

# Semiquantitation of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Human Esophagus by Immunohistochemistry and the Automated Cellular Imaging System

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## Abstract

It has been suggested that ingestion of polycyclic aromatic hydrocarbons (PAHs) may contribute to the high incidence and mortality of esophageal cancer in Linxian, China. To explore this relationship a semiquantitative immunohistochemical staining method was developed for localization of PAH-DNA adducts. Nuclear color intensity (bright field average pink intensity per nucleus for >1000 cells) was measured using the ChromaVision Automated Cellular Imaging System (ACIS). Paraffin-embedded sections of cultured human keratinocytes exposed to increasing concentrations of 7 $\beta$ ,8 $\alpha$ -dihydroxy-9 $\alpha$ ,10 $\alpha$ -epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) were incubated with BPDE-DNA antiserum and served as an internal positive control (standard curve). Values for nuclear staining intensity correlated directly with BPDE exposure concentration ( $r^2 = 0.99$ ) and were reproducible. DNA adduct levels determined by BPDE-DNA chemiluminescence immunoassay in DNA from BPDE-exposed keratinocytes, correlated with BPDE exposure concentrations ( $r^2 = 0.99$ ), showing that nuclear staining intensity determined by ACIS correlated directly with BPDE-DNA adduct levels determined by chemiluminescence immunoassay. The ACIS methodology was applied to 5 human samples from Linxian, and significantly positive nuclear PAH-DNA adduct staining was observed in this group when compared with esophageal tissue from 4 laboratory-housed monkey controls and 6 samples obtained at autopsy from smokers and nonsmokers in the United

States. Nuclear PAH-DNA staining was absent from Linxian samples when serial sections were incubated with normal rabbit serum (negative control) and was significantly reduced on incubation with BPDE-DNA antiserum absorbed previously with the immunogen BPDE-DNA. These results appear to support the hypothesis that high PAH exposure levels may be etiologically associated with the development of esophageal cancer in Linxian.

## Introduction

In the United States squamous cell esophageal cancer is relatively uncommon, and primarily associated with tobacco and alcohol use. In Linxian, China, where the cumulative mortality rate from esophageal and proximal stomach cancer is ~20%, the primary etiologic agents have yet to be determined. Several potential etiologic associations include nutritional deficiencies, poor oral hygiene, exposure to nitrosamines, and microbial contamination (1).

Recent studies suggest that exposure to carcinogenic PAHs<sup>2</sup> may play an etiologically significant role. Analysis of raw and cooked food obtained from Linxian revealed the presence of high levels of the carcinogenic PAH benzo(a)pyrene (2), and urines obtained from Linxian residents contained high concentrations of the PAH urinary metabolite 1-hydroxypyrene (3). To explore a potential association between PAH ingestion and the development of esophageal cancer, it is logical to evaluate PAH-DNA adduct formation in human esophagus, as PAH-DNA adduct formation is a necessary but not sufficient step in cancer induction in multiple animal models. Visualization of DNA adducts in human target tissue sections is arguably more versatile than DNA adduct quantitation in whole tissue DNA, as localization is possible in specific cells, and small quantities of paraffin-embedded tissues can be used.

We have developed and validated a new semiquantitative immunohistochemical method for PAH-DNA adduct determination. The data presented here describe the ACIS methodology for nuclear staining and adduct semiquantitation, the use of BPDE-exposed human keratinocytes as an internal positive control, and the determination of PAH-DNA adduct levels in esophageal epithelium obtained from Linxian, China. This pilot study is one of the first to demonstrate PAH-DNA adduct formation in archived paraffin-embedded samples of human esophagus and opens the possibility for a more extensive investigation designed to determine the relationship among

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<sup>2</sup>The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; ACIS, Automated Cellular Imaging System; AU, arbitrary unit; BPDE, 7 $\beta$ ,8 $\alpha$ -dihydroxy-9 $\alpha$ ,10 $\alpha$ -epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene; CIA, chemiluminescence immunoassay; KSM, keratinocyte serum-free medium; PBST, PBS with 0.05% Tween.

PAH exposure, PAH-DNA adduct formation, and cancer development.

