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Determining N7-Alkylguanine Adducts by Immunochemical Methods and HPLC with Electrochemical Detection: Applications in Animal Studies and in Monitoring Human Exposure to Alkylating Agents

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Many xenobiotics exert their toxic effects through interaction with DNA in the cells of the exposed organism. This interaction may lead to the formation DNA adducts. Some of these may give rise to mutations that initiate cell transformation and, ultimately, the formation of tumors. Sensitive methods for determining DNA adducts are indispensable for the study of chemical mutagenesis and carcinogenesis and for biomonitoring human exposure to genotoxic agents. Alkylating agents form an important class of genotoxic compounds. They react preferentially at the N7-position of guanine. Under neutral or acidic conditions, the adducts can be readily released from the DNA backbone as the free base N7-alkylguanine (N7-AlkGua). The imidazole ring of N7-alkyldeoxyguanosine (N7-AlkdGuo) can be opened under alkaline conditions, which results in formation of a more stable adduct in DNA. To develop immunochemical methods for the detection of N7-alkylations, we immunized mice with various alkylguanosines in the ring-opened form (RON7-AlkdGuo). Antibodies were selected to detect adducts in isolated DNA by competitive ELISA and in single cells by immunofluorescence microscopy (IFM). Various monoclonal antibodies were characterized in detail with respect to specificity and sensitivity toward methylated, ethylated, and hydroxyethylated DNAs. The antibodies showed extensive cross-reactivity toward N7-(m)ethyl- and N7-(2-hydroxyethyl)guanine modifications in the ring-opened form. The limits of detection in the direct and competitive ELISA were 5–10 and 1–2 adducts per 10^6 nucleotides, respectively. The detection limit of the IFM method was about 20 adducts per 10^6 nucleotides. To calibrate the immunochemical methods, we used an HPLC with electrochemical detection (HPLC-EC) to quantify N7-methyl-, N7-ethyl-, and N7-(2-hydroxyethyl)guanine released from DNA by neutral thermal hydrolysis. The base adducts were separated from nonmodified guanine and adenine on a reverse-phase HPLC column and quantified by use of an EC-detector at an oxidation potential of 1.35 V. For each of the alkylated guanines mentioned, the detection limit was about 0.3 adducts per 10^6 nucleotides. Results are presented of the application of the immunochemical assays and HPLC-EC to investigate the induction of methylations in liver DNA of rats treated *in vivo* with low dosages of hydrazine and the induction of DNA methylations in nucleated peripheral blood cells collected from a patient who received chemotherapy with the methylating cytostatic drug dacarbazine.

Introduction

It is generally accepted that most chemical carcinogens initiate neoplastic growth by interaction with the genetic material. DNA modification is the first step in the process that may finally lead to mutation, cell transformation, and tumorigenesis (*1*). Alkylating agents, including *N*-alkylnitroso compounds, can be regarded as an important group of environmental and occupational carcinogens (*2–4*). These chemicals give rise to the transfer of alkyl groups (methyl, ethyl, etc.) to nucleophilic sites in DNA, preferentially to the N7-atom of guanine (*5*). Sensitive methods for detecting and quantifying the resulting alkyl–DNA adducts are indispensable for mechanistic studies of mutagenesis and carcinogenesis induced by alkylating agents and for biomonitoring human populations with an increased risk for exposure to such genotoxicants (*6,7*). Among the various methods that are available for dosimetry of genotoxic damage are physicochemical and immunochemical techniques (*8,9*).

This paper examines the development and validation of immunochemical methods for detecting N7-alkylated guanines with specific monoclonal antibodies. As an independent method to corroborate the immunochemical data, an HPLC method was developed with sensitive electrochemical detection of several N7-alkylguanines. Both methods were set up for methyl (Me),

