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DNA-benzoquinone adducts analyzed by nuclease P1 mediated ^{32}P-postlabeling method

Jiang Xiangning, Liu Sufen and Xu Xiaobai

Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Abstract_ With the super high sensitivity, high reproducibility and accuracy, nuclease P1 mediated ^{32}P-postlabeling version has been successfully used to analyze the DNA-benzoquinone (DNA-BQ) adducts formed from in vitro cultured cells, reaction of benzoquinone with calf thymus DNA and nucleoside monophosphates. It has been proven that the major radioactive spot, contributing more than 70% of the total radioactivity of DNA-BQ adducts detected, is from deoxycytidine (dC) modified by benzoquinone while a minor one from deoxyguanosine (dG). The method is capable of detecting 1 adduct in 10^9 to 10^{10} DNA bases.

Keywords: P1 mediated ^{32}P-postlabeling; DNA-BQ adducts; dC-BQ adduct; dG-BQ adduct.

INTRODUCTION

Any chemical capable of forming covalent bonds with DNA is a potential mutagen, carcinogen or teratogen. These chemicals may exist in environment, because they are of either natural origins or man-made sources. Benzene is one such man-made chemical widely used as the major raw material in the production of pesticides, dyes, man-made resins and plastic products. Thus it is wide-spread in air, water, soil and so on. Consequently, exposure to benzene and its derivatives such as benzoquinone, phenols can not be completely eliminated from the environments. Chronic exposure of rat and mice to high dosage of benzene has been shown to induce tumors in Zymbal glands, oral, liver and nasal cavities and possibly in the mammary gland (Maltoni, 1985).

Mechanistic studies of the carcinogenesis of benzene have focused on the processes of transformation of benzene into its reactive metabolites and also damage to DNA possibly caused by these metabolites. It has been shown that benzene is subjected firstly to hydroxyling to phenols and catechol, and then to transform to hemiquinone and benzoquinone via hydroquinone. Hemiquinone and benzoquinone are toxic metabolites of benzene.

DNA adducts formed from the genotoxic chemicals are persistent in tissue and cells if they were not removed by automatic cell repairing and thus may become the main cause of carcinogenesis, mutagenesis or teratogenesis. Therefore, it is imperative to study DNA adducts

and its formation, and this is helpful for us, to some extend, to elucidate the mechanism and process of chemical carcinogenesis, mutagenesis and teratogenesis.

This article introduced a newly developed method (nuclease P1 enhanced sensitivity ^{32}P-postlabeling) used in the analysis of DNA adducts formed in vitro cultured cells and reactions. Reproducibility and accuracy of nuclease P1 mediated ^{32}P-postlabeling in benzoquinone adducts were investigated.

MATERIALS AND METHODS

Materials

Calf thymus DNA from Sigma (USA) was repurified with RNase and proteinase K and re-precipitated by ice-cooled alcohol before use.

Micrococcal endonuclease (EC. 3.1.31.1) and spleen exonuclease (EC. 3. 1. 16. 1) were from Washington Biochemical (USA) and used without any purification.

Nuclease P1 (EC. 3. 1. 30. 1) and T4 polynucleotide kinase (EC. 2. 7. 1. 78) were from Boehringer Mannheim (WG) and Bethesda Research Laboratories, respectively.

Apyrase (EC. 3. 6. 1. 5) from potato and 3'-deoxy-nuclosides were the products of Sigma.

High specific (>4000 Ci/mmol) carrier-free [γ-^{32}P] ATP was purchased from the China Institute of Atomic Energy, Radioisotope Division (Beijing).

Benzoquinone (AR) was from the Shanghai 3rd Chemicals Factory.

ODS C_{18} reversed phase thin layer chromatography (TLC) plate was the product of Whatman (USA). Polyethyeneimine (PEI) ion exchange cellulose sheets were from Machery-Negal (WG).

Additional chemicals and solvents were re-purified and redistilled if required. Buffers and solutions were prepared with deionized redistilled water.

