Immunochemical Visualization of Binding of the Chemical Carcinogen Benzo(a)pyrene Diol-Epoxide 1 to the Genome

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Abstract

Antisera against DNA modified with r-7,1,8-dihydroxy-7,9,10-oxo-7,8,9,10-tetrahydrobenzo(a)pyrene (BPDE-1) was elicited in rabbits. Such sera reacted with either single- or double-stranded modified DNA but not with unmodified DNA, free benzo(a)pyrene, or proteins modified by BPDE-1.

Indirect immunofluorescence studies indicated that the immunoglobulin G in the sera bound specifically to the nuclei of KD cells which were treated with BPDE-1. The intensity of fluorescence was proportional to the dose of BPDE-1 used to treat the cells. About 50% of the BPDE-1-DNA adducts remained bound to DNA 24 hr after the removal of the carcinogen. The location of BPDE-1-modified bases in Col E1 DNA was visualized by immunoelectron microscopy.

Introduction

The interaction of a carcinogen with the genome of a target cell may lead to transformation (19). To facilitate various studies on the mode of action of known carcinogens, it is necessary to have specific and sensitive methods for their detection and quantitation. Toward this goal, several laboratories have elicited antibodies which interact with free carcinogens (3, 4, 7, 14, 21) or DNA-bound carcinogenic adducts (4, 26, 28).

Thus, antibodies specific for benzo(a)pyrene-deoxyguanosine adducts have been elicited, and a sensitive radioimmunoassay has been developed (24). Antibodies which detect covalent adducts of N-2-acetylaminofluorene and DNA have been elicited (12, 26, 28) and used to detect formation and removal of adducts from DNA (23, 25), to study the distribution of acetylated and deacetylated deoxyguanosine C-8 adducts of N-acetoxy-2-acetylaminofluorene in various cells, and to visualize the binding sites of the adduct in circular Col E1 DNA (10). Immunochemical methods suitable for the detection of DNA components modified by alkylating carcinogens have also been developed (27).

Immunochemical studies on the organization of defined components in chromatin and chromosomes revealed that antibodies can serve as useful tools to study various aspects of the structure and function of the genome (5). Here, we report that DNA modified by the carcinogen BPDE-1, when injected into rabbits, elicits specific antibodies which can be used to detect and visualize the location of the bound carcinogen in cells by immunofluorescence and on DNA by immunoelectron microscopy. These antibodies also serve as tools to visualize the removal of the DNA adducts at the single-cell level.

Materials and Methods

Characterization of the Antigen. Commercial calf thymus DNA (Calbiochem) was further purified by digestion with Pronase and RNase followed by extraction with chloroform:isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase with 2 volumes of ethanol at 4°. BPDE-1 (obtained from the chemical repository of the National Cancer Institute) was dissolved in tetrahydrofuran at 3 mg/ml. When the DNA was modified with [3H]BPDE-1, the stock solution of [3H]BPDE-1 (466 mCi/mmol) was diluted with unlabeled BPDE-1 to reduce the specific activity 133-fold. The DNA was modified in vitro with BPDE-1 to 2.0% essentially as recommended by Jennette et al. (13). To 13.0 ml of DNA at 1.0 mg/ml, 3 additions of 0.6 ml of the BPDE-1 solutions were made at 15-min intervals. Two hr after the last addition, the unbound BPDE-1 was extracted with ether, and the DNA was dialyzed against 0.05 M Tris-HCl, pH 7.5:0.1 M NaCl.

To ensure removal of all possible single-stranded regions, the modified DNA was treated with S1 nuclease.

Immunization. Rabbits were immunized with modified DNA as recommended by Poirier et al. (24) except that an additional i.v. booster (1 mg modified DNA in 1 ml 0.05 M Tris-HCl, pH 7.5:0.1 M NaCl) was administered. Rabbits were bled weekly starting 1 week after i.v. boost.