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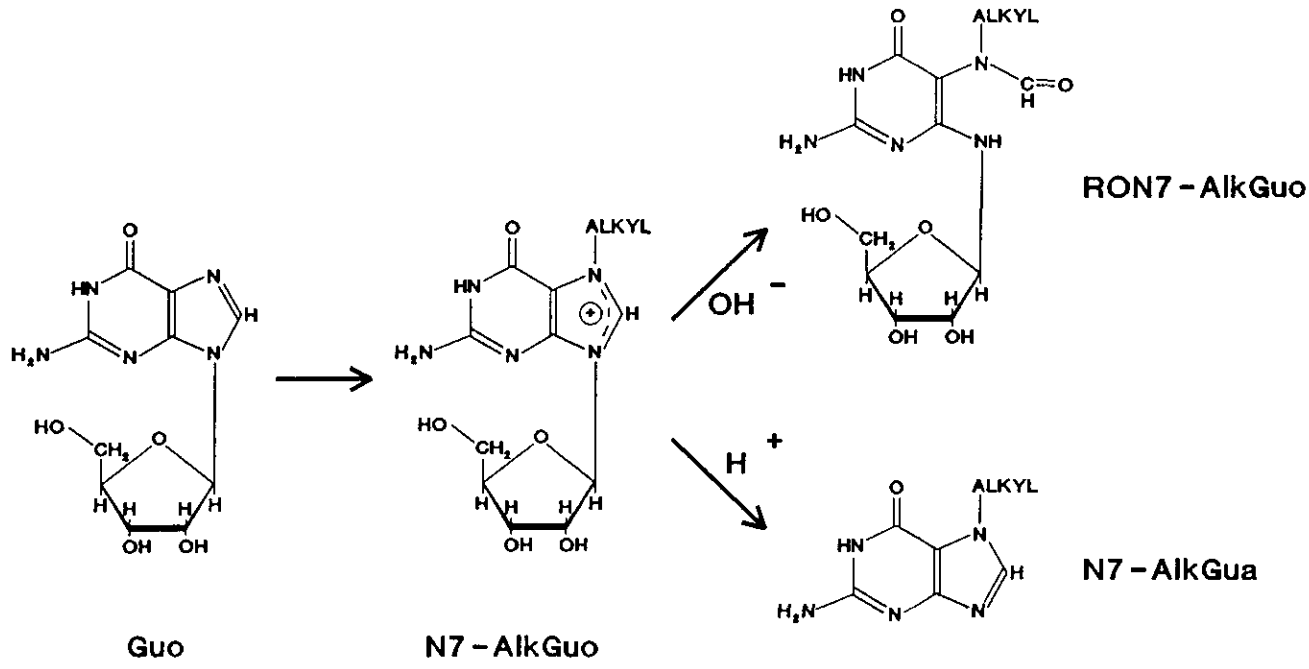

FIGURE 1. Formation of N7-alkylguanosine followed by ring-opening or depurination.

ethyl (Et), and 2-hydroxyethyl (EtOH) adducts. The latter adduct is formed on exposure of DNA to ethylene oxide. Results are presented of the comparative immunochemical and electrochemical analysis of N7-methylguanine adducts in liver DNA isolated from rats treated *in vivo* with hydrazine, an indirect methylating agent. Preliminary results are presented of the immunochemical analysis of alkyl–DNA damage in human blood cells obtained during chemotherapy of a patient with the alkylating cytostatic drug dacarbazine.

N7-alkylguanine (N7-AlkGua) is the most abundant form of base damage in alkylated DNA (*5*). Formation of this adduct involves the introduction of a positive charge in the imidazole ring of the purine, which leads to an unstable chemical structure. This instability can be resolved in one of two ways. The imidazole ring of N7-AlkGua can be opened under alkaline conditions, which results in the formation of a more stable adduct in DNA. Under neutral or acidic conditions hydrolysis can occur, during which N7-AlkGua is cleaved off (Fig. 1).

In the approach to immunochemical detection of N7-alkyldeoxyguanosine (N7-AlkdGuo) in DNA, the nucleoside adducts in their stable ring-opened form were used as haptens to obtain the specific antibodies needed. Prior to immunochemical detection, N7-alkylguanines in DNA were converted to the ring-opened form. Electrochemical detection of DNA alkylation was based on analysis of the free base adducts (N7-alkylguanines).

Materials and Methods

Preparation of N7-Alkylguanosines, N7-Alkylguanines, and Alkylated DNA

N7-Ethylguanosine (N7-EtGuo) and N7-hydroxyethylguanosine (N7-EtOHGuo) were synthesized by treating guanosine with *N*-ethyl-*N*-nitrosourea (ENU) and ethylene oxide (EtOx), respectively. N7-methylguanosine (N7-MeGuo) was purchased from Sigma (St. Louis, MO). After purification of the nucleoside adducts on a silica column, one part was converted into the imidazole ring-opened form by treatment with alkali, while another part was depurinated by heating at neutral pH. Some of the ring-opened (RO) product was also depurinated. The products RON7-AlkGuo, N7-AlkGua, and RON7-AlkGua were purified by reverse-phase or cation-exchange HPLC. The purity of several products was verified by ^{1}H-NMR spectroscopy and MS–MS analysis (courtesy of E. Marafante, Joint Research Centre of the European Community, Ispra).

DNA containing RON7-AlkGua was prepared by *in vitro* treatment of salmon sperm DNA with ENU, *N*-methyl-*N*-nitrosourea (MNU), or EtOx, followed by alkali-induced ring opening of the N7 adducts. Human white blood cells (WBC) with N7-alkyl-DNA adducts were obtained by *in vitro* exposure of WBC from a healthy donor to ENU or EtOx, followed by fixation in 70% ethanol. Later on, during the immunochemical procedures, the N7 adducts were transformed into the ring-opened form [see van Delft et al. (*10*) for experimental details].

