

Highly sensitive chemiluminescence immunoassay for benzo[*a*]pyrene-DNA adducts: validation by comparison with other methods, and use in human biomonitoring

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A chemiluminescence immunoassay (CIA) utilizing anti-serum elicited against DNA modified with (\pm)-7 β , 8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) has been developed and validated to study the formation of polycyclic aromatic hydrocarbon (PAH)–DNA adducts in human tissues. Advantages include a low limit of detection for 10b-(deoxyguanosin-*N*²-yl)-7 β ,8 α ,9 α -trihydroxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPdG, ~1.5 adducts/10⁹ nucleotides using 20 μ g DNA) and a high signal-to-noise ratio (\geq 100). The CIA BPDE–DNA standard curve gave 50% inhibition at 0.60 ± 0.08 fmol BPdG (mean \pm SE, $n = 30$), which was a 10-fold increase in sensitivity compared with the dissociation-enhanced lanthanide fluoroimmunoassay (DELFI). Calf thymus DNA modified with [1,3-³H]BPDE was assayed by radiolabeling, ³²P-postlabeling, DELFIA and CIA, and all assays gave similar values. Liver DNAs from mice exposed to 0.5 and 1.0 mg [7,8-³H]benzo[*a*]pyrene (BP) were assayed by the same four assays and a dose–response was obtained with all assays. The BPDE–DNA CIA was further validated in MCL-5 cells exposed to 4 μ M BP for 24 h, where nuclear and mitochondrial DNA adduct levels were associated with an increase in DNA tail length measured by the Comet assay. Human peripheral blood cell (buffy coat) DNA samples ($n = 43$) obtained from 25 individuals who were either colorectal adenocarcinoma patients or controls were assayed by BPDE–DNA CIA. Three samples (7%) were non-detectable, and the remaining 40 samples had values between 0.71 and 2.21 PAH–DNA adducts/10⁸ nucleotides. The intra-assay coefficient of variation (CV), for four wells on the same microtiter plate, was 1.85%. Sufficient DNA for two assays, on separate plates, was available for 38

the 43 samples, and the PAH–DNA adduct values obtained were highly correlated ($r^2 = 0.95$). Coded duplicate DNA samples from 15 individuals were assayed four times gave an inter-assay CV of 13.8%.

Introduction

Benzo[*a*]pyrene (BP) is a human carcinogen and a major carcinogenic component of multiple human exposures involving polycyclic aromatic hydrocarbons (PAHs) (1,2). The PAHs escape into the ambient atmosphere as a result of partial combustion, sources of which include cigarette smoking, vehicle exhaust, indoor heating, and industrial processes (1,2). In addition to inhalation, exposure to PAHs can occur by consumption of charbroiled food (3,4) and, in some cases, even uncooked food (5). Attempts to monitor human genetic damage resulting from such exposures have met with increasing levels of success during the past ~20 years (6,7), but the risk of human cancer conferred by PAH–DNA adduct formation is still a subject of much interest and investigation (8–12). The ultimate success of such endeavors will depend, to a significant extent, upon the sensitivity and validity of the methodologies applied.

The classic methods for PAH–DNA adduct determination have included radiolabeling, ³²P-postlabeling, and immunoassays utilizing antisera elicited against DNA samples modified with PAHs (7,13). Each of these approaches has advantages and disadvantages, but only occasionally have they been compared in the same study for DNA samples modified *in vitro* and *in vivo* (14,15). All of the aforementioned assays, with the exception of radiolabeling, are commonly used with human tissues, but the resulting data have been plagued with variability and difficulties in achieving specificity. The current work describes development and validation of an immunoassay for PAH–DNA adducts that is sufficiently sensitive to obtain measurable values for most human samples, and also has minimal variability and a good signal-to-noise ratio compared with similar immunoassays (16,17).

The highly sensitive BPDE–DNA chemiluminescence immunoassay (CIA) detects BPDE–DNA adducts in experimental samples exposed to BP, and PAH–DNA adducts in human samples, where typical exposures include multiple hydrocarbons. In this study the assay has been validated using a standard calf thymus DNA modified *in vitro* with [³H]-BPDE, by comparing the CIA data with results obtained using several other methods for BPdG adduct determination. In addition, we have examined DNA adducts in livers of BP-exposed mice, in BP-exposed cultured MCL-5 cells, and in human peripheral blood DNA samples. For the human samples, we have expressed the BPDE–DNA CIA measurements as PAH–DNA adducts, as the antiserum is specific for DNA samples modified with several carcinogenic PAHs (18).

Abbreviations: BP, benzo[*a*]pyrene; BPDE, (\pm)-7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPDE–DNA, DNA modified with BPDE having a single major adduct, BPdG; BPdG, 10b-(deoxyguanosin-*N*²-yl)-7 β ,8 α ,9 α -trihydroxy 7,8,9,10-tetrahydrobenzo[*a*]pyrene; CIA, chemiluminescence immunoassay; CV, coefficient of variation; DELFIA, dissociation-enhanced lanthanide immunoassay; MCL-5, multi competent human lymphoblastoid cell line expressing the cytochrome P450s 1A1, 1A2, 2A6, 3A4, and 2E1, and epoxide hydrolase; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate buffered saline; PBST, phosphate buffered saline with 0.05% Tween 20.

Materials and methods

Culture of MCL-5 cells and isolation of nuclear and mitochondrial DNA

MCL-5 cells (19) were purchased from Gentest Corporation (Woburn, MA) and maintained as suspension cultures in RPMI 1640 medium, without histidine but containing 2 mM 1-histidinol, supplemented with 9% horse serum, 30 µg/ml 5-aminolevulinic acid, and 200 µg/ml hygromycin B. Cells were passaged every 2–3 days to maintain a concentration of 2.5×10^5 cells/ml. Cells were exposed to 0 or 4.0 µM BP (in 2% dimethylsulfoxide) for 24 h.

