Formation and Removal of Specific Acetylaminofluorene-DNA Adducts in Mouse and Human Cells Measured by Radioimmunoassay

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ABSTRACT

Rabbit antiserum prepared against N-(guanosin-8-yl)-acetylaminofluorene was utilized in a radioimmunoassay to detect formation and removal of C-8 adducts from the DNA of cultured cells exposed to N-acetoxy-2-acetylaminofluorene. The assay was able to quantitate both acetylated and deacetylated C-8 adducts between 0.5 and 5 pmol while the N² adduct, 3-(deoxyguanosin-N²-yl)acetylaminofluorene, was not detected below 160 pmol. By varying the proportions of acetylated and deacetylated C-8 adducts in the radioimmunoassay, a series of standard curves were developed from which the relative proportion of each adduct could be determined in unknown mixtures. DNA from mouse epidermal cells and human skin fibroblasts exposed to N-acetoxy-2-acetylaminofluorene in culture contained only 3 and 5%, respectively, of the C-8 adduct in the acetylated form. Quantitation by radioimmunoassay of total C-8 adducts bound to DNA yielded values approximately 25% lower than total carcinogen binding determined by radiolabeling. When removal of C-8 adducts was followed over a 23-hr, carcinogen-free culture period, mouse and human cells removed 40 and 50%, respectively, of bound acetylated and deacetylated C-8 adducts. These studies demonstrate the versatility of radioimmunoassay as a molecular probe for studies of chemical carcinogens.

INTRODUCTION

The carcinogen 2-AAF, and its activated derivatives N-OH-AAF and N-Ac-AAF, bind covalently to guanine in DNA producing 3 addition products (11-13, 21-22). The C-8 adducts dG-8-AAF and dG-8-AF account for the major portion of the bound carcinogen and are presumably removed by excision repair mechanisms (11, 12). A minor adduct, dG-N²-AAF, has been observed only in an acetylated form and persists much longer than the other analogs in the DNA (11, 21). The relative proportions of each adduct formed, either in a particular organ of the whole animal or in cultured cells, will depend on the nature of the cells at risk and possibly the conditions under which the carcinogen is administered (3, 12, 16). Kriek (11, 12) has shown that, when radioactive N-OH-AAF is injected i.p. into rats, 5% of the total carcinogen bound to liver DNA is dG-N²-AAF, 20% is dG-8-AAF, and 70 to 75% is dG-8-AF. On the other hand, Cerutti (4) has exposed cultured human lung epithelial cells to N-Ac-AAF and observed 5% dG-N²-AAF, 65% dG-8-AAF, and 35% dG-8-AF. Such studies generally require the chromatographic resolution of individual adducts prior to quantitation and depend on relative changes in ratios of doubly labeled DNA. We have previously described (17, 19) a RIA capable of detecting ng (pmol) levels of dG-8-AAF in hydrolyzed, modified DNA obtained either from the chemical interaction between N-Ac-AAF and DNA or the exposure of cells to N-Ac-AAF in culture. The assay was highly specific for the C-8 adduct and did not detect N-Ac-AAF or guanosine independently. The present report demonstrates that the same antiserum does not cross-react with dG-N²-AAF but does contain antibodies which recognize the deacetylated C-8 adduct, dG-8-AF. Using this RIA, it is possible to follow the removal of C-8 adducts from DNA with time and, under certain conditions, to quantitate the relative proportions of acetylated and deacetylated C-8 adducts in DNA.