Isolation and Selection of Antibodies

Antibodies were raised in mice against the ring-opened form of N7-EtGuo (RON7-EtGuo). A series of monoclonal antibodies were obtained, which were characterized with respect to cross-reactivity with N7-methyl, N7-hydroxyethyl, and N7-sulfur mustard adducts. Antibodies were especially selected for good performance in *in situ* detection of DNA damage, by means of a cell ELISA (*10*). In a second series of immunizations, antibodies were raised against RON7-EtOHGuo. Suitable antibodies (N7E-026 and N7E-102, raised against RON7-EtGuo, and N7EO-E, raised against RON7-EtOHGuo) were selected and used to determine the level of DNA alkylation in single cells,

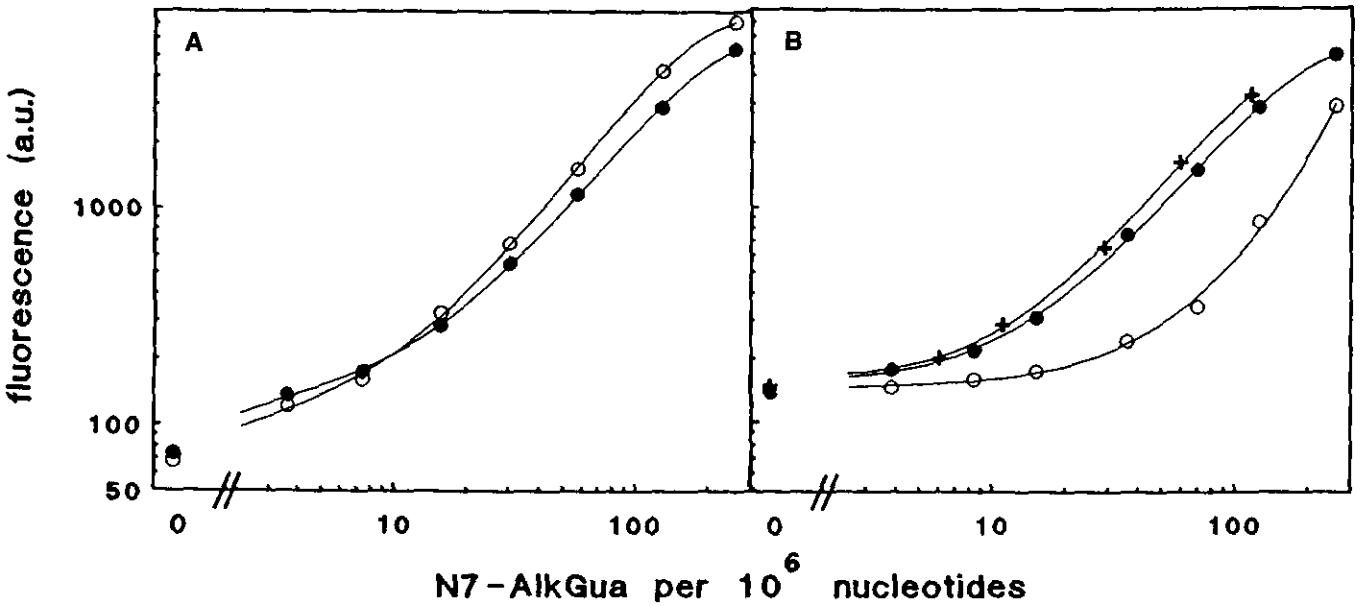

FIGURE 2. Characterization of monoclonal antibodies raised against ring-opened N7-ethylguanosine (RON7-EtGuo) or 2-hydroxyethylguanosine (RON7-EtOHGuo) by use of a direct ELISA. (*A*) Salmon sperm DNA was treated with various amounts of ethylene oxide and used as coating antigen. The antibodies tested were N7E-102, raised against RON7-EtGuo (○), and N7EO-E, raised against RON7-EtOHGuo (●). (*B*) Salmon sperm DNA treated with various amounts of *N*-methyl-*N*-nitrosurea (+) or ethylnitrosourea (○,●) was used as coating antigen. The antibodies tested were N7E-026 (+,●) and N7E-102 (○), both raised against RON7-EtGuo. Adduct levels in the coating DNAs (abscissa) were determined by HPLC-EC. The fluorescence indicated on the ordinate is a measure of the amount of antibodies bound to the immobilized antigen (see Materials and Methods). Averages of four wells are presented [see van Delft et al. (*10*) for details].

Table 1. Competitive ELISA of monoclonal antibodies against RON7-AlkGuo with alkylated DNA.

Competitor DNA		fmole N7-AlkGua at 50% inhibition for MAb[a]		
Alkylation	Adduct level[b]	N7E-026	N7E-102	N7EO-E
Methyl	11	57	ND	ND
	60	66	ND	ND
	117	43	ND	ND
Ethyl	11	150	ND	ND
	60	180	ND	ND
	259	130	ND	ND
2-Hydroxyethyl	16	ND	310	89
	57	ND	77	70
	256	ND	28	52

Abbreviations: Mab, monoclonal antibody; RO, ring opened; N7-AlkGuo, N7-alkylguanosine; N7-AlkGua, N7-alkylguanine; MNU, *N*-methyl-*N*-nitrosourea; DES, diethylsulfate; EtOx, ethylene oxide; EtGuo, ethylguanosine; EtOH, 2-hydroxyethyl.

[a]Expressed as the amount in inhibitor in a well of a microtiter plate that induces 50% inhibition of the binding of the MAb (culture supernatant diluted 6000- to 30000-fold) to immobilized antigen [DNA treated with MNU (1400 Me/10^6 nucleotides), DES (2600 Et/10^6 nucleotides), or EtOx (730 EtOH/10^6 nucleotides)]. Antibodies N7E-026 and N7E-102 were raised against RON7-EtGuo; N7EO-E was raised against RON7-EtOHGuo. For the three antibodies tested, the amount of unmodified DNA required for 50% inhibition of antibody binding varied between 5×10^7 and 2×10^8 fmole nucleotides.