Cells were suspended in three packed cell volumes of PBS containing 2 mM mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride, and centrifuged at 1000 g for 5 min at 22°C. Cell pellets were homogenized in two packed cell volumes of the above buffer using a tight-fitting Dounce homogenizer (12 strokes). Mitochondria were stabilized by adding two packed cell volumes of buffer containing 210 mM mannitol, 70 mM sucrose, 20 mM HEPES, pH 8.0, 2 mM EDTA, 2 µg/ml leupeptin, and 2 mM dithiothreitol. The nuclei were pelleted by centrifugation at 1600 g for 8 min at 22°C and the supernatant was subsequently centrifuged at 20 000 g at 4°C for 15 min to collect the mitochondrial pellet. DNA was isolated separately from nuclear and mitochondrial pellets using QIAamp DNA Mini Kits (Qiagen, USA), as per the manufacturer's protocol. The DNA concentration was determined by UV spectrometry.

In vitro modification of calf thymus standard DNA with [1,3-³H]BPDE

Calf thymus DNA (472 mg) in 400 ml 5 mM Bis-Tris, 0.1 mM EDTA, pH 7.1 was treated for 2 h with 4.2 µg [1,3-³H]BPDE (1744 mCi/mmol) that had been dissolved in 5.9 ml dimethylsulfoxide. The mixture was extracted three times with *n*-butanol and three times with isoamyl alcohol. These solvents had previously been saturated with 50 mM Bis-Tris, 1 mM EDTA, pH 7.1. The DNA was precipitated by the addition of ethanol and NaCl, washed with 70% ethanol, and redissolved in 5 mM Bis-Tris, 1 mM EDTA, pH 7.1 (15). The DNA concentration was determined by UV spectrometry and the extent of radiolabeled BPdG modification was established by liquid scintillation counting in sextuplicate.

In vivo modification of mouse liver DNA with [7,8-³H]BP

Eight-week-old male B6C3F1 mice (four/group) were injected intraperitoneally with 0, 0.5, or 1.0 mg [7,8-³H]benzo[*a*]pyrene (125 mCi/mmol) in 100 µl triolein. After 24 h, the mice were euthanized, livers were pooled by group, nuclei were prepared (20), and DNA was isolated by enzymatic digestion, extraction with organic solvents and precipitation (21). The DNA concentration was determined by UV spectrometry and the extent of radiolabeled BP-DNA modification was established by scintillation counting in triplicate.

HPLC analysis of DNA modified in vitro with [1,3-³H]BPDE

DNA reacted *in vitro* with [1,3-³H]BPDE was hydrolyzed to nucleosides by treatment with DNase I, followed by alkaline phosphatase and snake venom phosphodiesterase as described (22). The nucleoside adducts were extracted three times with water-saturated *n*-butanol. The *n*-butanol extracts were combined, back-extracted once with *n*-butanol-saturated water, evaporated, and the residue was redissolved in methanol for HPLC analysis. The *n*-butanol extracts contained 98% of the radioactivity associated with the DNA. Non-radioactive BPdG was added to the samples to serve as a UV marker and the samples were analyzed by reversed-phase HPLC using a Zorbax ODS C₁₈ column (9.4 × 250 mm) with an HPLC system consisting of two Waters Model 510 pumps, a Waters U6K injector, and a Waters Model 680 automated gradient controller. The peaks were monitored at 254 nm with a Hewlett-Packard 1040M diode array spectrophotometric detector. Samples were eluted with a 50 min linear gradient of 20–80% methanol, with a flow rate of 3 mL/min. Fractions were collected at 1 min intervals for measurement of radioactivity.

³²P-Postlabeling analyses of DNA adducts

The ³²P-postlabeling protocol used for the analysis of DNA adducts employed nuclease P1 enhancement as described by Phillips and Castegnarò (23), and was based on the approaches reported previously (24–26). Adducts were resolved on 10 × 10 cm polyethylenimine-cellulose thin layer chromatography plates by multi-directional chromatography and visualized by PhosphorImager. The quantitation has been reported (23).

Comet assay

The Comet assay, a single cell electrophoresis, was performed using the Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD). Briefly, cell suspensions were harvested by centrifugation and resuspended at 1×10^5 cells/ml in Ca²⁺ and Mg²⁺ free phosphate buffered saline (PBS). The cell suspension was mixed with low melting agarose at 42°C, transferred to chilled microscopic slides and incubated at 4°C for 10 min. The slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl

sarcosinate and 0.01% Triton X-100) and placed in the dark at 4°C for 1 h. The slides were then transferred to alkali buffer (0.3 M NaOH, 1 mM EDTA) and incubated at room temperature for 1 h. The slides were washed twice with 0.09 M Tris-borate, 2 mM EDTA, pH 8.3, subjected to horizontal electrophoresis using 0.09 M Tris-borate, 2 mM EDTA, pH 8.3 at 1 V/cm for 15 min, and then stained with SYBR Green. The cells were visualized by fluorescence microscopy and digitized using Image Pro Plus 3.0. Nuclear diameter, and tail length from the center of the nucleus, were expressed as µm, after measurements were taken by NIH image analysis software (<http://rsb.info.nih.gov/ni-image/index.html>) using the macros of Herbert M.Geller (<http://www2.umdj.edu/%7Egeller/lab/comet.htm>).

BPDE-DNA adducts by DELFIA

DNA samples were subjected to sonication and denaturation before conducting the DELFIA and the CIA. Specifically, aliquots of sample DNA in PBS were sonicated for 15 s using a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Plainview, NY), heat-denatured at 90°C for 2 min, and cooled immediately on ice.

The BPDE-DNA DELFIA was carried out as previously described (16). Essentially, 0.1 ng of DNA, modified to 35.9 pmol BPdG/µg DNA (1.2% modification), was used to coat microtiter plates. Non-specific binding was blocked with 1% fetal calf serum in PBS containing 0.05% Tween-20 (PBST). All washes were carried out with an automated plate washer (Ultra Wash Plus; Dynatech Laboratories, Gaithersburg, MD) using PBST. Diluted ($1 : 1 \times 10^6$) rabbit BPDE-DNA antiserum was mixed with equal volumes of either the standard BPDE-DNA (modified to 0.8 BPdG/10⁶ nucleotides (16)) or the sample DNA, and incubated on the plate. The DNA content in the standard curve wells was maintained equivalent to the amount of DNA used in the sample wells by adding appropriate amounts of sonicated and denatured carrier calf thymus DNA to normalize the matrix effect.

