity, specificity, growth, and productivity as determined by direct and competitive ELISA analysis (described below) and then subsequently subjected to two rounds of subcloning. The clones were subsequently cryopreserved.

Note. Under Vanderbilt Institutional Care and Use Committee Protocol #M-07-109, the Vanderbilt Antibody and Protein Resource Core is permitted to use Freund's complete adjuvant in the manner described above.

ELISA Procedure. The following solutions were prepared as follows and their use is described below: (a) carbonate-bicarbonate coating buffer (pH 9.6) was prepared from Na₂CO₃ (1.59 g/L), NaHCO₃ (2.39 g/L), and thimerosal (0.10 g/L); (b) PBS-Tween (pH 7.4) was prepared from NaCl (8.00 g/L), KH₂PO₄ (0.20 g/L), Na₂HPO₄ (1.15 g/L), KCl (0.20 g/L), Tween 20 (1.00 mL/L), and thimerosal (0.10 g/L); (c) BSA (5.0 g) mixed in PBS-Tween (500 mL); (d) 1 nM ABTS solution in 70 mM citrate-phosphate buffer (pH 4.2) was prepared from citric acid (5.64 g/L), Na₂HPO₄ (5.84 g/L), and AzBTS-(NH₄)₂ (0.548 g).

ELISA quality microtiter plates (Thermo Fisher Scientific; 96-well Immulon 2HB flat bottom microtiter plates or 384-well 4HB flat bottom plates) were coated with 50, 75, or 100 μ L of a 10 μ g/mL solution of 6-oxo-M₁Guo-BSA antigen in carbonate-bicarbonate coating buffer (a) and incubated at 4 $^{\circ}$ C overnight. Plates were washed three times with 100 μ L of PBS-Tween (b) using a Bio-Tek ELx405 automatic microplate washer (Winooski, VT, USA), then incubated with 100 μ L of PBS-Tween (b) for 30 min at 37 $^{\circ}$ C. After 30 min, the PBS contents were discarded. Aliquots of murine serum dilutions, hybridoma supernatants, or purified antibody (depending on the stage of the antibody development process) were then incubated in the coated wells with or without the addition of varying concentrations

of 6-oxo-M₁dG and structural analogues (Figure 1) in a final volume of 100 μ L PBS for 60 min at 37 $^{\circ}$ C. For wells that included 6-oxo-M₁dG or structural analogues, the serum dilution used was 1:5000 in PBS-Tween (b), and the compounds depicted in Figure 1 were preincubated in the dilute murine serum for 45 min prior to the serum addition step. This allowed the nonspecific antibodies to bind the structural analogues, theoretically leaving behind only 6-oxo-M₁dG-specific antibodies. Initial analysis revealed that xanthine was recognized nearly as well as 6-oxo-M₁dG, which led to the use of this structural analogue in further competition studies.

The plates were then washed three times with PBS-Tween (b). Aliquots of diluted horseradish peroxidase-conjugated goat antimouse IgG Fc region-specific or IgG (H + L) secondary antibody (diluted 1:5000 in PBS-Tween/BSA) were added, and the plates were incubated at 37 $^{\circ}$ C for 60 min. After 60 min, the plates were washed three times and 100 μ L of a freshly prepared ABTS solution (1.8 μ L of H₂O₂ per 1 mL of ABTS) was immediately added to each well. To determine peroxidase activity, the absorbance at 414 nm was measured after 15 and 30 min for each well using a Bio-Tek Powerwave HT 340 plate reader with Gen5 software.

Antibody Purification and Isotype Determination. Selected clones were scaled up, inoculated into one-liter bioreactors (Wilson Wolf Manufacturing, New Brighton, MN, USA) and grown for 3–4 wk. Purification of mAb from the bioreactor supernatants was achieved by affinity chromatography on Protein-G sepharose (GE Healthcare, Piscataway, NJ, USA) followed by a final desalting step into PBS. Purified mAb were isotyped and subsequently quantified by SDS-PAGE electrophoresis followed by infrared Coomassie staining.

