Biphasic Removal of DNA Adducts in a Repetitive DNA Sequence after Dietary Administration of 2-Acetylaminofluorene

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Dietary administration of the hepatocarcinogen 2-acetylaminofluorene (2-AAF) to rats results in the formation of a major hepatic DNA adduct, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF). In liver DNA, dG-C8-AF reaches steady-state conditions after approximately 2 weeks of feeding and is removed in a biphasic manner. In these experiments, we have quantified adduct concentrations in a 370 base-pair repetitive DNA fragment to determine if the adduct levels and kinetics of adduct removal were similar to those found in total genomic DNA. Male F344 rats were fed 0.02% 2-AAF for 28 days and were sacrificed at intermittent times up to 56 days after being returned to the control diet. Hepatic DNA adduct levels were measured by ^{32}P-postlabeling or radioimmunoassay (RIA) in total genomic DNA and in a 370 base-pair fragment obtained by digesting genomic DNA with Hind III. Biphasic removal of dG-C8-AF, which composed about 90% of the total adducts measured, was observed in total genomic DNA, with comparable rate constants being detected by both ^{32}P-postlabeling and RIA. ^{32}P-Postlabeling also showed analogous biphasic removal of dG-C8-AF in the 370 base-pair fragment. A second adduct, 3-(deoxyguanosin-N^2-yl)-2-AAF (dG-N^2-AAF), which accounted for about 10% of the total adducts measured, showed similar biphasic removal kinetics in the total genomic DNA and the 370 base-pair fragment; however, as compared to dG-C8-AF, little removal of dG-N^2-AAF was observed during the slow phase.

Introduction

The potential use of DNA damage as a marker for the risk assessment of cancer has increased interest in the formation and repair of DNA–carcinogen adducts. Because human exposure to carcinogens typically occurs on a continual basis, the DNA adduct concentrations detected in target tissues probably reflect an equilibrium between adduct formation and removal. To understand the dynamics of this process, we have been evaluating DNA adduct levels in rats chronically administered the hepatocarcinogen 2-acetylaminofluorene (2-AAF). In previous experiments the distribution of adducts among cell types in liver (*1*) and regions of hepatic chromatin (*2*) has been determined. The data demonstrated that the major adduct, N-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF), reached steady-state conditions after approximately 2 weeks of feeding and that adduct removal, measured during subsequent time on control diet, was biphasic. A rapid removal phase was observed for the first 7–14 days, with a slower removal phase occurring continuously, but only evident after the rapid removal phase was essentially com-

plete. Because a similar profile was observed within the various hepatic chromatin regions, the biphasic kinetics may indicate that adducts within certain nucleotide sequences are resistant to removal. As a first step to investigate this possibility, we have compared the concentration of dG-C8-AF and kinetics of dG-C8-AF removal in a 370 base-pair repetitive DNA fragment (*3*) to that observed in total genomic DNA. If both types of DNA showed similar adduct concentrations and removal kinetics, this would suggest that the repetitive fragment may be a suitable model to elucidate the identity of the resistant sequences.

Materials and Methods

Male F344 rats (~150 g; four per group; obtained from the breeding colony at the National Center for Toxicological Research) were fed NIH-31 meal containing 0.02% 2-AAF (Aldrich Chemical Co., Milwaukee, WI) for 28 days. Animals were then placed on a control diet and sacrificed by CO_2 exposure at 0, 3, 7, 14, 21, 28, or 56 days after completion of dosing. The livers were immediately excised, hepatic nuclei were prepared, and DNA was isolated as described previously (*2*). A 100-μg portion of the total genomic DNA was digested with 0.32 U of Hind III in a 50 mM NaCl, 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol buffer for 16 hr at 37 °C and fractionated on 2% agarose with an IBI HRH 54000 electrophoresis gel apparatus. The 370 base-pair band was excised from the gel and the DNA isolated by the Geneclean method (Bio 101, Inc., La Jolla, CA).

