Immunological Quantification by High-Affinity Antibodies of O°-Ethyldeoxyguanosine in DNA Exposed to N-Ethyl-N-nitrosourea

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ABSTRACT

Three immunological methods [radioimmunoassay (RIA), enzyme-linked immunosorbent assay, and radioimmunoadsorbent technique] were established for quantification of the potentially mutagenic O°-ethyldeoxyguanosine (O°-EtdGuo) in DNA treated with the carcinogen ethylnitrosourea in vivo or in vitro. To obtain high-affinity antibodies for specific detection of low levels of O°-EtdGuo in small amounts of DNA (cells), different schemes were applied for immunization of rabbits with the hapten O°-ethylguanosine coupled to various carrier proteins (rat serum albumin, bovine serum albumin, keyhole limpet hemocyanin).

Low-dose immunization with the hapten-keyhole limpet hemocyanin conjugate resulted in antibodies with an affinity constant of 1 × 10¹⁰ liters/mol and very low levels of cross-reactivity with normal as well as other alkylated DNA components. The RIA (the most sensitive of the three assays) detects 0.05 pmol of O°-EtdGuo at 50% inhibition of tracer (O°-ethyl[8,5'-3H]-2'-deoxyguanosine)-antibody binding. This permits quantification by RIA of O°-EtdGuo at an O°-EtdGuo:2'-deoxyguanosine molar ratio of ~3 × 10⁻⁶ in a hydrolysate of 100 μg of ethylated DNA. By chromatographic separation of O°-EtdGuo prior to the RIA, this value can be lowered to <5 × 10⁻⁸.

INTRODUCTION

Among the various reaction products of alkylating carcinogens with target cell DNA, the potentially mutagenic O°-alkyldeoxyguanosine deserves particular attention (21, 22, 29, 35, 37, 38). The extent of O°-alkyldeoxyguanosine formation in DNA by different compounds seems to correlate with their relative carcinogenicity (15, 29). Thus, exposure to EtNU, a potent pulse-carcinogen with a pronounced neurooocgenic effect in rats (20, 35), leads to a high O°-EtdGuo:7-EtdGuo molar ratio in DNA [~0.6 (15)], while the corresponding value, e.g., for the very weakly carcinogenic diethyl sulfate, is about 200 times lower (42). Furthermore, contrary to other ethylation products, O°-EtdGuo persists in the DNA of rat brain (high tumorigenic effect of EtNU) but is rapidly eliminated enzymatically from the DNA of other "low-risk" rat tissues (15, 35). A similar selective persistence of O°-EtdGuo has been observed in EtNU-treated human xeroderma pigmentosum fibroblasts in contrast to their normal counterparts (1, 3, 16).

The specific detection of carcinogen-modified DNA components and the kinetic analysis of their enzymatic elimination from (or persistence in) DNA require highly sensitive methods. This will be even more important if such studies are now able to be extended to very low levels of DNA modification (i.e., the effects of low doses of carcinogens) and/or small numbers of cells. Conventional radio-chromatographic techniques are, however, limited (a) by the specific radioactivity of radiolabeled carcinogens and (b) by the need to restrict the analyses to laboratory animals or cultured cells exposed to radiolabeled carcinogens. These shortcomings can possibly be circumvented by immunological detection methods, due to the high specificity of antibodies in recognizing minor alterations of molecular structure. As part of studies concerned with the development of immunological procedures for the quantification of DNA components structurally modified by N-nitroso carcinogens, we have recently described a RIA for O°-EtdGuo (27, 28). In an extension of this work, we now report on 3 highly sensitive immunological assays for O°-EtdGuo, using antibodies with an increased affinity and specificity.

MATERIALS AND METHODS

Animals

BDIX rats (8) were used for in vivo application of EtNU. Antisera were produced in rabbits (Graue Riesen; Kaninchenfarm H. Schriever, Iselersheim, Germany).

Ethynitrosourea

EtNU (Roth, Karlsruhe, Germany), twice recrystallized from methanol, was dissolved in citric acid/disodium phosphate buffer, pH 6.0 (24), to give a 0.1 M solution, immediately before i.v. injection into a tail or hind leg vein.