Methods

Experimental procedures before the radioactive labeling were performed in the biochemical laboratories, labeling procedures with sequential steps were carried out in the radioisotope laboratory in The Beijing Forestry University.

1. Formation of DNA adducts

(1) Formation of DNA adducts in vitro reaction

Calf thymus DNA (3mmol/L, 0.01 mol/L Tris, pH 7.5) re-purified with RNase and Proteinase K reacted in vitro with 9mmol/L benzoquinone at 37 °C for 12 hours. After salification benzoquinone adducted DNA was precipitated by ice-cooled alcohol (95−100%) and wrapped out with a glass rod. Then adducted DNA was washed twice with 70% alcohol and redissolved in 0.1 SSC buffer (15 mmol/L NaCl, 1.5 mmol/L sodium citrate, pH 7.6). Concentration of DNA solution was controlled in the range of 1.5−2 μg/μl. Accurate concentration of DNA was estimated with ultraviolet spectrophotometer (Shimadzu UV-120) at the wavelength of 260 nm and calculated according to the following formula (1.0 O. D. at 260 nm = 50 μg

DNA/ml, light path: 1.0 cm). The above procedures were used also in the formation and determination of benzoquinone adducted mononucleoside phosphates (dN-BQ).

(2) Formation of DNA-BQ adducts in cultured rabbit peripheral blood

20 ml of heparinized peripheral blood were sterilized and incubated with 10 ml o benzoquinone (4mg/ml) at 37 °C for 24 hours to form the DNA-BQ adducts in the nuclei of blood cells. The blood was used for isolation of leukocytes and lymphocytes.

2. Isolation of lymphocytes

10 ml of cultured peripheral rabbit blood were diluted with PBS (0.15 mol/L NaCl, 7.5 mmol/L Na_2HPO_4, pH 7.2) and then carefully transferred into the centrifugation tube containing Ficoll-paque (Shanghai Biochemicals Factory) and layered onto the Ficoll-Paque. When layering the sample do not mix with the Ficoll-Paque and the diluted blood sample. After centrifugation at 400 g for 30 minutes at 18–20 ℃, the upper layer was drawn off using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface. Care should be taken not to disturb the lymphocyte layer. Using a clean Pasteur pipette transfer the lymphocyte layer to a clean centrifuge tube. It is critical to remove all the interface but in minimum volume. At least 3 volumes of balanced salt solution are added to the lymphocytes in the test-tube. After centrifugation at 60–100 g for 10 minutes at 18–20 ℃ the supernatant is discarded. After twice centrifugation the lymphocytes should be suspended in the medium appropriate to the application.

Cells were collected by centrifugation at the speed of 1200 r/min for 30 minutes and red blood cells were lysed by washing twice with 0.5 mol/L NH_4Cl, each time followed by centrifugation. Collected cells were washed twice with PBS.

3. Isolation and purification of DNA

Cells were re-suspended and treated twice with 0.5% Triton X-100 and the nuclei were collected by centrifugation at 1000 g for 5 minutes DNA was isolated from the nuclei, using the procedure described by Gupta (1984), and purified by treatment with RNase and Proteinase K followed by extraction with phenol and chloroform: isoamyl alcohol (24: 1, v/v). After salification DNA was precipitated from the aqueous phase with cold ethanol, then redissolved with 0.1 SSC buffer. Concentration was determined as described above.

4. Nuclease P1 mediated ^{32}P-postlabeling

The procedure of nuclease mediated P1 ^{32}P-postlabeling and essentially described by Reddy (1986) was outlined in Fig. 1.