Linking Antibody to Sepharose Beads. Purified anti-6-oxo-M₁dG mAb was loaded onto PD-10 columns (GE Healthcare) equilibrated with 25 mL coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.0 in water). The anti-6-oxo-M₁dG mAb was eluted with 2.5 mL coupling buffer. Cyanogen bromide-activated sepharose beads (3.75 g) were prepared for conjugation by rapid swelling in 1 mM HCl followed by filtering, rinsing with 400 mL of 1 mM HCl and then 10 mL of coupling buffer. The sepharose beads were transferred to the antibody solution and gently rotated at 4 $^{\circ}$ C for about 20 h. The mixture was then filtered and washed with 500 mL of coupling buffer, and the residue was divided into four parts. Each quarter was placed in 14 mL of ethanalamine solution (1.0 M, pH 8.0) and gently rotated overnight at 4 $^{\circ}$ C. The sepharose beads were then filtered and washed with a low pH acetate buffer (0.1 M acetic acid, 0.1 M NaOH, and 0.5 M NaCl) followed by a 0.1 M Tris HCl buffer (0.5 M NaCl, pH 8.0). Each wash used 75 mL of buffer, and the wash cycle was repeated two more times. The resulting residue was suspended in 20 mL of 0.1 M Tris buffer with 0.5 M NaCl and 0.2% NaN₃ (pH 8.0), and the final product (the gel) was stored at 4 $^{\circ}$ C.

6-oxo-M₁dG Purification and Analysis. The gel was used to recover 6-oxo-M₁dG and [¹⁵N₅]-6-oxo-M₁dG from various fluids by the following general procedure. An aliquot of gel was prepared for use by rinsing with the following solutions: 50 mL of 0.1 M glycine (pH 2.7), 20 mL H₂O, 50 mL methanol, and 50 mL PBS. The gel was then resuspended in 3–5 mL of PBS. This cleaning procedure was necessary to remove any 6-oxo-M₁dG that had bound to the mAb during preparation.

The pH of the fluid to be analyzed was adjusted to 7.0–8.0 with acetic acid or ammonium hydroxide. An aliquot of the washed and reconditioned gel was added to the sample, and the suspension was mixed gently for 1–4 h. The sample/gel suspension was filtered through a precleaned empty polypropylene SPE cartridge fitted with a polypropylene frit (Sigma-Aldrich). The filtered gel was washed with 10 mL of PBS, and the analytes were eluted with 2 mL of methanol. The eluant was dried under N₂ gas, capped, and stored at –20 °C. The sample was reconstituted in 9:1 water/methanol (v/v) immediately prior to LC-MS/MS analysis.

Rat Urine and Fecal Sample Collection and Analysis. Male Sprague–Dawley rats were housed in metabolic cages. Urine and feces were collected at 24 h intervals and stored at –20 °C following collection. Urine samples were analyzed by thawing, adding 200–500 fmol of [¹⁵N₅]-6-oxo-M₁dG, and centrifuging the samples at 4000g for 25 min at 4 °C. An aliquot of supernatant was diluted 10–15-fold with PBS, and the pH of the final solution was adjusted to 7.5 ± 0.5 with either acetic acid or sodium hydroxide. The gel (prepared as described above) was introduced after pH adjustment, and the suspension was rotated gently end-over-end for 1–4 h. The suspension was filtered and the analyte eluted from the gel as described above.

Stored feces were thawed and weighed. The weighed sample was transferred to a clean vessel with 40 mL of PBS, and 200–500 fmol of [¹⁵N₅]-6-oxo-M₁dG were added. Dispersion of the sample was accomplished by rotating end-over-end for 2–3 h followed by sonication for 3–5 min. The suspension was then filtered through a mesh screen, with the collected solid material rinsed with 10 mL PBS. The filtrate was centrifuged at 4000g for 25 min at 4 °C. The supernatant was transferred to a clean vessel, diluted roughly 5 times with PBS, and the antibody gel (prepared as described above) was added. The suspension was rotated gently end-over-end for 1–4 h, filtered, and the analyte eluted from the gel as described above.

