Detection of Guanine-C8-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine Adduct as a Single Spot on Thin-layer Chromatography by Modification of the ^{32}P-Postlabeling Method

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N-(Deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-C8-PhIP) has been shown to be a major adduct in DNA of rats given [3H]PhIP. However, when DNA from organs of rats fed PhIP was analyzed by the ^{32}P-postlabeling method under standard and adduct-intensification conditions, four adduct spots were observed, and 3',5'-pdGp-C8-PhIP was detected as a minor, not a major, adduct spot. Since the three other major adduct spots were suspected to be those of adducted di- or oligo-nucleotides, the ^{32}P-labeled samples were further treated with nuclease P1 and phosphodiesterase I and found to yield only a single adduct spot. The material in this adduct spot was confirmed to be 5'-pdG-C8-PhIP. Thus, using this newly modified ^{32}P-postlabeling method, dG-C8-PhIP was detected as a major adduct in DNA of rats given PhIP.

Key words: PhIP-DNA adduct — ^{32}P-Postlabeling method — Major adduct — 5'-pdG-C8-PhIP

A variety of mutagenic and carcinogenic heterocyclic amines (HCAs) are formed during cooking of proteinaceous foods.[1–4] Of the HCAs examined, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) has been found at the highest level in various kinds of cooked foods.[4] PhIP is reported to be mutagenic in bacteria and cultured mammalian cells.[2,4,5] In long-term experiments in rodents, it has been demonstrated to induce lymphomas in mice and intestinal and mammary carcinomas in rats.[6–8] Like other HCAs, PhIP is converted to a proximate form, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-OH-PhIP), by cytochrome P450IA2.[9] The proximate form is then activated by N-2 esterification by O-sulfotransferase or O-acetyltransferase, and the resulting ultimate form reacts with DNA to form PhIP-DNA adducts.[10]

The chemically synthesized ultimate form, N-acetoxy-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (N-acetoxy-PhIP), reacts with 2'-deoxyguanosine to form N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (dG-C8-PhIP) in vitro.[11–13] This adduct has also been recovered as a major adduct from DNA of the liver of rats given [3H]PhIP.[12] On the other

hand, when DNA from rats treated with PhIP was analyzed by the ^{32}P-postlabeling method under standard conditions, several adduct spots were detected on thin-layer chromatography (TLC),[13,14] and the spot of authentic N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine 3',5'-diphosphate (3',5'-pdGp-C8-PhIP) coincided with a minor adduct spot, not a major adduct spot.[11,13] These results suggested that the other three spots were those of undigested dinucleotides or oligonucleotides including dG-C8-PhIP. In this study we, therefore, modified the ^{32}P-postlabeling method to digest the adducted di- or oligo-nucleotides more efficiently, and using this modified method we found that the PhIP adduct at position C-8 of guanine was a major adduct.

First, three authentic compounds, 3',5'-pdGp-C8-PhIP, N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine 3'-monophosphate (3'-dGp-C8-PhIP) and N-(deoxyguanosin-8-yl)-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine 5'-monophosphate (5'-pdG-C8-PhIP), were prepared by reported methods.[11] Briefly, 2'-deoxyguanosine phosphate ester (3',5'-pdGp, 3'-dGp or 5'-pdG) was treated with N-OH-PhIP in the presence of acetic anhydride, and the reaction mixture was separated by HPLC. The structures of 3',5'-pdGp-C8-PhIP, 3'-dGp-C8-PhIP and 5'-pdG-C8-PhIP were confirmed by their UV absorption spectra and also by their conversion to dG-C8-PhIP on treatment with alkaline phosphatase.[11]