After washing, plates were sequentially incubated with biotinylated rabbit anti-IgG (1 : 2500) and europium-labeled streptavidin (1 : 2000), and the final fluorescent signal was generated by the addition of Enhancement solution (Wallac, Gaithersburg, MD) before reading on the Wallac 1234 Research Fluorometer. The standard curve 50% inhibition (mean ± SD) was 6.3 ± 0.24 fmol BPdG per well ($n = 25$) and the lower limit of detection, using 20 µg of DNA per well, was ~ 1.3 adducts/10⁸ nucleotides (16).

BPDE-DNA determination by CIA

Microtiter plates for the CIA were 96-well high-binding LIA plates (Greiner Labortechnik, FRG). The CIA-specific reagents, including biotinylated anti-rabbit IgG, streptavidin-alkaline phosphatase, I block (casein), and CDP-Star with Emerald II, were obtained from Tropix (Bedford, MA).

DNA adducts were measured by BPDE-DNA CIA using an approach similar to that described previously for tamoxifen-DNA adducts (27). Briefly, 40 µg of sonicated BPDE-DNA (modified to 35.9 pmol BPdG/µg DNA, 1.2% modification (16)) or calf thymus DNA were coated on microtiter plates in 0.1 ml of Reactibind (Pierce) solution at room temperature for 48 h. Plates were washed with three cycles of PBST containing 0.05% of NaN₃ (see the BPDE-DNA DELFIA, above), and stored frozen until use. For assay, non-specific binding was blocked by incubating the wells with 310 µl casein (0.25%) in PBST for 1 h at 37°C. Equal volumes (50 µl) of anti-BPDE-DNA antibody (diluted to $1 : 3 \times 10^6$ in 0.25% casein) and serially diluted BPDE-DNA standard (0.03–27 fmol of BPdG in DNA modified to 1.0 BPdG per 10⁶ nucleotides (16)) or test sample, in PBS, were mixed and incubated at 37°C for 15 min prior to adding to the microtiter plate wells. The DNA content in the standard curve wells was maintained equivalent to the amount of DNA used in the sample wells by adding appropriate amounts of sonicated and denatured carrier calf thymus DNA to normalize the matrix effect. The final mixture of DNA and antibody (final dilution $1 : 6 \times 10^6$ in 0.25% casein) was incubated for 90 min on the microtiter plate. After washing three times with PBST, biotinylated anti-rabbit IgG (100 µl of $1 : 5000$ dilution in 0.25% casein in PBST) was transferred to wells and incubated for an additional 90 min at room temperature. After washing again, streptavidin-alkaline phosphatase (100 µl of $1 : 6000$ in 0.25% casein in PBST) was added and incubated at room temperature for 60 min, followed by washing with three cycles of PBST and two cycles of Tris buffer (20 mM Tris-1 mM MgCl₂, pH 9.5). Finally, CDP-Star containing Emerald II enhancer (100 µl) was transferred to wells, plates were incubated at room temperature for 30 min and at 4°C for 15 h, and luminescence was read at both times using a TR717 Microplate Luminometer (PE Applied Biosystems, Foster City, CA) at 542 nm. The standard curve 50% inhibition (mean ± SE) was 0.60 ± 0.08 fmol BPdG per well ($n = 30$) and the lower limit of detection, using 20 µg of DNA per well, was ~ 1.5 adducts/10⁹ nucleotides.

Human lymphocyte samples, source and DNA preparation

A series of 43 peripheral blood lymphocyte DNA samples, from 25 individuals who were either colorectal adenocarcinoma patients or controls, was obtained

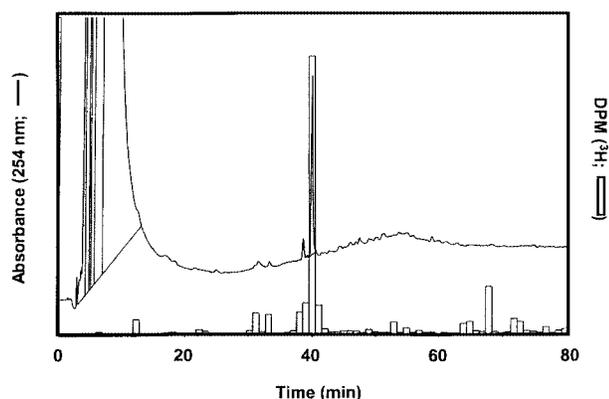


Fig. 1. HPLC analysis of the enzymatic hydrolysate from calf thymus DNA reacted with [³H]BPDE. Profile was obtained from 2 mg of DNA reacted at a molar ratio of 9.43×10^{-6} : 1 of BPDE:DNA nucleotides. The peak at 40 min shows co-chromatography of radiolabeling and UV absorbance.

from the Navy Colon Adenoma study. This case-control study of colorectal adenomas in active and retired military officers and their families was designed to evaluate meat cooking practices and tumor susceptibility. The study was approved by the institutional review boards of both the National Cancer Institute and the National Naval Medical Center and has been described in detail elsewhere (28). These 43 samples comprised a small subset of a much larger study, and were assayed for the purpose of obtaining quality control parameters. The sample set consisted of 36 samples (labeled 1–36) from 18 patients that were replicates or coded duplicates, two from each of the 18 patients. In addition, there were seven single samples from each of seven patients. The total number of samples was therefore 43. The samples assayed remain coded with respect to the original purpose of the study, so the numbers of cases and controls are currently unknown. Our intention here was to explore the usefulness of the BPDE-DNA CIA for human biomonitoring and to address specific quality control issues including sensitivity, background level, assay reproducibility, and fraction of samples measurable by the assay.