LC-MS/MS Analysis. Final LC-MS/MS analysis of gel-purified samples was accomplished on a Thermo Surveyor autosampler and MS pump in-line with a Thermo Quantum triple quadrupole mass spectrometer (Thermo Fisher Scientific). The mass spectrometer was equipped with an electrospray source and operated in positive ion mode. 6-oxo-M₁dG and its internal standard, [¹⁵N₅]-6-oxo-M₁dG, were detected via selected reaction monitoring (SRM) with the following transitions, respectively; *m/z* 320 → 204 and 325 → 209. In both cases, the transition corresponds to cleavage of the glycosidic bond and loss of the deoxyribose moiety, with the positive charge remaining on the base.

The analytes were chromatographed on the following reverse-phase gradient system; 2% B to 15% B in 5 min, 15% B to 80% B in 1.5 min, followed by a 1 min hold at 80% B. Component A was water and component B was methanol/acetonitrile (3:1), and each component contained 0.1% acetic acid (v/v). The flow rate was 300 μL/min. The column used was a Phenomenex C18 (Phenomenex, Torrance, CA, USA; 10.0 × 0.2 cm, 3 μm) held at 35–40 °C. The column was equilibrated at the initial conditions for 3 min prior to each injection. Under this chromatographic regime, 6-oxo-M₁dG eluted at approximately 5 min. 6-oxo-M₁dG was quantitated by stable isotope dilution against [¹⁵N₅]-6-oxo-M₁dG. LC-MS/MS data was acquired and processed by Xcalibur software (Thermo Fisher Scientific).

RESULTS

Selection and Purification of the Monoclonal Antibody. The overall scheme of the antibody generation and isolation process is depicted in Figure 2. Eight mice (four BALB/cJ and four A/J) were injected with 6-oxo-M₁G

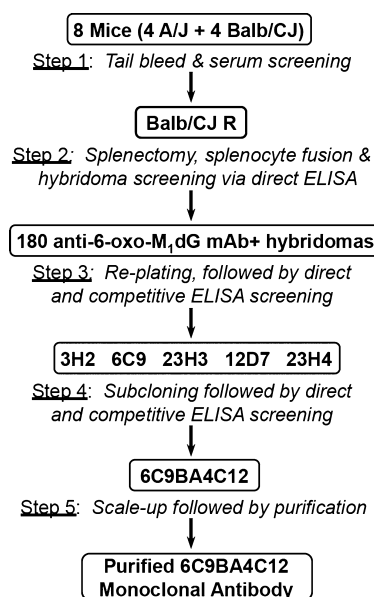


Figure 2. Schematic of monoclonal antibody generation and isolation procedure. Eight mice were inoculated with adduct/protein conjugate, followed by tail bleeding and testing for the presence of 6-oxo-M₁dG antibodies using direct ELISA analysis (step 1). Splenocytes from a single mouse, Balb/CJ R, were fused with myeloma cells, plated on twenty-four 96-well plates, and screened using direct and competitive ELISA analyses (step 2). From this screen, 180 hybridoma cell lines showed anti-6-oxo-M₁dG mAb production. These were replated and screened (step 3). Five parental cell lines were chosen for subcloning and further screening (step 4). On the basis of these final screenings, the cell line 6C9BA4C12 was chosen for scale-up and antibody purification (step 5).

conjugated to KLH as described in the Materials and Methods section. At 6, 10, and 35 wk after the initial inoculation, the mice were tail bled, and direct and competitive ELISA analyses of the sera were performed (Figure 2, step 1). All three bleeds from all 8 mice showed robust responses against the 6-oxo-M₁G-BSA antigen, indicating that the immune systems of the mice were producing significant amounts of antibodies against the 6-oxo-M₁G portion of the inoculant. Preinoculation tail bleeds detected no antibodies against 6-oxo-M₁G. Competitive ELISA screenings were executed to assess antibody specificity, with 6-oxo-M₁dG and a series of structural analogues (Figure 1) serving as competing antigens.