Synthesis of Alkylated Nucleic Acid Components

O°-EtGuA, O°-EtGuG, O°-EtGuC, O°-MeGu, and 7-EtGuA were synthesized according to published methods (2, 5, 11,
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7-EtGuo and 7-EtdGuo were obtained by reacting equal amounts (w/w) of guanosine or dGuo, respectively, with ethyl iodide in dimethyacetamide for 72 hr at 50°C. These products were purified by thin-layer chromatography on silica gel (developed with n-butyl alcohol:glacial acetic acid:H2O, 3:1:1). O6-EtGMP and O6-EtdGMP were synthesized according to a published procedure (44) as described previously (27). Nucleoside 3'-monophosphates and nucleoside 3',5'-diphosphates were destroyed by treatment of the products with Nuclease P1 (Boehniger, Mannheim, Germany; 10 μg/ml) in 15 mM sodium acetate buffer (pH 7.2) containing 0.4 mM ZnSO4 for 30 min at 37°C. The nucleoside 5'-monophosphates were purified on a column of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden), eluted with a linear gradient of 0 to 0.5 M triethyammonium bicarbonate (pH 8.0). O6-BuGuo was prepared by Dr. R. Saffhill (Manchester, England). All synthesized products showed >99% purity when analyzed by high-performance liquid chromatography on Aminex A6 (BioRad, Munich, Germany), eluted with 0.45 mM ammonium formate, pH 4.5 (6). They were stored in liquid N2 either in lyophilized form or dissolved in methanol. Natural nucleic acid components were purchased from Sigma-Chemie (Munich, Germany).

O6-Et[8,5'-3H]dGuo (specific activity, 14 Ci/mmol), the tracer in the RIA (see below), was synthesized from [8,5'-3H]dGTP (New England Nuclear; specific activity, 29.8 Ci/mmol). For hydrolysis of the PP1 linkages, [8,5-3H]dGTP was incubated in 0.1 N HCl for 30 min at 37°C. After neutralization, the α-phosphate was removed by incubation with 100 μg of AP (EC 3.1.3.1; Grade I; Boehringer) per ml for 30 min at 37°C. The enzyme was removed by adsorption to DE-52 cellulose (Whatman, Ferriers, France). Following evaporation, the labeled nucleoside was dissolved in absolute methanol and ethylated with diazoethane at 0°C for 2 hr (11), and the product was purified on a Sephadex G-10 (Pharmacia) column eluted with H2O (Chart 1).

**Nucleoside-Protein Conjugates**

O6-EtGuo was coupled to the carrier proteins RSA (Calbiochem, Marburg, Germany), BSA (99% purity; Serva, Heidelberg, Germany), or KLH (Calbiochem), respectively, according to the procedure or Erlanger and Beiser (10). One hundred mg of O6-EtGuo were stirred in 10 ml of 0.1 M NaOAc for 15 min at room temperature. The reaction was stopped by 20 μl of ethylene glycol, and the mixture was then added dropwise to 5 ml of the carrier protein solution (20 mg/ml). The pH was adjusted to 9.5 with 2 M Na2CO3 and was kept at this value for 45 min. The O6-EtGuo-protein complex (Schiff's base) was stabilized by 2.5 ml of a sodium borohydride solution (30 mg/ml). After 3 hr at 4°C, 1 drop of n-octanol was added to avoid foam formation, and the pH was adjusted to 6 to 7. The reaction mixture was dialyzed against PBS overnight and purified on a Sephadex G-50 (Pharmacia) column (30 x 1.5 cm) eluted with PBS (conjugate in the void volume). The nucleoside:protein molar ratios, calculated from difference spectra (19), were ~20 for RSA and BSA and ~200 for KLH (assuming a molecular weight of 800,000 for KLH (41)).

**Immunization Procedures**

**Procedure 1.** Three rabbits were immunized with nucleoside-protein conjugate (5 mg/animal) in 2.5 ml of PBS emulsified in 2.5 ml of complete Freund's adjuvant (Behring, Marburg, Germany), by injections into the hind foot pads and 5 to 10 other sites (i.m. and s.c.). Beginning 4 weeks later, the rabbits were boosted by i.v. injection of 500 μg of conjugate per animal in 1 ml of PBS at 2-week intervals. Sera were collected at 10 days after the second booster.

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**Chart 1.** Chromatographic separation of enzymatically hydrolyzed (Chart 2) DNA, (12 mg: deoxynucleosides, ADA treated) on a Sephadex G-10 column (1.6 x 85 cm) eluted with H2O. O6-Et[8,5'-3H]dGuo (~2 x 10^6 dpm) was added to the DNA hydrolysate. Flow rate, 0.6 ml/min. Fraction volume, ~9 ml. Separation characteristics remain unchanged over a pH range of 6 to 8. In a hydrolysate not treated with ADA, dAdo would appear in Fractions 31 to 35. Relative absorbance and 3H radioactivity per fraction are plotted against the fraction number. dCyd, 2'-deoxycytidine; dThd, 2'-deoxythymidine.
Procedure 2. A mixture of 500 μg of nucleoside-protein conjugate in 0.5 ml of PBS and 0.5 ml of aluminum hydroxide (Alugel S; Serva) was stirred for 1 hr at 4° and emulsified in 1 ml of complete Freund’s adjuvant. Three rabbits were immunized by injections into the hind foot pads and into about 50 i.c. sites (neck and axillae). Eight weeks later, the animals were boosted by the same procedure. After another 8-week interval, the rabbits received a second booster by i.m. injection of 500 μg of conjugate in 1 ml of PBS emulsified in 1 ml of incomplete Freund’s adjuvant (Behring). The sera were collected 2 weeks later.