(1) DNA digestion

DNA were first digested to 3'-deoxy-nucleoside monophosphates by micrococcal endonuclease and spleen exonuclease in digestion buffer consisting of 20 mmol/L sodium succinate, 8 mmol/L $CaCl_2$, pH 6.0, for 1 to 3 hours at 37 ℃. Digestions were then mediated further by adding nuclease P1 and corresponding buffer (pH 5.0) to destroy the normal 3'-deoxynucleoside monophosphates (3'-dNMP → Ni+Pi). Adducted deoxynucleoside monophosphates were remained undestroyed and enriched by means of nuclease P1 to enhance

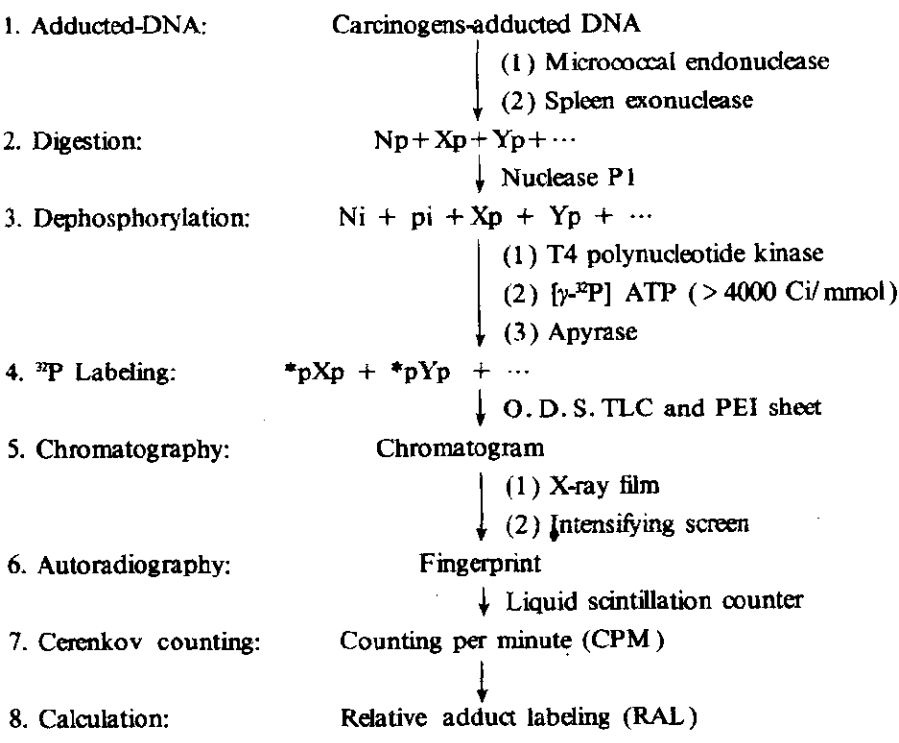

Fig. 1 Procedures of nuclease P1 mediated ^{32}P-postlabeling analysis of benzoquinone-DNA adducts

the detecting sensitivity.

(2) ^{32}P labeling

Digests were then treated with T4 polynucleotide kinase in a solution of 200 mmol/L Bicine (pH 9.6), 100 mmol/L $MgCl_2$, 100 mmol/L DDT, 10 mmol/L spermidine, containing 60 to 100 μ Ci [r-^{32}P] ATP at 37°C for 30 minutes subsequently 20 to 40 μg apyrase were added to the system to decompose excess ATP (ATP→AMP+2Pi).

(3) Purification and separation of adducts

Before sample application, the reversed-phase plate (20×20 cm) were prescored with a carbide wheel cutter and broken to obtain 10 × 10 cm plates, which were prepurified with 0.4 mol/L ammonium formate, pH 6.2, on to a filter paper wick attached to the top of the plate with paper clips, and then dried and developed for use. In adduct purification, the glass backing of the layer was scored according to Randerath *et al.* (1984). ^{32}P labeled digests were applied to each of the origins on the layer. After development, the plate was broken down along the prescored line (Fig. 2), then the top portion with the wick, containing the bulk radioactive

contaminants, was discarded. The bottom portion of the plated was dried uniformly in a current of cool air for 5–6 minutes, then warm air for 2 minutes, and broken down to the 1.2×1.2 cm^2 squares which contain the adducts along the scored lines (Fig. 2).