MATERIALS AND METHODS

Chemicals. The National Cancer Institute Chemical Repository (IIT Research Institute, Chicago, Ill.) was the source of both 9-[C]-N-Ac-AAF (40.6 mCi/mmol) and nonradioactive N-Ac-AAF. Both radioactive and nonradioactive dG-8-AAF were prepared as described previously (19). Synthesis of the deacetylated derivative, dG-8-AF, was accomplished by alkaline hydrolysis of dG-8-AAF at 85° for 3 hr (13) followed by Sephadex LH-20 chromatography (20 to 95% methanol gradient). A compound with the appropriate UV spectrum (13) eluted at about 75% methanol and was stable at 4° in the dark for 5 months. [3H]dG-8-AF was prepared from [3H]dG-8-AAF (6 or 12 Ci/mmol) (19) by incubating 1.5 to 2 x 10⁵ cpm [3H]dG-8-AAF, 25 ml water, 4 ml acetone, 8 ml methanol, and NaOH to pH 11 for 3 hr at 72°. After neutralization, the mixture was chromatographed on Sephadex LH-20 as above; the yield of [3H]dG-8-AF was variable but usually lower than 10%. The specific activity was assumed to be the same as the starting material. dG-N²-AAF was obtained from Dr. J. G. Westra (The Netherlands Cancer Institute, Amsterdam, Netherlands), and the purity of the compound was confirmed in this laboratory using thin-layer chromatography (11).

Cell Culture, Carcinogen Exposure, and DNA Preparation. Epidermal cells were isolated from newborn BALB/c mice as previously described (23, 24) and used as primary cultures grown in Medium 199 (NIH Media Unit) with 10%...
fetal bovine serum (Reheis Chemical Co., Phoenix, Ariz.) and 1% antibiotic-antimycotic solution (Grand Island Biological Co., Grand Island, N. Y.). Cells were plated at either 200 x 10⁶ cells in roller bottles (Bellico Glass, Vineland, N. J.; 825 sq cm) or 25 x 10⁶ cells in dishes (Falcon Plastics, Oxnard, Calif., 150 mm). Human fibroblasts (YDF) were obtained from a dermatofibroma excised from an adult male donor and dissociated with 0.35% collagenase (Worthington Biochemical Corp., Freehold, N. J.; type III) at 37° for 1 hr. The YDF cells (passages 8 to 10) were plated at 25 x 10⁶ cells/150-mm dish in Eagle's basal medium containing noessential amino acids (NIH Media Unit), 10% fetal bovine serum, and 1% antibiotic-antimycotic solution.

Cells were treated at confluence with N-Ac-AAF in 0.4% dimethyl sulfoxide for 1 hr at 37°. For removal experiments, one half of the treated dishes or bottles were harvested at 1 hr, and the other half were washed twice with phosphate-buffered saline (NIH Media Unit; 0.14 mM NaCl:2.0 mM KCl:8.3 mM Na₂HPO₄:1.4 mM KH₂PO₄:0.5 mM MgCl₂·6H₂O:0.8 mM CaCl₂·2H₂O), fresh medium was added and incubation continued for another 23 hr. Cells were harvested; DNA's were prepared on CsCl gradients as previously described (15, 18) and dialyzed against water. DNA isolated from untreated mouse cells, or calf thymus (Sigma Chemical Co., St. Louis, Mo.) was centrifuged on CsCl gradients and used as control. DNA from cells exposed to 9-[(4C)N-Ac-AAF was prepared as above, further treated with RNase and pronase, extracted with chloroform:isoamyl alcohol (5), and dialyzed against water.