Antibody Concentrations and Affinity Constants

The serum concentrations of antibodies directed against O6-EtdGuo and their affinity constants for O6-EtdGuo were determined by the method of Stewart and Petty (39). Binding curves were established for an antisemur dilution of ~5 × 10^{-10} mol of specific antibody-binding sites per liter, over a range of 5 to 500 × 10^{-10} mol of hapten (tracer) per liter.

Isolation of O6-EtdGuo-specific Antibodies

Thirty mg of O6-EtdGuo were incubated with 1.5 mg of epoxy-activated Sepharose 6B (Pharmacia), in 3 ml of 0.01 N NaOH for 20 hr at 50°. The gel was washed with H2O and 0.1 M borate buffer (pH 8.5) and incubated in 10 ml of 1 M ethanolamine (pH 9.0) for 12 hr at room temperature, in order to block remaining active groups. The immunosorbent was then washed with PBS and incubated in 5 ml of anti-O6-EtdGuo-KLH serum for 2 hr at room temperature, with gentle shaking. After centrifugation, the supernatant was removed, and the gel was washed extensively with PBS until the absorption (280 nm) of Lgof conjugate in 1 ml of PBS emulsified in 1 ml of incomplete Alugel S; Serva was stirred for 1 hr at 4° and emulsified in 1 ml of incomplete Freund’s adjuvant (Behring). The sera were collected 2 weeks later.

Enzyme-linked Immunosorbent Assay

The competitive ELISA was performed in 96-well microtiter plates (type M 129 A; Greiner, Nürtlingen, Germany). Wells were filled with 200 μl each of coating solution (2 μg of nucleoside-protein conjugate per ml of TBS) and incubated for 30 min at room temperature. The solution was removed, and the wells were washed twice with TBS. Remaining free binding sites on the plastic surface were saturated with a solution of 1% BSA (w/v) in TBS for 30 min at room temperature. The protein solution was removed, and the wells were incubated for 1 hr at 37° with 200 μl each of the AbAP conjugate (3 μg/ml TBS containing 0.1% BSA), premixed with either TBS or the inhibitor (i.e., a calibration standard or test sample). Thereafter, the AbAP solution was removed, the wells were washed 4 times with TBS, and 200 μl of a solution of 10 mm p-nitrophenyl phosphate (phosphatase substrate; Sigma-Chemie) in 50 mm sodium carbonate buffer (pH 9.5): 2 mm MgCl2 were pipetted into each well. After incubation for 1 hr at 37°, the reaction was stopped by addition of 100 μl of 1 N NaOH, and the absorption at 405 nm was determined using either a flow-through cuvet in a Gilford Model 250 spectrophotometer or a Multiscan spectrophotometer (Flow, Meckenheim, Germany). The degree of inhibition of AbAP binding to the solid phase was calculated by the formula

\[ \frac{E_2 - E_3}{E_2 - E_1} \times 100 = \text{inhibition (}) \%
\]

where \( E_1 \) is absorption of inhibitor-containing well; \( E_2 \) is absorption of well without inhibitor (0% inhibition); and \( E_3 \) is absorption of well without nucleoside-protein coat (100% inhibition). Samples were generally run as triplicates, and the inhibition values were within ±5% (S.D.) of the mean.

Radioimmunosorbent Technique

The competitive RIST was carried out in microtiter plates with 96 disposable wells (type M 74 of M 174; Greiner). Coating
of wells and saturation of free binding sites were performed as described for the ELISA. Fifty µl of a solution of O6-EtGua-specific antibodies (10 ng/well) in 0.1% BSA in TBS or inhibitor (calibration standard or test sample) were pipetted into each well. After incubation for 2 hr at 37°C, the antibody solution was removed, and the wells were washed 5 times with TBS and filled each with 100 µl of a solution of 125I-labeled specific goat anti-rabbit IgG antibodies (~50,000 dpm/well) in 0.1% BSA in TBS. After incubation at 4°C for ~15 hr, the antibody solution was removed, the wells were washed 5 times with TBS, and the remaining radioactivity was measured in a Packard Model 3375 gamma spectrometer. The degree of inhibition of antibody binding to the O6-EtGua-BSA on the plastic solid phase was calculated by the formula

\[
\text{inhibition} = \frac{\text{dpm}_2 - \text{dpm}_1}{\text{dpm}_2 - \text{dpm}_3} \times 100
\]

where dpm1 is dpm in inhibitor-containing well, dpm2 is dpm in wells without inhibitor (0% inhibition), and dpm3 is dpm in wells without O6-EtGua-BSA coat (100% inhibition). Samples were generally run as duplicates, and the inhibition values were within ±5% of the mean.

