Immunological Quantification by High-Affinity Antibodies of O\textsuperscript{6}-Ethyldeoxyguanosine in DNA Exposed to N-Ethyl-N-nitrosourea\textsuperscript{1}

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ABSTRACT

Three immunological methods [radioimmunoassay (RIA), enzyme-linked immunosorbent assay, and radioimmunosorbent technique] were established for quantification of the potentially mutagenic O\textsuperscript{6}-ethyldexoxyguanosine (O\textsuperscript{6}-EtGuo) 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\textsuperscript{6}-EtGuo in small amounts of DNA (cells), different schemes were applied for immunization of rabbits with the hapten O\textsuperscript{6}-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 to 2 \times 10\textsuperscript{10} 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\textsuperscript{6}-EtGuo at 50% inhibition of tracer (O\textsuperscript{6}-ethyl[\textsuperscript{8,5'-\textsuperscript{3}H]}-2'-deoxyguanosine)-antibody binding. This permits quantification by RIA of O\textsuperscript{6}-EtGuo at an O\textsuperscript{6}-EtGuo:2'-deoxyguanosine molar ratio of \textasciitilde 3 \times 10\textsuperscript{-7} in a hydrolysate of 100 \mu g of ethylated DNA. By chromatographic separation of O\textsuperscript{6}-EtGuo prior to the RIA, this value can be lowered to \textasciitilde 5 \times 10\textsuperscript{-8}.

INTRODUCTION

Among the various reaction products of alkylating carcinogens with target cell DNA, the potentially mutagenic O\textsuperscript{6}-alkyldeoxyguanosine deserves particular attention (21, 22, 29, 35, 37, 38). The extent of O\textsuperscript{6}-alkyldeoxyguanosine formation in DNA by different compounds seems to correlate with their relative carcinogenicity(15, 21, 37). Thus, exposure to EtNU, a potent pulse-carcinogen with a pronounced neurooncogenic effect in rats (20, 35), leads to a high O\textsuperscript{6}-EtGuo:7-EtGuo molar ratio in DNA [\textasciitilde 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\textsuperscript{6}-EtGuo 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\textsuperscript{6}-EtGuo 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 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\textsuperscript{6}-EtGuo (27, 28). In an extension of this work, we now report on 3 highly sensitive immunological assays for O\textsuperscript{6}-EtGuo, 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; Kaninchengarten H. Schriefer, Iselersheim, Germany).

Ethylnitrosourea

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\textsuperscript{6}-EtGuo, O\textsuperscript{6}-EtGuo, O\textsuperscript{6}-EtGuo, O\textsuperscript{6}-MeGuo, and 7-EtGuo were synthesized according to published methods (2, 5, 11, 37, 38). Synthesis was performed by coupling the alkylating agent to the appropriate 2'-deoxyguanosine compound, followed by incorporation into DNA helices. The resulting DNA was then subjected to various treatments, including limited digestion with restriction enzymes or digestion with DNA polymerase I to produce a mixture of DNA fragments containing different levels of alkylated guanine residues. The alkylated DNA fragments were then separated by chromatography or other methods, and the O\textsuperscript{6}-alkyldeoxyguanosine content was determined by immunological methods.

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as previously described (27). 7-EtGuo and 7-EtGuo were obtained by reacting equal amounts (w/w) of guanosine or dGuo, respectively, with ethyl iodide in dimethylacetamide 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-EtGMP 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 (Boehringer, 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 triethylammonium 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 0.01 M HCl for 30 min at 37°C. After neutralization, the a-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 diazomethane 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 NaIO4 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.
washed extensively with PBS until the absorption (280 nm) of Alugel S (Serva) was stirred for 1 hr at 4°C 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 x 10^{-9} mol of specific antibody-binding sites per liter, over a range of 5 to 500 x 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°C. 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-EtGuo-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 the supernatant decreased below 0.02. The antibodies were then eluted by incubating the gel in 2.5 ml of 1 M acetic acid for 3 x 5 min at room temperature. The combined supernatants were dialyzed against PBS overnight. Recovery of antibodies was >50%. The antibodies were stored at -20°C.

**AbAP Conjugate**

AP (type VII; Sigma-Chemie) was linked to O6-EtdGuo-specific antibodies according to a published procedure (9). Briefly, 1.5 mg of AP (calf intestine, type VII; Sigma-Chemie) were dissolved in 0.5 ml of a solution containing 1 mg of antibodies per ml of PBS and dialyzed against PBS (5 liters) for 2 hr. Glutaraldehyde (electron microcopy grade; Sigma-Chemie) was added to a final concentration of 0.2%. The reaction mixture was stirred for 2 hr at room temperature and then separated by elution with PBS on a Sephadex G-100 (Pharmacia) column (30 x 1.5 cm). The AbAP appeared in the void volume and was stored at 4°C after dialution to a final protein concentration of 3 µg/ml with TBS containing 1% BSA.