RIA. DNA samples were concentrated by nitrogen evaporation to approximately 300 μg DNA per ml, heated to 100° for 10 min, and cooled rapidly. In these experiments, S, nuclease (22) was used to hydrolyze the AAF DNA's and gave the same results as the DNase, venom phosphodiesterase, and alkaline phosphatase procedure used previously (19). S, nuclease digestion was performed in 25 mM sodium acetate buffer (pH 4.6):65 mM NaCl:4 mM ZnSO₄ for 3 hr at 37°. After the pH was adjusted to 6.0 with 0.1 M Tris (pH 9.2), 0.01 to 0.05 ml (3 to 15 μg DNA) of sample was routinely assayed using the more sensitive nonequilibrium conditions (19) and a final assay volume of 0.45 ml, where all the ingredients except the DNA were diluted in 10 mM Tris (pH 7.4). The RIA control using nonimmune serum, counts bound by immune serum without competition (B₀), and standard curve tubes contained an equivalent amount of control DNA prepared in a manner analogous to that of the carcinogen-exposed cells. Assays were performed in triplicate at 2 different sample volumes, and each DNA was assayed 2 or 3 times. Variability among assays for standard samples was ±8 to 9% when calculated for 16 or more assays run over a period of months. For example, 6.12 pmol dG-8-AF (saturation) gave 41.6 ± 0.51% (S.E.) inhibition. Similarly, 5.65 pmol of 5% dG-8-AAF-95% dG-8-AF assayed at 53.4 ± 0.73% inhibition. Variability in pmol/μg DNA among assays of hydrolyzed, modified DNA was ±10%. For experiments in which increasing concentrations of DNA were assayed by RIA (Chart 2B), the controls used at each level of DNA were the RIA control with nonimmune serum, B₀, B₁, plus 6.12 pmol dG-8-AF (saturating), and B₂, plus 5.5-6 pmol of other standard mixtures each containing an amount of DNA equal to that of the AAF-exposed sample. Unmodified, hydrolyzed DNA below 50 μg did not alter the assay.

The carcinogen binding to DNA from 9-[(4C)N-Ac-AAF-treated cells was determined on an aliquot of the same sample used for RIA; ³¹C activity was determined in Aquasol-2 (New England Nuclear, Boston, Mass.) in a Beckman LS-250 liquid scintillation counter at an efficiency of 85%. ³¹H activity in the RIA was counted at an efficiency of 40%.

RESULTS

Antiserum Specificity. The specificity of the G-8-AAF antiserum with respect to each of the 3 DNA-AAF adducts has been investigated. The standard curve for RIA competition between [³¹H]dG-8-AAF and dG-8-AF is shown in Chart 1A and has been reported previously (19). A similar standard curve was obtained under the same experimental conditions with [³¹H]dG-8-AF and dG-8-AF (Chart 1A). This indicates the presence of cross-reactivity and/or a second species of antibody specific for the deacetylated adduct. Additional evidence from Chart 1B supports the latter possibility. When mixtures of dG-8-AAF and dG-8-AF were assayed as inhibitor against [³¹H]dG-8-AAF, the increase in percentage of inhibition was always additive (Chart 1B), demonstrating a lack of competition for the same antibody-binding sites. Also, when increasing concentrations of dG-8-AF were assayed against [³¹H]dG-8-AAF (Chart 1A), maximum inhibition of antibody binding was approximately 40%. Thus, somewhat less than one-half of the total sites able to bind [³¹H]dG-8-AAF were also able to bind dG-8-AF. Chart 1A also indicates that only 25% of the total sites able to bind [³¹H]dG-8-AF could bind dG-8-AAF. Both ○-○ and ○-○ suggest that there is cross-reactivity among the 2 populations of antibodies. Specificity of the G-8-AAF antiserum towards the N² adduct, dG-N²-AAF, was assayed using [³¹H]dG-8-AAF. No significant inhibition was observed up to 160 pmol in an assay in which 1 pmol of dG-8-AAF inhibited by 50%. Therefore, the G-8-AAF antiserum does not recognize the
N² adduct at concentrations 2 orders of magnitude higher than the usual range for the C-8 adducts.

Quantitation of Acetylated and Deacetylated C-8 Adducts. Since the standard curves of Chart 1A did not allow for quantitative differentiation between C-8 adducts in a given mixture, a series of assays was performed with [³H]G-8-AAF and varying mixtures of the unlabeled C-8 adducts. Chart 2A shows that, above a dG-8-AAF:dG-8-AF ratio of 1:5, the standard curves have similar slopes with the usual RIA saturation plateau around 90% inhibition, but increasing concentrations of dG-8-AAF have shifted the curves in the direction of greater sensitivity. Below a dG-8-AAF:dG-8-AF ratio of 1:5, the standard curves have similar slopes with lower saturation plateaus with differences large enough to allow for quantitation based on relative concentrations when the dG-8-AAF is a minor component.