Assays for Serum Specificity. The specificity of the serum was tested by a filter binding assay (29). Supercoiled, circular 3H-Col E1 DNA (specific activity, 2.5 x 10⁴ cpm/μg) was prepared as described elsewhere (20). The 3H-Col E1 DNA was modified to yield 20 BPDE-1 adducts per DNA molecule. The filter assay is based on the finding that DNA to which antibodies are bound is retained on nitrocellulose filters while free DNA is not (29). To solutions containing about 5000 cpm of modified DNA, various dilutions of antibodies were added. The final reaction mixture contained 0.1 M Tris-HCl, 5 mM EDTA, and 5% dimethyl sulfoxide, pH 7.5, in a total volume of 200 μl. After 1 hr incubation at 25°, the solutions were filtered on nitrocellulose filters (Schleicher and Schuell; BA-90/1); washed with 0.1 M Tris-HCl; 5 mM EDTA:5% dimethyl sulfoxide, pH 7.5; dried; dissolved in 1.0 ml ethyl acetate; and counted in 10.0 ml Aquasol. In inhibition assays, modified, unlabelled Col E1 DNA or other test substances were included in the reaction mixtures.

Immunofluorescence. Slides containing attached cells were washed in Hanks' balanced salt solution and dipped for 5 min in methanol (precooled to -20°), dipped in acetone at -20°, and air dried. On a selected spot on the slides, 50 μl of a 10.0-μl solution of bovine serum albumin in PBS were added, and the slides were incubated in a moist chamber at 37° for 30
min. The slides were washed in PBS for 30 min with shaking. To the washed slides, 50 µl of 1:100 dilution of IgG were added. After 4 hr incubation at 37° in a moist chamber, the slides were extensively washed with PBS and treated with 50 µl fluorescein isothiocyanate-Protein A (Cappel Laboratories) diluted to 3.3 µg/ml in PBS for 1 hr at 37°. After being washed in PBS, coverslips were mounted with Aqua Mount (Lerner Laboratories). The slides were examined and photographed through a Zeiss photomicroscope III.

**Exposure of Cells to BPDE-1.** KD human fibroblasts were grown in tissue culture chamber slides (Lab Tek Products, Miles) as described (8).

The carcinogen dissolved in tetrahydrofuran at 1.0 mg/ml was diluted at selected concentrations to Eagle’s minimal essential medium without serum, and 1.0-ml aliquots of the various dilutions dispensed into the chambers. After 30 min at 37°, the chambers were rinsed twice with PBS, and the cells were reincubated in complete medium for timed periods. DNA was purified from cells essentially by the procedure of Marmur (18).

**Immunosedimentation.** †H-Col E1 DNA modified with BPDE-1 was incubated with IgG purified from anti-BPDE-1-DNA serum (diluted 1:20 in PBS) in a total volume of 0.1 ml. After a 2-hr incubation, the ferritin-labeled goat anti-rabbit IgG (Cappel) diluted 1:10 in PBS was added. The reaction mixture was applied to a 5 to 25% sucrose gradient, made in 0.02 M Tris-HCl:0.1 M NaCl:10 mM EDTA, pH 8.0, with 1.0 ml of 60% sucrose mixture cushion, and sedimented in a SW 50.1 rotor at 10° for 5 hr at 40,000 rpm. Thirty fractions were collected and counted.

**Immunoelectron Microscopy.** Fractions containing the DNA were diluted 10-fold with 0.1 M Tris-HCl buffer, pH 8.0, containing 20% formamide, 0.01 M EDTA, and 0.02% cytochrome c. Fifty µl of this solution were spread over the surface of a drop of 10 mM Tris-HCl:1 mM EDTA:0.7% formamide and picked up on an electron microscope grid coated with a collodion membrane. The grid was stained with 0.05 mM uranyl acetate in 90% ethanol for 2 min and washed twice with hexane. The grid was examined and photographed with a Hitachi HU-12 electron microscope.

**Results**

**Specificity of the Antiserum.** The data presented in Chart 1 illustrate that the antiserum bound to DNA which was modified with BPDE-1 but did not bind to unmodified DNA. Specific binding could be detected at sera dilutions as high as 1:10⁵. Nonspecific binding of IgG to DNA was not observed even at sera dilutions as low as 1:10⁷. IgG purified from nonimmune sera did not bind to BPDE-1-modified DNA. The binding of the IgG to the modified DNA was inhibited by nonradioactive modified DNA but not with unmodified, native, or single-stranded DNA. The antibody did not react with free BPDE-1 or proteins modified with BPDE-1. It is concluded that the antibodies are specific for BPDE-1-modified DNA. Further proof for this conclusion (including lack of detectable BPDE-1-RNA binding) is presented below.

