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Quantitation and Visualization of Alkyl Deoxynucleosides in the DNA of Mammalian Cells by Monoclonal Antibodies

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Conventional radiochromatographic procedures for the quantitation of carcinogen/mutagen-induced structural DNA modifications have a number of limitations. Thus, these techniques for the most part require application of radioactively labeled carcinogens and the use of relatively large amounts of DNA for analysis at low levels of DNA modification. Radiochromatographic methods also preclude analyses at the level of single cells and DNA molecules. Recently developed immunoanalytical methods have improved this situation considerably. Monoclonal antibodies (Mab) characterized by a high substrate specificity and affinity, in combination with radio- and enzyme-immunoassays, or with "immuno-slot-blot" techniques, now permit the detection of femtomole to subfemtomole amounts of, e.g., alkyldeoxynucleosides in small samples of DNA isolated from tissues or cultured cells previously exposed to nonradioactive *N*-nitroso compounds. Furthermore, selected Mab can be used to quantitate by direct immunofluorescence (with the aid of computer-based image analysis of electronically intensified fluorescence signals), specific alkyldeoxynucleosides in the nuclear DNA of single cells. With this method, the detection limit for the alkylation product O^6-ethyldeoxyguanosine (O^6-EtdGuo) is presently of the order of 10^2–10^3 O^6-EtdGuo residues per diploid mammalian genome. Individual cells can thus be monitored for the presence of specific carcinogen–DNA adducts, and with respect to their capacity for enzymatic removal of such modified structures from DNA (as exemplified here by the kinetics of the enzymatic elimination of O^6-EtdGuo from the DNA of malignant neurogenic rat cell lines). In combination with transmission electron microscopy, Mab also permit direct visualization (via Mab binding sites) of specific carcinogen-modified structures in individual DNA molecules. DNA strands of defined nucleotide sequence can thus be analyzed by immuno-electron microscopy for the presence of "hot spots" of specific structural alterations caused by defined carcinogens/mutagens.

Introduction

The sensitive detection and quantitation of defined reaction products of chemical carcinogens, mutagens, or chemotherapeutic agents, with target cell DNA represents an obligatory requirement for analysis of the genetic consequences of specific alterations of DNA structure, for measurements of cellular DNA repair capacity, and for biological dosimetry and risk estimation. Conventional radiochromatographic procedures for analysis of chemically modified DNA have a number of limitations. Thus, with the exception of ^{32}P-postlabeling methods (*1,2*), these techniques require application of radioactively labeled (i.e., laboratory-synthesized) DNA-reactive agents, and the use of relatively large amounts of DNA (cells) for analysis at low levels of DNA modification (*3,4*). This situation has been improved considerably by the recent introduction of immunoanalytical methods, notably those based on the use of monoclonal antibodies (Mab) (*5–16*). Selected high-affinity Mab can now be used for the sensitive and specific recognition and quantitation of DNA components structurally modified by nonradioactive (e.g., environmental) agents.

While other laboratories have focused on the production of antisera, and in some cases of Mab, directed against DNA components structurally altered by reaction with aflatoxin B_1, *N*-2-acetylaminofluorene, or benzo(a)pyrene (*7,9,12–15*), our group has concentrated on the development of Mab specific for alkyldeoxynucleosides produced in cellular DNA by alkylating *N*-nitroso compounds (*5,6,11,17–26*). We have thus exploited the exceptional capability of antibodies to recognize subtle alterations of molecular structure, in order to distinguish deoxynucleosides modified by the covalent attachment of single, small alkyl groups (methyl, ethyl, butyl, isopropyl) from their normal, unaltered counterparts. The results thus far obtained with this approach have been most encouraging. Mab with very high affinity and specificity for the respective alkyl-