Radioimmunoassay

In the competitive RIA (modified Farr assay [12]), the radio-labeled nucleoside (tracer) competes for the antibodies with the unlabeled nucleoside of the calibration standard or test sample (inhibitor). Each sample contained in a total volume of 100 µl of buffer [TBS with 1% BSA (w/v) and 0.1% normal rabbit IgG (w/v)], 105 dpm of tracer (O6-Et[8,5-I]dGua), antinucleoside serum at a dilution giving 50% binding of tracer in the absence of inhibitor, plus varying amounts of inhibitor. After incubation for 2 hr at room temperature (equilibrium), 100 µl of a saturated ammonium sulfate solution (pH 7.0) were added. Ten minutes later, the samples were centrifuged for 3 min at 10,000 x g. The radioactivity in 150 µl of supernatant was measured in 10 ml of a toluene-based scintillation fluid (Rotiszint 22; Roth) in a Searle-Nuclear Chicago Mark II liquid scintillation spectrometer. The degree of inhibition of tracer-antibody binding was calculated by the formula

\[
\text{inhibition} = \frac{\text{dpm}_3 - \text{dpm}_1}{\text{dpm}_3 - \text{dpm}_2} \times 100
\]

where dpm1 is dpm in the supernatant of inhibitor-containing sample, dpm2 is dpm in the supernatant of sample without inhibitor (0% inhibition), and dpm3 is dpm in the supernatant of sample without antibodies (100% inhibition). Samples were run as duplicates, and inhibition values were within ±2% of the mean.

Isolation and Enzymatic Hydrolysis of DNA

DNA of rat brain and liver was isolated using a modification of the method of Meinke et al. (25). The liquid N2-frozen tissue was thawed in a solution of 1% sodium dodecyl sulfate (10 ml/g tissue) and homogenized with a Polytron homogenizer (speed control Position 4) for 30 sec. After addition of proteinase K (Merck AG, Darmstadt, Germany) to a final concentration of 500 µg of enzyme per ml, the homogenate was incubated for 30 min at 37°C. A 10-fold volume of urea buffer (5 M urea:2 mM NaCl:0.18 M sodium phosphate, pH 6.8) was added, and the mixture was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). Hydroxylapatite (Bio-Gel HTP, DNA grade; BioRad Laboratories, Munich, Germany) was washed with urea buffer and added to the aqueous phase (3 g/g of tissue). The suspension was incubated with gentle shaking for 30 min at room temperature. After centrifugation (~10 sec at 500 x g), the supernatant was withdrawn, and the hydroxylapatite was washed 3 times with urea buffer and finally with 1 mM sodium phosphate, pH 6.8 (20 ml of buffer per g of hydroxylapatite). The DNA was eluted by incubating the hydroxylapatite in 0.5 mM sodium phosphate buffer (pH 6.8) for 5 min at room temperature (3 ml of buffer per g of hydroxylapatite). The elution was repeated twice, and the supernatants were combined, dialyzed against a 100-fold volume of H2O for 5 x 12 hr at 4°C, and lyophilized. The average yield of DNA was 72% of the theoretical value, and the contaminations with RNA (~7) and protein (protein assay kit; BioRad) were <0.1 and <0.5%, respectively. The ratios of E260/E280 and E260/E260 were ~0.42 and ~1.8, respectively. The isolated DNA had a molecular weight of ~107, as estimated by agarose:gel electrophoresis.

For enzymatic hydrolysis, the DNA (≤1 mg/ml) was first digested with DNase I (EC 3.1.4.5; Grade II; Boehringer; 100 µg/ml) in a buffer containing 10 mM Tris-HCl (pH 7.0):5 mM MgCl2 for 0 min at 37°C. The pH was then raised to 8.0 by addition of 1 M Tris-HCl buffer (pH 9.0). Snake venom phosphodiesterase (EC 3.1.4.5; Boehringer; 100 µg/ml) and AP (Grade I; Boehringer; 400 units/mg; 100 µg/ml) were added. Hydrolysis was complete after 45 min at 37°C (Chart 2), as measured by determination of free phosphate (13). In general, ADA (EC 3.5.4.4; Boehringer; 0.3 unit/ml) was used to convert dAdo to dino in the DNA hydrolysates, prior to chromatographic separation (Chart 1) or immunological analysis. The reaction was complete after 5 min at room temperature, as indicated by the decrease of absorption at 265 nm. This procedure does not lead to measurable deethylation of O6-EtGua. dGuo concentrations in the samples were determined UV spectrophotometrically as described previously (27). After chromatographic separation of the DNA hydrolysate, dGuo concentrations were calculated from the UV absorption values of the respective fractions (Chart 1), based on a molar absorption coefficient at neutral pH of ε253 = 13,700 for dGuo.