Blood was obtained, allowed to clot at room temperature and centrifuged to obtain serum (top), buffy coat (nucleated cells – middle), and hemoglobin (bottom). Buffy coat samples were retrieved and stored frozen at -80°C until DNA isolation was performed. DNA was prepared as described by Daly *et al.* (29). Briefly, nuclei were collected by centrifugation of buffy coat samples lysed in sucrose buffer. The nuclear pellet was resuspended in neutral Tris-HCl buffer, incubated at 65°C for 30 min, and extracted with 2 ml chloroform. Following centrifugation, the aqueous DNA-containing upper phase was transferred to a fresh tube, and the DNA was precipitated in ethanol and spooled. The spooled DNA was washed in 70% ethanol and allowed to dry at room temperature for 20 min. In the absence of visible spooled material, DNA was recovered by centrifugation. DNA was resuspended in 50 to 400 μl 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 by incubation at 60°C for 8–16 h. DNA was stored, following aliquoting, at -80°C . DNA purity was checked by electrophoresis on ethidium bromide-containing agarose minigels using Tris-Borate-EDTA buffer (30). Molecular weight markers (either 100 bp ladder from Gibco-BRL or lambda *Hind*III digest) were also included on each gel. Bands were visualized on a UV transilluminator, and gels photographed using a Kodak DC40 digital camera.

Results

In vitro modified calf thymus [1,3-³H]BPDE-DNA

A large quantity of calf thymus DNA was modified with [1,3-³H]BPDE (23) with the intention of preparing a standard that could be utilized by many laboratories for assay validation. Digestion of the DNA and subsequent analysis by HPLC demonstrated a single major peak of radioactivity at 40 min that co-chromatographed with an authentic BPdG adduct standard (Figure 1). The modification level determined by radiolabeling (Table I) was 110.7 ± 2.1 adducts/ 10^8 nucleotides (mean \pm SD, six measurements). When the same DNA was measured by ³²P-postlabeling (Figure 2A), BPDE-DNA DELFIA, and BPDE-DNA CIA, the values obtained (Table I) were very similar to the radiolabeling value.

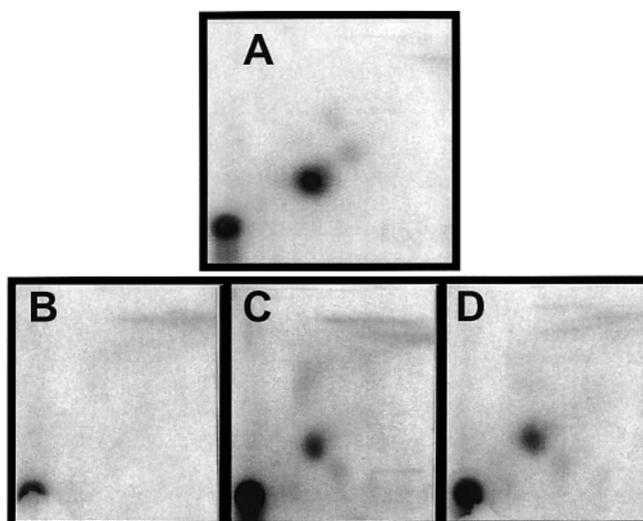


Fig. 2. ³²P-Postlabeling analysis of: (A) calf thymus DNA modified *in vitro* with [³H]BPDE as described in Figure 1; (B, C, D) liver DNA from mice treated with 0.0, 0.5 or 1.0 mg of [7,8-³H]BP, respectively. The adducts were visualized with a phosphorimager using attenuation factors of 500, 250 and 400, respectively.

In vivo modification of mouse liver DNA with [7, 8-³H]BP

A dose-response for binding of BP to mouse liver DNA was obtained with the intraperitoneal injection of 0.5 and 1.0 mg [7,8-³H]BP into mice (Table II); radiolabeling values were 86.4 and 137.6 BP molecules/ 10^8 nucleotides, respectively (23). These numbers reflect the total quantity of radiolabeled BP bound to mouse liver DNA. Both the BPDE-DNA DELFIA and BPDE-DNA CIA determinations showed dose-responses, however, both underestimated the total amount of BP bound to DNA. The DELFIA and CIA detected 5–6% and 12–13% of the total signal, respectively, suggesting that most of the DNA-bound BP radioactivity was not in a form structurally similar to BPdG, or at least recognizable by the BPDE-DNA antiserum. The ³²P-postlabeling assay (23) detected 13–16% of the total hepatic DNA-bound radioactivity in the form of the BPdG adduct, which was visualized by thin layer chromatography (Figure 2B, C, D, by comparison with A). Overall, the values obtained by the immunoassays and the ³²P-postlabeling method were very comparable, and data from all of these assays indicate that much of the DNA-bound radioactivity was in a form structurally different from the BPdG adduct.

Validation using the MCL-5 cells and Comet assay

MCL-5 cells are a stable multi-competent human lymphoblastoid cell line expressing cytochrome P450s 1A1, 1A2, 2A6, 3A4 and 2E1, as well as epoxide hydrolase (19). The MCL-5 cells were cultured for 24 h in the presence of 4 μM BP and the samples assayed for genotoxicity by two different methodologies. From one set of flasks, DNA was isolated and used for determination of BPdG adducts by the CIA. The data showed values of 5.8 BPdG adducts/ 10^8 nucleotides for nuclear DNA and 63.5 BPdG adducts/ 10^8 nucleotides for mitochondrial DNA (Table III). Cells in a second set of flasks were examined by Comet assay, and there was a substantial increase in comet tail length (Figure 3 and Table III), accompanied by a decrease in nuclear size, in the BP-exposed cells, compared with the unexposed cells (Table III). Thus, DNA damage observed as

Table I. DNA adduct levels (per 10⁸ nucleotides) induced in calf thymus DNA modified with [1,3-³H]BPDE^a and measured by different methods

	[1,3- ³ H]BPDE radiolabelling	³² P-Postlabeling ^b	BPDE-DNA DELFIA	BPDE-DNA CIA
Adducts	110.7 ± 2.1	103.1 ± 67.4	135 ± 79	121 ± 25
Number of assays	6	54	11	10
% of [³ H]	100	93	122	110

^aThe data are reported as the mean ± SD.

