Immunohistochemical Detection of O\textsuperscript{6}-Ethyldeoxyguanosine in the Rat Brain after In Vivo Applications of N-Ethyl-N-nitrosourea\textsuperscript{1}

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ABSTRACT

An immunohistochemical procedure was developed which allows the localization of the DNA lesion O\textsuperscript{6}-ethyldeoxyguanosine (O\textsuperscript{6}-EtdGuo) within tissues and organs. The method permits the detection of 24,000 residues of O\textsuperscript{6}-EtdGuo per diploid nucleus. We have used this procedure to localize N-ethyl-N-nitrosourea (ENU)-induced O\textsuperscript{6}-EtdGuo in the rat brain. Shortly after a single injection of ENU, we observed O\textsuperscript{6}-EtdGuo in most or all of the rat brain nuclei. After repeated injections of small doses of ENU, with intervals of 1 or 2 weeks between the injections and between the last injection and sacrifice, we could demonstrate O\textsuperscript{6}-EtdGuo only in part of the rat brain nuclei. Oligodendrocytes, granular neurons and endothelial cells, and part of the pyramidal neurons and astrocytes had accumulated O\textsuperscript{6}-EtdGuo, while in all other cells this lesion was not detectable after repeated injections of small doses of ENU. We found no obvious correlation between the putative sensitivity of rat brain cells to tumor induction and the accumulation of O\textsuperscript{6}-EtdGuo in their DNA.

INTRODUCTION

The development of antisera which recognize specific carcinogen-induced DNA lesions has facilitated the study of chemical carcinogenesis in several respects (28). One possible application of these antisera is the localization of DNA adducts in cells and tissues by means of immunocytochemical techniques (29, 30). This is important in those carcinogenesis model systems in which only part of the cells of the target tissue is sensitive to tumor induction. An example of such a system is the ENU-induced tumorigenesis in the rat brain (12, 37); most of the ENU-induced brain tumors seem to arise from glial cells and/or their precursors (5, 12, 27). The sensitivity of the rat brain to tumor induction has been correlated with the persistence of the ENU-induced DNA lesion O\textsuperscript{6}-EtdGuo in total brain DNA (7). Whether the persistence of this lesion is the same in the separate cell types of the brain was not analyzed, however. The development of antibodies, specific for O\textsuperscript{6}-EtdGuo (20, 36), has made it possible to study this question. We have analyzed the persistence of O\textsuperscript{6}-EtdGuo in the separate cell types of the rat brain by means of immunohistochemical techniques. We found that O\textsuperscript{6}-EtdGuo is not equally persistent in the different types of rat brain cells and that there is no obvious correlation between the putative sensitivity of rat brain cells to tumor induction and the persistence of O\textsuperscript{6}-EtdGuo.

MATERIALS AND METHODS

Schedules of ENU Administration. The persistence of O\textsuperscript{6}-EtdGuo in the DNA of rat brain cells was deduced from the accumulation of this lesion after repeated injections of ENU. A survey of the experiments is given in Table 1. We used male Sprague-Dawley rats from the Central Institut für Versuchstierzucht (Hannover, German Federal Republic). ENU (Fluka, Buchs, Switzerland) was dissolved in 0.9% NaCl solution: 1% NaH\textsubscript{2}PO\textsubscript{4}:Na\textsubscript{2}HPO\textsubscript{4}, pH 5.1, to a final concentration of 12.5 mg/ml immediately prior to i.p. injection. The first injection was given on postnatal Day 90. In each experiment, 2 animals were given injections of the ENU solution and one was given solvent only.

Tissue Fixation and Sectioning. At the end of each experiment, the animals were killed by decapsulation, and the brains were isolated quickly from the skulls. Each brain was divided in 2 halves. One half was stored at −30°C until DNA isolation, and one half was cut into 1- to 1.5-mm-thick slices. These slices were cooled on ice and then frozen in liquid N\textsubscript{2} which was cooled below its boiling point. Subsequently, freeze substitution was carried out for 14 days at −40°C with constant shaking in chloroform:methanol:acetic acid (1:2:1). The slices were then brought via −20°C to 4°C in about 3 hr. Subsequently, they were incubated for 20 hr at 4°C in chloroform with 3 changes of the chloroform, brought to room temperature, and embedded in paraffin (Merck, Darmstadt, German Federal Republic). From each slice, 8- or 12-μm-thick sections were cut and mounted on ovalbumin-coated slides.