**Isolation of Specific Goat Anti-Rabbit IgG Antibodies**

Rabbit IgG was isolated from normal rabbit sera as previously described (27). One hundred mg of IgG were dissolved in 0.1 M sodium acetate buffer (pH 5.0) at a concentration of 15 mg/ml. After addition of 2 ml of a 2.5% glutaraldehyde solution (electron microcopy grade; Sigma-Chemie), the mixture was gently stirred at room temperature overnight. The polymerized gel was homogenized (Polytron homogenizer; Kinematica, Lucerne, Switzerland), incubated in a solution of 1 M glycine in 0.1 M borate buffer (pH 8.5) for 10 min, and finally washed 5 times with PBS. The isolation of specific antibodies from goat anti-rabbit IgG serum (Behring) was carried out as described for the isolation of O6-EtdGuo-specific antibodies with the exception that 0.1 M glycine-HCl buffer was used instead of 1 M acetic acid for the elution of antibodies.

**125I Labeling of Antibodies**

After evaporation of the benzene solvent, 1 µCi of 125I-labeled Bolton-Hunter reagent (specific activity, 1,500 Ci/mmol; New England Nuclear, Dreieichenhain, Germany) was dissolved in 10 µl of a solution of specific goat anti-rabbit IgG antibodies in 0.1 M borate buffer (pH 8.5) and incubated for 3 hr at 0°C. After addition of 500 µl of a solution of 0.2 M glycine in borate buffer and ~1 mg of phenol red, the mixture was separated on a Sephacry G-50 column (1 x 12 cm) eluted with PBS. The iodinated antibodies were found in the first peak (void volume), while free 125I appeared in the second, phenol red-containing peak. About 95% of the radioactivity in the protein peak were precipitable with 5% trichloroacetic acid. About 20% of 125I was incorporated into the protein; the specific radioactivity of the iodinated antibodies was ~2,000 Ci/mmol.

**Enzyme-linked Immunosorbent Assay**

The competitive ELISA was performed in 96-well microtiter plates (type M 129 A; Greiner, Nurtingen, 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°C 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°C, 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

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

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-EtdGuo-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°, 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° 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-EtdGuo-BSA on the plastic solid phase was calculated by the formula

\[
\text{inhibition (\%)} = \left(1 - \frac{dpm_2 - dpm_3}{dpm_1 - dpm_3}\right) 
\times 100
\]

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-EtdGuo-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 radiolabeled 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 1% BSA (w/v) and 0.1% normal rabbit IgG (w/v). 10⁶ dpm of tracer (O6-Et[8,5'-3H]dGuo), 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

\[
\text{inhibition (\%)} = \left(1 - \frac{dpm_1 - dpm_2}{dpm_1 - dpm_3}\right) 
\times 100
\]

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-EtdGuo-BSA coat (100% inhibition). Samples were generally run as duplicates, and the 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°. A 10-fold volume of urea buffer (5 M urea; 2 M 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 M sodium phosphate, pH 6.8 (20 ml of buffer per g of hydroxylapatite). The DNA was eluted by incubating the hydroxylapatite in 0.5 M sodium phosphate buffer (pH 6.8) for 5 min at room temperature (3 ml of buffer per g of hydroxyapatite). 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°, 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/E₂₅₀ and E₂₆₀/E₂₅₀ 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 10 min at 37°. 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° (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-EtdGuo. 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-EtdGuo 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-EtdGuo, while the concentration of O6-EtdGuo-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-EtdGuo, 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°-EtGuo, and by different immunization procedures

Rabbits were immunized with conjugates of O°-EtGuo 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 RIA.

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<th>Antibody affinity constant, K (liter/mol)</th>
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<tr>
<td>O°-EtGuo-RSA</td>
<td>1</td>
<td>4</td>
<td>&lt;1:3</td>
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<td>ND</td>
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<tr>
<td>O°-EtGuo-BSA</td>
<td>1</td>
<td>4</td>
<td>1:20,000-1:40,000</td>
<td>1.9-4.3</td>
<td>6-8 x 10⁸</td>
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<tr>
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<td>2</td>
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<td>1.1</td>
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<tr>
<td>O°-EtGuo-KLH</td>
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<td>3</td>
<td>1:50,000-1:200,000</td>
<td>0.9-1.5</td>
<td>1-2 x 10⁻⁵</td>
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ND, not done.

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).