RESULTS

Evaluation of Immunization Procedures and Characteristics of Antibodies. To obtain anti-O6-EtGua antibodies of high affinity and high specificity (i.e., low cross-reactivity with normal DNA components and other ethylation products in DNA), 2 different immunization procedures (Procedures 1 and 2; see “Materials and Methods”) were compared, as well as 3 carrier proteins (RSA, BSA, KLH) of different molecular weight and phylogenetic resemblance to rabbit proteins. The results are shown in Table 1. Immunization Procedure 2 was superior to Procedure 1 with respect to the affinity of the antibodies for O6-EtGua, while the concentration of O6-EtGua-specific antibodies was higher in the antiserum obtained by Procedure 1. The phylogenetically most dissimilar carrier (KLH) produced the antibodies with the highest affinity (antibody association constant for O6-EtGua, 1 to 2 x 1010 liters/mol) and the
Table 1

Properties of anti-O°-EtdGuo antisera obtained with the use of different carrier proteins for the hapten O°-EtdGuo, and by different immunization procedures

Rabbits were immunized with conjugates of O°-EtdGuo and various carriers (RSA, BSA, KLH), using Immunization Procedures 1 and 2 (see "Materials and Methods"). Antibody titers in the antisera are expressed as final serum dilutions giving 50% tracer binding in the RIA. Relative antibody reactivities for O°-EtdGuo as compared with dGuo and dAdo were calculated from the respective inhibition values in the competitive AlA.

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<th>Immunization procedure</th>
<th>No. of rabbits</th>
<th>Antibody titer (lgG) (mg/ml)</th>
<th>Fraction of DNA hydrolyzed (%)</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>O°-EtdGuo-RSA</td>
<td>1</td>
<td>4</td>
<td>&lt;1:3</td>
<td>ND</td>
<td>0%</td>
</tr>
<tr>
<td>O°-EtdGuo-BSA</td>
<td>1</td>
<td>4</td>
<td>1:20,000–1:40,000</td>
<td>1:10</td>
<td>80%</td>
</tr>
<tr>
<td>O°-EtdGuo-KLH</td>
<td>2</td>
<td>2</td>
<td>1:60,000</td>
<td>1:10</td>
<td>80%</td>
</tr>
</tbody>
</table>

ND, not done.

Table 2

Chart 2. Kinetics of enzymatic DNA hydrolysis to deoxynucleosides. Calf thymus DNA was digested with DNase I and then treated with phosphodiesterase and AP (see "Materials and Methods"). Extent of hydrolysis determined by measurement of the liberated phosphate (13) (100% value calculated from input DNA).

Immuno!ologiCa! Quantification of O°-EtdGuo

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<tr>
<td>O°-EtdGuo-KLH</td>
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<td>2</td>
<td>1:60,000</td>
<td>1:10</td>
<td>80%</td>
</tr>
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</table>

ND, not done.

Sensitivity and Specificity of the RIA. As expected, O°-EtdGuo was the strongest inhibitor in the RIA. With Antiserum E3, 0.05 pmol of O°-EtdGuo was sufficient to inhibit tracer-antibody binding by 50% (Table 2; Chart 3). Using a probability grid to linearize the sigmoid inhibition curve over the range between 10 and 90% inhibition, the O°-EtdGuo concentration required to inhibit tracer-antibody binding by, e.g., 20% can be determined, thus lowering the detection limit for O°-EtdGuo to ~0.01 pmol (Chart 4).

With Antiserum E3, the 50% inhibition value for O°-EtdGuo remained unaltered up to concentrations of ~300 μg of hydrolyzed DNA, O°-EtdGuo concentrations can be determined by comparing the inhibition values with calibration curves constructed for the respective DNA concentrations (Chart 6). In 100 μg of DNA (equivalent to the DNA content of ~1.6 x 10⁷ diploid cells), O°-EtdGuo can thus be measured at an O°-EtdGuo:dGuo molar ratio of ~3 x 10⁻⁷ (i.e., ~700 O°-EtdGuo molecules per diploid genome).