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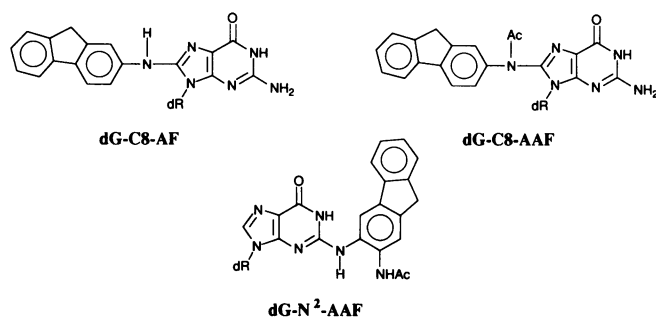
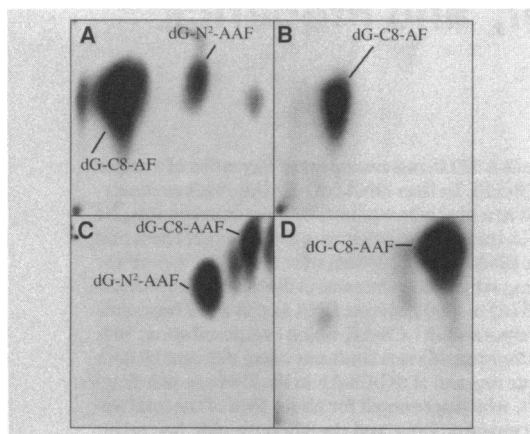FIGURE 1.   Structures of DNA adducts found in livers of male F344 rats fed 2-acetylaminofluorene.

FIGURE 2.   ^{32}P-Postlabeling autoradiograms of DNA adducts obtained from (*A*) liver genomic DNA from a male F344 rat fed 0.02% 2-acetylaminofluorene (2-AAF) for 28 days followed by control diet for 3 days; (*B*) calf thymus DNA reacted with *N*-hydroxy-2-aminofluorene; (*C*) calf thymus DNA reacted with *N*-acetoxy-2-AAF; and (*D*) deoxyguanosine 3'-monophosphate reacted with *N*-acetoxy-2-AAF.

This procedure yielded approximately 1 µg of the 370 base-pair DNA fragment.

Approximately 1–5 µg of either the 370 base-pair fragment or total genomic DNA was digested to 3'-nucleotides (*4*). The adducts were then ^{32}P-postlabeled using an *n*-butanol enrichment procedure (*4*) and separated by thin layer chromatography as described previously (*5*). Adducts were identified by comparison to a dG-C8-AF DNA standard, derived from the reaction of calf thymus DNA with [ring-^{3}H]*N*-hydroxy-2-aminofluorene, and to a *N*-(deoxyguanosin-8-yl)-2-AAF (dG-C8-AAF) and 3-(deoxyguanosin-*N*[2]-yl)-2-AAF (dG-N[2]-AAF) DNA standard, derived from the reaction of [ring-^{3}H]*N*-acetoxy-2-AAF with calf thymus or salmon testes DNA (Fig. 1). Adduct levels were quantified by comparison to the dG-C8-AF DNA standard.

Radioimmunoassays (RIA) were conducted on total genomic DNA according to the procedures outlined by Poirier et al. (*6*) using rabbit antisera that recognize dG-C8-AF and dG-C8-AAF.

Results

Identification of Adducts

Figure 2*A* shows the adduct profile as determined by ^{32}P-postlabeling in total genomic liver DNA 3 days after discon

tinuing the 2-AAF feeding. A qualitatively similar adduct pattern was observed in both total genomic DNA and the 370 base-pair fragment after 0, 7, 14, 21, 28, and 56 days on control diet. A major adduct, which had the same elution characteristics as the dG-C8-AF standard (Fig. 2*B*), accounted for about 90% of the ^{32}P detected. A second more polar adduct (~10% of the ^{32}P) was also separated by thin layer chromatography (Fig. 2*A*). By comparison with the acetylated adduct standards, dG-C8-AAF and dG-N[2]-AAF (Fig. 2*C*), this adduct was identified as dG-N[2]-AAF. The dG-C8-AAF and dG-N[2]-AAF were distinguished through comparison to the adduct obtained from reacting *N*-acetoxy-2-AAF with deoxyguanosine 3'-monophosphate, which yields only dG-C8-AAF (Fig. 2*D*).