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deoxynucleosides were obtained by immunization with alkylribonucleosides as haptens coupled to keyhole limpet hemocyanin (KLH) as a carrier protein (*5,6,22*). With the use of different immunoanalytical methods, specific alkyldeoxynucleosides can now be quantitated with high sensitivity in small amounts of DNA or in DNA hydrolyzed to monodeoxynucleosides, in the DNA of individual cells, and in single DNA molecules (*19,21,22,24–26*). In the present report, we describe the properties of Mab specific for the DNA alkylation products O^6-methyldeoxyguanosine (O^6-MedGuo), O^6-ethyldeoxyguanosine (O^6-EtdGuo), O^6-butyldeoxyguanosine (O^6-BudGuo), O^6-isopropyldeoxyguanosine (O^6-iProdGuo), O^4-methyldeoxythymidine (O^4-MedThd), and O^4-ethyldeoxythymidine (O^4-EtdThd). In addition, the principles as well as the particular advantages of different types of immunoassays will be compared, and an example will be presented showing the application of a competitive radioimmunoassay (RIA) for measuring the kinetics of the enzymatic elimination of O^6-EtdGuo from the DNA of a number of malignant neural rat cell lines.

Immunoanalytical Methods Using Anti-Alkyldeoxynucleoside Antibodies

Production and Characterization of Anti-Alkyldeoxynucleoside Monoclonal Antibodies (Mab)

For immunization, alkylribonucleosides (haptens) were coupled to the carrier protein keyhole limpet hemocyanin (KLH; Calbiochem, Marburg, Germany) (*5,22,27*). Adult female rats of the inbred BDIX strain (*28*), or adult female Balb/c mice, were immunized by intracutaneous injections of the immunogen emulsified in aluminium hydroxide (Alugel S; Serva, Heidelberg, Germany) and Freund's adjuvant (Behring-Werke, Marburg, Germany), following published procedures (*5,22*). Spleen cells isolated from the immunized animals were fused with cells of the mouse myeloma cell line P3-X63-Ag8.653 (*29*) or with cells of the rat myeloma cell line X3-Ag1.2.3 (*30*), respectively, polyethylene glycol (PEG 4000; Roth, Karlsruhe, Germany) being used as the fusion reagent (*5,6*). Hybridoma cell cultures secreting anti-alkyldeoxynucleoside Mab were identified with the aid of an enzyme immunoassay (*6*) or by competitive RIA (see below), and subsequently cloned and recloned. Positive rat × rat and mouse × mouse hybridoma clones were maintained in cell culture for antibody production. When larger amounts of Mab were required, hybridoma cells were injected intraperitoneally either into Pristan-pretreated Balb/c mice or into Pristan-pretreated, X-irradiated (4 Gy) BDIX-rats for antibody production into the ascitic fluid (*5,6*). Mab isotype analyses were carried out with the use of anti-rat isotype antisera (Miles, Frankfurt am Main, Germany) and antimouse isotype antibodies kindly donated to us by A. Radbruch (Institut für Genetik, Universität Köln, Germany). Antibody concentrations in cell culture media or ascitic fluid, and antibody affinity constants for the respective alkyl-deoxynucleosides, were calculated from data obtained by competitive RIA (*31*). When required, Mab were isolated with the aid of specific hapten-immunosorbents (hapten coupled to epoxy-activated Sepharose 6 B; Pharmacia, Uppsala, Sweden) at acid or alkaline pH (*22*). The affinity constants of the anti-alkyldeoxynucleoside Mab thus far produced in our laboratory range from 3×10^6 to 3×10^{10} L/mole.

Competitive Radioimmunoassay (RIA)