**Immunofluorescence Studies.** Indirect immunofluorescence studies on cells treated with various concentrations of BPDE-1 indicated that the antibodies specifically bind to the nuclei of cells treated with the carcinogen (see Fig. 1). Controls were done using nonimmune sera on BPDE-1-treated cells or anti-BPDE-1-DNA sera on cells which were not treated with BPDE-1. No fluorescence was detectable in any of the control cells.

The concentration of the BPDE-1 to which the cells were exposed seems to affect the intensity of fluorescence. Comparison of the intensity of fluorescence displayed in Fig. 1D to that displayed in Fig. 1, E and F, indicates that the nuclei of cells treated with BPDE-1 (5.0 µg/ml) fluoresce with an intensity comparable to that of cells treated with BPDE-1 (1.0 µg/ml). Cells exposed to BPDE-1 (0.5 µg/ml) displayed significantly lower fluorescence intensity than did those treated with higher doses. Weak nuclear fluorescence could be detected with 0.25-µg/ml doses of carcinogen (not shown).

Comparison of the intensity of fluorescence among the various cells present on a slide did not reveal significant cell-to-cell variability (e.g., Fig. 2). It is concluded, therefore, that the cells do not differ in their susceptibility to modification by BPDE-1.

**Visualization of Repair.** The possibility that the time course of repair of the damaged DNA can be followed and visualized at the single-cell level has been explored. In these experiments, after the cells were treated for 30 min with various concentrations of carcinogens, the medium was replaced. At selected times after removal of the carcinogen, the cells were examined by indirect immunofluorescence using anti-BPDE-1-DNA and fluorescein-labeled Protein A.

The results indicate that the intensity of the nuclear fluorescence in BPDE-1-treated cells was diminished in samples taken 24 hr after removal of the carcinogen (Fig. 2). After this time, cells exposed to 5.0-µg/ml doses of carcinogen displayed nuclear fluorescence (Fig. 2C); while in cells exposed to 0.5 µg/ml, the fluorescence was predominantly associated with the peripheral regions of the nuclei (Fig. 2D). Additional studies revealed that significant amounts of residual BPDE-1-modified DNA could be detected by immunofluorescence even 48 hr after the removal of the carcinogen. The residual BPDE-1-DNA adducts at various times after removal of the carcinogen from the cells were quantitated in a double-label experiment.

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In these experiments, the cells were prelabeled by a 24-hr incubation with [3H]thymidine. The cells were then treated with [14C]BPDE-1 (0.5 μg/ml) for 30 min. The medium was replaced, and the cells were allowed to continue to grow. Cells were harvested at various times after removal of the carcinogen, and the 14C:3H ratios in the DNA purified from these cells were determined. The 14C:3H ratio is a measure of the residual BPDE-1 present in the cells. The data presented in Table 1 indicate that 12 hr after the removal of the carcinogen 59% of the adducts still remain bound to DNA. Doubling the repair time did not significantly diminish the percentage of adducts remaining bound to DNA. Thus, the residual immunofluorescence observed in cells which were allowed to repair the damage induced by the carcinogen for 12 to 24 hr is indeed due to the presence of the adduct on the DNA.

**Immunoelectron Microscopy.** The availability of antibodies specific to BPDE-1-modified DNA raises the possibility that the location of the adduct on a DNA molecule can be pinpointed using immunoelectron microscopic techniques. To minimize adherence of free IgG molecules to the electron microscope grids, the IgG molecules bound to BPDE-1-DNA were separated from unbound IgG and from unmodified DNA by immunosedimentation. To this end, [3H]-Col E1 DNA (or anti-BPDE-1-[3H]-Col E1 DNA) was mixed with ferritin-labeled goat anti-rabbit IgG, and the reaction mixture was sedimented in a sucrose gradient. The data presented in Chart 2 clearly indicate that specific binding of IgG to the modified DNA results in a complex which is easily separable from unmodified DNA (run in a parallel gradient).