Antibodies. The development and characterization of the anti-O\textsuperscript{6}-EtdGuo antisera has been described earlier (36). The average affinity constant of the antibodies for O\textsuperscript{6}-EtdGuo was 9.5 × 10\textsuperscript{9} liters/mol, and the constant of the antibodies for O\textsuperscript{6}-EtdGuo was 9.5 × 10\textsuperscript{9} liters/mol, and the constant of the antibodies for O\textsuperscript{6}-EtdGuo was 9.5 × 10\textsuperscript{9} liters/mol.

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\textsuperscript{3}The abbreviations used are: ENU, N-ethyl-N-nitrosourea; O\textsuperscript{6}-EtdGuo, O\textsuperscript{6}-ethyldeoxyguanosine; PBS, phosphate-buffered saline (140 mm NaCl, 9 mm Na\textsubscript{2}HPO\textsubscript{4}, and 1.3 mm Na\textsubscript{2}HPO\textsubscript{4}, pH 7.4); PAP, peroxidase-(rabbit)antiperoxidase; GAR, goat-anti-rabbit; GFAP, glial fibrillary acidic protein; DAB, 3,3-diaminobenzidine-4HCl; HS-ELISA, high-sensitivity enzyme-linked immunosorbent assay; SEL, subependymal layer of the lateral ventricles of the cerebrum.

\textsuperscript{4}L. A. Aarden, personal communication.
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Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>ENU injection (mg/kg)</th>
<th>No. of injections</th>
<th>Interval between injections</th>
<th>Interval between injection and sacrifice</th>
<th>DNA (μmol/ml DNA- phosphate)</th>
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<td>1</td>
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<td>1.4</td>
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<tr>
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<td>1</td>
<td>NR*</td>
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<td>1 wk</td>
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<tr>
<td>5</td>
<td>50</td>
<td>5</td>
<td>2 wk</td>
<td>2 wk</td>
<td>3.3</td>
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* NR, not relevant.

the gel-purified M, 51,000 protein from bovine brain intermediate filaments (18). This antiserum reacts specifically with astrocytes and ependymal cells; its characterization will be described elsewhere.6

Immunohistochemical Staining. Prior to immunohistochemical staining, the sections were always subjected to the following treatments: deparaffination in xylene for 20 hr with 3 changes of the xylene; 5 min in absolute ethanol; 45 min in methanol with 3% H2O2 to inactivate endogenous peroxidase (34); rehydration via graded ethanol; 5 min in deionized water; 45 min at 37°C in 10 mM Tris-HCl:10 mM EDTA, pH 7.6, with RNase A (200 μg/ml), from bovine pancreas; Sigma Chemical Co., St. Louis, Mo.), pretreated for 10 min at 70°C, and RNase T1 (50 units/ml) (from Aspergillus oryzae; Boehringer Mannheim, German Federal Republic); 2 short rinses in deionized water; 3 min in 0.07 M NaOH (to denature the DNA (20, 36)); 15 min in wash buffer [50 mM Tris-HCl:150 mM NaCl:0.25% gelatin (Merck); 5 mM EDTA:0.05% Triton X-100, pH 7.6]; 2 short rinses in PBS; and 5 min in PBS.

In some control experiments, the sections were treated with DNase after the RNase incubation. The sections were incubated for 4 hr at 4°C in 20 mM CH3COONa:1 mM MgSO4, pH 6.5, with DNase I (1.5 mg/ml; DN-CS, Sigma).

Incubation in the first antisera (anti-O6-EtdGuo, anti-DNA, or anti-GFAP) was carried out for 30 min at room temperature, then for 16 hr at 4°C, and again for 30 min at room temperature (31).

Binding of anti-O6-EtdGuo antibodies was visualized by "double PAP" staining (24). This procedure involves incubation of the sections in, successively, GAR, PAP, GAR, and PAP. All of these incubations were carried out for 45 min at room temperature. After each of these incubations, including the last PAP incubation, the following washing steps were carried out: 2 short rinses in PBS; three 5-min rinses in PBS; 15 min in wash buffer; 2 short rinses in PBS; and 5 min in PBS. Finally, the sections were incubated for 5 min in 0.05 M Tris-HCl, 0.01 M imidazole (Sigma) (33), DAB [0.5 mg/ml (Fluka) (22), pH 7.5], and then for 60 min in the same medium with 0.01% H2O2 (Merck). After two 5-min washes in deionized water, the sections were either dehydrated through graded ethanol and xylene and mounted in Dépex (Searle, High Wycombe, United Kingdom) or stained with the anti-GFAP serum for the identification of astrocytes. In the latter case, the anti-O6-EtdGuo antibodies were first removed by 3 washes of 3 min each in 0.01 M NaOH.