The conditions of the competitive RIA, a modified Farr assay (*32*), have previously been described (*21,22*). A typical RIA sample contains in a total volume of 100 μL of Tris-buffered saline supplemented with 1% bovine serum albumin (w/v) and 0.1% bovine lgG (w/v), ~2.5 $\times 10^3$ dpm of [^{3}H]-labeled tracer, an antibody solution diluted to give 50% binding of tracer in the absence of inhibitor, and varying amounts of inhibitor (i.e., either alkylated DNA hydrolyzed enzymatically to monodeoxynucleosides or other natural or modified DNA constituents to be analyzed for cross-reactivity). After incubation at room temperature for 2 hr (equilibrium), 100 μL of a saturated ammonium sulfate solution (pH 7.0) are added. After 10 min, the samples are centrifuged at 10,000*g* for 3 min. Thereafter, the [^{3}H]-activity is measured by liquid scintillation spectrometry in a 150 μL-aliquot. The degree of inhibition of tracer-antibody binding (ITAB) is calculated as described (*22*). For quantitation of unknown amounts of alkyldeoxynucleosides in DNA isolated from tissues or cultured cells, DNA is hydrolyzed to monodeoxynucleosides with DNase I (EC 3.1.4.5; Boehringer Mannheim, Mannheim, Germany), snake venom phosphodiesterase (EC 3.1.4.1; Boehringer), and alkaline phosphatase (EC 3.1.3.1; Boehringer), as described (*22*). Concentrations of deoxyguanosine (dGuo) and deoxythymidine, respectively, in the DNA hydrolyzates are determined by peak integration after separation by reverse-phase high pressure liquid chromatography (HPLC). Antigen concentrations in the DNA hydrolyzates are determined by comparing their ITAB-values with those of standard curves for the particular alkyldeoxynucleosides in question. Reverse-phase HPLC is also used for separation of different alkylation products from the same DNA sample followed by concentration of the respective fractions by evaporation in a Speed Vac concentrator (Savant Instruments, Hicksville, NY, USA), prior to their analysis by RIA (*6*), (see Fig. 1 and Table 1). Under these conditions, the sensitivity of the competitive RIA is limited only by the total amount of DNA available for analysis. Table 1 shows the detection limit and sensitivity of the competitive RIA, using selected Mab specific for dGuo with different alkyl groups covalently

attached to the O^6-atom, and for deoxythymidine methylated or ethylated in the O^4-position.

An example of the application of the competitive RIA for analysis of alkyldeoxynucleosides in small amounts of DNA is shown in Figure 2. By using Mab ER-6 (Table 1), the capacity for enzymatic removal of O^6-EtdGuo from DNA was determined in a number of cultured malignant neuroectodermal cell lines (BT- and V-lines) induced by *in vivo* exposure of fetal rat brain cells (FBC) to the *N*-nitroso carcinogen *N*-ethyl-*N*-nitrosourea (EtNU) (*33–36*). Interestingly, these FBC-derived malignant cell lines remove O^6-EtdGuo from their DNA very efficiently; in fact, more efficiently than rat liver, which among normal rat tissues is characterized by the highest capacity for enzymatic removal of O^6-EtdGuo (*34,37*). The BT- and V-lines originate from BDIX-rat FBC (18th day of prenatal development), and have either undergone tumorigenic conversion in cell culture after exposure to EtNU *in vivo* (BT-lines) or are derived from neural tumors that had developed *in vivo* after prenatal exposure to EtNU (V-lines) (*33,34,38*). Both pre- and postnatal rat brain cells are, however, deficient with respect to enzymatic removal of O^6-alkyldeoxyguanosine from DNA (*34,37,39*). In this cell system malignant transformation (or some as yet undefined stage of the process of malignant conversion preceding the ultimate development of tumorigenic phenotypes) may, therefore, be associated with the activation of O^6-alkyldeoxyguanosine repair (*36*). Since the O^6-EtdGuo elimination-proficient, malignant neuroectodermal BT- and V-cells were maintained and analyzed in cell culture, it will, however, also be important to test whether the expression of DNA repair enzymes may be modified by *in vitro* cultivation of (malignant) cells. The present studies have also provided information on the stability of the "O^6-EtdGuo repair-phenotype" of the malignant neurogenic rat cells (*36*). Subcloning in semisolid agar medium of one of the repair-proficient clonal BT-lines (BT3Ca) (Fig. 2*A*) resulted in a panel of eight subclones which, upon re-analysis by competitive RIA, again exhibited varying degrees of O^6-EtdGuo removal from DNA (Fig. 2*B*). This rapid diversification of cellular capacity for O^6-EtdGuo elimination in the course of the cell generations required for subcloning, indicates considerable instability of the "O^6-EtdGuo repair phenotype." Malignant cell subpopulations varying with respect to DNA repair capacity may, therefore, continuously develop in the course of tumor growth and progression. The implications of tumor cell heterogeneity in terms of cellular DNA repair capacity have been discussed elsewhere (*35*).

