Adducts from in Vivo Action of the Carcinogen 4-Hydroxyaminoquinoline 1-Oxide in Rats and from in Vitro Reaction of 4-Acetoxyaminoquinoline 1-Oxide with DNA and Polynucleotides

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Native DNA was obtained from chicken erythrocytes. It had the following characteristics: s20,w = 22S; hyperchromicity at 260 nm, 41%. The polynucleotides poly(dG-dC)-poly(dG-dC) and poly(dA-dT)-poly(dA-dT) were purchased from PL Biochemicals. All the enzymes (bovine pancreatic DNase I, snake venom phosphodiesterase, calf spleen phosphodiesterase, alkaline phosphatase of calf intestine) were purchased from Boehringer-Mannheim, Mannheim, Federal Republic of Germany.

Preparation of in Vitro Ac-4-HA[2-3H]QO-modified DNA and Polynucleotides. Native DNA and polynucleotides were modified by the ultimate carcinogen Ac-4-HA[2-3H]QO in the following manner. The reactions were performed in 2 mM sodium citrate buffer (pH 7); approximately 1 mg of polymer (concentration, 0.5 mg/ml) was incubated at 37° for 0.25 hr under a nitrogen atmosphere, in the dark, and in the presence of the equivalent amount of Ac-4-HA[2-3H]QO solubilized in dimethyl sulfoxide. The modified DNA was purified by diethyl ether extractions and extensive ethanol precipitations (0.1 volume of 5 M NaCl in 2 volumes of precooled ethanol were added to each DNA solution. The DNA precipitated after shaking.). The amount of bound quinoline ring was calculated as described previously (5). It was 2.5, 3.3, and 2% of modified bases for the modified DNA, poly(dG-dC)-poly(dG-dC), and poly(dA-dT)-poly(dA-dT), respectively.

Preparation of in Vivo 4-HA[2-3H]QO-modified DNA. The in vivo-modified DNA was prepared as follows. Approximately 105 ascites tumor cells (H.Z. line) were injected i.p. into 3-month-old male Wistar rats weighing about 300 g. Seven days after transplantation, an acidic 0.9% NaCl solution of 0.034 mM 4-HA[2-3H]QO in HCl was injected i.p. (0.5 ml/rat). The rats were killed by decapitation 1 or 2 hr later. The ascitic fluid was drained, and the cells were harvested by centrifugation (5 min at 1500 rpm). Cells were washed in a 0.25 w-0.88 mM sucrose gradient containing 10% phosphate-buffered saline in order to remove contaminating RBC. Then DNA was extracted using the method of Marmur (11). We obtained a modified DNA sample with 0.04% modified base pairs (2 molecules of carcinogen for 10,000 nucleotides).

Enzymatic Hydrolysis of the Modified DNA to Nucleosides and Chromatographic Analysis of the Hydrolysate. In vitro Ac-4-HA[2-3H]QO- and in vivo 4-HA[2-3H]QO-modified DNAs were hydrolyzed under the following conditions. One mg of modified DNA was solubilized (concentration, 2 mg/ml) in a buffer of 5 mM Tris-HCl, 2 mM MgCl2, and 2 mM CaCl2 (pH 7.4) and incubated at 37° for 2 hr with 45 /ig of DNase I; then 45 /ig of each phosphodiesterase were added for an additional incubation of 17 hr at 37°. The pH was adjusted to 8.0, 35 ng of alkaline phosphatase were added, and dephosphorylation of the hydrolysis products was performed for 7 hr. Thus, the total incubation time was 26 hr.

Chromatography on Bio-Gel P-2 (minus 400 mesh) was performed using a column (0.6 x 60 cm) equilibrated with 2 mM sodium citrate buffer (pH 7). The flow rate was 4 ml/hr. Fractions of 1 ml were collected, and the radioactivity measured. Each P-2 tritiated fraction was collected and concentrated, and its analysis was carried out by HPLC. A C18-Bondapak column was used, with a methanol-water linear gradient (during 20 min) from water to 95% methanol-water. The flow rate was 2 ml/min. Conditions under which the water was acidified with H3PO4 (pH 2.8) were also used.