In an attempt to quantitate the amount of each C-8 adduct on DNA’s from N-Ac-AAF-exposed BALB/c mouse and YDF human cells, increasing concentrations of each DNA were assayed against [³H]G-8-AAF and the profiles matched to lines ○—○, □—□, and △—△ in Chart 2A, plus a mixture of 8.7% dG-8-AAF and 91.3% dG-8-AF not shown. A typical profile for BALB/c DNA is shown in Chart 2B (●—●). The percentage of inhibition at saturation for the BALB/c DNA’s, when expressed as a fraction of the percentage of inhibition at dG-8-AF saturation in the same assay, was 0.131 ± 0.013 after 7 experiments. Similarly, for the YDF DNA’s, 5 experiments yielded 0.247 ± 0.018. Since similar data from 8 standard curves gave a value of 0.273 ± 0.016 for 5.65 pmol of a mixture of 5% dG-8-AAF and 95% dG-8-AF, it was determined that 3% of the C-8 substitution in the case of BALB/c DNA (●—●) and 5% for YDF DNA (○—○) is the acetylated adduct.

For routine assay, a standard curve mixture containing 5% dG-8-AAF:95% dG-8-AF was used to quantitate C-8 substitution on BALB/c and YDF DNAs with [³H]G-8-AAF (Chart 1A, □—□). The amount of DNA was chosen to give percentage of inhibition values on the linear portion of the standard curve (between 15 and 35% inhibition). If [³H]G-8-AAF was utilized in the RIA (data not shown), the BALB/c and YDF DNA’s gave a linear increase up to 80% inhibition, as in standard curve ●—● of Chart 1A. The C-8 substitution (pmol/μg DNA) observed under these assay conditions and that calculated from values on the linear portion of the standard curve of Chart 1A (○—○) were the same.

A Comparison of DNA Binding by RIA and ¹⁴C after Exposure of BALB/c Cells to 9-[¹⁴C]-N-Ac-AAF. Binding of 9-[¹⁴C]-N-Ac-AAF to DNA of cultured mouse epidermal cells after 1 hr exposure is shown in Table 1. One aliquot of each DNA was assayed in RIA and another by liquid scintillation counting. For each experiment, the RIA value was about 25% lower than the ¹⁴C value. When the [¹⁴C]-AAF-DNA’s from the first experiment were pooled, 75% (2241 cpm) of the total counts (3016 cpm) were precipitable by the G-8-AAF antiserum. The last line of Table 1 represents DNA for Specific AAF-DNA Adducts

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Binding of 9-[¹⁴C]-N-Ac-AAF to mouse DNA as detected by ¹⁴C and RIA</td>
</tr>
<tr>
<td>BALB/c epidermal cells were treated at confluence for 1 hr in medium containing serum, and DNA’s were prepared on CsCl gradients, treated with RNase and pronase, and hydrolyzed with S, nuclease. Aliquots of the same DNA were used for RIA and radioactivity determination.</td>
</tr>
<tr>
<td>µmol carcinogen/mol DNA-phosphate</td>
</tr>
<tr>
<td>Concentration N-Ac-AAF (m)</td>
</tr>
<tr>
<td>1.0 x 10⁻⁴</td>
</tr>
<tr>
<td>0.9 x 10⁻⁴</td>
</tr>
<tr>
<td>(24 hr)⁶</td>
</tr>
<tr>
<td>a Mean ± S.E. of triplicate experiments.</td>
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<tr>
<td>b The carcinogen was removed at 1 hr, and samples were incubated for 23 hr.</td>
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<tr>
<td>c Mean ± range of duplicate experiments.</td>
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<table>
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<th>Table 2</th>
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<tr>
<td>RIA determination of binding and removal of C-8 adducts from the DNA of BALB/c and YDF cells</td>
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<tr>
<td>Confluent cells were treated with N-Ac-AAF in medium containing serum. After a 1-hr exposure, one-half of the dishes were harvested, while the other half were washed twice with phosphate-buffered saline and incubated for another 23 hr in fresh medium. DNA’s were prepared on CsCl gradients, hydrolyzed with S, nuclease, and assayed by RIA.</td>
</tr>
<tr>
<td>pmol C-8 adduct/µg DNA</td>
</tr>
<tr>
<td>Concentration of N-Ac-AAF (m)</td>
</tr>
<tr>
<td>BALB/c</td>
</tr>
<tr>
<td>2.0 x 10⁻⁴</td>
</tr>
<tr>
<td>YDF</td>
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<tr>
<td>1.2 x 10⁻⁴</td>
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<tr>
<td>1.5 x 10⁻⁴</td>
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<tr>
<td>a Mean ± S.E. from 4 to 5 replicate experiments; each experiment represents DNA extracted from four to five 150-mm dishes of cells or 1 roller bottle.</td>
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A Mean ± range of duplicate experiments.
from BALB/c cells allowed to incubate for 23 hr after the removal of the carcinogen and indicates that by both assays about 40% of the bound products were removed from the DNA during the first 24 hr after exposure. Additional data are shown in Table 2.