Figure 3 depicts a competitive ELISA analysis of the 35-week serum from mouse BALB/cJ R. This analysis employed 6-oxo-M₁dG as the competing antigen and a robust concentration dependent decrease in optical density is observed, indicating the presence of anti-6-oxo-M₁dG antibodies in the sera from this mouse. The results of similar analyses of the sera of all 8 inoculated mice can be seen in Supporting Information Figure C. Supporting Information Figure D displays the results from competitive ELISA analyses of the 35-week serum from all 8 inoculated mice where M₁dG is employed as the competing antigen. Increasing amounts of M₁dG do not result in a decrease in optical density, indicating that M₁dG does not bind to the anti-6-oxo-M₁dG antibody(ies) present in the sera.

On the basis of the ELISA analysis of murine sera, a single mouse (BALB/cJ R) was chosen for hybridoma production. Splenocytes from BALB/cJ R were fused with both Sp/20 and NS1 myeloma cells (Figure 2, step 2), and 1670 hybridomas were formed from 2304 fusions wells. ELISA analysis revealed

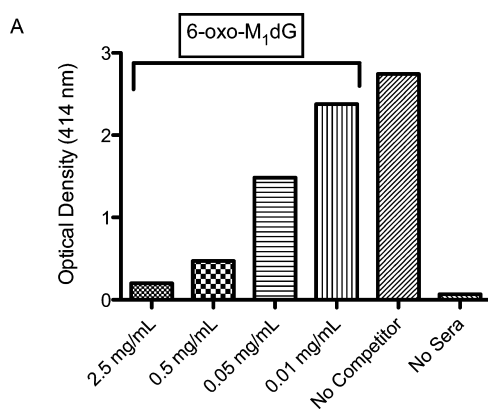


Figure 3. 6-oxo-M₁dG antibodies are present in the serum of mouse BALB/cJ R. The serum of mouse BALB/cJ R was subjected to competitive ELISA analysis with 6-oxo-M₁dG used as the competitor. The displayed results represent the average of spectroscopic readings at 15 and 30 min postaddition of ABTS substrate.

that of the 1670 hybridomas, 180 showed production of anti-6-oxo-M₁dG antibodies. These were grown in 24-well plates (Figure 2, step 3), and the supernatants were subjected to further ELISA screenings. On the basis of the screening data, five hybridomas were selected for subcloning and further screenings (Figure 2, step 4).

Figure 4 shows a representative ELISA analysis of the supernatants from two subclones (B and E2) of parental

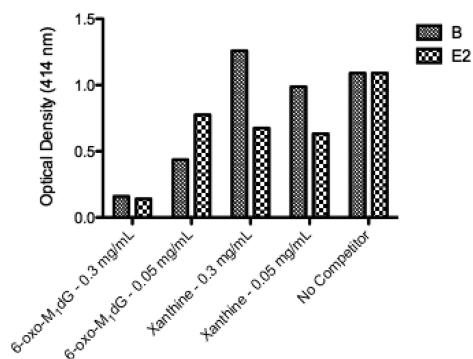


Figure 4. ELISA analysis of specificity of various subclonal antibodies. The specificity of antibodies produced by two hybridoma cell lines (B and E2) subcloned from the parental hybridoma cell line 6C9 were assessed using competitive ELISA analysis. Xanthine and 6-oxo-M₁dG were used as competing antigens. The displayed results represent the average of spectroscopic readings of subclones E2 and B at 15 and 30 min postaddition of ABTS substrate.

hybridoma cell line 6C9 in which xanthine and 6-oxo-M₁dG are employed as competing antigens. The presence of unbound 6-oxo-M₁dG decreases the optical density of both B and E2 supernatants, indicating the presence of anti-6-oxo-M₁dG antibodies. For subclone E2, xanthine has a similar effect, demonstrating that E2's mAb are not specific to 6-oxo-M₁dG. However, xanthine has no effect on the optical density of subclone B supernatant, suggesting that mAb from subclone B are specific to 6-oxo-M₁dG. On the basis of these data, subclone B was subjected to further subcloning but E2 was not. Supporting Information Figure E illustrates that cell line 6C9BA4, a subclone of hybridoma 6C9B, retained activity against 6-oxo-M₁dG but did not react to xanthine.