Male F344 rats, 7 weeks old, were given 500 ppm PhIP in the diet for 4 weeks, then killed, and the major organs were excised. Since in our previous study[14] the highest adduct levels were detected in the lung and heart, we used DNA samples from the lung in the present study. The DNA, isolated by phenol-chloroform extraction, was digested with micrococcal nuclease and spleen phosphodiesterase (Worthington Biochemical Co., Freehold, NJ) at 37°C for 3 h. The DNA digest was ^{32}P-labeled under both standard and adduct-intensification conditions: a sample of 0.17 μg of the DNA digest was ^{32}P-labeled by T4 polynucleotide kinase (Takara Shuzo Co., Ltd., Kyoto) with [γ-^{32}P]ATP (92 Ci/mmol, 58 μM, ICN Biomedicals, Irvine, CA) under standard conditions, and 5 μg of the DNA digest with [γ-^{32}P]ATP (650 Ci/mmol, 2.4 μM) under adduct-intensification conditions, at 37°C for 1 h. Both reaction mixtures were then treated with potato apyrase solution (Sigma, St. Louis, MO, 40 mU). The ^{32}P-labeled samples thus obtained were spotted on a polyethyleneimine (PEI)-cellulose TLC sheet (POLYGRAM CEL 300 PEI, Machery-Nagel, Duren, Germany) and developed under the same conditions as used in previous studies,[14] except that development in direction D4 was carried out twice. The TLC sheet was exposed to a Fuji imaging plate (Fuji Photo Film Co., Tokyo) and the radioactivities of ad-

duct spots were analyzed with a Bio Imaging Analyzer (BAS2000, Fuji Photo Film Co.). As shown in Fig. 1A, four adduct spots were detected under the standard conditions. The mean levels of adduct spots a, b, c and d, respectively, in three independent experiments were estimated to be 1.7, 1.6, 1.4 and 4.9 (total 9.6) per 10^7 nucleotides. Four adduct spots were also observed under adduct-intensification conditions (Fig. 1B), and the intensification factors for spots a–d were calculated to be 220, 231, 331 and 255, respectively.

The position of spot a (Fig. 1A and B) on TLC coincided with that of 3′,5′-pdGp-C8-PhIP, as we reported previously.[11] We also confirmed this by HPLC. Spot a in Fig. 1B, detected under adduct-intensification conditions, was cut out and extracted twice with 0.5 ml of 4 M pyridinium formate (pH 4.5) with shaking for 30 min.[15] The mixture was centrifuged at 17,500 rpm for 30 min and the supernatant was passed through a 0.2-μm filter (Millipore Corp., Bedford, MA). An aliquot of the extract thus obtained was mixed with authentic samples of 3′,5′-pdGp-C8-PhIP, 3′-dGp-C8-PhIP, 5′-pdG-C8-PhIP and dG-C8-PhIP and subjected to HPLC on a TSKgel ODS-80Ts column (5 μm particle size, 4.6×250 mm, Tosoh Corp., Tokyo). As shown in Fig. 2, radioactivity was detected in a peak fraction corresponding to 3′,5′-pdGp-C8-PhIP.