The ^{32}P-postlabeling intensities for dG-N[2]-AAF and dG-C8-AAF in Figure 2*C* did not reflect the amounts of the two adducts in the sample. Reaction of *N*-acetoxy-2-AAF with DNA typically gives about 75% dG-C8-AAF and about 25% dG-N[2]-AAF (*7*). The proportion of dG-C8-AAF to dG-N[2]-AAF in the *N*-acetoxy-2-AAF-modified DNA used in the present study was determined to be 85:15 by HPLC analysis of an enzymatic hydrolysate of the DNA (100 µg DNAse/mg DNA and 0.08 U snake venom phosphodiesterase/mg DNA for 3 hr at 37°C, followed by 5.0 U alkaline phosphatase/mg DNA for 3 hr at 37°C). However, when the DNA standard shown in Figure 2*C* was analyzed by ^{32}P-postlabeling, 14% of the ^{32}P was associated with dG-C8-AAF, whereas 86% was found with dG-N[2]-AAF;
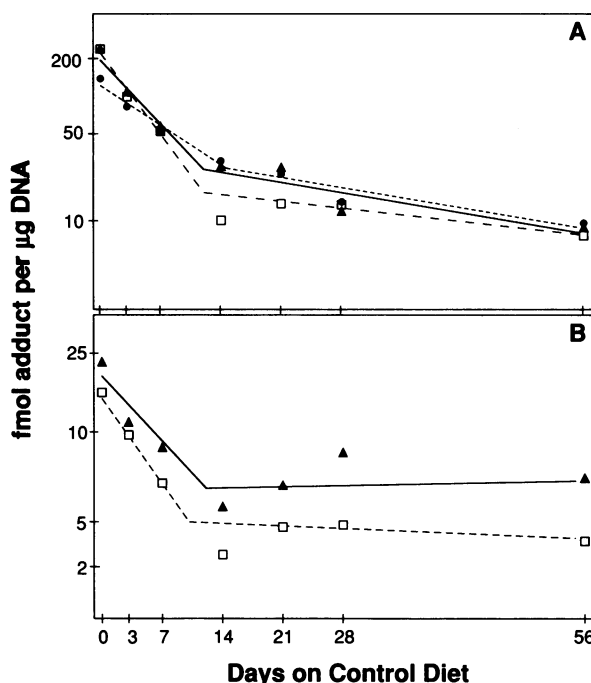

FIGURE 3.   Concentration of *N*-(deoxyguanosin-8-yl)-2-aminofluorene (*A*) and 3-(deoxyguanosin-N[2]-yl)-2-acetylaminofluorene (*B*) in total genomic DNA (. . . □) and in a 370 base-pair fragment obtained from digesting genomic DNA with *Hind* III (——▲) as assayed by ^{32}P-postlabeling, and in total genomic DNA (. . .●)as assayed by radioimmunoassay. Each point represents the adduct levels found in two to four rats sacrificed at the specified time. The relative standard deviations for the data in *A* and *B* were 36% and 46%, respectively. The data were fit by linear regression analyses to a two-phase adduct removal model described by the differential equation $-d[A]/dt = k_{fast}[A] + k_{slow}[A]$.

thus, the ^{32}P-postlabeling intensity of dG-N^2-AAF was 40-fold greater than that of dG-C8-AAF. Further investigation suggested that the DNA surrounding the dG-C8-AAF adduct was not completely digested by the micrococcal nuclease and spleen phosphodiesterase used during ^{32}P-postlabeling. This was shown by HPLC analysis of the DNA standard after digestion using ^{32}P-postlabeling conditions (1 μg micrococcal nuclease/μg DNA and 1 μg spleen phosphodiesterase/μg DNA for 3 hr at 37 °C) and followed by 5.0 U alkaline phosphatase/mg DNA for 3 hr at 37 °C indicated 56% dG-C8-AAF and 44% dG-N^2-AAF.