The anti-GFAP antibodies were visualized by the same procedure as that for the anti-O6-EtdGuo antibodies except that 4-chloro-1-naphthol (Merck) rather than DAB was used as a substrate for the peroxidase reaction (21). Thus, we obtained double-stained sections with a brown DAB precipitate in the O6-EtdGuo-containing nuclei and a blue precipitate in astrocytes and ependymal cells. Sections stained with 4-chloro-1-naphthol were mounted in chrome glycerin jelly (25).

Anti-O6-EtdGuo staining was always accompanied by anti-DNA staining on serial sections. These sections were pretreated in the same way as that for the anti-O6-EtdGuo-stained sections. Binding of the anti-DNA antibodies was visualized by means of a rabbit-anti-mouse peroxidase conjugate and DAB staining. The incubation conditions for the first and second antibody and for the DAB reaction were the same as those described above. The anti-DNA staining was often followed by the anti-GFAP staining (see above).

The following dilutions were used: anti-O6-EtdGuo, 1:4000; anti-GFAP, 1:100; anti-DNA (ascites fluid), 1:2000; GAR, 1:50; PAP, 1:400; and rabbit-anti-mouse peroxidase conjugate, 1:40. All dilutions were made in PBS with 10% normal goat serum. For the optimization of the procedure, experiments were carried out on sections which had been treated in vitro with ENU. Deparaffinized paraffin sections or air-dried unfixed cryostat sections were treated for 15 min at room temperature with 0.01 M ENU in PBS. ENU was first dissolved in absolute ethanol and was added to the PBS immediately prior to incubation. The final concentration of ethanol was 0.5%. After the ENU incubation, the sections were rinsed in distilled water, fixed, and treated further for immunohistochemical staining of O6-EtdGuo. ELISA. The amount of O6-EtdGuo in DNA was determined by HS-ELISA (36). DNA was isolated by the procedure of Kirby and Cook (13). The DNA was dissolved in PBS to a final concentration of 40 μg/ml, denatured by heating for 10 min in a boiling water bath, and then cooled rapidly on ice. Subsequently, the DNA concentration was adjusted to 35 μg/ml with PBS. Microtiter wells (3040F, Microtest II; Falcon, Oxnard, Calif.) were coated with denatured DNA by evaporation of 80 μl of this solution per well at 37°C. As a standard, we used calf thymus DNA which was modified in vitro with ENU (32) and which contained 300 μmol O6-EtdGuo per mg DNA-phosphate (36). To obtain a standard curve, we coated a series of microtiter wells with increasing amounts (0, 8.25, 17.5, 35, 70, 140, and 280 ng) of standard DNA; the total amount of DNA in these wells was adjusted to 2.8 μg/ml with unmodified denatured rat brain DNA. The amount of O6-EtdGuo/well was determined by HS-ELISA as described by Van Der Laken et al. (38). The HS-ELISA experiments were run in triplicate. The values of different determinations varied by less than 10%.

RESULTS

Optimization of the Immunohistochemical Procedure. Of the current immunohistochemical procedures, the PAP method is probably the most sensitive (31). Therefore, we started from this procedure to optimize the immunohistochemical detection of O6-EtdGuo. We used rat brain sections which had been treated in vitro with 0.01 M ENU to find the optimal fixation procedure. Freeze substitution in chloroform:methanol:aceton (1:2:1) was chosen for the following reasons. (a) After paraformaldehyde fixation, the anti-O6-EtdGuo staining is impaired strongly (not shown). (b) Cryostat sections fixed in methanol, acetone, or chloroform:methanol:aceton (2:1:1) could not stand the lengthy incubation procedure; moreover, nuclei in unexposed cryostat sections reacted aspecifically with the anti-O6-EtdGuo serum (not shown). (c) Acid fixatives were not tested because of the risk of hydrolysis and loss of DNA. (d) Freeze substitution in ethanol resulted in extensive damage from ice crystals in large neuronal nuclei (not shown). (e) Freeze substitution in chloroform:methanol:aceton (1:2:1) had none of these disadvantages and was therefore used as a fixation in all further experiments. Experiments to find the most sensitive method of detection of O6-EtdGuo were carried out on brains from rats which had received a single i.p. injection of ENU (250 or 50 mg/kg) 4 hr prior to sacrifice. We made no efforts to quantify the DAB precipitate. We merely modified the original PAP procedure (31) if this resulted in a directly visible intensification of the anti-O6-EtdGuo staining. This was the case with the denaturation of the
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DNA (cf. Refs. 20 and 36), the prolonged deparaffinization in xylene, the use of the double PAP (24) rather than the single PAP procedure (31), and the addition of imidazole to the DAB peroxidation reaction medium (33) (Fig. 1).