RESULTS

Comparative Chromatographic Analysis of the Enzymatic Hydrolysates of in Vivo 4-HA[2-3H]QO- and in Vitro Ac-4-HA[2-3H]QO-modified DNAs. Samples of in vivo 4-HA[2-3H]QO-modified DNA and in vitro Ac-4-HA[2-3H]QO-modified native DNA were incubated with the enzymes, at 37° for 26 hr. The hydrolysates were analyzed on a Bio-Gel P-2 minus 400 mesh column. The UV detection at 254 nm (not shown) exhibited 4 peaks corresponding to the 4 deoxyribonucleosides as expected after the hydrolysis. The chromatographic radioactive patterns are shown in Chart 1. In both cases, similar profiles were obtained. There were 5 distinct tritiated fractions, which we called A, B, C, D, and E. They were corresponding to the same elution volumes for in vivo- and in vitro-modified DNA. Moreover, the percentage of each fraction was almost the same in both cases. The compound corresponding to Peak E was previously proved by HPLC to be the dGuo-C8-AOQ (5).

For in vitro- and in vivo-modified DNAs, every other P-2 fraction was collected, concentrated, and submitted to HPLC analysis. We used an absorbance detection at 2 wavelengths: 254 nm, the characteristic wavelength of both nucleosides and quinoline ring; and 365 nm, the characteristic wavelength of the quinoline moiety. The quinoline was also detected by tritiated radioactivity measurements of 500-yl aliquots. Fractions B and C, which were the quantitatively less important, were studied first. The study of P-2 Fraction B was difficult because its UV characterization at 254 nm was completely hindered by the 3 deoxyribonucleosides.
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dAdo, dThd, and dCyd) which were present in this fraction. Some tentative separations of the unlabeled nucleosides on a Sephadex LH-20 column could not be effected. However, we observed that the 2 HPLC radioactive profiles (not shown) exhibited 2 peaks which were recovered, whatever the origin, in vivo or in vitro, of the modification of DNA. P-2 Fraction C of the in vivo- and in vitro-modified DNAs contained only one compound which was the same in both cases (HPLC profiles not shown). Its percentage was very weak: about 6 and 8% of the total radioactivity of in vivo- and in vitro-modified DNA, respectively.

Chart 2 shows the HPLC tritiated profiles of the quantitatively more important P-2 Fractions A and D for in vivo (Chart 2, a and c) and in vitro (Chart 2, b and d) modified DNAs. In the P-2 Fraction A, 2 identical compounds were recovered from the in vivo- and in vitro-modified DNAs, which we called A1 and A2. Another peak, A3, was detected only in the hydrolysate of the in vitro-modified DNA. P-2 Fraction D was composed in both cases of 4 major compounds, D1, D2, D3, and D4, which were well individualized on the UV profiles at 365 and 254 nm. On the tritiated profile, the 2 peaks corresponding to D3 and D4 were not as well separated, due to the very similar retention time of the 3 compounds, D2, D3, and D4. We also detected in this fraction 2 minor radioactive peaks with a retention time at about 15 min. Therefore, we can claim that all the HPLC radioactive peaks obtained from the hydrolysates of the in vivo-modified DNA were also recovered from the in vitro-modified DNA, with the same chromatographic characteristics (identical retention times on Bio-Gel P-2 and HPLC; identical ratios (Δ) of the absorbances at 254 and 365 nm in neutral and acidic conditions in HPLC). We will now focus our attention on the HPLC profiles obtained from the in vitro-modified DNA, and we will consider the more quantitatively important P-2 fractions, Fractions A and D. In the case of Fraction D, the corresponding subfractions will be studied by comparison with the HPLC profiles obtained with modified polynucleotides.

HPLC Analysis of the Hydrolysates of DNA and Polynucleotides Modified in Vitro by Ac-4-HA[2-3H]QO (P2 Fractions A and D). The 2 compounds A1 and A2 (see Chart 2) (which account for about 15% of the total radioactivity of DNA) were identified as 2 degradation products of the dGuo-C8-AQO. Indeed, when incubated at 37° during 48 hr under the conditions of the enzymatic hydrolysis, the dGuo-C8-AQO gave a P-2 chro-

Chart 2. HPLC tritiated elution profiles of P-2 Fractions A and D of the DNA hydrolysates. Chromatographies were carried out with a linear gradient (during 20 min) from water to 95% methanol-water, 0.5-ml aliquots were collected, and radioactivity was measured. Each tritiated peak gave absorbance signals at 254 and 365 nm and was characterized by its ratio (Δ) of the absorbance at these 2 wavelengths. a and c, P-2 Fractions A and D of in vivo 4-HA[2-3H]QO-modified DNA; b and d, P-2 Fractions A and D of in vitro Ac-4-HA[2-3H]QO-modified DNA.

