

Sensitive Radioimmunoassay for Detection of O⁶-Ethyldeoxyguanosine in DNA Exposed to the Carcinogen Ethylnitrosourea *in vivo* or *in vitro*

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O⁶-ethyl-2'-deoxyguanosine (O⁶-EtdGuo) is a major premutational product formed in both intracellular DNA and in purified DNA *in vitro*, after exposure to the potent alkylating carcinogen N-ethyl-N-nitrosourea (EtNU). Antibodies directed against O⁶-EtdGuo were obtained by immunizing rabbits with a conjugate of O⁶-EtGuo and bovine serum albumin. In a competitive radioimmunoassay (RIA), with O⁶-Et[8,5'-³H]dGuo as a tracer and various alkylated and natural nucleic acid components as inhibitors, these antibodies show high specificity for O⁶-EtdGuo and detect this product at a level of 0.3 picomol (antibody association constant, 7×10^8 l/mol). In a sample of 130 μ g of hydrolyzed DNA, O⁶-EtdGuo can thus be measured at a molar ratio of O⁶-EtdGuo/2'-deoxyguanosine of about 3×10^{-6} , *i. e.*, about 5×10^3 O⁶-EtdGuo molecules per diploid cell. Examples are given for the quantitation of O⁶-EtdGuo in DNA exposed to EtNU *in vivo* or *in vitro*.

Introduction

Present evidence suggests that structural alterations of target cell DNA play an important role in the initiation of the process of neoplastic transformation by chemical carcinogens [1]. The reaction products formed in DNA by some of the alkylating N-nitroso carcinogens have been characterized to a remarkable extent [2–4]. N-ethyl-N-nitrosourea (EtNU), a highly potent, direct acting, neural-tissue-specific carcinogen in the rat [5, 6], predominantly ethylates the oxygen atoms in both intracellular DNA and purified DNA exposed to EtNU *in vitro* [7]. In comparison with other, less carcinogenic alkylating compounds, EtNU causes the formation of O⁶-ethylguanine (O⁶-EtGua) in DNA with a particularly high relative yield (molar ratio of O⁶-/7-EtGua, ~ 0.6) [6–8]. This premutational lesion is highly persistent in the DNA of rat brain (high tumorigenic effect of EtNU), but eliminated more rapidly from the DNA of other rat tissues [6, 8]. The specific detection of alkylation products in DNA and the monitoring of their enzymic elimination from or persistence in DNA require highly sensitive analytical techniques. Immunochemical methodology – given equal or greater sensitivity – would obviate the need to utilize high specific activity radiolabelled carcinogens (*i. e.*, a condition

restricted to carcinogen exposure of laboratory animals or cultured cells [6–8]). Recently, several groups have successfully demonstrated the applicability of immunological techniques for the specific analysis of minor bases in DNA [9], or of a N-acetoxy-acetylaminofluorene-DNA adduct [10]. As part of studies concerned with the production of antibodies directed against DNA components structurally modified by N-nitroso carcinogens, we report here a radioimmunoassay (RIA) for the specific detection of low levels of O⁶-ethyl-2'-deoxyguanosine (O⁶-EtdGuo).

Materials and Methods

O⁶-EtGua was prepared from 6-chloroguanine [11]. O⁶-methylguanosine (O⁶-MeGuo) and O⁶-EtGuo were synthesized from 6-chloroguanosine (Pharma Waldhof) [12], and further purified by chromatography on a silica gel column eluted with *n*-butanol: glacial acetic acid: H₂O (3:1:1). O⁶-EtGuo was obtained by alkylation of 2'-deoxyguanosine (dGuo) with diazoethane [13] and purified on a Sephadex G-10 column eluted with H₂O. 7-ethylguanine (7-EtGua) was prepared by ethylation of 2'-deoxyguanosine-5'-phosphate with diethylsulfate [14] and purified on a Dowex WX 50 cation exchange column eluted with a linear gradient of 1–8 N HCl. 7-ethylguanosine (7-EtGuo) was synthesized by reacting equal amounts (w/w) of guanosine (Guo) with ethyl iodide in dimethylformamide for 72 h at 50 °C, and purified by pre-