[b]Determined by HPLC-EC; values expressed as RON7-AlkGua per 10^6 nucleotides.

e.g., in human WBCs by immunofluorescence microscopy (IFM) and in isolated DNA by (competitive) ELISA. The various immunoassays are briefly described in the following paragraphs. Full experimental details of these procedures have been previously published (*10*).

Immunochemical Methods

The ELISA is a well-known method for quantifying antigens. In the assay as applied in the present work, DNA (with or without adducts) was used as immobilized antigen. The amount of monoclonal anti-RON7-alkylGuo antibody bound to the coating antigen was determined by binding of an alkaline phosphatase/goat-anti-mouse immunoglobulin conjugate and incubation with a colorigenic or fluorogenic substrate. In the competitive ELISA, the amount of antigen is determined that causes 50% inhibition of the binding of antibodies to immobilized alkylated DNA. This value is a measure of the relative preference of the antibody for the inhibitor.

For the IFM procedure, fixed WBC were deposited on glass slides, treated with alkali to induce denaturation of DNA and ring opening of N7-alkyl adducts, and incubated with anti-adduct monoclonal antibody and goat-anti-mouse immunoglobulin-fluorescein isothiocyanate (FITC) conjugate. With a scanning laser fluorescence microscope and computer processing of the emission signal, the amount of FITC fluorescence per nucleus can be determined (*11*).

HPLC with Electrochemical Detection

A physicochemical method, HPLC with electrochemical detection (HPLC-EC), was developed for the quantification of the free base adducts N7-MeGua, N7-EtGua (*12*), and N7-EtOHGua (*13*). Electrochemical detection of compounds is based on redox reactions, which occur at the appropriate oxidation potential. Detection was achieved by use of an amperometric detector cell (Antec, Leiden, The Netherlands), containing a glassy carbon working electrode and an Ag/AgCl reference elec-

trode. Changes in electric current (in nA) through the cell are recorded. The amount of N7-AlkGua in DNA samples was quantified by comparing peak heights with those observed upon analysis of standard samples. DNA samples for HPLC-EC were prepared as follows. N7-AlkGua was liberated from DNA by neutral thermal hydrolysis, whereafter DNA was removed by precipitation with HCl and centrifugation. The supernatant was neutralized and analysed by HPLC-EC. Full experimental details of the HPLC-EC method have been published elsewhere (*13*).

Isolation of Rat Liver DNA Samples

Rats (Wistar) were fasted overnight and dosed by gavage with various amounts of hydrazine (0.01–10 mg/kg body weight). After 16 hr, the animals were sacrificed and the livers were removed, frozen, and pulverized (these experiments were performed by J. Lewalter at Bayerwerke, Leverkusen, FRG). DNA isolation was performed in several steps, through sequential isolation of nuclei, chromatin, and nucleic acids. RNA was then degraded by incubations with RNAse A and T1. The absence of RNA was checked by chromatographic determination of the amount of 3′-riboguanosine monophosphate in an enzymic DNA digest (M.-J.S.T. Steenwinkel, manuscript in preparation). Before immunochemical analysis, the DNA was alkali treated for 30 min at 37 °C to induce ring opening of N7-methylguanine adducts. Prior to electrochemical analysis, the DNA was incubated for 30 min at 70 °C, to release N7-MeGua.

Processing of Blood Samples from Patients Receiving Chemotherapy

Blood was collected from a patient who received chemotherapy with the methylating cytostatic drug dacarbazine (DTIC; 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide). The dose, administered by infusion, was 400 mg/m^2. Blood samples were taken at various time points following the treatment and immediately cooled on ice. DNA was isolated, alkali treated, and analyzed for the presence of N7-MeGua adducts in a direct ELISA [see van Delft et al. (*10*) for experimental details].

Results

Immunochemical Detection

Monoclonal antibodies were raised against RON7-EtGuo and RON7-EtOHGuo. The antibodies were selected for their suitability to recognize these adducts in DNA *in situ* by IFM and in isolated DNA by ELISA. Several antibodies were characterized with respect to specificity, by investigation of their cross-reactivity with other N7-alkyl adducts, and to sensitivity by determination of the detection limits in various immunochemical assays (*10,14*). In addition to antibodies that recognize ethyl and/or 2-hydroxyethyl adducts in the ring-opened form, a suitable monoclonal antibody was obtained for the detection of methyl modifications (N7E-026). Here, data are presented of antibodies N7E-026 and N7E-102 (raised against RON7-EtGuo) and N7EO-E (raised against RON7-EtOHGuo). Alkylation levels in *in vitro* modified DNA were determined by means of ELISA with the various antibodies. The results, presented in Figure 2, show that ethyl, methyl, and 2-hydroxyethyl adducts can be detected with about equal sensitivities. In all cases a dose-dependent increase of the response was observed for adduct levels above 10 N7-alkylations per 10^6 nucleotides.