Spots b, c and d were thought possibly to be those of ^{32}P-labeled di- or oligo-nucleotides ([^{32}P]pX(pN)$_n$p: where X is an adducted nucleoside, and N is a normal nucleoside). On incubation with nuclease P1, such adducted di- or oligo-nucleotide might be 3′-dephosphorylated, producing [^{32}P]pX(pN)$_n$.[16] This [^{32}P]pX(pN)$_n$ could then be digested further with phosphodiesterase I (PDE I) to yield [^{32}P]pX plus npN.[17] On similar treatments with nuclease P1 and PDE I, 3′,5′-pdGp-C8-PhIP should be converted to 5′-pdG-C8-PhIP. Therefore, samples ^{32}P-labeled under standard and adduct-intensification conditions were treated with nuclease P1 and PDE I as described below. Of the 15 μl volumes of reaction mixtures after kination, 2 μl aliquots were used for total nucleotide analysis after treatment with apyrase. The remaining 13 μl of each mixture was mixed with 1.8 μl of 0.3 M HCl to adjust the pH to about 5, and then incubated with 3.5 μl of 0.13 M sodium-citrate buffer (pH 5.3) containing 2 μg of nuclease P1 (Yamasa Shoyu Co., Ltd., Choshi) and 0.3 mM ZnCl$_2$ at 37°C for 10 min. The resulting reaction mixture was adjusted to about pH 9 by addition of 3 μl of 0.5 M Trizma base, and treated with 3 μl of an aqueous solution containing 30 mU of PDE I (Worthington Biochemical Co.) at 37°C for 30 min. Then, an aliquot was applied to a PEI-cellulose sheet and developed with the same solvent systems as used in standard and adduct-intensification conditions.
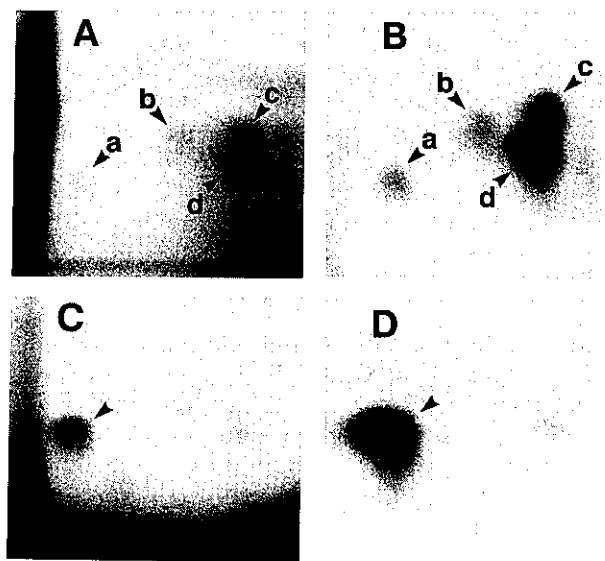

Fig. 1. Autoradiograms of PhIP-DNA adducts in the lung of rats fed PhIP, analyzed by four methods. (A) Standard method, (B) adduct-intensification method, (C) modified standard method, (D) modified adduct-intensification method. The exposure time of the imaging plate was 3 days for (A) and (C), and 1 h for (B) and (D).
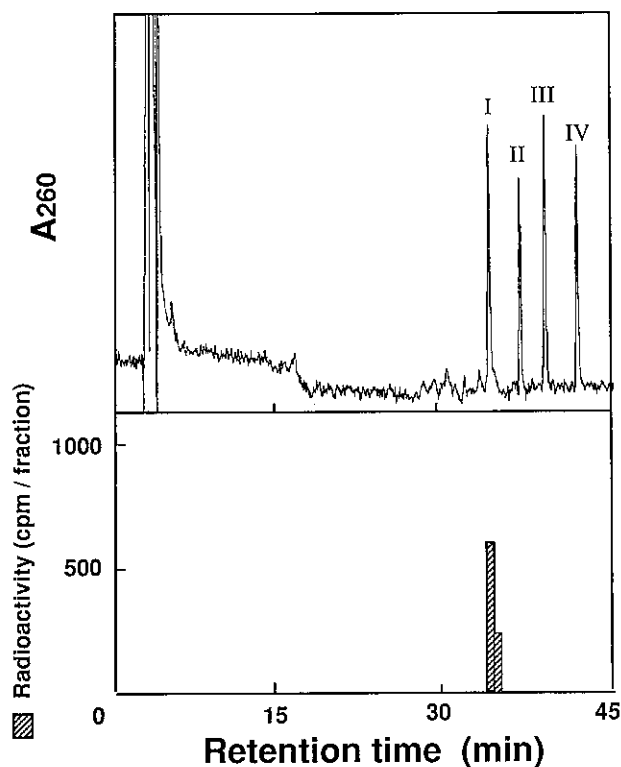
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Fig. 2. HPLC pattern of an extract of spot a (Fig. 1B) with four authentic compounds, 3',5'-pdGp-C8-PhIP (I), 3'-dGp-C8-PhIP (II), 5'-pdG-C8-PhIP (III) and dG-C8-PhIP (IV). A sample was injected into a TSKgel ODS-80Ts column (5 μm particle size, 4.6×250 mm), and eluted with the following solvent system at a flow rate of 1 ml/min at the ambient temperature: 0–10 min, 10% CH_3CN in 25 mM phosphate buffer (pH 2.0); 10–70 min, a linear gradient of 10 to 50% CH_3CN in 25 mM phosphate buffer (pH 2.0). The eluate was monitored by measuring its UV-absorbance at 260 nm. The radioactivity of each fraction collected at 1 min intervals was measured in a Beckman LS1801 liquid scintillation counter.