On the basis of a review of the cumulative ELISA data, a final daughter cell line was selected from each parental hybridoma line, and these daughter cell lines were cryopreserved. The ELISA data indicated that antibodies from the cell line 6C9BA4C12 displayed the most promising expression and specificity. Thus, this cell line was subjected to large-scale expression and antibody purification. The final antibody was isotyped as IgG1-kappa.

Figure 5 shows the ELISA responses of the mAb purified from 6C9BA4C12 in the presence of 6-oxo-M₁dG and structural analogues. Only 6-oxo-M₁dG attenuated the optical density, indicating a specificity of the purified mAb for 6-oxo-M₁dG over close structural analogues.

Performance of Covalently Linked mAb Gel and LC-MS/MS Analysis. The purified anti-6-oxo-M₁dG mAb was covalently linked to sepharose beads as described above. The purification and analysis protocol outlined in Materials and Methods was used to quantify aliquots of 10 mM potassium phosphate (pH 8.0) spiked with varying amounts of 6-oxo-M₁dG (0.25, 2.5, and 25 pmol) and 1.0 pmol of the internal standard, [¹⁵N₅]-6-oxo-M₁dG ($n = 3$ for each concentration of 6-oxo-M₁dG). As shown in Table 1, the experimentally determined amount of 6-oxo-M₁dG was within 12% of the known amount at each level. The average percent recovery of 6-oxo-M₁dG ranged from 94% to 74%, while the internal standard was recovered at an average rate of 79%. Thus, the gel was able to bind 6-oxo-M₁dG from an aqueous solution and released the analyte in the presence of methanol.

The limit of detection (LOD) of the described LC-MS/MS system was established at approximately 10 fmol 6-oxo-M₁dG on-column (Supporting Information Figure F, top) by analyzing a series of increasingly dilute 6-oxo-M₁dG solutions. Given this LOD and the percent recovery observed from urine and fecal samples (approximately 30%), the limit of quantification of the assay was estimated to be 50 fmol per sample. Additionally, the LC-MS/MS system provided a linear response to 6-oxo-M₁dG solutions over a 5000-fold concentration range and a single [¹⁵N₅]-6-oxo-M₁dG concentration (Supporting Information F, bottom).

These results indicate that the gel provided a sufficient level of recovery of the analyte and its internal standard as well as sufficient capacity for the expected levels of 6-oxo-M₁dG present in rat urine and feces. They also demonstrate that the LC-MS/MS analysis of the purified sample provided sensitive and accurate quantification.

Analysis of Rat Urine and Feces for the Presence of 6-oxo-M₁dG. Urine and feces collected from male Sprague-Dawley rats over 3 days were analyzed as described in the Materials and Methods section. Table 2 lists the results of the analysis. 6-oxo-M₁dG was detected in urine from only one subject, animal 3, at a rate of 188 fmol/kg·d. However, 6-oxo-M₁dG was eliminated in feces in all the animals at a rate of 350–1893 fmol/kg·d. Thus, it appears fecal elimination is the main route by which that 6-oxo-M₁dG is excreted from the body.