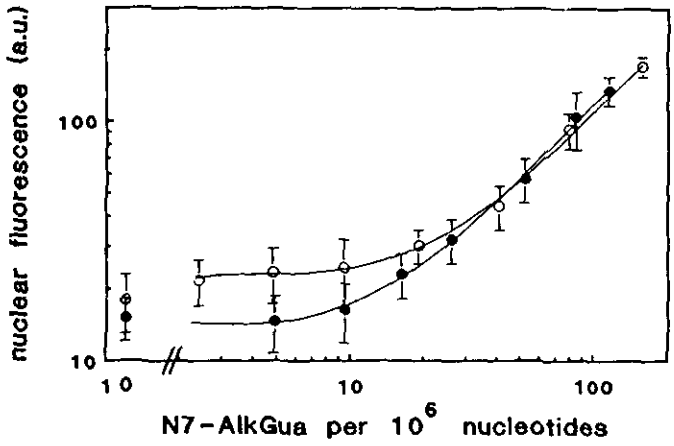

FIGURE 3. Detection of N7-ethylguanine (N7-EtGua) in the nuclei of white blood cells (WBC) by means of immunofluorescence microscopy with antibody N7E-026 (●) and of 2-hydroxyethylguanine (N7-EtOHGua) with N7EO-E(○). The dose–response relationship was established with peripheral human blood cells that had been treated with ethylnitrosourea or ethylene oxide. DNA adduct levels in these cells were determined in purified WBC–DNA by HPLC-EC. Nuclear fluorescence is a measure of the number of antibodies bound to nuclear DNA per cell. Averages with SDs of about 30 cells are presented.

The competitive ELISA can be used to determine the level of N7-alkylation in an unknown DNA sample by comparison of the response with the inhibition curves obtained with standard alkyl-DNA samples. Data in the literature indicate that the binding efficiency of antibodies to competitor DNA may strongly depend on the modification level of this DNA (*15,16*). Experiments were carried out to investigate the effect of the alkylation level of the competitor DNA on the total amount of adducts required for 50% inhibition of anti-alkyl-adduct antibody-binding. The results are shown in Table 1. Adduct levels in methylated, ethylated, and hydroxyethylated DNA (standard preparations) were determined electrochemically (see below). These DNAs were then alkali treated and used as competitors in the competitive ELISA. The 50% inhibition points were determined for the monoclonal antibodies N7E-026 (tested with Me- and Et-DNA), N7E-102, and N7EO-E (tested with EtOH-DNA). Only in the case of antibody N7E-102 was a strong effect of the modification level of the competing DNA observed.

The sensitivity of the *in situ* detection of N7-EtGua and N7-EtOHGua in the nuclei of WBC was investigated by IFM with monoclonal antibodies N7E-026 and N7EO-E. The microscope images were computer processed to identify the nuclei, and the nuclear immunofluorescence was quantified (*11*). A linear relationship was observed between nuclear fluorescence and alkylation levels above 10 N7-EtGua and above 20 N7-EtOHGua per 10^6 nucleotides, respectively. Results are shown in Figure 3.

HPLC with Electrochemical Detection

In order to calibrate the immunochemical assays, quantification of N7-methyl-, N7-ethyl-, or N7- (2-hydroxyethyl)-guanine adducts in the various coating DNAs was carried out by means of HPLC-EC. After neutral thermal hydrolysis of the DNA,