^b³²P-Postlabeling values are from Phillips and Castegnaro (23) and shown here for comparison.

Table II. DNA adduct levels (per 10⁸ nucleotides)^a in liver DNA from mice given 0.5 or 1.0 mg [7,8-³H]BP by intraperitoneal injection: comparison of different methods

[7,8- ³ H]BP dose	[7,8- ³ H]BP radiolabeling	³² P-postlabeling ^c	BPDE-DNA DELFIA	BPDE-DNA CIA
Number of assays	3	33	6	6
0.5 mg	86.4 ± 2.4 (100%) ^b	11.4 ± 4.1 (13.2%)	4.3 ± 2.0 (5.0%)	11.0 ± 1.1 (12.8%)
1.0 mg	137.6 ± 3.4 (100%)	22.1 ± 9.4 (16.0%)	7.9 ± 4.9 (5.7%)	16.6 ± 3.9 (12.1%)

^aThe data are reported as the mean ± SD.

^bPercent of radiolabeled value in parentheses.

^c³²P-postlabeling values are from Phillips and Castegnaro (23) and shown here for comparison.

Table III. Determination of BPdG values and Comet assay parameters in MCL-5 cells exposed to 4 μM BP for 24 h

Assay	Unexposed cells	BPDE cells
BPdG in nuclear DNA (Adducts/10 ⁸ nucleotides)	0	5.8 ± 0.5 ^a
BPdG in mitochondrial DNA (Adducts/10 ⁸ nucleotides)	0	63.5 ± 0.5 ^b
Comet tail length (μm)	0	16.40 ± 0.85 ^a
Diameter of nucleus (μm)	6.04 ± 0.30 ^a	2.73 ± 0.23 ^a

^aMean ± SE, *n* = 3 assays for BPdG, and *n* ≥ 11 cells for Comet tail and nuclear size.

^bMean ± range, *n* = 2 assays.

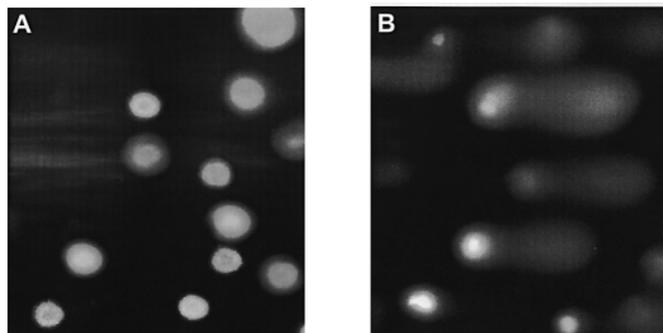


Fig. 3. Comet assay of MCL-5 cells. (A) Unexposed cells and (B) cells exposed for 24 h to 4 μM BP. Values for the decrease in nuclear size and the increase in Comet tail length are presented in Table III.

tail lengthening in the Comet assay associated with DNA adduct formation.

Determination of BPdG in human lymphocyte DNA

A series of 43 peripheral blood cell (buffy coat) DNA samples, from 25 individuals who were either colorectal adenocarcinoma patients or controls, was obtained from the Navy Colon

Table IV. Human blood cell DNA samples from the Navy Colon Adenoma Study

Number of patients	Number of DNA samples	Number of DNA samples assayed twice
18	36 (18 coded replicates)	31
7	7	7
Total = 25	Total = 43 40 detectable 3 (7%) non-detectable	Total = 38 <i>r</i> ² for two assays, on different plates, of DNA from 1 tube = 0.948

Adenoma study (28). The sample set (Table IV) consisted of 18 duplicates that were received as 36 separate coded samples, and seven single samples from each of seven patients that were submitted without replicates. Each sample was analyzed by BPDE-DNA CIA, using 10 μg of DNA per microtiter plate well and three experimental wells plus one control well per plate. Of the total 43 samples, 38 were assayed twice, as there was insufficient DNA to assay the remaining five samples a second time. For the 38 samples assayed twice, the two assays were performed on different days using separate plates and the correlation between the two assays (Figure 4) was excellent (*r*² = 0.948). The intra-assay coefficient of variation (CV) for microtiter wells on the same plate was 1.85%. Three samples out of the 43 (7%) were below the limit of detection, and for the 40 measurable samples, values ranged from 0.71 to 2.21 adducts/10⁸ nucleotides.

Among the total of 43 samples, 36 samples were coded duplicates from 18 individuals provided to us with 36 different numbers as if they were separate samples (Table IV). Fifteen pairs of coded duplicates (30 samples) were assayed twice. When the pairs were identified, there were 15 samples each assayed a total of four different times (two coded duplicates with two assays each) on different microtiter plates; the coefficient of variation for these assays was 13.8%. As this is much larger than the intra (three wells on one plate)- or inter