While the amounts of 6-oxo-M₁dG are low, the assay provided a sufficient signal-to-noise ratio to permit accurate quantification. Figure 6 displays a sample chromatogram from a processed fecal sample. The upper trace (m/z 320 → 204) shows the analyte, while the bottom trace (m/z 325 → 209) represents the [¹⁵N₅]-6-oxo-M₁dG internal standard. Both compounds gave chromatographic peaks that were well above the background noise. The inset of Figure 6 is the internal

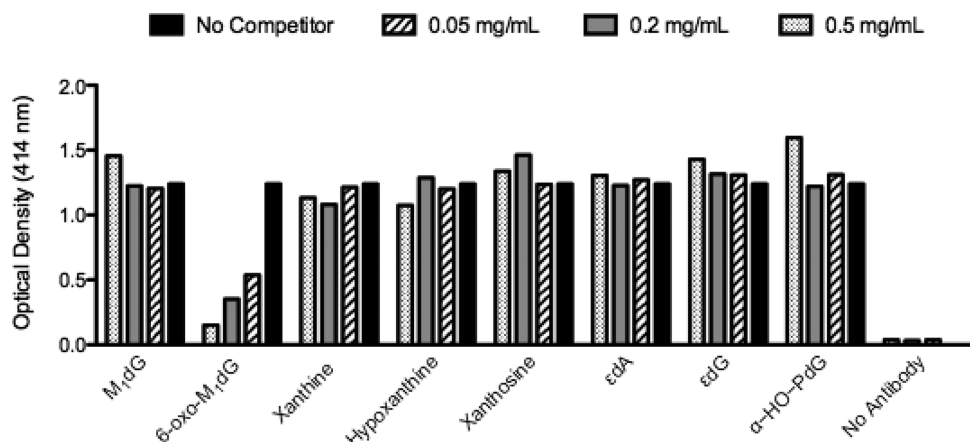


Figure 5. Specificity determination of antibodies produced from subclone 6C9BA4C12. Purified antibodies from the hybridomal subclone (6C9BA4C12) were screened for specificity for 6-oxo-M₁dG in the presence of structural analogues using competitive ELISA analyses.

Table 1. Results of the Analysis of Buffer Spiked with Varying Amounts of 6-oxo-M₁dG and 1.0 pmol of the Internal Standard [¹⁵N]-6-oxo-M₁dG^a

6-oxo-M ₁ dG per sample (pmol)	amount detected (pmol)	% accuracy	% recovery
0.25	0.28 ± 0.02	11.7 ± 7.7%	93.5 ± 7.2%
2.5	2.73 ± 0.2	9.0 ± 6.0%	82.2 ± 6.0%
25.0	26.18 ± 0.6	4.7 ± 2.2%	74.3 ± 2.5%

^aThe amount detected, % accuracy, and % recovery values are shown as the mean ± S.D. ($n = 3$ in all cases). The % recovery of the internal standard was 78.7 ± 3.4%.

Table 2. Amount of 6-oxo-M₁dG Eliminated by Sprague–Dawley Rats via Urine and Feces^a

subject	urine	feces
animal 1	<LOQ	1893 ± 1963
animal 2	<LOQ	350 ± 314
animal 3	188 ± 62	534 ± 404

^aValues are given as the mean amount observed in fmol/kg·d ± S.D. ($n = 3–5$ for feces and 2 for urine).

standard alone after recovery from PBS. There is no peak in the 6-oxo-M₁dG trace of the inset, which is illustrative of the fact that there was no isotopic impurity in the internal standard that could contribute to the 6-oxo-M₁dG signal.

DISCUSSION

M₁dG is an endogenous DNA adduct detectable in the genomic DNA of humans and rodents.^{2,3} Its presence is associated with peroxidative damage to cellular constituents, particularly polyunsaturated fatty acids, and DNA. Although many studies have shown that the lipid peroxidation product, malondialdehyde, can react in vitro with deoxyguanosine to form M₁dG,¹ recent studies in *E. coli*, with defined polyunsaturated fatty acid content in membrane phospholipids, indicate that this is not a major source of M₁dG.¹³ Rather, the DNA peroxidation products, base propenals, appear to account for nearly all of the M₁dG generated when bacteria are exposed to peroxynitrite (ONOO⁻).¹³ Thus, although M₁dG appears to be a reliable marker of oxidative damage to cells, it primarily reflects oxidative damage to DNA.