## Materials and Methods

**Chemicals, Reagents, and Antisera.** BPDE was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute (Kansas City, MO). RNase and proteinase K were from Sigma Chemical Co. (St. Louis, MO). PBS without calcium and magnesium ( $10\times$  concentrate) and KSM were from Life Technologies, Inc. (Rockville, MD). Quality Biological, Inc. (Gaithersburg, MD) supplied 0.5 M EDTA (pH 8.0). Tween 20 was from Bio-Rad (Hercules, CA). Casein, Avidin AP, CPD-Star Emerald-II substrate, Assay Wash buffer and anti-rabbit biotinylated antibody were from Tropix (Bedford, MA). Supermount, antirabbit StrAviGen Super Sensitive Alkaline phosphate, Antigen Retrieval Citra Solution, hematoxylin counterstain, and normal rabbit serum were from BioGenex, (San Ramon, CA). Fast Red was from DAKO A/S (Glostrup, Denmark). The DNA extraction kits were from Intergen (Purchase, NY). White, high-binding, 96-wells microtiter plates were purchased from Greiner Labortechnik GmbH (Frickenhäusen, Germany). Permout was from Fisher Scientific (Fair Lawn, NJ). DNA binding solution was from Pierce (Rockford, IL). Reacti-Bind DNA Coating Solution was from Pierce.

Antiserum specific for DNA modified with BPDE (rabbit #31 bleed 8/16/78) was elicited and characterized as described (4). To prepare immunogen-absorbed BPDE-DNA antiserum, unabsorbed BPDE-DNA antiserum (0.4 ml) was absorbed two times with a total of 388  $\mu\text{g}$  BPDE-DNA (1.4% modified, 13.9 nmol BPDE-DNA adducts). Cross-reactivity of this antiserum for DNA samples modified with chrysene, benz(a)anthracene, and other PAHs has been reported (5). When the antiserum was incubated with cells exposed to BPDE, the data are expressed as BPDE-DNA adducts. However, in human samples, where adducts of multiple PAHs are likely to be present, the values obtained are designated PAH-DNA adducts.

**Patients, Tissue Samples, and Primate Controls.** The 5 human esophageal samples from China were obtained from esophagectomies performed in Linxian in the spring/summer of 1995. All of the patients were diagnosed with squamous carcinoma *in situ* or early invasive squamous cell carcinoma. All of the esophagectomy tissues were fixed in 10% formalin, cut in a grid pattern, and embedded in paraffin. Blocks with normal-appearing esophageal tissue were used for this experiment. The study was approved by the Institutional Review Boards of the collaborating institutions: The Cancer Institute of the Chinese Academy of Medical Sciences and the National Cancer Institute, NIH.

The 6 human esophageal samples from the United States, from 2 smokers and 4 nonsmokers, were obtained from autopsies performed at the National Cancer Institute Laboratory of Pathology in 1999 and 2000. The immediate causes of death for these individuals included fungal bronchopneumonia, pneumonia, septic shock, hypotension, myocardial infarction, and diffuse alveolar damage, but all of the patients had either cancer (other than esophageal) or multiple sclerosis. The samples were fixed in 10% formalin and embedded in paraffin. The use of these samples was approved by the Office of Human Subjects Research and the Institutional Review Board of the National Cancer Institute, NIH.