Immuno-Slot-Blot (ISB)

The ISB, a noncompetitive solid-phase immunoassay, was designed particularly for the quantitation of low levels of modified deoxynucleosides in very small samples of DNA, e.g., in DNA contained in small numbers of cells, in selected fractions of chromatin or in DNA restriction fragments (*26*). With the presently available Mab, the ISB requires single-stranded DNA. Application of the ISB is, therefore, restricted to the analysis of DNA containing modified structures that are stable during denaturation of DNA by heat or alkali treatment, e.g., O^6-EtdGuo or O^4-EtdThd (*26*).

For analysis by ISB, samples of alkylated DNA ($\leq$ 3 μg of DNA in a volume of 100 μL) are heat-denatured for 10 min, immediately chilled on ice, and mixed with equal volumes of 2 M ammonium acetate. The single-stranded DNA is then immobilized on nitrocellulose (NC) filters (BA 52; Schleicher and Schüll, Dassel, Germany) using a 72-slot Minifold II vacuum filter device (Schleicher and Schüll). The NS filters are presoaked in 1 M ammonium acetate prior to use. After application of DNA, the slots are rinsed with 1 M ammonium acetate (200 μL/slot). Thereafter, the NC filters are soaked in 5 × SSC (0.75 M NaCl, 0.075 M trisodium citrate) for 5 min, dried, and baked in a vacuum oven for 2 hr at 80°C. Prior to incubation with an anti-alkyldeoxynucleoside Mab (first antibody), the NC filters are treated for 2 hr with phosphate-buffered saline (PBS) containing 0.1–0.5% casein (Sigma, St. Louis, Mo, USA) and 0.1% deoxycholate, to prevent nonspecific Mab-binding. The NC filters are then incubated for 1 hr in the same type of solution as above in a heat-sealed plastic bag with an Mab solution containing 15 μg of Mab per milliliter and per 10–15 cm^2 of NC filter area (first antibody). Mab concentrations are determined by RIA titration (*31*).

Table 1. Detection limit and sensitivity of the competitive RIA using various anti-alkyldeoxynucleoside Mab.

Designation of Mab (isotype)	Alkyldeoxy-nucleoside	Mab affinity constants, L/mole	Detection limit of RIA, fmole[a]	Sensitivity of RIA (lowest measurable alkyldeoxynucleoside/ deoxynucleoside molar ratio in DNA)[b]
ER-7 (IgG_1)	O^6-MedGuo	1.0×10^9	250	1.9×10^{-7}
ER-6 (IgG_{2b})	O^6-EtdGuo	2.0×10^{10}	40	3.1×10^{-8}
ER-11	O^6-BudGuo	9.1×10^9	60	4.6×10^{-8}
ER-1005 (IgG_{2a})	O^6-iProdGuo	1.2×10^{10}	50	3.8×10^{-8}
EM-051 (IgG_{2a})	O^4-MedThd	4.0×10^7	7040	3.9×10^{-6}
ER-01 (IgG_{2a})	O^4-EtdThd	1.3×10^9	240	1.3×10^{-7}

[a] Standard conditions for RIA: 100 μL assay volume; 3×10^{-10} M [^{3}H]-labeled tracer; 50% inhibition of tracer-antibody binding (ITAB).

[b] Analysis by RIA of the respective alkyldeoxynucleoside fraction only, separated by HPLC from a sample of 2 mg of DNA enzymatically hydrolyzed to monodeoxynucleosides. Values calculated for RIA conditions as described in footnote a, assuming a content of 6.5×10^{-7} mole of dGuo and 9.0×10^{-7} mole of dThd per mg of DNA. In practice, a hydrolyzate of 5 × 2 mg of DNA must be separated by HPLC for more precise analysis by RIA (duplicate assay, and a dilution series of five samples).