Thus, it is possible to obtain an immune complex free of nonbound antibodies and unreacted DNA. An electron micrograph of such a complex is presented in Fig. 3B. Examination of the electron micrograph reveals that the ferritin molecules are exclusively located on the modified DNA. The areas surrounding the DNA or control, unmodified DNA were devoid of ferritin molecules. In the example presented in Fig. 3B, 6 areas containing antibodies can be resolved along the treated DNA molecule. The spacing of these areas indicates that the binding of the carcinogen to the DNA is not cooperative (i.e., that the BPDE-1 adducts are not clustered).

**DISCUSSION**

It is now well established that immunization with DNA modified by a variety of adducts elicits antibodies which specifically bind to the modified DNA. Sensitive immunoassays have been developed which can detect and quantitate DNA containing 10^{-15} mol of adducts (12, 24, 26, 27, 28). With these assays, it is possible to quantitate the formation and removal of DNA adducts in various types of cultured cells (23). The results presented in this manuscript indicate that such antisera also can be used to visualize the cellular location of the adduct by immunofluorescence and by immunoelectron microscopy.

Poirier et al. (24) elicited a serum which by radioimmunoassay was specific for BPDE-1-modified DNA. In the present study, we demonstrate specificity of a serum elicited in a similar manner by a filter binding radioimmunoassay in which the sera can be diluted to about 1:10^5. Further evidence for the specificity of the antiserum is provided by the immunofluorescence studies, the immunosedimentation studies, and immunoelectron microscopy.

It is known that BPDE-1 forms covalent adducts not only with DNA but also with proteins and RNA (15, 17, 22). Since the immunofluorescence studies localized the fluorescence to the nucleus of the cell, it can be reasoned that the antibodies recognized only adducts formed on the DNA and did not bind to modified proteins and RNA. Immunofluorescence studies allow examination of the formation and removal of adducts at the single-cell level. The results presented suggest that when a population of cells is initially exposed to BPDE-1 there is little variability among cells in the amount of carcinogen bound. Application of this approach to cells treated with benzo(a)pyrene will allow estimation of the variability of individual cells in their ability to metabolize benzo(a)pyrene. The KD cells display some variability in their ability to remove the DNA adduct. The data obtained by the filter binding technique suggests that a 12-hr repair period is sufficient to remove most of the "repairable" adducts from DNA. Similar results were obtained in several laboratories which studied the removal of adducts from various cells treated with derivatives of acetylaminofluorene (1, 2, 6, 23). About 50% of the N-acetoxy-2-acetylaminofluorene adducts are removed within 24 hr. In some cases, however, most of the BPDE-1 adducts can be removed at a significantly greater rate (9). Apparently, the repair process is influenced by conditions of cell growth and carcinogen exposure.

The ability to obtain a complex of antibodies and BPDE-1-modified Col E1 DNA by immunosedimentation greatly facil-
tated the visualization of the location of the carcinogen by immunoelectron microscopic techniques. The electron micrographs obtained are similar to those previously presented by De Murcia et al. (10) using antibodies to N-acetoxy-2-acetylaminofluorene.

The specificity and titer of the antiserum ensured that under appropriate conditions specific binding to modified DNA can be demonstrated by these techniques. Since the adducts were not clustered, it can be concluded that the carcinogen bound to the purified DNA essentially in a random manner.

In the eukaryotic cell, DNA is packaged as a nucleoprotein which exhibits distinct structural features (11, 16). The influence of the structural features of the chromatin fiber on the interaction of a chemical carcinogen with the genome are not well studied. Our findings that the antibodies are suitable to visualize and quantitate adducts not only on purified DNA but also on intact cells and that the location of the carcinogen can be visualized with the resolution provided by the electron microscope suggest that immunochromosomal techniques can be used to study various aspects of the interaction of a carcinogen with the eukaryotic genome.

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REFERENCES

Fig. 3. Electron micrographs of Col E1 plasmid DNA incubated with anti-BPDE-1-DNA antibodies and ferritin-labeled goat anti-rabbit IgG. Bar, 0.1 μm.
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