The presence of O6-EtdGuo in brain nuclei of rats which had received an i.p. injection of ENU (50 mg/kg) 4 hr prior to sacrifice was clearly detectable by the described procedure (Table 2, Experiment 1). Brain DNA from these rats contained 2 O6-EtdGuo residues per 10⁸ nucleotides (Table 1) or, on an average, 24,000 per diploid nucleus.

Controls. On the basis of the following experiments, we assume that the binding of anti-O6-EtdGuo antibodies to rat brain nuclei is due to a specific reaction with O6-EtdGuo residues in DNA. (a) Under our experimental conditions, anti-O6-EtdGuo antibodies do not bind detectably to brain nuclei of unexposed rats (Fig. 2F). (b) Addition of 0.1 µM O6-EtdGuo to the 1:4000 diluted anti-O6-EtdGuo antiserum immediately prior to incubation completely inhibits the reaction of this serum with brain nuclei of ENU-treated rats (Fig. 2D); this corresponds to an 18-fold excess of added free antigens over antigen binding sites and to a reduction of the concentration of free anti-O6-EtdGuo antibodies by a factor of 1250 [given the average antibody affinity constant of 9.5 x 10⁹ liters/mole (36)]. If 0.01 µmol O6-EtdGuo is added (a 1.8-fold excess), oligodendrocytes and granular neurons are still stained faintly (sections of Experiment 4, not shown). Under this condition, the concentration of free anti-O6-EtdGuo antibodies is reduced by a factor of 50. (c) Control rabbit sera do not react with brain nuclei of ENU-exposed rats (not shown). (d) Anti-O6-EtdGuo antibodies do not bind detectably to DNAse-treated sections of brains from ENU-treated rats (not shown).

As a control for the presence and immunological reactivity of DNA, we always carried out an anti-DNA staining in parallel with the anti-O6-EtdGuo staining (see "Materials and Methods"). Anti-DNA antibodies reacted with almost all nuclei in rat brain sections (Fig. 2A and E) and did not react with sections which had been treated with DNAase (Fig. 2B).

Localization of O6-EtdGuo after a Single Injection of ENU.

In brain sections from rats which had received a single injection of ENU shortly before sacrifice, almost all nuclei reacted with the anti-O6-EtdGuo antiserum (Table 2; Fig. 3, B, F, and I). O6-EtdGuo was detectable in astrocytes, oligodendrocytes, endothelial nuclei, nuclei of the meninges, the SEL and the choroid plexus, and in all types of neuronal nuclei that we could discriminate on the basis of nuclear morphology and localization. We found no difference between the distribution of the anti-O6-EtdGuo reactivity in these brain sections and the distribution of anti-DNA reactivity in serial sections (Fig. 3, A and B). Thus, the DNA lesion O6-EtdGuo is induced in all nuclei of the rat brain after an i.p. injection of ENU.

Localization of O6-EtdGuo after Repeated Injections of ENU. Using the above-described immunohistochemical procedure, we analyzed whether there are differences in the persistence of O6-EtdGuo between the different cell types of the brain. For this purpose, we studied the accumulation of this lesion in rat brain cells after repeated injections of ENU. The intervals between the injections (1 or 2 weeks; see Table 1) were chosen so that they were about equal to the half-life of O6-EtdGuo in total brain DNA, namely, about 10 days (8); under that condition, differences in the persistence of O6-EtdGuo are most likely to show up. Table 2 summarizes the results of the experiments.