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parative thin layer chromatography on silica gel (developed with *n*-butanol: glacial acetic acid: H₂O: 3:1:1). All synthesized compounds showed >99% purity when analyzed by thin-layer chromatography on silica gel (various solvents) and by high resolution liquid chromatography on Aminex-A6 eluted with 0.45 M ammonium formate, pH 4.5 [15]. O⁶-ethylguanosine-5'-monophosphate (O⁶-EtGMP) and O⁶-ethyl-2'-deoxyguanosine-5'-monophosphate (O⁶-EtdGMP) were obtained by reacting equimolar amounts of 2'-deoxynucleoside and phosphoryl chloride in triethylphosphate [16]. The products were purified on a column of DEAE-Sephadex A-25 eluted with a linear gradient of 0-0.6 M triethylammonium bicarbonate, pH 8. All other compounds were purchased from Sigma. O⁶-Et[8-³H]Guo and O⁶-Et[8,5'-³H]dGuo were obtained by alkylation with diazoethane [13] of [8-³H]-Guo (Amersham; spec. act. 10 Ci/mmol) and [8,5'-³H]dGuo (prepared by hydrolysis of [8,5'-³H]dGTP; NEN; spec. act. 29.8 Ci/mmol), respectively. The products were purified by chromatography on a Sephadex G-10 column eluted with H₂O. The specific activities of the ethylated nucleosides were 3 Ci/mmol for O⁶-Et[8-³H]Guo and 10 Ci/mmol for O⁶-Et[8,5'-³H]dGuo, respectively.

O⁶-ethylguanosine (O⁶-EtGuo) was reacted with bovine serum albumin (BSA; Serva) [17] at a nucleoside/BSA ratio of 1:1 (w/w). The conjugate was purified by dialysis against phosphate buffered saline (PBS), followed by gel filtration on Sephadex G-50. By this procedure about 20 nucleosides were coupled per BSA molecule, as confirmed by difference spectra and the incorporation of ³H-labeled nucleoside.

Six rabbits were immunized with 5 mg/animal of O⁶-EtGuo-BSA in 2.5 ml of PBS emulsified in 2.5 ml of complete Freund's adjuvant, by injection into the foot pads and into about 10 intramuscular sites. Four weeks later, the 3 animals with the highest titers were boosted by intravenous (i. v.) injection of 500 µg of conjugate/animal in 1 ml of PBS at 2-week intervals.

IgG-fractions were purified by precipitation from the rabbit sera with 33% ammonium sulphate. The precipitate was redissolved and dialyzed against 20 mM phosphate buffer, pH 8.0. Remaining impurities were removed by passage through a column

of DEAE-cellulose (Whatman DE 52) equilibrated and eluted with the same buffer.

The RIA was carried out under equilibrium conditions as a modified Farr assay [18], at an antibody concentration sufficient to bind 50% of tracer in the absence of inhibitor. Each sample contained in a total volume of 50 µl of buffer (140 mM NaCl; 20 mM Tris-HCl, pH 7.5; 3 mM NaN₃; 1% BSA [w/v], and 0.1% normal rabbit IgG [w/v]), 4500 dpm of O⁶-Et[8,5'-³H]dGuo, purified IgG from antinucleoside sera, plus varying amounts of inhibitor. After incubation for 5 min at 20 °C, 50 µl of a saturated ammonium sulphate solution were added. Ten min later the samples were centrifuged for 3 min at 10,000 × *g*. The ³H-radioactivity in 50 µl of the supernatant was counted in 10 ml of toluene-based scintillation fluid (Rotiszint 22; Roth) in a Mark II (Searle) liquid scintillation spectrometer. The inhibition of tracer-antibody binding was calculated by the following formula:

$$\text{inhibition (\%)} = \frac{\text{dpm}_{S1} - \text{dpm}_{S2}}{\text{dpm}_{S3} - \text{dpm}_{S2}} \times 100;$$

dpm_{S1} = dpm in the supernatant of inhibitor-containing sample (test sample); dpm_{S2} = dpm in the supernatant of sample without inhibitor (0% inhibition); dpm_{S3} = dpm in the supernatant of sample without antibodies (taken as value for 100% inhibition). Samples were run as triplicates, and the measured values were within ±2% of the mean.