M₁dG is repaired by nucleotide excision repair⁴ and is excreted in urine at rather low levels in humans (12 ± 3.8 fmol/

kg·d).⁸ A major contributor to the low levels of M₁dG is its oxidative metabolism to 6-oxo-M₁dG.^{9–11} This suggests 6-oxo-M₁dG may be superior to M₁dG as a noninvasive marker of oxidative damage. In the present manuscript, we describe the generation of a monoclonal antibody that exhibits sensitive and specific binding to 6-oxo-M₁dG and its use in a quantitative assay suitable for analysis of urine and feces.

In the assay, the sepharose-bound mAb is used to isolate 6-oxo-M₁dG from urine and feces. The purified sample is subjected to LC-MS/MS analysis where 6-oxo-M₁dG is quantified via stable isotope dilution against the synthetic internal standard, [¹⁵N₅]-6-oxo-M₁dG, which is introduced to the sample prior to antibody purification.

The antibody gel shows excellent recovery of 6-oxo-M₁dG from buffer solutions. The analyte is recovered at 74% or greater from 10 mM potassium phosphate over a 100-fold range of 6-oxo-M₁dG, 0.25 pmol to 25.0 pmol. The internal standard was recovered at 79% (1.0 pmol) from these same solutions, and spiked buffer solutions were quantified accurately to ±12%. These data demonstrate that 100 μL of the antibody gel is able to bind at least 26 pmol of 6-oxo-M₁dG from solution, and LC-MS/MS detection of the purified sample is quantitative.

The recovery of [¹⁵N₅]-6-oxo-M₁dG from saline solutions was greater than 78% but its recovery from urine and feces was only ~29%. This suggests a matrix effect where other nucleosides, deoxynucleosides, and/or endogenous congeners compete with 6-oxo-M₁dG for antibody binding sites. Although there was minimal cross-reactivity of the antibody with individual members of a panel of exocyclic adducts and purine oxidation products (Figures 1 and 5), it is possible that high levels of low-avidity nucleosides in urine and feces compete with the low levels of endogenous 6-oxo-M₁dG for binding sites on the mAb.

6-oxo-M₁dG was detected in the feces of each of three different Sprague–Dawley rats but was detected in the urine of only one of the rats (Table 2). The fecal levels of 6-oxo-M₁dG (350–1893 fmol/kg·day) were significantly higher than the urinary levels (188 fmol/kg·day). This is consistent with previous findings that demonstrate M₁dG is excreted mainly in urine, whereas 6-oxo-M₁dG is excreted mainly in feces.^{10,11}

Because 6-oxo-M₁dG is present endogenously in rats, it suggests that its metabolic precursor, M₁dG, is also produced endogenously in rats and metabolized to 6-oxo-M₁dG. This