**Binding and Removal of N-Ac-AAF in BALB/c Mouse Epidermal Cells and YDF Human Skin Fibroblasts Determined by RIA.** BALB/c mouse epidermal cells and YDF human fibroblasts were treated at confluence with N-Ac-AAF as described in Materials and Methods. The carcinogen was removed 1 hr after exposure, and binding was assayed immediately or after an additional culture period of 23 hr. The results are shown in Table 2. Initial binding levels varied from experiment to experiment probably reflecting differences in culture conditions, inherent variations in different preparations of primary cultures (23, 24), and variations in carcinogen potency. Experiments presented in Tables 1 and 2, as well as others not included, indicate that mouse epidermal cells removed about 40% of the bound C-8 adducts in 24 hr. Values observed for YDF human fibroblasts in similar experiments showed approximately 50% removal (Table 2) during that time period.

**DISCUSSION**

The G-8-AAF RIA was developed using antiserum obtained in rabbits upon immunization with a G-8-AAF-bovine serum albumin conjugate (19). Results presented in this paper show that the RIA will detect pmol quantities of the C-8 adducts, dG-8-AAF and dG-8-AAF, formed by the interaction of DNA with the carcinogen 2-AAF. The data presented in Chart 1 demonstrate the existence in the antiserum of antibody which recognizes dG-8-AAF and dG-8-AAF. The antibodies do not recognize the minor adduct dG-N2-AAF at concentrations 2 orders of magnitude higher than the usual range for the C-8 adducts; thus, the N2 adduct does not interfere during routine quantitation of C-8 adducts by RIA. In 2 other studies, antibodies have been raised against either 2-AAF alone (7) or native DNA-AAF (5% modified) (14). A RIA was established with the 2-AAF antiserum using nonradioactive 2-AAF as inhibitor, but the assay was also inhibited by N-OH-AAF and most of the ring-hydroxylated 2-AAF metabolites, over a range of 10^5 to 10^8 pmol (7). The anti-native DNA-AAF was specific for native DNA-AAF, denatured DNA-AAF, and GMP-AAF, with similar binding affinities to Fab fragments for all 3 haptens (14). Whether this antiserum can distinguish between C-8 and N2 adducts remains to be determined.