The degree of inhibition by O°-EtdGuo of tracer-antibody binding was comparatively measured with Antiserum E3 in SS, DS, and hydrolyzed DNA (nucleosides; ADA treated), ethylated by EtNU in vitro (O°-EtdGuo:dGuo, 2.5 x 10⁻⁵). The following ratios were obtained (relative O°-EtdGuo concentrations required for 50% inhibition of tracer-antibody binding): DNA hydrolysate:SS, 1:13; SS:DS, 1:20; DNA hydrolysate:DS [S, nuclease (Boehringer)-treated], 1:250 (Chart 7).

Sensitivity and Specificity of ELISA and RIST. Both the RIST and the ELISA can be carried out in a microtiter system and are, therefore, preferable to the RIA in case of large series of measurements. In both assays, free O°-EtdGuo in the test sample or in calibration standards, respectively, inhibits the binding of O°-EtdGuo-specific antibodies to an O°-EtdGuo-BSA

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Table 2
Inhibition of tracer-antibody binding by various alkylated and natural nucleic acid components in the competitive RIA: comparison of three antisera (E1, E2, and E3) directed against O\textsuperscript{6}-EtdGuo

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiserum E1 (pmol)</th>
<th>Antiserum E2 (pmol)</th>
<th>Antiserum E3 (pmol)</th>
<th>Multiple of O\textsuperscript{6} \text{-} EtdGuo</th>
</tr>
</thead>
<tbody>
<tr>
<td>O\textsuperscript{6}-EtdGuo</td>
<td>0.6</td>
<td>0.12</td>
<td>0.05</td>
<td>1</td>
</tr>
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<td>O\textsuperscript{6}-EtGua</td>
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<td>0.7</td>
<td>0.15</td>
<td>3</td>
</tr>
<tr>
<td>O\textsuperscript{6}-EtGMP</td>
<td>20</td>
<td>1.8</td>
<td>0.7</td>
<td>14</td>
</tr>
<tr>
<td>O\textsuperscript{6}-BuGuo</td>
<td>60</td>
<td>6</td>
<td>1.4</td>
<td>28</td>
</tr>
<tr>
<td>O\textsuperscript{6}-MeGuo</td>
<td>276</td>
<td>30</td>
<td>12</td>
<td>240</td>
</tr>
<tr>
<td>7-EtdGuo</td>
<td>NT\textsuperscript{a}</td>
<td>420</td>
<td>170</td>
<td>3400</td>
</tr>
<tr>
<td>7-EtGua</td>
<td>~4 \times 10\textsuperscript{4}\textsuperscript{b}</td>
<td>1 \times 10\textsuperscript{4}</td>
<td>4 \times 10\textsuperscript{3}</td>
<td>8 \times 10\textsuperscript{3}</td>
</tr>
<tr>
<td>dGuo</td>
<td>~1 \times 10\textsuperscript{5}\textsuperscript{c}</td>
<td>~1 \times 10\textsuperscript{5}\textsuperscript{c}</td>
<td>~3 \times 10\textsuperscript{5}\textsuperscript{c}</td>
<td>~5 \times 10\textsuperscript{5}\textsuperscript{c}</td>
</tr>
<tr>
<td>dAdo</td>
<td>~8 \times 10\textsuperscript{4}\textsuperscript{b}</td>
<td>~5 \times 10\textsuperscript{4}\textsuperscript{b}</td>
<td>~6 \times 10\textsuperscript{4}\textsuperscript{b}</td>
<td>~1 \times 10\textsuperscript{4}\textsuperscript{b}</td>
</tr>
<tr>
<td>dIno</td>
<td>~1.5 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~6 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{c}</td>
</tr>
<tr>
<td>2'-Deoxyctidine</td>
<td>~5 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~3 \times 10\textsuperscript{6}\textsuperscript{d}</td>
</tr>
<tr>
<td>2'-Deoxythymidine</td>
<td>~5 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{d}</td>
<td>~3 \times 10\textsuperscript{6}\textsuperscript{d}</td>
</tr>
<tr>
<td>DNA hydrolysate (ADA-treated)</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~5 \times 10\textsuperscript{6}\textsuperscript{c}</td>
<td>~1 \times 10\textsuperscript{6}\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} NT, not tested.
\textsuperscript{b} 20% inhibition at this concentration.
\textsuperscript{c} 10% inhibition at this concentration.
\textsuperscript{d} <10% inhibition at this concentration.

Chart 3. Inhibition of tracer-antibody binding by various alkylated and natural nucleic acid components in the competitive RIA. Antiserum E3 (see Table 2). The enzymatic DNA hydrolysate (deoxynucleosides; see Chart 2) was treated with ADA. Immunogen, O\textsuperscript{6}-EtGuo-KLH; tracer, O\textsuperscript{6}-Et[8,5'-3H]-2'-dGuo.