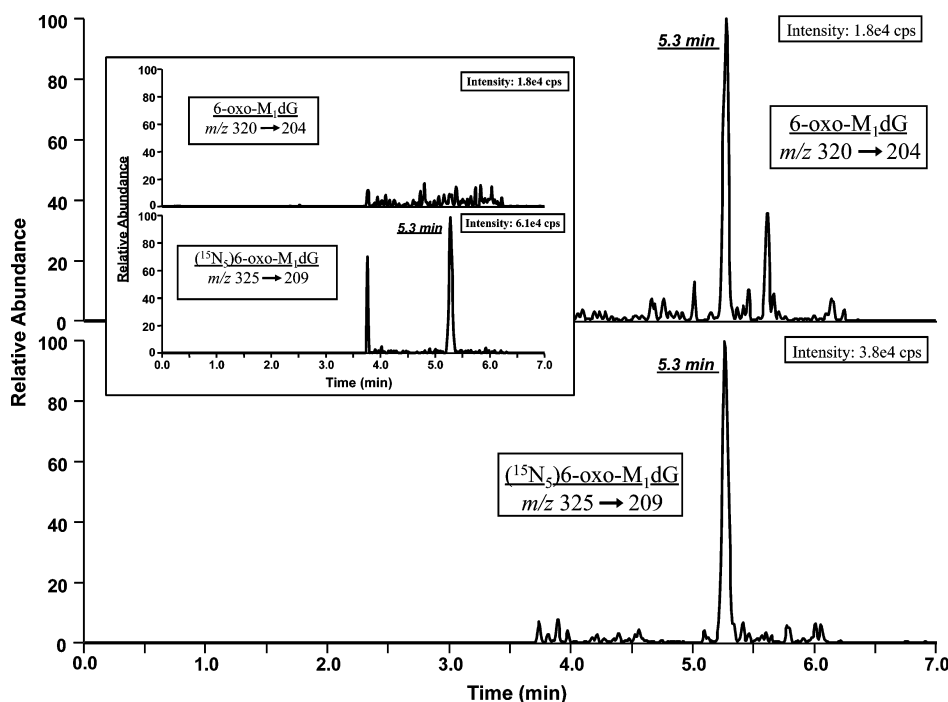


Figure 6. 6-oxo-M₁dG is present in rat feces. A representative LC-MS/MS chromatogram of 6-oxo-M₁dG and the internal standard [¹⁵N₅]-6-oxo-M₁dG isolated from rat feces is displayed. The inset represents a chromatogram of internal standard alone, purified from PBS.

idea is consistent with the finding that M₁dG is present in rodent and human genomic DNA^{2,3} and with studies from this laboratory demonstrating that 6-oxo-M₁dG is the sole metabolite of M₁dG.^{10,11,14}

It may be argued that endogenous 6-oxo-M₁dG is generated in the nucleoside pool rather than arising from direct damage to DNA. However, this seems unlikely if one considers that base propenals are the principal precursors to M₁dG.¹³ Base propenal formation is triggered by abstraction of the hydrogen from the C-4' position of the deoxyribose unit of double-stranded DNA. The base propenal produced then reacts with a deoxyguanosine residue in duplex DNA to form M₁dG. In comparison, the yields of base propenals are much lower when deoxynucleosides are oxidized instead of duplex DNA. Furthermore, M₁dG formation as a reaction of base propenals and free deoxyguanosine is a bimolecular process. If base propenals are formed in duplex DNA, they are generated in close proximity to deoxyguanosine residues in the DNA duplex, which should maximize the probability of their reaction with a deoxyguanosine residue. This is not the case for base propenals generated in the nucleoside pool.

Another possible source of both 6-oxo-M₁dG and M₁dG is diet. One or both of these compounds may have been ingested, then excreted or metabolized. It was not possible to control for this possibility under the reported experimental regime, so future studies with animals fed nucleic acid-free diets will be required to test this hypothesis.

The ability to measure excreted 6-oxo-M₁dG has the potential for clinical relevance in assessing the levels of oxidative DNA damage in humans. Since the basal levels of both deoxynucleoside adducts are low, they should provide high sensitivity to increases triggered by oxidative damage. Given that the analytical method described herein is applicable to feces and urine, the possibility exists for preclinical or clinical studies conducted in a noninvasive fashion.

■ ASSOCIATED CONTENT

📄 Supporting Information

Proton NMR of 6-oxo-M₁Guo; scheme of the generation of the 6-oxo-M₁Guo/protein conjugate; sera reactivity with 6-oxo-M₁dG; lack of reactivity of sera with M₁dG; specificity of sera antigens for 6-oxo-M₁dG; limit of detection and linearity of the LC-MS/MS system. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

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■ ABBREVIATIONS

M₁dG, 3-(2'-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-α]purine-10(3H)-one; 6-oxo-M₁dG, 3-(2-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-f]purine-6,10(3H,5H)-dione; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); equiv, equivalents; mAb, monoclonal antibody; mCKLH, mariculture keyhole limpet hemocyanin; ELISA, Enzyme-Linked Immunosorbent Assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SRM, selected reaction monitoring

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