One week after a single i.p. injection of ENU (50 mg/kg), most of the rat brain nuclei do not contain detectable anti-O6-EtdGuo reactivity; only in part of the oligodendrocytic and endothelial nuclei did we observe a weak anti-O6-EtdGuo staining (Table 2, Experiment 2). However, after repeated injections of ENU (50 mg/kg), with intervals of 1 week between the injections and between the last injection and sacrifice (Table 2, Experiment 4), an intense anti-O6-EtdGuo staining was observed in nuclei of several cell types; these cell types, namely, oligodendrocytes (Fig. 1, A to C), endothelial cells (Fig. 3K), granular neurons (Fig. 2C), and part of the astrocytes and pyramidal neurons (Table 2), must have accumulated O6-EtdGuo; in these cells, the half-life of O6-EtdGuo therefore exceeds 1 week. Other cells, namely, part of the astrocytes and pyramidal neurons (Fig. 3H) and all of

<table>
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<th>Table 2</th>
<th>Immunohistochemical localization of O6-EtdGuo in the rat brain after repeated injections of ENU</th>
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<tr>
<td>Anti-O6-EtdGuo reactivity</td>
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<tr>
<td>Stratum pyramidal hippocampus</td>
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<td></td>
<td>Oligodendrocytes</td>
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<td>+</td>
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<td>Unscheduled DNA synthesis</td>
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* ±, staining just detectable in part of the cells; ±, no staining; +, staining clearly detectable; ++, intense staining.
* In part of the cells.
* In internal granular layer of the cerebellum.
* In dentate gyrus.
* From Ref. 9; data for 26-day-old rats.
* +, unscheduled DNA synthesis-positive; −, unscheduled DNA synthesis-negative.
the cells of the ependyma (Fig. 3W), the SEL, and the choroid plexus (Table 2), do not react with anti-O6-EtdGuo antibodies after this ENU treatment. The half-life of O6-EtdGuo is therefore shorter than 1 week in these cells. The results, obtained with 2-week intervals between the injections, are identical to those obtained with 1-week intervals (Table 2). Thus, in part of the rat brain nuclei the half-life of (immunodetectable) O6-EtdGuo is longer than 2 weeks, and in part of the nuclei the half-life is shorter than 1 week. Rat brain cells are therefore heterogeneous as to the persistence of O6-EtdGuo.

DISCUSSION

The experiments described in this paper show, for the first time, the precise immunohistochemical localization of a DNA lesion induced by in vivo applications of a carcinogen. The procedure followed is relatively sensitive (cf. Ref. 29) and has the advantage of yielding permanent preparations which allow an easy comparison of the immunohistochemical staining with microanatomical structures. The procedure has 2 limitations: (a) it allows the detection only of those DNA lesions that are accessible for antibodies; and (b) it is, for a variety of reasons (31), unsuitable for the exact quantitation of DNA lesions. For this purpose, assays like radioimmunoassay (10, 20) or enzyme-linked immunosorbent assay (11, 36) are probably more reliable. For several reasons, the localization of DNA lesions is important in chemical carcinogenesis studies (16). The separate cell types within an organ might differ in their sensitivity to tumor induction (5, 12, 27), their capacity for DNA repair (9, 17), their capacity for metabolic activation of carcinogens (17), and their accessibility for carcinogens (16).

As for the ENU-induced tumorigenesis in the rat brain, it is unlikely that rat brain cells differ largely in the accessibility for ENU or in the metabolic activation (if any) of ENU (cf. Ref. 4). Our data do not provide evidence for this either (Table 2, Experiment 3). However, rat brain cells do differ in their sensitivity to tumor induction (12, 27) and their capacity for ENU-induced unscheduled DNA synthesis (9). The persistence of O6-EtdGuo in rat brain cells does not correlate with an inability to carry out ENU-induced unscheduled DNA synthesis (Table 2). This is not surprising, because repair of O6-EtdGuo might occur by deethylation (26) which is not necessarily accompanied by DNA repair synthesis. Tumor induction in the rat brain has been correlated with the persistence of O6-EtdGuo in total brain DNA (7, 8). However, the different types of rat brain cells are not equally susceptible to carcinogenesis by ENU. The majority of ENU-induced tumors in the rat brain are gliomas of several types; in addition, meningiomas and ependymomas are induced (12, 37). The susceptibility to ENU-induced tumorigenesis in the brain decreases sharply during postnatal development of the rat, but the spectrum of ENU-induced tumor types does not vary strongly with age (12). Chronic administration of ENU precursors in food (14) or drinking water (15) to adult Sprague-Dawley rats resulted in mixed gliomas and oligodendrogliomas of the brain. Administration of ENU in various ways [including i.p. injections (35)] to adult rats of other strains resulted in oligodendrogliomas, mixed gliomas, astrocytomas, and meningiomas of the brain (6, 12, 23, 35). To our knowledge, there are no published examples of ependymomas which were induced by ENU in the adult rat brain, but precursors of N-methyl-N-nitrosourea induced ependymomas of the brain in adult Sprague-Dawley rats (14). Thus, possible target cells for ENU-induced tumorigenesis in the adult rat brain are cells of the SEL (5), the meninges, and the ependyma as well as (precursors of) oligodendrocytes and astrocytes. Of these cells, part of the astrocytes and all cells of the SEL, ependyma, and meninges lose O6-EtdGuo relatively rapidly from their DNA (t1/2 < 1 week). In the SEL, this might be entirely the result of dilution because of cell division; but in the meninges, astrocytes and ependyma, probably not (1, 2). Thus, our data do not provide support for the idea that persistence of O6-EtdGuo is of main importance for ENU-induced tumorigenesis in the rat brain. However, a definitive conclusion on this subject must await a precise definition of the target cell population within the rat brain and an analysis of the persistence of O6-EtdGuo within this population.