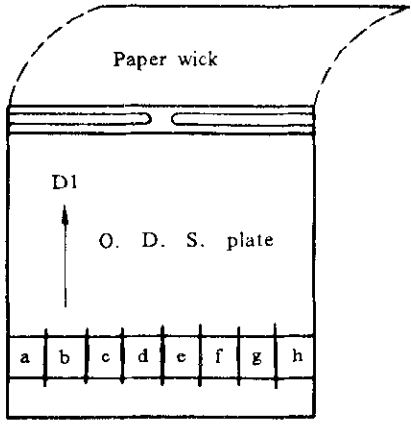

Fig. 2 Scheme of prescoring and development of ODS C_{18} reversed-phase thin-layer plate for benzoquinone-DNA adducts purification

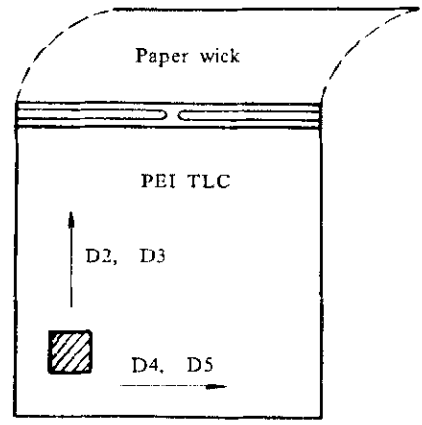

Fig. 3 Scheme of adduct transfer from ODS C_{18} to PEI-cellulose sheet

^{32}P-labeled digests were applied at each origins on a 10×10 cm plate with a wick attached to it, and contaminants were removed by chromatography in 0.4 mol/L ammonium formate, pH 6.2. The plate was dried and broken down along the prescored lines to isolate the minor squares containing origins for subsequent adduct transfer to PEI-cellulose thin-layer as described in the text.

To contact-transfer the adducts, the PEI TLC sheets were developed with clips in ascending fashion at 50°C in preheated tank with n-propanol/H_2O (1 : 1, v/v) containing 1% Nonidet P-40 to the top (ca. 45 minutes). After development, the detached wet sheets were taken out of the tank. The wet sheets were soaked twice with frequent vigorous agitation in distilled water for 7 minutes each, and then further developed before drying (Fig. 3).

To resolve the benzoquinone-DNA adducts, the TLC chromatograms were developed in the direction of the preceding development to the top in 0.36 mol/L lithium formate, 4.25 mol/L urea, pH 3.5. The sheets were then soaked twice in water for 7 minutes each and dried. The chromatogram was then developed in two dimensions first in 0.36 mol/L LiCl, 0.22 mol/L Tris HCl, 3.8 mol/L urea, pH 8.0, and then in 1.7 mol/L sodium phosphate, pH 6.0. After the wick had been removed, the chromatograms were washed in water to remove the radioactive contaminants and dried for autoradiography on the X-ray film. For calculation of radioactivity, radioactive nucleotide spots and adjacent blank areas were excised from maps and counted by

Cerenkov assay. Calculations were done according to:

$$\text{Relative adduct labeling (RAL)} = \frac{\text{CPM in adduct spot (s)}}{3240 \times \mu\text{gDNA} \times 3.75 \times 10^6}$$

These calculations were based on the assumption that the adducts investigated were completely resistant to 3'-dephosphorylation by nuclease P1 and completely labeled (assuming 1μg DNA = 3240 pmol dNp and specific activity of [γ-^{32}P] ATP was 3.75 $\times 10^6$ CPM/pmol dNp).

RESULTS

Analysis of DNA-BQ adducts

Four adducts can be obviously identified on the X-ray autoradiography film via ^{32}P-postlabeling. One of them was major while others were minor. This proved that benzoquinone can react with calf thymus DNA in vitro to form the covalent bond adducts. The radioactivity of the major one can account for 70% of total radioactivity. All auto-radiographic spots cannot be found either on the sheet of blank DNA as control or on the sheet of benzoquinone as a check. This has further proven that all these spots identified on the autoradio-graphied X-ray films are from DNA adducts ensuing radioactive labeling.

Relative radioactive density of each spot, relative adduct labeling and base modified rate are shown in Table 1.