An autoradiogram of PhIP-DNA adducts, which were ^{32}P-labeled under standard conditions and treated with nuclease P1 and PDE I, is shown in Fig. 1C. A single spot was detected and its level was estimated to be 9.1 adducts/10[7] nucleotides, which is 93% of that detected by the standard method. Similarly, a single spot was observed by the modified adduct-intensification method (Fig. 1D) and the level of the spot was almost the same as that detected by the original adduct-intensification method. Moreover, when the extract of the adduct spot in Fig. 1D was subjected to HPLC on a TSKgel ODS-80Ts column, 86% of the radioactivity was recovered in a peak fraction corresponding to that of 5'-pdG-C8-PhIP (Fig. 3). The structure of 5'-pdG-C8-PhIP is shown in Fig. 4.
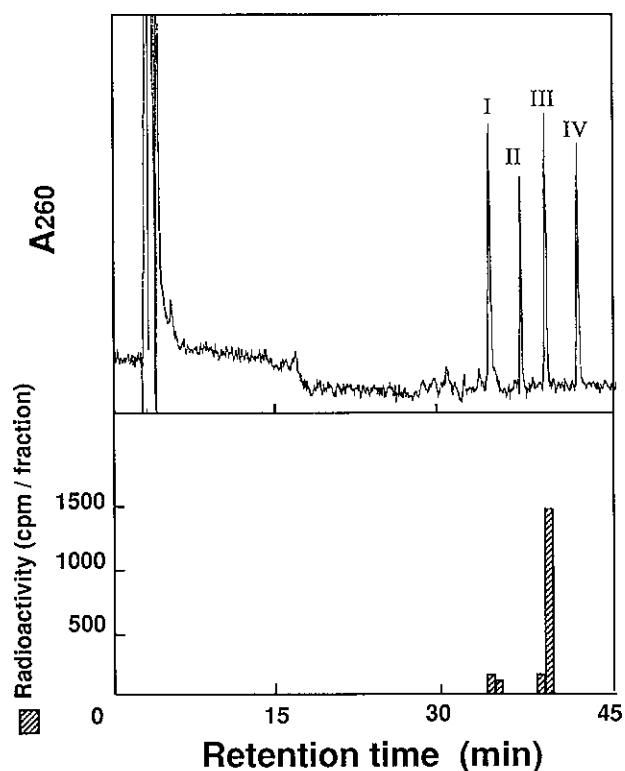
Fig. 3. HPLC elution profiles of a mixture containing an extract of an adduct spot (Fig. 1D) and four authentic compounds, 3',5'-pdGp-C8-PhIP (I), 3'-dGp-C8-PhIP (II), 5'-pdG-C8-PhIP (III) and dG-C8-PhIP (IV). HPLC conditions were the same as those used for Fig. 2.
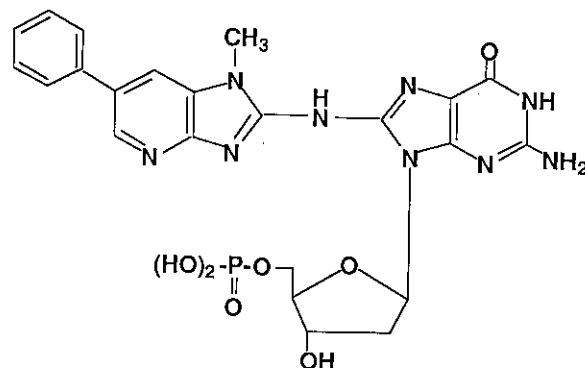

Fig. 4. Structure of 5'-pdG-C8-PhIP.