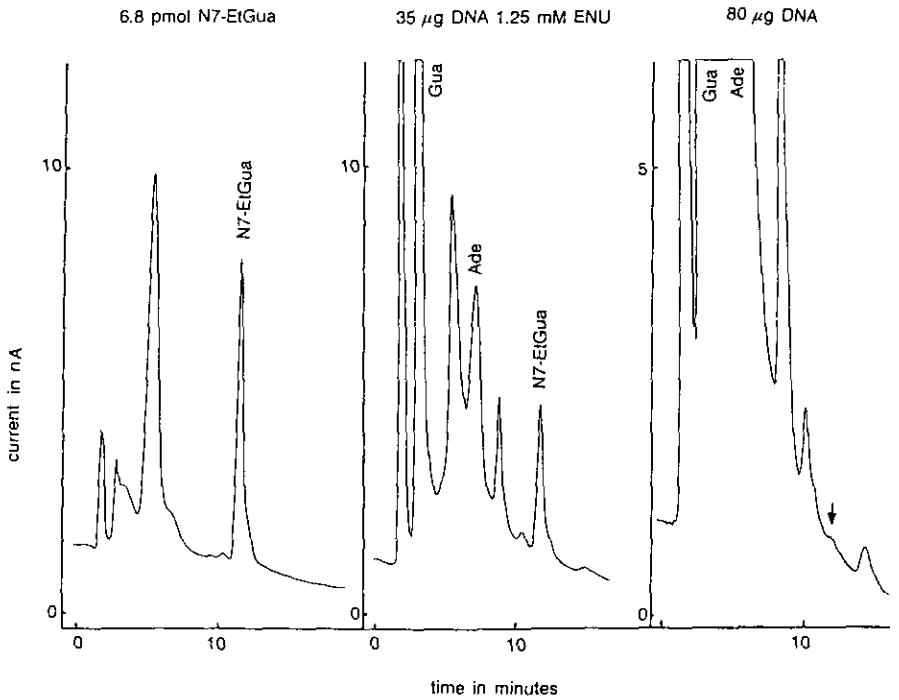

FIGURE 4. HPLC-EC analysis of N7-ethylguanine (N7-EtGua) in DNA. Salmon sperm DNA was treated with 1.25 mM ethylnitrosourea (ENU) for 30 min at 37 °C. After depurination and acid precipitation of the DNA, the supernatant was neutralized and run on reverse-phase HPLC. Electrochemical detection occurred at an oxidation potential of 1.35 V. Analyses are shown of authentic N7-EtGua (left panel), 35 μg ENU-treated DNA (middle panel), and 80 μg control DNA that had been processed in the same way (right panel). The positions of the guanine, adenine, and N7-EtGua peaks were identified by HPLC analysis with a diode-array UV-detector and that of the N7-EtGua peak also by co-chromatography with authentic N7-EtGua.

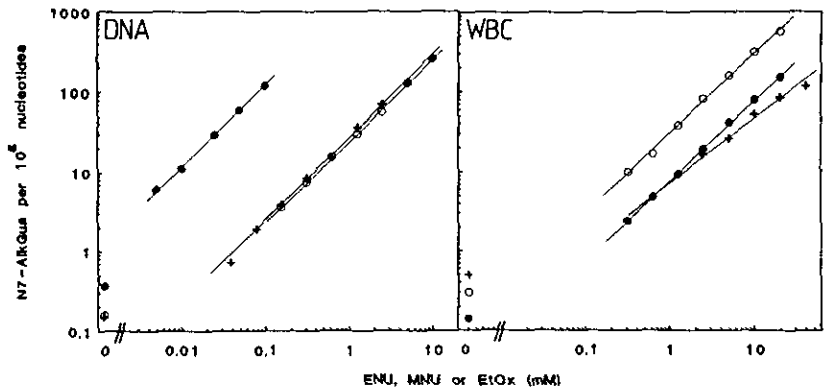

FIGURE 5. Determination of N7-alkylguanine (N7-AlkGua) by HPLC-EC. (Left) Salmon sperm DNA was treated with various amounts of *N*-methyl-*N*-nitrosourea (●), ethylnitrosourea (ENU) (+) or ethylene oxide (EtOx) (○). After thermal hydrolysis at neutral pH and precipitation of DNA, the liberated base adducts (N7-AlkGua) were separated from other nucleobases by reverse-phase HPLC and detected with the electrochemical detector set at 1.35 V. The background current was 15–30 nA. Data are given as the average result of two analyses. The slope of the log/log plots is about 1.02. (Right) White blood cells (WBC) from a healthy donor were incubated at 37 °C with various amounts of ENU (30 min, +) or EtOx (15 min, ●; 60 min, ○). DNA was isolated from the cells, whereafter N7-AlkGua was liberated, purified, and analyzed as described above. The detection limit of this HPLC-EC analysis is about 0.3 AlkGua per 10^6 nucleotides in isolated DNA (*10,13*).

N7-AlkGua was separated from other nucleobases by chromatography on a reverse-phase HPLC column and analyzed electrochemically. A typical elution pattern obtained with ENU-treated DNA is shown in Figure 4. The peaks were quantified on the basis of the signals obtained with known amounts of authentic N7-AlkGua (Fig. 4, left panel).

Determination of adducts in a series of DNA samples exposed *in vitro* to an alkylating agent (MNU, ENU or EtOx) also showed a linear dose-dependent increase in the level of N7 modifications (Fig. 5, left panel). Similar results were obtained upon teatment of nucleated blood cells with ENU or EtOx, isolation of DNA, and analysis of N7-alkylguanine (Fig. 5, right

panel). The detection limit of this HPLC-EC analysis for DNA samples is about 0.3 N7-AlkGua per 10^6 nucleotides.

Applications of ELISA and HPLC-EC for Analysis of DNA alkylation

Analysis of N7-Methylguanine Adducts in Liver DNA of Rats Treated with Hydrazine. Hydrazine, (NH_2NH_2), was shown to induce methylations in DNA by an indirect mechanism, which probably involves *S*-adenosyl-methionine (*17*). It belongs to a group of compounds for which there is evidence that the *in vivo* genotoxic effects become manifest only upon exposure to acutely toxic dose levels (*18*). Experiments were carried out to investigate whether this phenomenon is reflected in the pattern of dose-dependent DNA methylation after *in vivo* exposure of rats to various doses of hydrazine. Sixteen hours after treatment with a single oral dose of the compound, the animals were killed and DNA was isolated as described in Materials and Methods. Part of the DNA was treated with alkali to induce ring opening of the N7-linked guanine adducts. This material was further analyzed by competitive ELISA with antibody N7E-026, which shows a strong (cross)reactivity with N7-MeGuo lesions in DNA. Another part of the DNA was heated at pH 1 and 70°C for 30 min to release N7-MeGua. After prepurification by cation-exchange HPLC, the fraction containing the alkylguanine was analyzed by reverse-phase HPLC-EC (the protocol for this prepurification and analysis was kindly provided by A. de Groot, State University, Leiden). Details of these experiments will be published elsewhere (Van Delft et al., manuscript in preparation). The combined results, presented in Figure 6, show a dose-dependent increase in the level of DNA methylation after hydrazine doses of 1 mg/kg or higher. Furthermore, there appears to be a background level of methyl DNA adducts in untreated animals.