Table 2

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

Rabbits were immunized with conjugates of O°-EtGuo 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°-EtGuo as compared with dGuo and dAdo were calculated from the respective inhibition values in the competitive RIA.

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<td>2</td>
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<td>5 x 10⁻⁶; 1</td>
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ND, not done.

Sensitivity and Specificity of the RIA. As expected, O°-EtGuo was the strongest inhibitor in the RIA. With Antiserum E3, 0.05 pmol of O°-EtGuo 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°-EtGuo concentration required to inhibit tracer-antibody binding by, e.g., 20% can be determined, thus lowering the detection limit for O°-EtGuo to ~0.01 pmol (Chart 4).

With Antiserum E3, the 50% inhibition value for O°-EtGuo remained unaltered up to concentrations of ~300 μg of hydrolyzed DNA, O°-EtGuo 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°-EtGuo can thus be measured at an O°-EtGuo:dGuo molar ratio of ~3 x 10⁻⁷ (i.e., ~700 O°-EtGuo molecules per diploid genome).

The degree of inhibition by O°-EtGuo 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°-EtGuo:dGuo, 2.5 x 10⁻⁵). The following ratios were obtained (relative O°-EtGuo 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 microtitre system and are, therefore, preferable to the RIA in case of large series of measurements. In both assays, free O°-EtGuo in the test sample or in calibration standards, respectively, inhibits the binding of O°-EtGuo-specific antibodies to an O°-EtGuo-BSA
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 O6-EtdGuo

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Antiserum E1 (pmol)</th>
<th>Antiserum E2 (pmol)</th>
<th>Antiserum E3 (pmol)</th>
<th>Multiple of O6-EtdGuo</th>
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<td>0.05</td>
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<td>3.6</td>
<td>0.7</td>
<td>0.15</td>
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<tr>
<td>O6-EtdGMP</td>
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<td>1.8</td>
<td>0.7</td>
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<td>6</td>
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<td>28</td>
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<td>O6-BuGuo</td>
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<td>NT</td>
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<td>30</td>
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<td>240</td>
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<tr>
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<td>170</td>
<td>3400</td>
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<td>4 × 10^−3</td>
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<tr>
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<td>−3 × 10^−6</td>
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<tr>
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<td>−1 × 10^−6</td>
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<tr>
<td>dino</td>
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<td>−1 × 10^−6</td>
<td>−6 × 10^−6</td>
<td>−1 × 10^−6</td>
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<tr>
<td>2′-Deoxyctydine</td>
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<td>−1 × 10^−6</td>
<td>−1 × 10^−6</td>
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<td>−1 × 10^−6</td>
<td>−1 × 10^−6</td>
<td>&gt;3 × 10^−6</td>
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<tr>
<td>DNA hydrolysate (ADATreated)</td>
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<td>−1 × 10^−6</td>
<td>−5 × 10^−6</td>
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</table>

a NT, not tested.
b 20% inhibition at this concentration.
c 10% inhibition at this concentration.
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, O6-EtGua-KLH; tracer, O6-[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 O6-EtdGuo was sufficient to inhibit antibody binding by 50% (Table 3; Chart 8). O6-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 O6-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 O6-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
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 5. Influence of the DNA hydrolysate concentration in the test sample on the inhibition of tracer-antibody binding by O⁶-EtdGuo in the RIA. Samples contained a constant amount of O⁶-EtdGuo (giving 50% inhibition of tracer-antibody binding in the absence of DNA hydrolysate) and varying amounts of enzymatically hydrolyzed DNA. Inhibition of tracer-antibody binding plotted against DNA (hydrolysate) concentration in the test sample.

~100 μg of DNA, O⁶-EtdGuo could thus be determined at a O⁶-EtdGuo:dGuo molar ratio of ~3 x 10⁻⁶ (i.e., ~7000 O⁶-EtdGuo molecules per diploid genome).

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 10⁻⁶ 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
the use of a high-specific-activity (14 Ci/mmol) 3H-labeled tracer. Furthermore, the methyl group in the O6-position of guanine may represent a more antigenic site than did the methyl group in the 4-position of adenine. As demonstrated by the RIA, the antibodies of Antisera E1, E2, and E3 show considerably lower levels of sensitivity and specificity than does the present RIA for O6-EtdGuo.

Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount required for 50% inhibition of antibody binding</th>
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<tr>
<td></td>
<td>RIST</td>
</tr>
<tr>
<td>O6-EtdGuo</td>
<td>1</td>
</tr>
<tr>
<td>O6-EtGuo</td>
<td>3</td>
</tr>
<tr>
<td>dGuo</td>
<td>3 x 10^{-6}</td>
</tr>
<tr>
<td>dAdo</td>
<td>3 x 10^{-8}</td>
</tr>
<tr>
<td>dIlo</td>
<td>3 x 10^{-8}</td>
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"20% inhibition at this concentration.