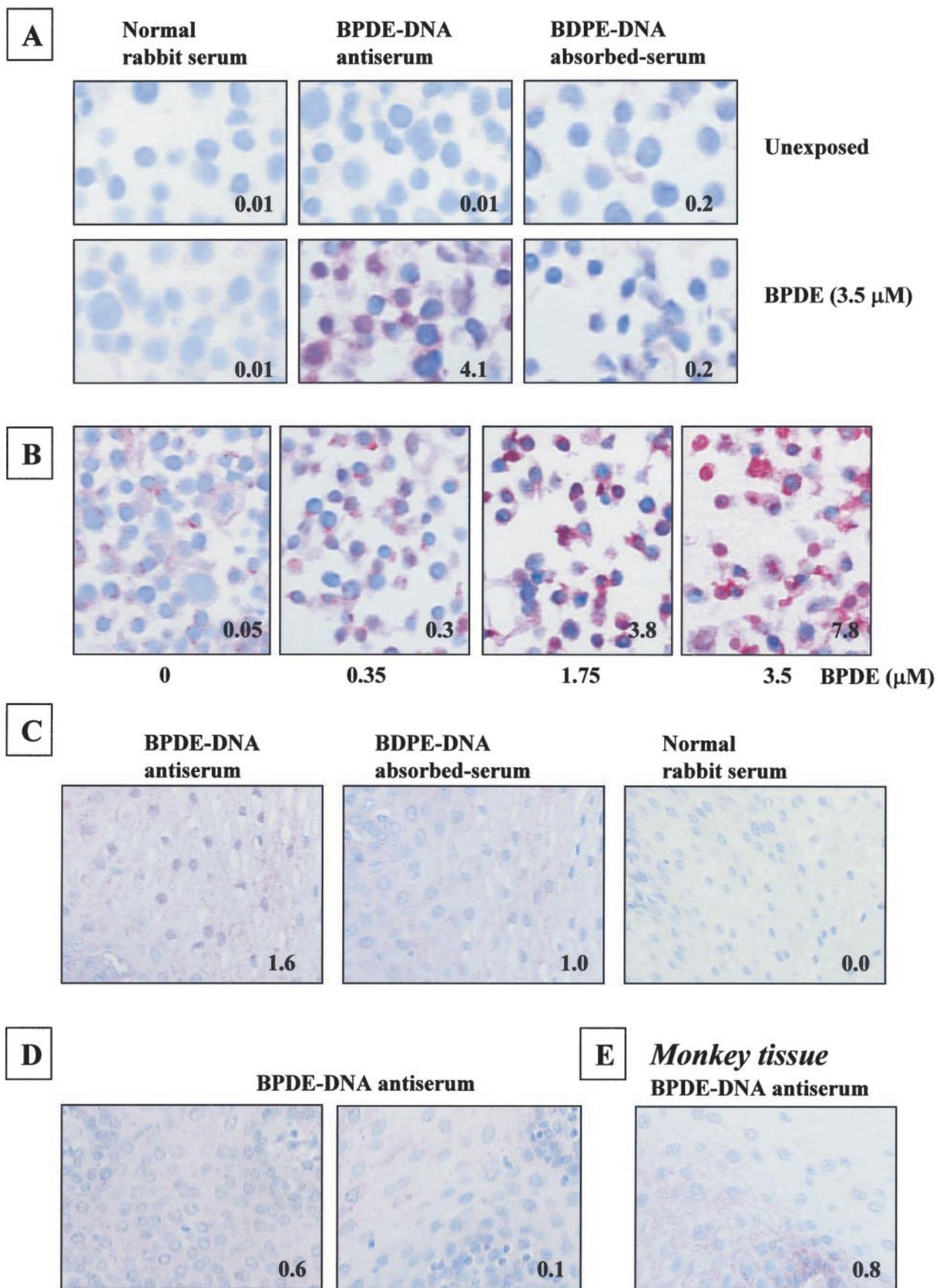
*Erythrocebus patas* monkeys were maintained under American Association for Accreditation of Laboratory Animal Care-approved conditions at BioQual, Inc., (Rockville, MD) in

accordance with humane principles for laboratory animal care. Protocols were reviewed and approved by the BioQual, Inc. Animal Care and Use Committee. The 4 monkeys used as controls for this study were retired breeders, and tissues were obtained at autopsy.

**Exposure of Cultured Human Keratinocytes.** Immortalized normal human cervical keratinocytes (6) were cultured in 150-mm dishes using KSM supplemented with antibiotics at 37°C and 5% CO<sub>2</sub>. Cells were allowed to grow to near confluency before exposure. BPDE was diluted in DMSO and added to fresh medium immediately before exposure. The cells were exposed to several concentrations of BPDE between 0.035 and 3.5  $\mu\text{M}$  BPDE. For the immunohistochemistry standard curve the BPDE concentrations were 0, 0.035, 0.35, 1.75, and 3.5  $\mu\text{M}$ . For the quantitative BPDE-DNA adduct immunoassay cells were exposed to 0, 0.18, 0.35, and 1.75  $\mu\text{M}$  BPDE. These were two separate experiments performed with different batches of cells having the same level of confluence. After 1 h cells were washed once with PBS and harvested in PBS. Cells from 10 plates were combined, centrifuged for 20 min at 2500 rpm, and the supernatant was removed. The cell pellet was either fixed in formalin and embedded in paraffin or DNA was isolated using the Intergen DNA-extraction kit according to the instructions of the manufacturer.