The above results indicate that all the adduct spots except that of 3',5'-pdGp-C8-PhIP detected under the standard and adduct-intensification conditions were undigested di- or oligo-nucleotides containing dG-C8-PhIP.

To confirm this, we examined an additional digest of spots b–d (Fig. 1B) detected under adduct-intensification conditions. Each spot was cut out and extracted with 4 M pyridinium formate (pH 4.5) as mentioned above, the extract was evaporated and the residue was dissolved in 15 μl of 30 mM Tris-maleate buffer (pH 6.5). This solution was incubated with 4 μg of nuclease P1 and 1 μl of 1 mM ZnCl$_2$ at 37°C for 1 h. The digest was then mixed with 3 μl of Trizma base to adjust the pH to about 9 and incubated at 37°C for 30 min with 10.5 mU of PDE I and 1 μl of 0.5 M MgCl$_2$. Each reaction mixture was then applied to a PEI-cellulose sheet and developed under the same conditions as used in the standard and adduct-intensification methods. These treatments resulted in almost complete disappearance of spots b–d with the appearance of a single spot coinciding in position with that of 5′-pdG-C8-PhIP. Furthermore, the compound in this new spot was confirmed to be 5′-pdG-C8-PhIP by HPLC analysis.

In the present study, using a modified ^{32}P-postlabeling method with nuclease P1 and PDE I we identified dG-C8-PhIP as a major adduct in lung DNA of rats treated orally with PhIP. A single adduct spot corresponding to 5′-pdG-C8-PhIP was also detected in DNA samples from the colon, which is a target organ of PhIP carcinogenesis in male F344 rats. The level of adduct in the colon was around two-thirds of that in the lung (3.8 adducts/10^7 nucleotides), in animals given 400 ppm PhIP in the diet for 4 weeks. Recently, Pfau et al. reported that additional nuclease P1 hydrolysis following the labeling reaction detected PhIP-DNA adduct as one major spot.[18] However, in our study, additional treatment with nuclease P1 alone was not sufficient to digest adducted di- or oligonucleotides. Other HCAs, 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) and 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx) produced several adduct spots *in vivo* when analyzed by the ^{32}P-postlabeling method under standard and adduct-intensification conditions, and the most abundant adducts in both cases were identified as C8-guanine adducts.[19-21] The structures of the remaining adducts have not yet been clarified, and some of them may be undigested dinucleotides or oligonucleotides including C8-guanine adducts, as was the case for the PhIP-DNA adduct. This modification method should be useful in resolving such problems in analyzing DNA adducts with IQ and MeIQx.

Three of seventeen mammary gland caricnomas that developed in F344 rats fed PhIP were found to have point mutations of G-to-A transition in codon 12 of Ha-*ras*.[22] Moreover, a p53 gene mutation due to G-to-T transversion in codon 130 was also detected in one of ten cases.[22] These observations suggest that modification of a guanine base is involved in mammary gland carcinogenesis induced by PhIP. This is in accord with the finding that PhIP primarily binds to position C-8 of guanine *in vivo*. Since humans are exposed to PhIP in everyday life, it is very likely that dG-C8-PhIP is formed in DNA of human organs. The new modification of the ^{32}P-postlabeling method is more sensitive than the original method and can detect PhIP-DNA adducts at levels of 5 per 10^{10} nucleotides under intensification conditions. Thus, this method in combination with HPLC should be useful for detection of PhIP-DNA adducts in human organs.

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