Analysis of DNA Methylation Induced by Chemotherapeutic Treatment. An interesting application of the various methods for dosimetry of DNA damage in humans *in vivo* is the analysis of the effects of iatrogenic exposure, *i.e.*, during chemotherapeutic treatment of patients with genotoxic cytostatic compounds (*14*). We investigated the formation of methyl adducts in DNA of peripheral blood cells obtained from a patient who received a single dose of dacarbazine, an indirectly methylating cytostatic drug. Immunochemical detection of N7-MeGua in DNA isolated from these cells was carried out by direct ELISA, with antibody N7E-026. Results are shown in Figure 7. During the first hour after the end of the treatment, a rapid increase in the level of methylation was observed, followed by a slow decrease during the following days.

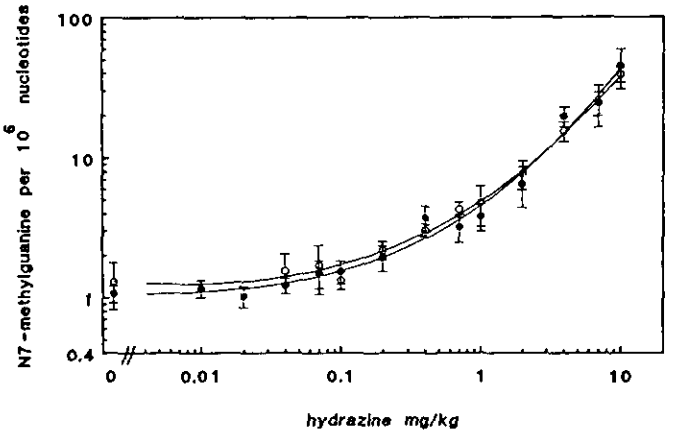

FIGURE 6. Formation of N7-methylguanine (N7-MeGua) in liver DNA of rats exposed to hydrazine. Rats received a single dose of hydrazine by gavage. The animals were sacrificed 16 hr after the treatment. Liver DNA was isolated and processed for immunochemical or electrochemical detection of N7-methylguanine, as described in Materials and Methods. Bars represent range of values of duplicate experiments from three or six rats at each hydrazine concentration. The detection limit, i.e., the response observed with unmodified salmon sperm DNA, was 1 N7-MeGua for the ELISA, and 0.4 N7-MeGua for the HPLC-EC method.

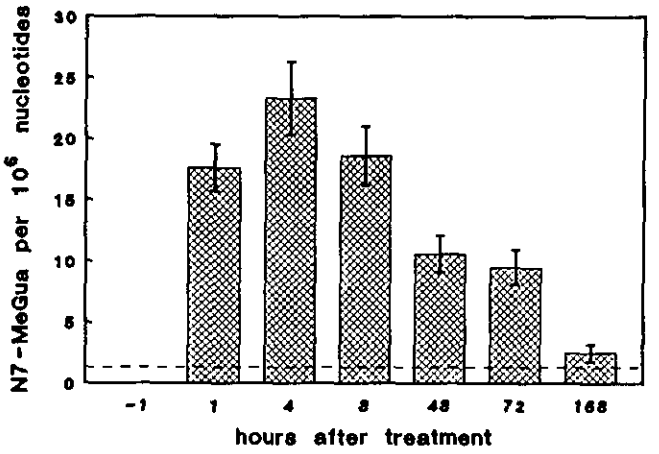

FIGURE 7. Analysis of N7-methylguanosine (N7-MeGua) adducts in peripheral blood cells of a patient who received 400 mg/m^2 decarbazine by IV infusion during 30 min. The first blood sample was collected 1 hr after the end of the infusion period. The analysis of N7-MeGua in DNA was carried out in a direct ELISA as described in Materials and Methods. The signal observed with the sample taken before the start of the treatment, indicated by −1 on the abscissa, was below the limit of detection (1.2 N7-MeGua per 10^6 nucleotides; dashed line).

Discussion

This paper has presented the characterization of several monoclonal antibodies directed against various N7-alkylguanosines. During immunization of the mice and screening of the antibodies, the chemically stable imidazole ring-opened form of the alkylated nucleosides was used (*10*; see Fig. 1). The antibodies were compared with respect to their performance in the detection of alkylations in isolated DNA, by direct and competitive ELISA, and *in situ*, at the single-cell level by IFM. As an independent assay to confirm the immunochemical data, HPLC with electrochemical detection (HPLC-EC) was developed for various N7-alkylguanines, released from DNA as free base adducts by neutral thermal hydrolysis.