The *in vitro* reaction of calf-thymus DNA (Serva; 2 mg/ml) with EtNU (Roth; twice recrystallized from methanol; 190 µg/ml) was carried out in 25 mM potassium phosphate buffer, pH 7.25, 37 °C. The reaction was stopped by precipitating the DNA with cold ethanol. For *in vivo* ethylation of liver and brain DNA, EtNU was administered i. v. to 5-week-old BDIX-rats, and the DNA isolated 1 h later, as previously described [8, 19]. Enzymic hydrolysis of DNA was performed in a buffer containing 20 mM Tris-HCl, 5 mM MgCl₂, pH 7.5, at 37 °C. DNA (1–3 mg/ml) was first digested with 100 µg of DNase I (EC 3.1.4.5; Boehringer; 1000 Kunitz units/mg) per mg of DNA for 30 min. Snake venom phosphodiesterase (EC 3.1.4.1; Boehringer, 50 µg/mg of DNA) and alkaline phosphatase (EC 3.1.3.1; Boehringer, 35 units/mg; 50 µg/mg of DNA) were added. Hydrolysis was complete after 4 h, as confirmed by determination of free phosphate [20]. 2'-deoxyadenosine (dAdo) in the DNA hydrolysate

was deaminated by addition of adenosine deaminase (ADA; EC 3.5.4.4; Boehringer; 0.3 units/mg of DNA). After 5 min of incubation at 20 °C, dAdo was completely converted to 2'-deoxyinosine (dIno), as shown by thin layer chromatography on silica gel and the decrease in absorption at 265 nm [21]. This procedure does not lead to measurable deethylation of O⁶-EtdGuo. DNA concentrations in the samples were determined spectrophotometrically at 260 nm ($E_{260}^{1\text{cm}} = 28$, for 1 mg of hydrolyzed DNA/ml), or by the diphenylamine reaction [22]. dGuo represents 21.8 and 21.5%, respectively, of total nucleosides in bovine and rat DNA [23].

Results and Discussion

The antibody activity in the rabbit sera was measured with the RIA. Antibody titers were expressed as the final serum dilutions giving 50% binding of tracer (O⁶-Et[8,5'-³H]dGuo) in the absence of inhibitor. Four weeks after the initial immunisation the sera had antibody titers of 1:1400 – 1:3000. The antibody titers then increased from 1:14 000 – 1:18 000 on the 10th day after the 1st booster, to 1:20 000 – 1:60 000 on the 10th day after the 2nd booster. Thereafter no further increase of antibody titers was observed, and the rabbits were exsanguinated at 10 days after the 5th booster. The molar antibody concentrations of the sera and the antibody association constants were determined by the ammonium sulphate precipitation method [24]. Binding curves were established for at least two antiserum dilutions (containing 3 – 50 × 10⁻⁹ mol of specific antibody binding sites/l), over a range of 3 – 300 × 10⁻⁷ mol of hapten (tracer)/l. The concentrations of antibody binding sites varied from 1.5 – 4.3 × 10⁻⁵ mol/l of serum. For all 3 sera, the antibody association constants, as determined at 50% saturation of the antibody binding sites, were 1 × 10⁸ l/mol and 7 × 10⁸ l/mol, respectively, for the tracers O⁶-Et[8-³H]Guo and O⁶-Et[8,5'-³H]dGuo.

Using the antibodies described above and O⁶-Et[8,5'-³H]dGuo as the tracer in the RIA, 0.3 picomoles of O⁶-EtdGuo were sufficient to inhibit tracer-antibody binding by 50%. As shown in Table I, all other alkylated DNA components analyzed, exhibited considerably lower degrees of inhibition. The lower inhibition of tracer-antibody binding by O⁶-EtGuo, O⁶-EtGua, and O⁶-ethyl-2'-deoxyguanosine-5'-monophosphate indicates a spe-

Table I. Radioimmunoassay for O⁶-ethyl-2'-deoxyguanosine (O⁶-EtdGuo). Inhibition of tracer (O⁶-Et[8,5'-³H]-2'-dGuo)-antibody binding by various alkylated and natural nucleic acid components.