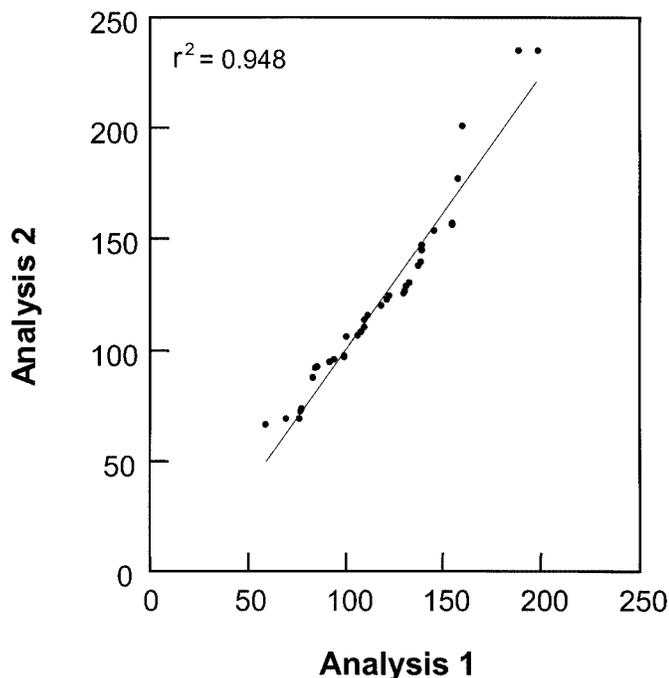


Fig. 4. Comparison between two different BPDE-DNA CIA values (Analysis 1 and Analysis 2) performed on different microtiter plates and on different days but using DNA from the same tube. The figure shows PAH-DNA adduct values for 38 samples, where the inter-assay correlation coefficient (r^2) is 0.948. The units for the ordinate and abscissa are PAH-DNA adducts/ 10^{10} nucleotides.

(two assays of DNA from the same tube)-assay variation (Figure 4), it would appear that the error associated with handling and aliquotting the DNA samples comprises a large source of variability. For three pairs of coded duplicate samples, provided to us as six samples, there was unacceptable variation in the values for the separate-sample duplicates (but not for the replicate assays from the same sample tube). For each of the three pairs, one sample had a measurable value and the second sample was undetectable. When third and fourth assays were performed on one pair of samples, the discrepancy disappeared.

Discussion

The purpose of this study was two-fold: first to develop a highly sensitive and stable immunoassay (CIA) for the determination of PAH-DNA adducts in human samples, and second to validate the CIA by comparison with previously established DNA adduct detection methods using modified DNA standards and biological samples obtained *in vitro* and *in vivo*.

The newly-developed BPDE-DNA CIA appears to be superior to similar immunoassays (16,17), with a level of sensitivity (1.5 BPdG adducts/ 10^9 nucleotides) that is usually seen with ^{32}P -postlabeling. The high signal-to-noise ratio gives a consistent assay with a stability that provides a considerable advantage compared with the BPDE-DNA DELFIA. In the DELFIA and previous immunoassays using this antiserum, high backgrounds resulted in variability that was constantly a problem. In contrast, in this assay the standard curves are more reproducible, largely because the problem of high background has been solved. One general disadvantage of the immunoassays, the fact that they require large quantities of

DNA, has not been completely eliminated. However, using 10 μg of DNA per microtiter plate well in the BPDE-DNA CIA we found only three out of 43 human samples that gave values below the limit of detection. This constitutes a substantial improvement over the earlier immunoassays that used 35 μg of DNA per microtiter plate well (17), as the total DNA requirement has been reduced from ~ 250 μg to ~ 80 μg . The fact that most human samples were in the detectable range for this assay suggests that we may have eliminated the problem of performing studies where a substantial fraction of samples assayed have non-detectable adduct levels. In this set of samples, most of the individuals are not considered to have consistently received high PAH exposures, such as those encountered in some polluted occupational environments, and yet PAH-DNA adducts were measurable in most samples.

The radiolabeled BPDE-DNA modified to 1.1 BPdG adducts/ 10^6 nucleotides was prepared as part of an inter-laboratory effort to synthesize DNA standards modified with chemical carcinogens in the range of human biological samples (23). The idea was that many laboratories could use such modified DNA samples as standardization reagents when assaying human DNA by multiple different methods. A previous publication by Beland *et al.* (15) has described the synthesis and validation of a DNA modified with 4-aminobiphenyl designed to be made available to many laboratories. Other efforts to use standards for ^{32}P -postlabeling assays have been published previously (14,23,31) and attest to the difficulties of such endeavors. For the radiolabeled *in vitro*-modified BPDE-DNA standard described here we compared the modification levels by radiolabeling, ^{32}P -postlabeling (23), and the two immunoassays, the BPDE-DNA DELFIA and the BPDE-DNA CIA. The radiolabeling value was similar to those obtained using all the other methods, suggesting that good inter-laboratory standardization for PAH-DNA adduct formation is potentially achievable using this calf thymus DNA modified *in vitro* with BPdG.

To investigate the usefulness of shared experimental model standards, mice were exposed to 0.5 and 1.0 mg of [7,8- ^3H]-BP, and the DNA modification determined by radiolabeling, BPDE-DNA DELFIA, and BPDE-DNA CIA. These were the same samples that had been shared earlier in the postlabeling trial (23), the data for which are shown in Table II. This experiment was complicated by the fact that much of the radioactivity bound to liver DNA was not in the form of the BPdG adduct and therefore not recognizable by the BPDE-DNA antiserum. In addition much of the radiolabeling was not measurable by ^{32}P -postlabeling, confirming the previous supposition. Unlike mouse skin DNA, which contains mainly different enantiomeric forms of BPdG after topical BP exposure (32,33), the liver appears to metabolize BP to multiple different DNA-adducted forms, only a small fraction of which comprise the BPdG adduct (34,35). Nonetheless, a comparable dose-response was observed for the two different doses by ^{32}P -postlabeling and both immunoassays.

The use of MCL-5 cells in this study has provided an opportunity to compare multiple different genotoxic end points in this unique cell line. MCL-5 cells are human B-lymphoblastoid cells transfected with cDNAs encoding for carcinogen activating enzymes (19). In several previous studies (36-38) these cells have been exposed to BP and monitored for genotoxicity using end points that include DNA fragmentation by Comet assay, DNA adducts by ^{32}P -postlabeling, and mutagenesis. In this study the Comet assay data provide a further

validation of the BPDE–DNA CIA, as the Comet tail lengths were similar to those previously reported (38). In addition, by comparison with nuclear BPdG adduct levels, 10-fold higher BPdG adduct levels were found in mitochondrial DNA. These data reproduce literature findings (39,40) and give further validation to the BPDE–DNA CIA.