For three of the antibodies obtained, the specificities and sensitivities toward different ring-opened N7-alkylguanine adducts were investigated in detail (*14*). One of the anti-RON7-EtGuo antibodies (N7E-026) appeared to be suitable for detecting methyl adducts, while another (N7E-102) recognized hydroxyethyl modifications even better than ethyl adducts. In this respect the latter antibody was comparable to that raised directly against RON7-EtOHGuo (N7EO-E; compare the results of the direct

ELISA, Fig. 2). These data indicate that the combination of these antibodies may be useful for analyzing DNA samples that contain various N7-alkylguanine lesions. Such a situation may occur in practical applications, e.g., in biomonitoring studies of human populations exposed to a mixture of uncharacterized alkylating chemicals. Combination of assays with different antibodies may give more information, for instance, with N7E-026 about the total of methyl and ethyl adducts and with N7EO-E more specificallyabout hydroxyethyl guanine.

The sensitivities of the different immunochemical assays appeared to vary substantially. A 5- to 10-fold difference was seen in the two ELISAs. In the direct ELISA the limit of detection was 10 N7-methyl- or N7-ethyl-, and 5–10 N7-hydroxyethylguanine adducts per 10^6 nucleotides, for antibodies N7E-026 and N7EO-E, respectively (see Fig. 2). The competitive ELISA with alkylated DNA as inhibitor was the most sensitive method, allowing the detection of 1–2 N7-alkylguanines per 10^6 nucleotides [data not shown, compare van Delft et al. (*10*)]. This limit is set by the binding of the antibody to untreated DNA. This binding is nonspecific and not indicative of a real alkylation level, because the maximum amounts of N7-EtGua and N7-MeGua in this DNA, as determined by HPLC-EC, are 0.15 and 0.36 alkylations per 10^6 nucleotides, respectively (Fig. 6). The direct ELISA is less sensitive than the competitive ELISA, but it has the great advantage that much smaller amounts of DNA (1 versus 100 μg) are required and that the assay takes less time. The analysis by immunofluorescence microscopy showed the highest detection limit of the methods applied, *viz.* ≈ 20 N7-EtGua per 10^6 nucleotides (Fig. 3). It is not unusual for assays such as ELISA to be one or two orders of magnitude more sensitive than immunocytochemical techniques. The latter assays, however, offer the unique opportunity to analyze DNA modifications at the level of the single cell.

HPLC-EC proved to be a simple and sensitive method for the detection of N7-alkylguanines. It permits a quantitative determination of these adducts over a wide concentration range. The detection limit of the HPLC-EC analysis is in most cases determined by a small electrochemical signal in the HPLC pattern of untreated DNA at the expected elution position of the N7-alkylguanine. In Figure 4 (right panel), the response would correspond to about 0.3 N7-EtGua per 10^6 nucleotides. It is unclear, however, whether this signal really represents N7-EtGua or an unknown component.

Because of its strong cross-reactivity with RON7-MeGuo, antibody N7E-026 was used for the analysis of methylations in liver DNA of rats exposed to a single dose of hydrazine, a compound shown to induce methylations of DNA by an indirect mechanism (*17*). Evidence has been presented that hydrazine can be considered a genotoxic and carcinogenic compound, but probably only at acutely toxic dose levels (*18*). To investigate whether this phenomenon is reflected in the pattern of adduct induction, liver DNA of hydrazine-treated rats was analyzed for the presence of methyl adducts, with the immunoassay and HPLC-EC. The preliminary results of these experiments, shown in Figure 6, demonstrate a good correlation between the two methods. On the basis of the low detection limit of the HPLC-EC technique, the conclusion can be drawn that a certain level of DNA methylation is already present in untreated rats. This would confirm data presented by other authors (*19*). A more detailed account of these experiments and a discussion of the results will be published elsewhere (van Delft et al., manuscript in preparation).

The antibody N7E-026 also appears suitable for analyzing methyl–DNA adduct formation and removal *in vivo*, as was shown with peripheral blood cells of a patient receiving chemotherapy with the methylating cytostatic drug dacarbazine (see Fig. 7). These experiments are part of a study on the *in vivo* kinetics of the formation and persistence of methyl-DNA adducts in man. In this study, both N7-methyl- and O^6-methyl adduct levels in human lymphocytes and granulocytes will be investigated in relation to the capacities of these cells to metabolically activate the methylating genotoxicant and to repair methyl–DNA lesions.

The methods reported in this paper for the detection of N7-EtOHGua may also be applied for the quantification of N7-(2-oxoethyl)guanine (N7-EtOGua), as this base modification can be transformed quantitatively into N7-EtOHGua by reduction with sodium borohydride. N7-EtOGua is the predominant DNA lesion induced *in vivo* by many chemicals, such as vinyl chloride, urethane, and other compounds.

The determination of N7-AlkGua by means of the assays described here may be a useful tool to study the relationship between the induction and repair of damages in cellular DNA on the one hand, and the resulting biological effects, e.g., cytogenetic aberrations, mutagenesis and carcinogenesis, on the other. Furthermore, these techniques can be of value for assessing human exposure to occupational and environmental genotoxicants, e.g., nitrosamines, EtOx, vinyl chloride, and other compounds, by analysis of blood or urine samples. The choice of an analysis method depends on the requirements, such as sensitivity and detection *in situ* or in isolated DNA, and on the amount of material that is available.

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