Compound	Amount required for 50% inhibition	
	picomol	multiples of O ⁶ -EtdGuo
O ⁶ -Ethyl-2'-deoxyguanosine	0.3	1
O ⁶ -Ethylguanosine	1.8	6
O ⁶ -Ethyl-2'-deoxyguanosine-5'-MP	10	33
O ⁶ -Ethylguanosine-5'-MP	20	67
O ⁶ -Ethylguanine	30	100
O ⁶ -Methylguanosine	138	460
7-Ethylguanosine	2.5 × 10 ⁴ *	~3 × 10 ⁵
7-Ethylguanine	1 × 10 ⁴ *	~1 × 10 ⁵
7-Methylguanine	n. i. d.	>10 ⁶
2'-Deoxyadenosine	2.5 × 10 ⁴ *	~3 × 10 ⁵
2'-Deoxyguanosine	1 × 10 ⁵ *	~1.5 × 10 ⁶
2'-Deoxyinosine	1.5 × 10 ⁵ *	~2 × 10 ⁶
Adenosine	7.5 × 10 ⁴ *	~1 × 10 ⁶
Guanosine, Inosine	1 × 10 ⁵ **	>3 × 10 ⁶
2'-Deoxycytidine, Thymidine	2.5 × 10 ⁵ **	>10 ⁷
Cytidine, Uridine	2.5 × 10 ⁵ **	>10 ⁷
Adenine, Guanine	2.5 × 10 ⁴ **	>10 ⁶
Cytosine, Thymine, Uracil	n. i. d.	>10 ⁷
DNA-Hydrolysate (Nucleosides) ¹	5 × 10 ⁴ **	>10 ⁶
DNA-Hydrolysate, ADA-treated	1 × 10 ⁵ **	>3 × 10 ⁶

n.i.d. no inhibition detectable,

* 15% inhibition at this concentration,

** ≤ 5% inhibition at this concentration,

¹ considerable extent of conversion of dAdo into dIno during hydrolysis, due to trace amounts of adenosine deaminase (ADA) in alkaline phosphatase used.

cific recognition in O⁶-EtGuo not only of the O⁶-ethyl group but also of the deoxyribose moiety (Table I). Ribonucleosides oxidized by periodate and subsequently reduced by borohydride during coupling to BSA, structurally resemble deoxyribonucleosides [17]. The O⁶-EtGuo-BSA conjugate used as an immunogen in the present study was prepared according to this procedure. This could explain the significantly higher inhibition of tracer-antibody binding by O⁶-ethylated deoxyguanosine as compared to O⁶-ethylated guanosine.

All naturally occurring bases and nucleosides showed inhibition of tracer-antibody binding only at very high concentrations (Table I; Fig. 1). dAdo exhibited the comparatively highest degree of inhibition, but could easily be converted to dIno by addition of ADA to the DNA hydrolysates (see

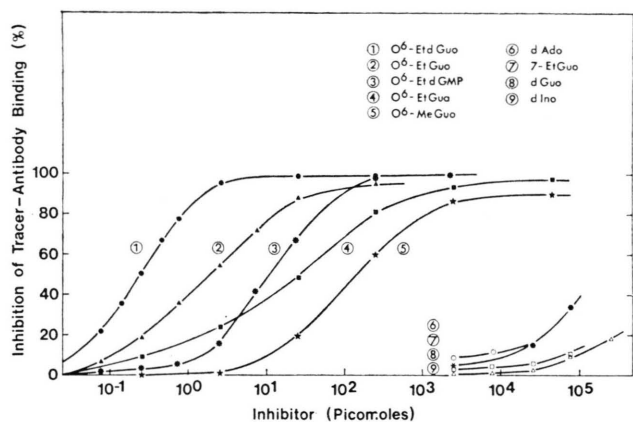


Fig. 1. Inhibition of tracer (O⁶-Et[8,5-³H]dGuo) binding to rabbit-anti-(O⁶-EtGuo-BSA) antibodies by various alkylated and natural nucleic acid components.