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matographic tritiated pattern which exhibited 2 major peaks, the nonhydrolyzed dGuo-C8-AQO and an A fraction which contained A1 and A2. HPLC analysis showed that, whatever the conditions under which the HPLC profiles were recorded (neutral and acidic media), the 2 hydrolysis compounds were recovered at the same retention times as those of A1 and A2 when obtained from the DNA hydrolysate.

Three samples, native DNA, poly(dA-dT)-poly(dA-dT), and poly(dG-dC)-poly(dG-dC), were modified in vitro with Ac-4-HAQO, enzymatically hydrolyzed, and submitted to the chromatographic analysis. The HPLC profiles (absorbance at 365 nm, detection of the quinoline moiety) of the 3 P-2 Fractions D are shown in Chart 3. Chart 3a, which corresponds to the modified DNA, exhibits 4 peaks as mentioned previously, D1, D2, D3, D4, and another compound (D5). By using the method of Dische (2), we showed that the 2 compounds D1 and D3 which contained the quinoline moiety also contained a deoxyribosyl residue, indicating that they are adducts. The D5 component arose from the degradation of the adduct D1; indeed, the stability of this isolated adduct was checked by HPLC and allowed to follow the appearance of this product with a retention time of about 15 min. D2 was identified as a quinoline compound, 4-AQO. Indeed, D2 comigrated with the reference standard (R, 10.5 min) with an identical ratio (Δ), which is 1.5 and 8.8 in near neutral and acidic media, respectively. The possible origin of 4-AQO in the DNA hydrolysates will be discussed below. We can only observe here that it was not recovered from modified poly(dA-dT)-poly(dA-dT). Any information was obtained about the D2 compound (R, 11.8 min; Δ 2.3). The observation of the HPLC profile of the modified poly(dG-dC)-poly(dG-dC) (see Chart 3b) indicated that Compounds D1, D2, D4, and D5 were recovered from this modified polymer. Their retention times and their ratios Δ were identical to those of the equivalent compounds obtained from the DNA hydrolysate. Chart 3c indicates that P-2 Fraction D obtained after hydrolysis of the modified poly(dA-dT)-poly(dA-dT) contained only one compound which was the D2 compound that we recovered from the modified DNA. Indeed, the retention time and Δ values were exactly the same, and the 2 isolated D2 compounds [either from modified poly(dA-dT)-poly(dA-dT) or from modified DNA] comigrated in HPLC.

As we mentioned previously D1 and D2 are quinoline adducts. We previously checked the reactivity of the 4 deoxyribonucleosides with Ac-4-HAQO, and we showed that any reaction was obtained with dCyd and dThd when positive reaction was obtained with dGuo and dAdo (1). Moreover, in their previous works about the NQO adducts, Ikegami et al. (8) and Tada and Tada...
(17, 18) indicated that 4-NQO reacted preferentially with guanine and adenine in nucleic acids in vivo. Thus, we can claim that D₁ is a guanyl adduct, dGuo-2-AQO, and that D₃ is an adenyl adduct, dAdo-AQO.

DISCUSSION

In this study, an important observation was made. The main NQO adducts (the dGuo-C₈-AQO, the dGuo-2-AQO, and the dAdo-AQO), which are formed on in vivo 4-HAQO-modified DNA, are recovered from the in vitro Ac-4-HAQO-modified DNA. The in vivo system that we used (ascite cells) is open to criticism. Nevertheless, in terms of metabolism of 4NQQO, the fact that the same 3 main adducts were recovered from this in vivo system and from the in vitro material strengthens the choice of Ac-4-HAQO as a ultimate carcinogen model in our studies of chemical carcinogenesis by 4-NQO.