Overall, this study describes the validation of a new, highly-sensitive immunoassay for the determination of PAH–DNA adducts. The major changes, compared with the BPDE–DNA DELFIA, include the use of different microtiter plates, a different blocking agent, more highly-diluted antiserum, and a chemiluminescent end signal. It should be noted that for any immunoassay, the sensitivity depends upon a particular combination of plate, coating, blocking, antibody, and standard, and the sensitivity of this CIA may change if the conditions vary. In this study we have applied and validated the BPDE–DNA CIA using a DNA sample modified with BPDE *in vitro*, and *in vivo*-modified samples from mouse liver, cultured human cells and human blood cell DNA. Because the BPDE–DNA CIA is highly sensitive (detecting measurable values in >90% of the samples assayed), and reproducible, it is a promising method for human biomonitoring in molecular epidemiology studies.

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References

- Phillips,D.H. (1983) Fifty years of benzo(a)pyrene. *Nature*, **303**, 468–472.
- Gelboin,H.V. and Ts'o,P.O.P. (eds) (1978) *Polycyclic Hydrocarbons and Cancer*. Academic Press, New York.
- Rothman,N., Poirier,M.C., Baser,M.E., Hansen,J.A., Gentile,C., Bowman,E.D. and Strickland,P.T. (1990) Formation of polycyclic aromatic hydrocarbon–DNA adducts in peripheral white blood cells during consumption of charcoal-broiled beef. *Carcinogenesis*, **11**, 1241–1243.
- Rothman,N., Poirier,M.C., Haas,R.A., Correa-Villasenor,A., Ford,P., Hansen,J.A., O'Toole,T. and Strickland,P.T. (1993) Association of PAH–DNA adducts in peripheral white blood cells with dietary exposure to polycyclic aromatic hydrocarbons. *Environ. Health Perspect.*, **99**, 265–267.
- Roth,M.J., Strickland,K.L., Wang,G.Q., Rothman,N., Greenberg,A. and Dawsey,S.M. (1998) High levels of carcinogenic polycyclic aromatic hydrocarbons present within food from Linxian, China may contribute to that region's high incidence of oesophageal cancer. *Eur. J. Cancer*, **34**, 757–758.
- Poirier,M.C. and Weston,A. (1996) Human DNA adduct measurements: state of the art. *Environ. Health Perspect.*, **104** (Suppl. 5), 883–893.
- Poirier,M.C., Santella,R.M. and Weston,A. (2000) Carcinogen macromolecular adducts and their measurement. *Carcinogenesis*, **21**, 353–359.
- Rundle,A., Tang,D., Hibshoosh,H., Estabrook,A., Schnabel,F., Cao,W., Grumet,S. and Perera,F.P. (2000) The relationship between genetic damage from polycyclic aromatic hydrocarbons in breast tissue and breast cancer. *Carcinogenesis*, **21**, 1281–1289.
- Tang,D., Santella,R.M., Blackwood,A.M., Young,T.-L., Mayer,J., Jaretski,A., Grantham,S., Tsai,W.-Y. and Perera,F.P. (1995) A molecular epidemiological case–control study of lung cancer. *Cancer Epidemiol. Biomarkers Prev.*, **4**, 341–346.
- Rundle,A., Tang,D., Zhou,J., Cho,S. and Perera,F. (2000) The association between glutathione-S-transferase M1 genotype and polycyclic aromatic hydrocarbon–DNA adducts in breast tissue. *Cancer Epidemiol. Biomarkers Prev.*, **9**, 1079–1085.
- Tang,D., Rundle,A., Warburton,D., Santella,R.M., Tsai,W.-Y., Chiamprasert,S., Hsu,Y.Z. and Perera,F.P. (1998) Associations between both genetic and environmental biomarkers and lung cancer: evidence of a greater risk of lung cancer in women smokers. *Carcinogenesis*, **19**, 1949–1953.
- Tang,D., Phillips,D.H., Stampfer,M., *et al.* (2001) Association between carcinogen–DNA adducts in white blood cells and lung cancer risk in the Physicians Health Study. *Cancer Res.*, **61**, 6708–6712.
- Phillips,D.H., Farmer,P.B., Beland,F.A., Nath,R.G., Poirier,M.C., Reddy,M.V. and Turteltaub,K.W. (2000) Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ. Mol. Mutagen.*, **35**, 222–233.
- Phillips,D.H., Castegnaro,M. and Bartsch,H. (eds) (1993) *Postlabelling Methods for Detection of DNA Adducts*. IARC Scientific Publications No. 124, Lyon, France.
- Beland,F.A., Doerge,D.R., Churchwell,M.I., Poirier,M.C., Schoket,B. and Marques,M.M. (1999) Synthesis, characterization, and quantitation of a 4-aminobiphenyl–DNA adduct standard. *Chem. Res. Toxicol.*, **12**, 68–77.
- Schoket,B., Doty,W.A., Vincze,I., Strickland,P.T., Ferri,G.M., Assennato,G. and Poirier,M.C. (1993) Increased sensitivity for determination of polycyclic aromatic hydrocarbon–DNA adducts in human DNA samples by dissociation-enhanced lanthanide fluoroimmunoassay (DELFLIA). *Cancer Epidemiol. Biom. Prev.*, **2**, 349–353.
- Santella,R.M., Weston,A., Perera,F.P., Trivers,G.T., Harris,C.C., Young,T.L., Nguyen,D., Lee,B.M. and Poirier,M.C. (1988) Interlaboratory comparison of antisera and immunoassays for benzo[a]pyrene-diol-epoxide-I-modified DNA. *Carcinogenesis*, **9**, 1265–1269.
- Weston,A., Manchester,D.K., Poirier,M.C., Choi,J.-S., Trivers,G.E., Mann,D.L. and Harris,C.C. (1989) Derivative fluorescence spectral analysis of polycyclic aromatic hydrocarbon–DNA adducts in human placenta. *Chem. Res. Toxicol.*, **2**, 104–108.
- Crespi,C.L., Gonzalez,F.J., Steimel,D.T., Turner,T.R., Gelboin,H.V., Penman,B.W. and Langenbach,R. (1991) A metabolically competent human cell line expressing five cDNAs encoding procarcinogen-activating enzymes: application to mutagenicity testing. *Chem. Res. Toxicol.*, **4**, 566–572.
- Basler,J., Hastie,N.D., Pietras,D., Matsui,S.-I., Sandberg,A.A. and Berezney,R. (1981) Hybridization of nuclear matrix attached deoxyribonucleic acid fragments. *Biochemistry*, **20**, 6921–6929.
- Beland,F.A., Fullerton,N.F. and Heflich,R.H. (1984) Rapid isolation, hydrolysis and chromatography of formaldehyde-modified DNA. *J. Chromatogr.*, **308**, 121–131.
- Heflich,R.H., Morris,S.M., Beranek,D.T., McGarrity,L.J., Chen,J.J. and Beland,F.A. (1986) Relationships between the DNA adducts and the mutations and sister-chromatid exchanges produced in Chinese hamster ovary cells by *N*-hydroxy-2-aminofluorene, *N*-hydroxy-*N'*-acetylbenzidine and 1-nitrosopyrene. *Mutagenesis*, **1**, 201–206.
- Phillips,D.H. and Castegnaro,M., on behalf of the trial participants. (1999) Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trials and production of recommended protocols. *Mutagenesis*, **14**, 301–315.
- Reddy,M.V. and Randerath,K. (1986) Nuclease P1-mediated enhancement of sensitivity of ³²P- postlabeling test for structurally diverse DNA adducts. *Carcinogenesis*, **7**, 1543–1551.
- Culp,S.J. and Beland,F.A. (1994) Comparison of DNA adduct formation in mice fed coal tar or benzo[a]pyrene. *Carcinogenesis*, **15**, 247–252.
- Culp,S.J., Warbritton,A.R., Smith,B.A., Li,E.E. and Beland,F.A. (2000) DNA adduct measurements, cell proliferation and tumor mutation induction in relation to tumor formation in B6C3F1 mice fed coal tar or benzo[a]pyrene. *Carcinogenesis*, **21**, 1433–1440.
- Divi,R.L., Osborne,M.R., Hower,A., Phillips,D.H. and Poirier,M.C. (1999) Tamoxifen–DNA adduct formation in rat liver determined by immunoassay and ³²P-postlabeling. *Cancer Res.*, **59**, 4829–4833.
- Sinha,R., Chow,W.H., Kulldorff,M., Denobile,J., Butler,J., Garcia-Closas,M., Weil,R., Hoover,R.N. and Rothman,N. (1999) Well-done, grilled red meat increases the risk of colorectal adenomas. *Cancer Res.*, **59**, 4320–4324.
- Daly,A.K., Steen,V.M., Fairbrother,K.S. and Idle,J.R. (1996) *CYP2D6* multiallelism. *Methods Enzymol.*, **272**, 199–210.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Baan,R.A., Steenwinkel,M.-J.S.T., van Asten,S., Roggeband,R. and van Delft,J.H.M. (1997) The use of benzo[a]pyrene diol-epoxide-modified DNA standards for adduct quantification in ³²P-postlabelling to assess exposure to polycyclic aromatic hydrocarbons: application in a biomonitoring study. *Mutat. Res.*, **378**, 41–50.
- Ashurst,S.W., Cohen,G.M., Nesnow,S., DiGiovanni,J. and Slaga,T.J. (1983) Formation of benzo(a)pyrene/DNA adducts and their relationship to tumor initiation in mouse epidermis. *Cancer Res.*, **43**, 1024–1029.
- Suh,M., Ariese,F., Small,G.J., Jankowiak,R., Hower,A. and Phillips,D.H. (1995) Formation and persistence of benzo[a]pyrene–DNA adducts in mouse epidermis *in vivo*: importance of adduct conformation. *Carcinogenesis*, **16**, 2561–2569.
- Kulkarni,M.S. and Anderson,M.W. (1984) Persistence of benzo(a)pyrene