Materials and Methods). The inhibitory effect of dIno is 6 times lower than that of dAdo. The 50% inhibition value of O⁶-EtdGuo remained uninfluenced over a concentration range of up to 50 μ g of ADA-treated DNA hydrolysate added to the 50 μ l RIA-samples. Therefore, the O⁶-EtdGuo concentrations of the test samples, at dilutions giving 50% inhibition, could be determined without background correction by comparison with the standard inhibition curve (Fig. 1). For determinations of O⁶-EtdGuo in the presence of higher amounts of DNA (*e. g.*, in the case of lower molar ratios of O⁶-EtdGuo/dGuo), calibration curves were constructed for the respective DNA concentrations (≤ 130 μ g of DNA/sample). Thus, in an amount of DNA (130 μ g) equivalent to the DNA content of about 2×10^7 diploid cells, O⁶-EtdGuo can be determined at a molar ratio of O⁶-EtdGuo/dGuo $\sim 3 \times 10^{-6}$, *i. e.*, about 5×10^3 O⁶-EtdGuo molecules per diploid cell.

Recently developed RIAs for O⁶-methyl-deoxyguanosine ([25]; S. A. Kyrtopoulos and P. F. Swann, personal comm.) gave lower levels of sensitivity and specificity than the RIA for O⁶-EtdGuo described here. However, aside from differences in immunization schemes and the use of tracer structurally more dissimilar to the inhibitor to be analyzed [20], the ethyl group at the O⁶ of dGuo may represent a better recognizable antigenic site than the corresponding methyl substitution.

In Fig. 2 we give two examples of the application of the present RIA for the determination of the

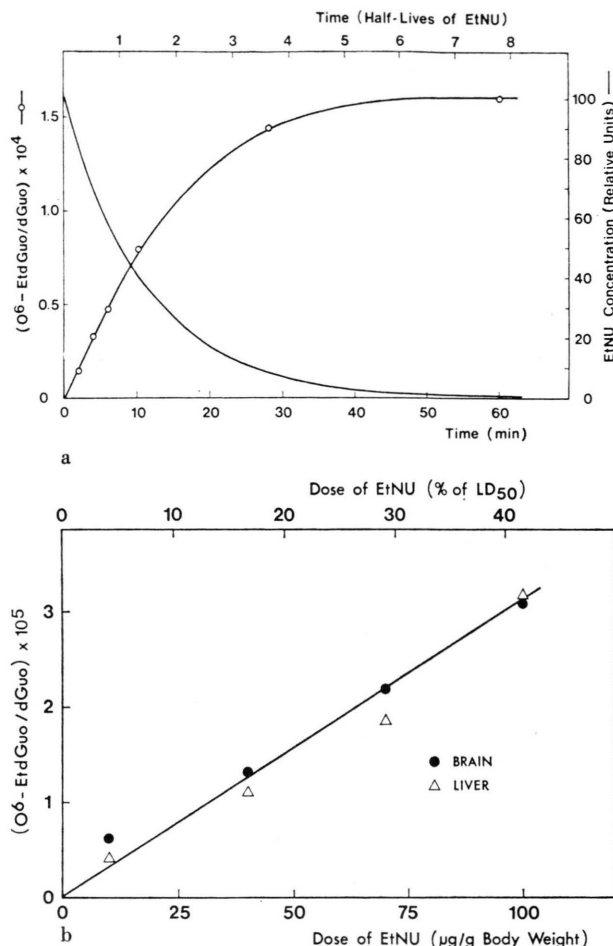


Fig. 2. a) Ethylation by EtNU of O⁶-dGuo in double-stranded DNA *in vitro*. For comparison, heterolytic decomposition of EtNU, as indicated by the decrease in absorption at $\lambda = 235$ nm (rel. units) [19]. b) Ethylation by EtNU of O⁶-dGuo in brain and liver DNA in 5-week-old BDIX-rats.

molar content in DNA of O⁶-EtdGuo, after exposure of DNA to EtNU. Fig. 2 a shows that the kinetics of O⁶-guanine ethylation in DNA mirror the rate of heterolytic decomposition of EtNU (*i. e.*, the generation of the reactive ethyl cation) under quasi-physiological conditions *in vitro*. In Fig. 2 b, the initial (1 h) molar fraction of O⁶-EtdGuo/dGuo in the DNA of brain and liver is demonstrated to be linearly dependent on the dose of EtNU applied to BDIX-rats *in vivo*, over a dose-range of 10–100 μ g of EtNU/g body weight. These data underline the potential of immunological methods for the specific recognition of molecular structures in DNA, and for the detection and quantitation of structural altera-

tions of DNA by carcinogenic and mutagenic agents in particular.

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