Another important feature is the identification of the 2 hydrolysis products arising from the decomposition of the dGuo-C₈-AQO during enzymatic hydrolysis. With regard to the total radioactivity of the modified DNA, these 2 compounds were recovered at about 16% (in vitro) and 20% (in vivo). This finding explains the fact that the percentage of dGuo-C₈-AQO (13%) was underestimated when determined from an enzymatic hydrolysis, as compared to the determination that we recently made from a spectrophotometric titration of the C₈-guanyl adduct on a nonhydrolyzed modified DNA (6). Using this spectrophotometric determination, we found that a modified native DNA and a modified single-stranded DNA contained, respectively, 30 and 70% of dGuo-C₈-AQO. Taking into account the degradation of this adduct in its titration after enzymatic hydrolysis, we obtain the same values of 30 and 70%. The nature of the hydrolysis products of dGuo-C₈-AQO may be discussed in light of the results obtained previously by Kriek and Westra (10) with the C₈-guanine adduct of aminofluorene. They observed its hydrolysis through guanine 7–8 bound at basic pH, in the presence of cationic ions such as Mg²⁺ and Mn²⁺. The presence of alkaline phosphatase in the medium increased the hydrolysis. Due to the chemical similarity between the C₈-guanyl adducts of 4-NQO and aminofluorene, an identical reaction may be possible with dGuo-C₈-AQO during its hydrolysis since it is performed under the same conditions (basic pH, Mg²⁺, and alkaline phosphatase). Studies were carried out very recently in our laboratory about the NQO adduct, Tada and Tada (17, 18) showed that there were 3 guanine adducts, one of them being very quickly decomposed during the chemical hydrolysis of their modified DNA, leading to a release of 4-AQO. Although our hydrolysis conditions were milder than those of Tada and Tada, it is possible that this phenomenon occurred. It is also possible that more than one guanine adduct were decomposed to release 4-AQO during and after the enzymatic hydrolysis. For example, we think that, after the enzymatic hydrolysis and during the chromatographic P-2 separation, some interactions with Bio-Gel P-2 could lead to the emergence of degradation compounds (4-AQO and others) which were then recovered in the different P-2 fractions. We rapidly tested this hypothesis by making a direct HPLC analysis of the total enzymatic hydrolysate of an in vitro Ac-4-HAQO-modified DNA. 4-AQO was still detected but in a smaller amount, indicating a possible bad effect of the P-2 column chromatography.

By comparison between the amounts of the 3 adducts (evaluated by the intensities of the 3 HPLC peaks at 365 nm), we observed that the dGuo-2-AQO seemed to be the major adduct formed on the Ac-4-HAQO-modified DNA. The same direct HPLC analysis of an in vitro-modified poly(dG-dC)-poly(dG-dC) indicated the presence on this polymer of the 2 main guanine adducts, dGuo-C₈-AQO and dGuo-2-AQO.

In conclusion, we point out that Ac-4-HAQO is a very adequate model for the study of the in vitro carcinogenesis by 4-NQO. The decomposition of different adducts was elucidated (the C₈-guanyl adduct led to ring-opened dGuo-C₈-AQO; one (or more) guanine adduct(s) led to 4-AQO). Moreover, we established the breaking of the quinoline-base bond. In their investigations on the NQO adducts, Tada and Tada (17, 18) showed that there were 3 guanine adducts, one of them being very quickly decomposed during the chemical hydrolysis of their modified DNA, leading to a release of 4-AQO. Although our hydrolysis conditions were milder than those of Tada and Tada, it is possible that this phenomenon occurred. It is also possible that more than one guanine adduct were decomposed to release 4-AQO during and after the enzymatic hydrolysis. For example, we think that, after the enzymatic hydrolysis and during the chromatographic P-2 separation, some interactions with Bio-Gel P-2 could lead to the emergence of degradation compounds (4-AQO and others) which were then recovered in the different P-2 fractions. We rapidly tested this hypothesis by making a direct HPLC analysis of the total enzymatic hydrolysate of an in vitro Ac-4-HAQO-modified DNA. 4-AQO was still detected but in a smaller amount, indicating a possible bad effect of the P-2 column chromatography.

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existence of a second guanine adduct, dGuo-2-AQO, which seemed to be a major one for in vitro-modified DNA. The yield of dGuo-2-AQO formation is secondary structure DNA dependent, like the dGuo-C8-AQO (6), but a greater extent of this guanine adduct was observed on the native DNA (5). In other words, the double helix facilitates its formation. The identification of the structure of the dGuo-2-AQO was just recently achieved in our laboratory, and all our data on this adduct strongly support its characterization as 3-(deoxyguanosine-N7-yl)-4-aminoquinoline 1-oxide.

We also investigated the structure of the dAdo-AQO and showed that it is the same compound as the adenine adduct QA II previously isolated by Kawazoe et al. (9), but while these authors proposed either the N1 or the N6 position of adenine our data strongly support the structure of dAdo-AQO as the 3-(deoxyadenosine-N6-yl)-4-aminoquinoline 1-oxide.* Chart 4 illustrates our actual knowledge about the DNA-NQO adducts.

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