Conjugate on the plastic surface of the reaction vessels. In the RIST, antibody binding is measured via the radioactivity of 125I-labeled goat anti-rabbit IgG antibodies, while the same parameter in the ELISA is measured via the cleavage of p-nitrophenyl phosphate by the AP covalently linked to the antibodies (i.e., by the color reaction resulting from the formation of dinitrophenol).

Using antibodies purified from Antiserum E2 in the RIST and the ELISA, 1 or 0.25 pmol, respectively, of O\textsuperscript{6}-EtdGuo was sufficient to inhibit antibody binding by 50% (Table 3; Chart 8). O\textsuperscript{6}-EtGua and 7-EtdGuo exhibited considerably lower degrees of inhibition. As in the RIA (Table 2; Chart 3), the naturally occurring nucleosides showed inhibition of antibody binding only at very high concentrations. dAdo exhibited the comparatively highest degree of inhibition but could easily be converted to dIno by addition of ADA to the DNA hydrolysates prior to the assay. The inhibitory effect of dIno is 5 and 20 times lower than that of dAdo in the RIST and in the ELISA, respectively. In the ELISA, the inhibition value for O\textsuperscript{6}-EtdGuo remained uninfluenced over a concentration range of up to ~350 μg of ADA-treated hydrolyzed DNA per ml in the test sample. For analysis of O\textsuperscript{6}-EtdGuo in the presence of higher amounts of DNA, calibration curves were constructed for the respective DNA concentrations (350 to 1500 μg/ml). In an amount of
Immuno!ogical Quantification of O6-EtdGuo

Chart 4. RIA calibration curve for O6-EtdGuo. Inhibition of tracer-antibody binding by O6-EtdGuo plotted against O6-EtdGuo (inhibitor) concentration in a probability grid. Reading at 20% inhibition gives an O6-EtdGuo value of ~0.01 pmol.

Chart 6. RIA calibration curves for O6-EtdGuo in the presence of ADA-treated DNA hydrolysate. Inhibition of tracer-antibody binding is shown as a function of the O6-EtdGuo concentration in the presence of different amounts of hydrolyzed DNA in the test samples.

Determination by RIA of O6-EtdGuo in DNA. Charts 9 and 10 show examples of the application of the RIA (Antiserum E3) for determination of the molar concentration of O6-EtdGuo in DNA ethylated by EtNU in vitro or in vivo. In order to measure the O6-EtdGuo content of DNA at very low molar concentrations (i.e., after exposure of DNA to low levels of the carcinogen), O6-EtdGuo was quantitatively separated and concentrated from the remaining nucleosides in the DNA hydrolysate by chromatography on Sephadex G-10 (Chart 1) and subsequent evaporation of the solvent (H2O), prior to the RIA. By this procedure, O6-EtdGuo:dGuo molar ratios in DNA of <5 x 10^-8 can be easily quantitated (Chart 9). Chart 10 indicates that the molar content of O6-EtdGuo in the DNA of brain and liver is approximately identical and linearly dependent on the dose of EtNU over a dose range of 2.5 to 100 µg of EtNU per g body weight, when measured by RIA at 1 hr after an i.v. EtNU pulse to 5-week-old BDIX rats.

DISCUSSION

The present and previous studies (27, 28) on antibodies specifically directed against O6-EtdGuo demonstrate that immunological methods represent powerful tools for overcoming...
some of the shortcomings of conventional radiochromato-
graphic techniques for the specific quantification of carcino-
gen-induced, structural modifications of DNA. Similar, although
less sensitive and less specific assays have been reported for
immunological detection of 2-acetylaminofluorene-DNA ad-
ducts (17, 31, 32, 34), benzo(a)pyrene-DNA adducts (33), O6-
fold-lower amount of conjugate at 8-week intervals, the immu-

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>RIST</th>
<th>ELISA</th>
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<tbody>
<tr>
<td>O6-EtdGuo</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>O6-EtGlu</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>dAdo</td>
<td>3 x 10^{-6}</td>
<td>~2 x 10^{-5}</td>
</tr>
<tr>
<td>dGlu</td>
<td>10^{-8}</td>
<td>~5 x 10^{-8}</td>
</tr>
<tr>
<td>dIno</td>
<td>10^{-8}</td>
<td>~5 x 10^{-8}</td>
</tr>
</tbody>
</table>

* 20% inhibition at this concentration.