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REFERENCES

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Fig. 1. Optimization of the detection of O$^6$-EtGuo. Coronal sections of the corpus callosum of a rat which has been given an injection of ENU (1 x 250 mg/kg) (Table 1, Experiment 3). Sections were incubated with anti-O$^6$-EtGuo antiserum, and binding of the antibodies was visualized by PAP procedure (31) (A); double PAP procedure (24) (B); and double PAP procedure with imidazole added to the DAB-peroxidation reaction mixture (33) (C). Interference contrast optics, x 610.

Fig. 2. Characterization of the anti-O$^6$-EtGuo staining. Parasagittal sections of the cerebella of rats which have been treated with ENU (5 x 50 mg/kg) (A-D) or solvent (E, F) (Table 1, Experiment 4). Sections were incubated as follows: A, E, anti-DNA; B, anti-DNA after treatment of the section with DNase; C, F, anti-O$^6$-EtGuo; D, anti-O$^6$-EtGuo in the presence of 0.1 µM O$^6$-EtGuo. For details of the incubation conditions, see "Materials and Methods." Binding of the antibodies was visualized as described in "Materials and Methods." gl, granular layer; ml, molecular layer; p, Purkinje cell. Interference contrast optics, x 610.
Fig. 3. Localization of O6-EtdGuo in coronal sections of the rat brain. A to D, overview of the parietal cortex; A, B, from a rat which has been treated with ENU (1 x 250 mg/kg) (Table 1 Experiment 3); C, D, from a rat which has been treated with ENU (5 x 50 mg/kg) (Table 1 Experiment 4); A, C, incubated with anti-DNA antiserum; B, D, incubated with anti-O6-EtdGuo antiserum; note the similarity in staining pattern in A and B; D, only a few scattered nuclei bind anti-O6-EtdGuo antibodies. E to H, detail of the parietal cortex; E, F, from a rat which has been treated with ENU (1 x 250 mg/kg) (Experiment 3); G, H, from a rat which has been treated with ENU (5 x 50 mg/kg) (Experiment 4); E, G, incubated with anti-DNA antiserum; F, H, incubated with anti-O6-EtdGuo antiserum; the anti-DNA or anti-O6-EtdGuo staining was followed by an anti-GFAP staining as described in “Materials and Methods”; as, astrocyte; n, neuronal nucleus; ol, oligodendrocytic nucleus; note absence of anti-O6-EtdGuo staining in astrocytic and neuronal nuclei in H. I to K, meninges and blood vessels; I, from a rat which has been treated with ENU (1 x 250 mg/kg); J, K, from a rat which has been treated with ENU (5 x 50 mg/kg) (Experiment 4); I, J, incubated with anti-O6-EtdGuo antiserum; J, incubated with anti-DNA antiserum; m, meninges; bl v, blood vessel; note absence of anti-O6-EtdGuo staining in meningeal nuclei in K. L to N, ependyma of the lateral ventricle; L, from a rat which has been treated with ENU (1 x 250 mg/kg) (Experiment 3); M, N, from a rat which has been treated with ENU (5 x 50 mg/kg) (Experiment 4); ep, ependyma; A to D and I to K, bright-field optics; E to H and L to N, interference contrast optics; A to D and I to K, x 130; E to H and L to N, x 500.
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