- metabolite: DNA adducts in lung and liver of mice. *Cancer Res.*, **44**, 97–101.
35. Anderson, M.W., Boroujerdi, M. and Wilson, A.G.E. (1981) Inhibition *in vivo* of the formation of adducts between metabolites of benzo(a)pyrene and DNA by butylated hydroxyanisole. *Cancer Res.*, **41**, 4309–4315.
36. Scates, D.K., Spigelman, A.D., Phillips, R.K.S. and Venitt, S. (1996) Differences in the levels and pattern of DNA-adduct labelling in human cell lines MCL-5 and CCRF, proficient or deficient in carcinogen-metabolism, treated *in vitro* with bile from familial adenomatous polyposis patients and from unaffected controls. *Carcinogenesis*, **17**, 707–713.
37. Busby, W.F., Jr., Smith, H., Crespi, C.L. and Penman, B.W. (1995) Mutagenicity of benzo[a]pyrene and dibenzopyrenes in the *Salmonella typhimurium* TM677 and the MCL-5 human cell forward mutation assays. *Mutat. Res.*, **342**, 9–16.
38. Martin, F.L., Cole, K.J., Orme, M.H., Grover, P.L., Phillips, D.H. and Venitt, S. (1999) The DNA repair inhibitors hydroxyurea and cytosine arabinoside enhance the sensitivity of the alkaline single-cell gel electrophoresis ('comet') assay in metabolically-competent MCL-5 cells. *Mutat. Res.*, **445**, 21–43.
39. Backer, J.M. and Weinstein, I.B. (1980) Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative of benzo[a]pyrene. *Science*, **209**, 297–299.
40. Stairs, P.W., Guzelian, P.S. and Van Tuyle, G.C. (1983) Benzo[a]pyrene differentially alters mitochondrial and nuclear DNA synthesis in primary hepatocyte cultures. *Res. Commun. Chem. Pathol. Pharmacol.*, **42**, 95–106.

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