**Immunohistochemistry.** Staining for BPDE-DNA adducts in cultured human keratinocytes and for PAH-DNA adducts in human esophagus was performed in a similar fashion to that described previously for tamoxifen-DNA adducts (7). In short, sections (5  $\mu\text{m}$ ) of paraffin-embedded cells or tissues were deparaffinized, hydrated, and washed once in PBS (pH 7.4) with 10 mM EDTA (PBS/EDTA). Subsequently, the slides were placed in Antigen Retrieval Citra Solution. The solution was heated in a microwave oven for 2 min to a quick boil (microwave on full power), maintained near the boiling point for an additional 15 min (microwave 10% power), and allowed to cool down to 37°C. The slides were rinsed in PBS/EDTA and put into blocking solution [0.25% Casein in 10 mM EDTA,  $1\times$  PBS, and 0.05% Tween (PBST)] for 60 min at 37°C. After rinsing the slides in PBST for 10 min, slides were rinsed in deionized water and equilibrated in PBS/EDTA solution. Slides were incubated overnight at 4°C with rabbit BPDE-DNA antiserum (dilution 1:1000–2500). After washing (rinse in deionized water, agitate for 10 min in PBST, rinse with deionized water, and equilibrate for 3 min in PBS/EDTA), slides were incubated with anti-rabbit biotinylated antibody (BioGenex) for 30 min at room temperature. The slides were washed (see above) and incubated with Streptavidin-Alkaline-Phosphatase (BioGenex) for 30 min at room temperature. After washing (see above) slides were equilibrated in Tris-HCl buffered saline for 10 min before applying the Fast Red staining solution. The color was developed in 2–3 min. Slides were rinsed in deionized water and counterstained with Mayer's hematoxylin for 15 s. After rinsing with 3% ammonia and water, slides were mounted with Supermount (BioGenex), and 16 h later, coverslips were applied using permount. Slides incubated with normal rabbit serum (BioGenex) and immunogen (BPDE-DNA)-absorbed serum served as controls for the staining. Staining was evaluated using an E400 Nikon Eclipse microscope and prepared for evaluation by ACIS.

The Linxian samples were all stained on four separate occasions, and on each occasion both specific BPDE-DNA antiserum and the immunogen-absorbed BPDE-DNA antiserum were used. On each occasion one or more monkey



samples were stained simultaneously with both specific and absorbed antisera.

**Quantification of Nuclear Staining Intensity by ACIS.** The intensity of the nuclear BPDE-DNA or PAH-DNA adduct staining (purple color) was measured using ACIS (7). This system consists of a bright field microscope with  $\times 4$  to  $\times 60$  objectives, a charge coupled display digital camera, an automated slide loading system, and a computer. ACIS software (version 1.81b and 1.82) regulates microscope calibration and slide loading, and runs applications that identify and quantify immunohistochemical stainings of cellular organelles.

Characterization of the immunohistochemical staining is based on three color parameters: the color defined by hue (purple is 190–224, blue is 130–190), the “darkness” defined as luminosity, and density of the color defined as the saturation. The three parameters together comprise color intensity, given in AUs. By increasing or decreasing the thresholds for hue, luminosity, and saturation it is possible to eliminate the contribution of background staining. In the applications used in these experiments the ACIS software was instructed, by setting the color-specific thresholds for hue, luminosity, and saturation in combination with the settings for morphological features, to determine the intensity only in round-shaped organelles within the standard size range for nuclei in squamous epithelium. The “threshold tool,” which is part of the ACIS software, made it possible to set thresholds for hue, luminosity, and saturation, and to visualize regions recognized by the software as positive for nuclear intensity determination.

For the quantitation of PAH-DNA adduct staining, two different ACIS software applications were used. The first application scans the whole section, including all of the tissue layers, and the second application scans, captures, and saves a picture of the whole section as a “histological reconstruction.” Within the histological reconstruction, the squamous cell layer can be selected as the “area of interest.” The ACIS software is programmed to focus on nuclei, defined as round or elliptical areas of a certain size range, and it determines the total number of pixels contained within all of the nuclei that are either pink (indicating PAH-DNA adducts) or blue (indicating nuclei). It also separately calculates the intensity of the pink and blue colors. From these parameters the computer determines the average pink staining intensity per nucleus and the percentage of nuclei that are positive for pink (PAH-DNA adducts) within the area of interest. For each section,  $\sim 1000$  cells were counted.