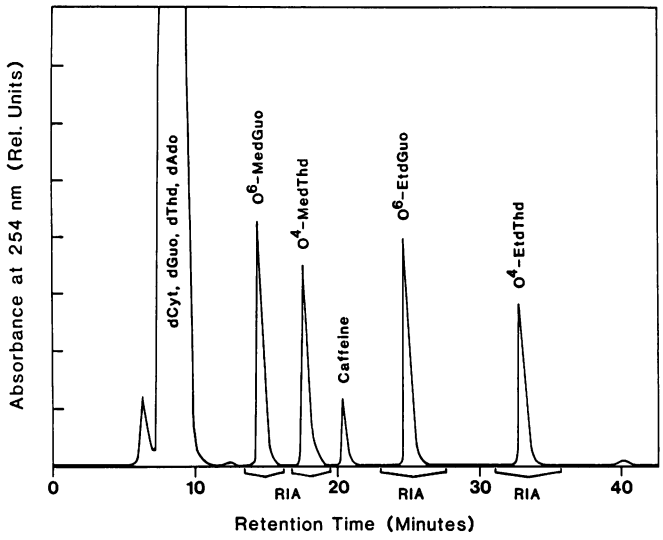

FIGURE 1. Separation by reverse-phase HPLC of O^6-alkyldeoxyguanosine and O^4-alkyldeoxythymidine from the same sample of DNA enzymatically hydrolyzed to monodeoxynucleosides (alkyl methyl, ethyl),prior to analysis by competitive RIA. HPLC fractions collected for RIA analysis following concentration by evaporation, are indicated in the graph. Caffeine was added to the DNA hydrolyzate as a marker. Due to the absence of cross-reactants in the final RIA test samples, the detection limit of the RIA is improved considerably (see Table 1). HPLC conditions: column, 2 × 25 cm (Merck Lichrosorb RP_{18}, 10 μm); flow rate, 1 mL/min; temperature, 37°C; buffer A (start): 0.2 M NH_4-acetate, pH 5.0, 25% MeOH; buffer B: MeOH. Elution program: (i) 0%B–15%B, 25 min; (ii) 100% B–15% B; 10 min.

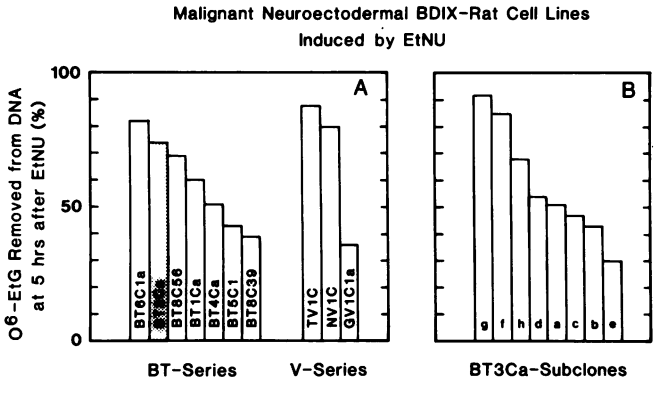

FIGURE 2. Malignant neuroectodermal rat cell lines induced by *in vivo* exposure of fetal BDIX-rat brain cells to the *N*-nitroso carcinogen *N*-ethyl-*N*-nitrosourea (EtNU) (*33,34*). Enzymatic removal from DNA of the alkylation product O^6-ethyldeoxyguanosine (O^6-EtdGuo), as measured by competitive RIA using Mab ER-6 (Table 1). From Rajewsky and Huh (*35*) with permission of the publishers.