and low-specificity antibodies. In contrast, Procedure 2 could
stimulate a more limited number of immunocytes preferentially
carrying high-specificity and high-affinity receptors for O6-
EtdGuo. The antisem (E3) with the highest association con-
stant for O6-EtdGuo (1 to 2 x 10^{10} liters/mol) and the lowest
degree of cross-reactivity with normal DNA constituents (Table
2; Chart 3) were indeed obtained by Immunization Scheme 2,
using KLH as the carrier protein for O6-EtGlu (i.e., a carrier phyl-
genetically very distinct from the proteins of the im-
munized animal species, with a molecular weight of about
800,000).
The RIA for O6-EtdGuo established with Antiserum E3 is
highly sensitive (50% inhibition of tracer-antibody binding at
0.05 pmol of O6-EtdGuo) and specific. The normal nucleosides
dGuo, dAdo, and dIno (produced by deamination of dAdo by
ADA during DNA hydrolysis) and the deoxypynimidines reacted
with the antibodies about 10^{7}-fold less than did O6-EtdGuo.
The RIA will thus detect ~700 O6-EtdGuo molecules per diploid
gene in a DNA sample of 100 µg (equivalent to ~1.6 x 10^{7}
diploid cells). In combination with chromatographic separation
of O6-EtdGuo from the DNA hydrolysate prior to the RIA (Chart
2), O6-EtdGuo:dGuo molar ratios of <5 x 10^{-8} can be meas-
ured without difficulty. Recently published RIA's for O6-meth-
ydeoxyguanosine (4)^\textsuperscript{a} show considerably lower levels of sen-
sitivity and specificity than does the present RIA for O6-EtdGuo.
This may be ascribed to the higher affinity and higher specificity
of the antibodies obtained with Immunization Procedure 2 and
the use of a high-specific-activity (14 Ci/mmol) \textsuperscript{3}H-labeled
tracer. Furthermore, the ethyl group in the O6-position of guanine may represent a more antigenic site than did the

\textsuperscript{a} S. A. Kyrtopoulos and P. F. Swann, personal communication.
E2, and E3 have greater affinity for O6-EtdGuo than for the corresponding base, deoxynucleotide, or ribonucleoside (Tables 2 and 3; Charts 3 and 8). Therefore, the antibodies recognize not only the O6-ethylated purine but also the sugar moiety. The lower affinity of the antibodies for O6-EtGuo can be explained by the structure of the O6-EtGuo-protein conjugate used for immunization. O6-EtGuo is oxidized by periodate, thus yielding 2 carbonyl groups at the 2',3'-ribose positions. These react spontaneously with the free amino groups in the carrier protein, especially with the ε-amino group of lysine. The resulting unstable Schiff's base is stabilized by reduction with borohydride. Therefore, the immunogen structurally resembles more closely the deoxyribonucleoside than the ribonucleoside.

Tracer-antibody binding in the RIA is inhibited by O6-EtdGuo-containing denatured DNA to the same extent as by O6-EtdGMP [O6-EtdGuo:O6-EtdGMP, 1:14 (Table 2); hydrolyzed DNA (nucleosides):SS = 1:13 (Chart 7)]. The antibodies thus seem to recognize O6-EtdGuo contained in SS DNA equally well as the free modified nucleotide. The antibodies react with O6-EtdGuo in denatured DNA about 20 times better than with O6-EtdGuo in DS DNA. This indicates that due to steric hindrance only a fraction of the O6-EtdGuo in native DNA is recognized by the antibodies. Antibodies to other naturally occurring modified (18, 26, 30) and carcinogen-modified (34) nucleosides also recognize these residues preferentially in denatured rather than in native DNA.

A comparison of the 3 immunological assays for O6-EtdGuo (RIA, RIST, and ELISA) shows that the ELISA and the RIST are somewhat less sensitive than is the RIA (Tables 2 and 3; Charts 3 and 8). On the other hand, the ELISA has the advantage of being carried out more readily in large-scale experiments. Furthermore, since the ELISA does not require a radiolabeled tracer, it can be performed with DNA samples radioactively prelabeled for other purposes.

The measurements by RIA of the molar content of O6-EtdGuo in DNA after ethylation by EtNU in vivo or in vitro (Charts 9 and 10) show the potential of immunological detection methods for analyses of carcinogen-modified, potentially mutagenic DNA components at low levels of carcinogen exposure. These techniques will also permit studies on the persistence (or rate of elimination) of DNA lesions in small numbers of cells or in defined fractions of chromatin.
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REFERENCES

Immunological Quantification by High-Affinity Antibodies of $O^6$-Ethyldeoxyguanosine in DNA Exposed to $N$-Ethyl-$N$-nitrosourea

Rolf Müller and Manfred F. Rajewsky