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 dIlo (produced by deamination of dAdo by ADA during DNA hydrolysis) and the deoxyuridines reacted with the antibodies about 10^{12}-fold less than did O6-EtdGuo. The RIA will thus detect ~700 O6-EtdGuo molecules per diploid genome in a DNA sample of 100 µg (equivalent to ~1.6 x 10^{17} 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^{-6} can be measured without difficulty. Recently published RIA's for O6-methyldeoxyguanosine (4)^9 show considerably lower levels of sensitivity 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) ^3H-labeled tracer. Furthermore, the ethyl group in the O6-position of guanine may represent a more antigenic site than did the corresponding methyl substitution. In this respect, it is interesting to note that in the present RIA Antiserum E3 showed a higher degree of cross-reactivity with butyrylated and natural nucleic acid components as a function of the inhibitor concentration in the RIST and the ELISA, respectively.

some of the shortcomings of conventional radiomicrographic techniques for the specific quantification of carcinoma-induced, structural modifications of DNA. Similar, although less sensitive and less specific assays have been reported for immunological detection of 2-acetylaminofluorene-DNA adducts, benzo(a)pyrene-DNA adducts, and methyldeoxyguanosine (4), 47-methylguanine (36), and N6-methyladenosine (32, 34, 36). The pioneering work of Erlanger and Beiser, and their coworkers (10, 23, 26) on the production of antibodies specific for normal and modified minor bases in DNA has provided important guidelines for this work.

The sensitivity of an immunological assay for a specific, structurally modified DNA component mainly depends on the affinity and specificity of the antibodies directed against the respective purified product. Therefore, phylogenetically different carrier proteins for the hapten O6-EtGuo with varying molecular weights (RSA, BSA, KLH), as well as 2 different immunization procedures, were compared with respect to the properties of the resulting antibodies. In Immunization Procedure 1, high amounts of conjugate (5 mg/rabbit) were administered at rather short intervals (i.e., 2 to 4 weeks) by routes (i.m., s.c.) permitting fast release of the immunogen into the lymphatic system. In Procedure 2, rabbits were immunized i.c. with a 10-fold-lower amount of conjugate at 8-week intervals, the immunogen being adsorbed to aluminum hydroxide to ensure slow release. Procedure 1 should produce high serum antibody concentrations, with a considerable proportion of low-affinity 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 antisera used in this work were indeed obtained by Immunization Scheme 2, using KLH as the carrier protein for O6-EtGuo (i.e., a carrier phylogenetically very distinct from the proteins of the immunized 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 dIlo (produced by deamination of dAdo by ADA during DNA hydrolysis) and the deoxyuridines reacted with the antibodies about 10^{12}-fold less than did O6-EtdGuo. The RIA will thus detect ~700 O6-EtdGuo molecules per diploid genome in a DNA sample of 100 µg (equivalent to ~1.6 x 10^{17} 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^{-6} can be measured without difficulty. Recently published RIA's for O6-methyldeoxyguanosine (4)^9 show considerably lower levels of sensitivity and specificity than does the present RIA for O6-EtdGuo.
Chart 9. O²-Ethylolation of guanine in double-stranded calf thymus DNA in vitro, as a function of EtNU concentration. The reaction was carried out for 1 hr at 37° in 0.11 M Na₂HPO₄; 0.01 M citric acid buffer, pH 7.2, at a DNA concentration of 2 mg/ml. RIA.

E2, and E3 have greater affinity for O²-EtdGuo than for the corresponding base, deoxynucleotide, or ribonucleoside (Tables 2 and 3; Charts 3 and 8). Therefore, the antibodies recognize not only the O²-ethylated purine but also the sugar moiety. The lower affinity of the antibodies for O²-EtGuo can be explained by the structure of the O²-EtGuo-protein conjugate used for immunization. O²-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 O²-EtdGuo-containing denatured DNA to the same extent as by O²-EtdGMP [O²-EtdGuo:O²-EtdGMP, 1:14 (Table 2); hydrolyzed DNA (nucleosides):SS = 1:13 (Chart 7)]. The antibodies thus seem to recognize O²-EtdGuo contained in SS DNA equally well as the free modified nucleotide. The antibodies react with O²-EtdGuo in denatured DNA about 20 times better than with O²-EtdGuo in DS DNA. This indicates that due to steric hindrance only a fraction of the O²-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 O²-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 O²-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.
ACKNOWLEDGMENTS

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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