After extensive washing in PBS supplemented with 0.16 M NaCl and 0.1% Triton X-100 (with several buffer changes), the NC filters carrying specifically bound first antibody are again sealed in plastic bags containing PBS supplemented with 0.1–0.5% casein and 0.1% deoxycholate and reacted for 1 hr with an ^{125}I-labeled second antibody specific for the immunoglobulin (Ig) of the first Mab. Thereafter, the NC filters are washed, dried, and exposed to Kodak X-Omat AR film. Alternatively, the degree of binding of the antialkyldeoxynucleoside Mab to the DNA on the NC filters may be determined by a three-step procedure resulting in a colored precipitate. Following incubation with the first Mab, excess antibody is removed by extensive washing in PBS supplemented with 0.16 M NaCl (PBS-NaCl). The NC filters are then incubated for 30 min at room temperature with a biotinylated second antibody specific for the first Mab (Vectastain; biotinylated rabbit anti-rat IgG Ig; Camon, Wiesbaden, Germany; diluted 1:200 in PBS–NaCl), washed three times for 10 min in PBS–NaCl, and once for 5 min in Tris–NaCl buffer (0.1 M Tris-HCl, pH 7.5, 1.0 M NaCl, 2-mM $MgCl_2$, 0.05% Triton X-100). Thereafter, the NC filters are incubated for 30 min at room temperature with a complex of avidin and biotinylated, polymerized alkaline phosphatase (*40*), and washed in Tris–NaCl buffer four times for 5 min at room temperature. The NC-filters are then incubated in a solution of nitro blue tetrazolium (0.33 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.17 mg/mL) in 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5 (*40*). Color development is terminated by a final wash in PBS containing 10 mM EDTA. Prior to densitometric evaluation, the NC filters are stored wet in heat-sealed plastic bags (*26*).

The extraordinary sensitivity of the ISB derives from the fact that the measured signals (color development on the NC filter or silver grains on the sensitive X-ray film) are further amplified with the time of incubation or exposure, respectively. A direct comparison of the ISB with the competitive RIA illustrates the performance of the ISB. Thus, the detection limit for O^6-EtdGuo obtained with Mab ER-6 (*5,26*) in the RIA (40 fmole of O^6-EtdGuo at 50% ITAB, Table 1) is reduced to $\geq$ 0.3 fmole in the ISB when $\leq$ 3 μg of DNA are analyzed per slot. In comparison with the RIA, much less DNA is required for ISB analysis (e.g., 3 μg DNA for the ISB instead of 100 μg DNA for the RIA, of a DNA sample containing O^6-EtdGuo at an O^6-EtdGuo/dGuo molar ratio of $\sim 3 \times 10^{-7}$). As the noncompetitive ISB does not operate under equilibrium conditions, there is less influence of the antibody affinity constant on the detection limit in comparison with the competitive RIA. For example, the best of the anti-(O^4-EtdThd) Mab, ER-01 (*6*) is characterized by an antibody affinity constant of 1×10^9 L/mole and a detection limit of 240 fmole in the RIA (Table 1). In contrast, the detection limit of Mab ER-01 in the ISB ($\geq$0.1 fmole in a sample of $\leq$3 μg DNA) is even lower than the ISB-detection limit of ER-6 for O^6-EtdGuo (antibody affinity constant, 2×10^{10} L/mole).

Immunocytological Analysis (ICA)

The particular advantage of ICA (which also represents a noncompetitive, solid-phase type of immunoassay) lies in the fact that specific alkyldeoxynucleosides can be detected directly in the genomic DNA of individual cells (e.g., in squash preparations of small tissue samples or in cell culture, in the case of frozen tissue sections even in cells in their proper histological environment). However, compared with the RIA and the ISB, ICA requires much more sophisticated equipment and experience (*24*).

For ICA, cell samples (smears, squash preparations, cytocentrifuged cell preparations, sections of frozen or paraffin-embedded tissues) are fixed for 10 min in Carnoy's ethanol:chloroform:acetic acid (6:3:1), washed in ethanol, and rehydrated in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate). After treatment with RNase A (EC 3.1.4.22; Sigma, München, Germany; 200 μg/mL of 2 × SSC) and T1 RNase (EC 3.1.4.8; Boehringer; 50 units/mL of 2 × SSC) for 1 hr at 37°C, the cell preparations are washed in 0.15 M NaCl, and nuclear DNA is denatured by dipping the slides into 0.07 M NaOH for exactly 4 min at room temperature. This is immediately followed by a wash in cold TEA buffer (10 mM triethanolamine, 150 mM NaCl, 100 mM $MgCl_2$, 10 mM EDTA, 0.02% NaN_3, pH 7.2; 10 min), and layering of the antibody solution onto the preparations. For visualization of O^6-EtdGuo in cellular DNA by direct immunofluorescence, cell preparations are incubated overnight at 4°C with a tetramethylrhodamine isothiocyanate (TRITC)-labeled Mab (ER-14) (*24,41*) at an Mab concentration of 20 μg/mL of TEA buffer supplemented with 4% polyethylene glycol 4000 (Roth) and the Fc-fragment of rat IgG (0.25 mg/mL). Subsequently, the cell preparations are washed for 2 × 10 min in TEA buffer and embedded in 10% (w/v) Elvanol (DuPont, Niagara Falls, NY) dissolved in PBS and 30% glycerol, (pH 8), containing *p*-phenylenediamine (1 mg/mL). For quantitation of nuclear fluorescence, fluorescence images are amplified by an image intensifier and fed into an image analysis system via a high sensitivity television camera (*24,41*).