Rate Constants for Removal of dG-C8-AF and dG-N^2-AAF

dG-C8-AF was removed from total genomic DNA with biphasic kinetics (Fig. 3A) as measured by both ^{32}P-postlabeling (fast, $t_{1/2}$ = 2.8 days; slow, $t_{1/2}$ = 40.8 days) and RIA (fast, $t_{1/2}$ = 4.6 days; slow, $t_{1/2}$ = 25.7 days). In addition, a biphasic removal profile with similar rate constants (fast, $t_{1/2}$ = 3.7 days; and slow, $t_{1/2}$ = 24.8 days) was demonstrated via ^{32}P-postlabeling for the 370 base-pair fragment. Insufficient amounts of the 370 base-pair DNA fragment were isolated to determine rate constants by RIA.

The acetylated adduct, dG-N^2-AAF, was also removed in a biphasic manner in both total genomic DNA and the 370 base-pair fragment (Fig. 3B), and the phase change occurred in the same time period (7–14 days after dosing) as that of the major adduct, dG-C8-AF. The fast rate for total genomic DNA was $t_{1/2}$ = 3.5 days and $t_{1/2}$ = 4.1 days for the 370 base-pair fragment, which is comparable to that calculated for dG-C8-AF; however, dG-N^2-AAF displayed negligible removal after about 14 days ($t_{1/2}$ = 126 and 267 days for total genomic DNA and 370 base-pair DNA, respectively). The measurements from ^{32}P-postlabeling data could not be compared to those from RIA, as the antibody did not recognize dG-N^2-AAF.

Discussion

Using ^{32}P-postlabeling, two adducts have been found in liver DNA from rats administered 2-AAF in the diet for 28 days. In confirmation of assays previously conducted by RIA, dG-C8-AF was the major adduct detected, accounting for 90% of the adducts at the completion of the feeding period. The second adduct, dG-N^2-AAF, occurred at approximately 10% of the extent of dG-C8-AF. Preferential binding to the 370 base-pair fragment as opposed to total genomic DNA was not observed for either adduct, unlike that reported by Gupta after administering a single dose of N-hydroxy-2-AAF (8). A second acetylated adduct, dG-C8-AAF, that is formed during the initial dosing with 2-AAF was not found after 28 days of feeding, which also confirms previous results obtained by RIA (2). Interestingly, it appears that the two acetylated adducts formed from 2-AAF are assayed by the ^{32}P-postlabeling technique with very different efficiencies, with dG-N^2-AAF being labeled to approximately 40 times the extent of dG-C8-AAF. This suggests that caution should be exercised when quantifying unknown adducts based simply on the extent of incorporation of ^{32}P.

Both dG-C8-AF and dG-N^2-AAF showed biphasic removal kinetics, with a rapid phase lasting 7–14 days followed by a slower phase. The rate constants measured for the fast phases of both adducts are in agreement with rates reported previously for dG-

C8-AF (2); however, unlike dG-C8-AF, dG-N^2-AAF showed negligible removal during the slow phase. Biphasic removal kinetics of both adducts, with similar rate constants, was also observed in a 370 base-pair repetitive fragment obtained by digestion of total genomic DNA with Hind III. As the removal kinetics of the adducts were found to be similar in both total genomic DNA and the 370 base-pair fragment, the latter can serve as a model in which to study the biphasic removal of the adducts and to determine if certain nucleotide sequences may be more susceptible to adduct formation or resistant to DNA repair. The nature of these sequences is currently being investigated.

These experiments combined with previous ones (1,2,5,6) indicate that, during the continuous administration of carcinogens, adduct levels reach a dose-dependent steady state that presumably reflects an equilibrium between adduct formation and removal. The results further suggest that adduct removal is nonrandom. Recent data indicate that DNA adducts can persist in human tissue for a considerable time after exposure (9). Our results with animal models suggest that this may be due to the resistance of certain nucleotide sequences to DNA repair.

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