Table 1 Data analysis of DMA-BQ adducts*

Spots	CPM	RRD, %	RAL	BMR, adduct (s)/10^{10}Ns
1	387.4	8.44	7.97×10^{-9}	79.65
2	434.3	9.46	8.93×10^{-9}	89.29
3	506.3	11,03	10.42×10^{-9}	104.20
4	3252.9	71.07	67.09×10^{-9}	670.90

*CPM: counting per minute, average of 7 replicated experiments subtracting the background;
RRD: relative radioactive density; RAL: relative adduct labeling;
BMR: base modification rate, adduct (s) / 10^{-10} bases

Reproducibility and accuracy of nuclease P1 mediated ^{32}P-postlabeling method

Results of seven-replicated experiments showed that the nuclease P1 mediated ^{32}P-postlabeling used in the analysis of DNA-BQ adduct is well reproducible and highly accurate. Based on the data from 7 replications the accuracy is statistically 70% at the obvious variance

level (t_α=0.2), 50% or higher at the significant level (t_α=0.1) and most significant level (t_α=0.01), respectively. The accuracy of the major adduct spot is enhanced by exclusion of two terminal data, while accuracies of other spots were either enhanced or reduced to some extent (Table 2).

Table 2 Accuracy of ^{32}P-postlabeling

Spot	CPM	t_α=0.2		t_α=0.1		t_α=0.05		Remarks
		±CPM	Accuracy, %	±CPM	Accuracy, %	±CPM	Accuracy, %	
1	387.4	116.7	69.88	157.4	59.40	198.3	48.82	without
2	434.3	107.2	75.31	144.7	66.69	182.2	58.05	exclusion
3	506.6	150.2	70.35	202.7	59.99	255.3	49.61	of data.
4	3252.9	868.0	73.40	1171.3	64.11	1475.1	54.79	
1	368.6	145.5	60.53	202.3	45.11	263.4	28.53	with exclusion
2	473.6	151.7	67.93	211.0	55.41	274.8	41.94	of two terminal
3	490.4	116.9	76.17	162.6	66.85	211.7	56.84	data.
4	3342.2	630.8	81.13	877.2	73.75	1142.3	65.83	

Base determination of DNA-BQ adducts

In comparison of DNA-BQ adducts with the nucleoside monophosphates-benzoquinone adducts on TLC, the major adduct can be recognized as deoxycytidine modified by benzoquinone and confirmed by co-chromatography, while a minor one is from deoxyguanosine. On the autoradiographied X-ray film of the co-chromatograms, the major spot from the components of both DNA-BQ and dC-BQ samples located at the equivalent positions (R_{fx}. and R_{fy}. values were 0.29±0.01 and 0.29±0.01, respectively). Fractions collected from HPLC corresponding to dC-BQ standard were labeled and applied onto the TLC and then developed under the same conditions of DNA-BQ adduct.

DNA-BQ adducts formed in cultured blood cells

DNA-BQ adducts can also be detected on the chromatograms of DNA samples isolated from nuclei of lymphocytes in vitro BQ-incubated rabbit peripheral blood. The results are shown in Table 3. However, discrepancies between BQ-adducted DNA sample and DNA from BQ-incubated blood cells were present in the relative radioactive densities of 4 detectable spots and their RAL values. These differences may result from biological factors inner cells of in vitro cultured rabbit peripheral blood.

Table 3 DNA-BQ adducts in lymphocytes

Spots	CPM	RRD, %	RAL	BMR, adduct (s)/10^{6}Ns
1	113.7	29.30	9.36×10^{-9}	93.6
2	84.0	21.64	6.91×10^{-9}	69.1
3	84.7	21.82	6.97×10^{-9}	69.7
4	105.7	27.24	8.23×10^{-9}	82.3

DISCUSSION

A new super high sensitive and highly reproducible ^{32}P-postlabeling version has been used to detect DNA-BQ adducts formed both in vitro reaction and cultured cells. Both the sensitivity and reproducibility of the new version have been documented in this paper.