The detection limit of ICA is defined by the number of modified deoxynucleosides detectable in the nuclear DNA of single cells, and is strongly dependent on the nonspecific "background binding" produced by individual Mab. Only selected Mab from our collection of anti-alkyldeoxynucleoside Mab can be used for ICA; the majority of the Mab either do not stain well, or they produce nonspecific staining, probably due to stickiness of the Mab molecules, or due to their binding to cellular epitopes not identical with the antigenic determinant on the particular DNA alkylation product to be detected. At present, ~700 O^6-EtdGuo residues per diploid genome (corresponding to an O^6-EtdGuo/dGuo molar ratio in DNA of $\sim 3 \times 10^{-7}$) can be detected using TRITC-labeled Mab ER-14 (*24,41*). Due to its limitation by nonspecific antibody binding, ICA is a particular domain of the ultrapure Mab. In view of the obvious importance of ICA for the demonstration of specific DNA adducts of carcinogens and mutagens, as well as chemotherapeutic agents in individual (e.g., human) cells, and for comparative analyses of the DNA repair capacity of phenotypically differing cells (including the assessment of intercellular and inter-individual variability), continued efforts are necessary to further optimize ICA methodology and to expand its range to a larger number of structurally modified DNA constituents.

Immuno-Electron Microscopy (IEM)

With the use of immuno-electron microscopy (IEM) in conjunction with a protein-free DNA spreading technique (*25*), specific alkyldeoxynucleosides can be visualized in double-stranded DNA molecules via Mab-binding sites. In a typical preparation for IEM of DNA containing O^6-EtdGuo, and anti-(O^6-EtdGuo) Mab (e.g., Mab ER-6; 5, 25; 80 μg/mL (is incubated with DNA (50 μg/mL) in TMS-buffer (10 mM triethanolamine, 100 mM Mg-acetate, 150 mM NaCl, 10 mM EDTA, 0.02% NaN_3, pH 7.2). In the case of Mab ER-6, incubation is for 30 min at 37°C. Glutardialdehyde may then be added to give a final concentration of 0.01%. Thereafter, control DNA, or ethylated DNA carrying bound Mab, is separated from unreacted Mab molecules by gel filtration on Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) in TMS-buffer. Aliquots of DNA-containing fractions are diluted with 10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (pH 7.2) to give a concentration of 5–10 μg of DNA/mL and immediately mounted onto freshly cleaved mica as described (*25*). The mica supports with the adherent material are washed in H_2O for 1 min, stained for 30 sec in a 1% aqueous solution of uranyl acetate, and washed again in H_2O. For subsequent analysis by transmission electron microscopy, the DNA preparations are shadowed with Pt/C at 7°C, and the replicas are enforced with carbon conditioned by NaCl. Using IEM, a highly nonrandom formation of O^6-EtdGuo has recently been demonstrated in chromosomal DNA of fetal rat brain isolated 1 hr after transplacental exposure to EtNU *in vivo* (*25*). IEM can thus be applied to localize possible "hot spots" of specific structural modifications caused by DNA-reactive agents within DNA strands (genes) of known nucleotide sequence.

In summary, various types of immunoassays, in conjunction with the use of monoclonal antibodies, have opened new possibilities for the sensitive detection, quantitation, and visualization of low levels of specific structural modifications in cellular DNA. The new immunoanalytical methodology may be of particular relevance for research on molecular mechanisms of carcinogenesis and mutagenesis (including DNA repair), on the interactions of chemotherapeutic agents with the DNA of cancer cells, and for cancer epidemiology.

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