The RIA described in the present report can be used to determine the proportion of each C-8 adduct in AAF-modified DNA's where the dG-8-AAF content is below 20%. This can be accomplished by comparing the profile obtained with increasing concentrations of a particular AAF-modified DNA with a set of standard curves derived by utilizing mixtures of the acetylated and deacetylated C-8 adducts with or without appropriate concentrations of control DNA (Chart 2). These types of experiments have indicated that the proportion of C-8 adducts as dG-8-AAF in the DNA of treated BALB/c epidermal cells is about 3%, that of YDF human fibroblasts is about 5%, the remaining 97 and 95%, respectively, being dG-8-AF (Chart 2B). The profiles obtained with increasing concentrations of AAF-DNA's were remarkably consistent from experiment to experiment in cells grown and treated under identical conditions. However, preliminary data suggest that mouse epidermal cells treated with N-Ac-AAF in the absence of serum gave a profile indicating >3% dG-8-AAF; hence, the possibility arises that the conditions of carcinogen exposure will influence the distribution of DNA adducts. The data of Kriek and Cerutti (see "Introduction") and those presented here show that the proportions of different adducts can vary in in vivo model systems. One consistent feature of the in vivo systems is that the fact that a large portion of the total carcinogen bound is in the form of deacetylated C-8 adducts. Deacetylation activity can be cytoplasmic (8), nuclear membrane bound (20), and/or transacetylation (3, 9, 10), and each of these activities can vary considerably from species to species (3, 10). Deacetylation has also been proposed as an early step in N-Ac-AAF-induced mutagenesis (20), but its ultimate importance in carcinogenesis is currently unclear. There is notable lack of deacetylation when N-Ac-AAF is reacted chemically with native DNA. Under these conditions, 9 to 20% of the total adducts are in the form of dG-N2-AAF, and 80 are dG-8-AAF (6, 21, 22).

It was our intention, by comparing the binding of 9-[14C]-N-Ac-AAF with C-8 adducts measured by RIA, to measure independently the accuracy of the RIA. The results differed by about 25%, which is reasonable considering the sources of error inherent in each technique. It is not possible to determine what percentage of the total counts represent the N2-adduct or to rule out the possibility that binding of the radiolabeled carcinogen to nuclear proteins has rendered them refractory to pronase treatment. Neither of these conditions would influence the RIA. However, the basic assumption underlying the use of RIA standard curves with hydrolyzed control DNA plus the chemically synthesized adducts is that the profiles will be the same as curves obtained with hydrolyzed, in vivo-modified DNA's. The hydrolysis of denatured, modified DNA's has not been proven to be complete (22). However, previous data (19) have suggested that it is adequate for detection of most C-8 modification since hydrolysis of a chemically modified AAF-DNA allowed for detection of the same level of modification by RIA and spectrophotometry. Identical results were obtained when DNA's were hydrolyzed with DNase, alkaline phosphatase, and venom phosphodiesterase or the S, nuclease procedure.

Assayed by RIA, the removal of C-8 adducts from mouse epidermal cells was found to be approximately 40% over a 24-hr repair period (Tables 1 and 2). The human fibroblasts, YDF, removed approximately 50% of the total C-8 adducts during the same time interval (Table 2). These results are similar to data obtained in other laboratories utilizing different techniques. Amacher et al. (1. 2), using 9-[14C]-N-Ac-AAF, have shown 25 and 50% removal of bound carcinogen in 24 hr from BALB/c 3T3 fibroblasts and human skin fibroblasts, respectively. Levinson and McCormick2 have reported that approximately 50% of label is removed from human fibroblasts treated with [3H]-N-Ac-AAF in 24 hr. Cerutti (4) has observed a 30% removal of C-8 adducts from

\(^{2}\) J. W. Levinson and J. J. McCormick, personal communication.
A549 human lung cells in 21 hr (one doubling time) and indicated that the dG-8-AF is removed faster than the dG-8-AAF. In our studies, preliminary evidence suggests that the rate of removal of acetylated and deacetylated C-8 adducts is similar in each cell line since the relative amount of each adduct was the same at 1 hr and 24 hr.

The data presented in this paper indicate that RIA is a sensitive and specific means of measuring carcinogen-DNA adducts and should enhance the study of mechanisms of carcinogenesis by allowing the identification of adducts present in DNA during preneoplasia. At the present time, with one antiserum, it is possible to measure 90 to 95% of the carcinogen bound to the DNA of N-Ac-AAF-treated cells. In due time, hopefully, it will be possible to characterize the other 5 to 10% by RIA and quantitate each adduct during the process of carcinogenesis.

ACKNOWLEDGMENTS

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