The sensitivity of the previous version is capable of only detecting 1 adduct 10^{7} nucleotides, while the new version is capable of detecting 1 adduct in 10^{10} nucleotides. The new version is 3 orders of magnitude higher than the previous version. A specific enzyme, nuclease, nuclease P1 which has both 3',5'-phosphodiesterase activity and 3'-monophosphatase activity, is introduced to destroy the normal nucleotides in the new version, since the bulk of normal nucleotides will compete the [γ-^{32}P]ATP with adducted nucleotides in the labeling reaction and thus reduce the rate of labeling of adducted nucleotides. Bulk of nucleotides in the system makes it difficult to purify and separate the adducted nucleotides from normals. This is why the previous version has the lower sensitivity.

Jowa *et al.* (1986) reported one of two DNA-benzoquinone adducts was from deoxyguanosine and characterized it as (3'-hydroxy)-benzetheno-(1, N-2)-deoxyguanosine adduct by means of NMR, MS and ultraviolet and fluorescence spectra. Reddy *et al.* (1989) documented that in vitro reaction of DNA and benzoquinone may yield several adducts observed by ^{32}P-postlabeling method. But they referred to the major spot as the spot of Jowa's characterization (1986), without the further confirmation experiments. After conducting series of experiments repeatedly including HPLC separation of dC-BQ adduct and PEI cochromatography of DNA-BQ and dN-BQ components, we have discovered and confirmed that the major adduct was formed from the deoxycytidine modified by benzoquinone, and not from deoxyguanosine. One of the minor spots was the adduct from dC-BQ as described by Jowa *et al.* (1986). None of them formed from thymidine and one may form from deoxyadenosine. The structural characteristics of identified adducts are under way to be recognized.

In our study we use cell culture system, to investigate the DNA-BQ adduct formations under biological conditions. We find some discrepancies of DNA-BQ adducts formation in the leukocytes of cultured rabbit peripheral blood and in vitro reactions of DNA and benzoquinone. There were four adducts identified in both, but their relative densities were different. The major

one in vitro reactions counted 70% radioactivity of total radioactivities, while in cultured blood only a quarter. The possible explanation of these differences may relate to the biological factors such as entirely doubled helix structure of DNA and histone coat of DNA in cells.

The results suggest that benzene and its derivatives might become genotoxic substances in human being's environments and this may help us to understand the mechanism of chemical carcinogenicity at the molecular level.

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REFERENCES

Huff, J. E., Toxicology and carcinogenesis studies of benzene in F344/N rats and B6C3F, mice (gavage studies). Technical Report No. 289. National Toxicology Program, Research Triangle Park, NC., 1986

Jowa, L., Winkle, S., Kalf, G. F., Witz, G. and Snyder, R., Deoxyguanosine adducts from benzoquinone and hydroquinone (Eds. by Kocsis, J., Jollow, D. J., Witmer, C. M., and Nelson, J. O), Advances in experimental medicine and biology., Biological intermediates III. New York: Plenum Press, 1986: 825

Liu, S-F., Rappaport, S. M., Rasmussen, J. and Bodell. W. J., Carcinogenesis, 1988, 9(8): 1401

Maltoni, C., Conti, B., Cotti, G. and Belpoggi, F., Am. J. Ind. Med., 1985. 7: 415

Mazzulo, M., Environ. Health Perspect., 1986, 82: 259

Reddy, M. V., Blackburn, G. R., Irwin, S. E., Kommineni, C., Mackerer, C. R. and Mehlman, M. A., Environ. Health Perspect., 1989, 82: 239

Reddy, M. V. and Randerath, K., Carcinogenesis, 1986, 7: 1543

Reddy, M. V., Blackburn, G. R., Schreiner, C. A., Mehlman, M. A. and Mackerer, C. R., Environ. Health Perspect., 1989, 82: 253

Reddy, M. V., Gupta, R. C. and Randerath, K., Anal. Biochem., 1981, 117: 271

Reddy, M. V. and Randerath, K., Carcinogenesis, 1986, 7(9): 1543

Ranserath, K., Reddy, M. V. and Gupta, R. C., Proc. Natl. Acad. Sci. USA, 1981, 78: 6126

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