**CIA.** Quantitation of BPDE-DNA adducts was performed by the BPDE-DNA CIA similar to that described previously for tamoxifen-DNA adducts (8). In short, Greiner 96-well high-binding plates were coated for 48 h with 1.5 fmol BPDE-DNA (1% modified) per well using Reacti-Bind DNA Coating Solution. After washing with PBST, plates were incubated at 37°C for 1 h with 0.25% casein in PBST to reduce nonspecific antibody binding. After washing with PBST, biological sample DNA or standard BPDE-DNA (diluted in Tris/EDTA buffer) was mixed with an equal volume of PAH-DNA antiserum (1:3,000,000 diluted in PBST containing 0.25% casein) and

added to the wells. Serial dilutions of the standard BPDE-DNA in carrier calf-thymus DNA were prepared such that each well contained an equal quantity of DNA. The plates were incubated for 1.5 h at 37°C. Subsequently plates were washed and incubated with anti-rabbit biotinylated antibody (1:5,000 in 0.25% casein solution; Tropix) for 1.5 h at room temperature. After washing, plates were incubated with Avidin Alkaline Phosphatase (avidin-AP: 1:8,000; Tropix) for 60 min. The plates were washed with PBST, distilled water, and Assay buffer. CDP star with Emerald II solution was added, and the plates were stored overnight at 4°C. The next day plates were allowed to warm to room temperature before measuring luminescence. Luminescence was measured at 37°C using the Tropix Microplate Luminometer TR717.

**Statistical Methods.** We compared the mean values (AU) for BPDE-DNA staining measured by ACIS for samples from Linxian and the laboratory housed monkeys using a linear mixed model (9) with sample (individual) and experimental (assay) crossed random effects, and a human *versus* monkey fixed effect. A likelihood ratio test was used to test whether the mean values for humans were different from those for monkeys. All of the *P*s were two-sided. A two-factor variance components model (with crossed effects) was used to decompose the sources of variation for both BPDE-DNA antiserum and absorbed BPDE-DNA antiserum. Restricted maximum likelihood was used for variance components estimation.

## Results

### Validation and Standardization of the ACIS Methodology.

For the initial experiment, immortalized normal human cervical keratinocytes were exposed to either 0 or 3.5  $\mu\text{M}$  BPDE for 1 h and were subsequently embedded in paraffin. Sections of unexposed and exposed cells were incubated with BPDE-DNA antiserum, immunogen (BPDE-DNA)-absorbed serum, or normal rabbit serum (Fig. 1A). Purple nuclear staining, indicating the presence of BPDE-DNA adducts, was absent in sections of unexposed and exposed keratinocytes that were incubated with normal rabbit serum (Fig. 1A, *left photos*), whereas the localization of the nuclei is indicated by hematoxylin (blue) staining. Purple nuclear staining, indicating the presence of BPDE-DNA adducts, was found in sections of exposed keratinocytes incubated with the BPDE-DNA antiserum, but absent in sections of unexposed keratinocytes incubated with the BPDE-DNA antiserum (Fig. 1A, *middle photos*). Nuclear staining was removed in sections of BPDE-exposed keratinocytes incubated with immunogen (BPDE-DNA)-absorbed serum (Fig. 1A, *right bottom photo*). Values for intensity of nuclear purple staining, obtained by ACIS and expressed as AUs, are shown on the photos in Fig. 1A. The intensity of the purple nuclear staining in unexposed keratinocytes (0.01 AU) was much lower than that measured in BPDE-exposed keratinocytes (4.1 AU). Nuclear staining intensity in slides (unexposed and exposed) incubated with immunogen-absorbed BPDE-DNA antiserum was 0.2 AU.

For preparation of a standard curve, human cervical kera-

**Fig. 1.** Photographs ( $\times 600$  magnification) of cultured human keratinocytes (A and B), human esophagus (C and D), and monkey esophagus (E). Numbers on the photos are in AUs and refer to intensity of purple nuclear staining measured by ACIS. A, staining for BPDE-DNA adducts in human keratinocytes unexposed (top row) or exposed to 3.5  $\mu\text{M}$  BPDE (bottom row). Paraffin-embedded cells were incubated with either normal rabbit serum (left row), BPDE-DNA antiserum (middle row), or BPDE-DNA-absorbed BPDE-DNA antiserum (right row). B, dose-response for intensity of nuclear BPDE-DNA staining in cultured human keratinocytes exposed to 0, 0.035, 0.35, and 3.50  $\mu\text{M}$  BPDE. C, PAH-DNA adduct staining in the squamous cell layer of human esophageal sample number 5 from Linxian, China. Sections were incubated with BPDE-DNA antiserum (left panel), BPDE-DNA-absorbed BPDE-DNA antiserum (middle panel), or normal rabbit serum (right panel). D, PAH-DNA adduct staining in the squamous cell layer of human esophageal samples from the United States. Shown are human samples 1 and 6. E, PAH-DNA adduct staining in the squamous cell layer of esophageal tissue from monkey number 2.

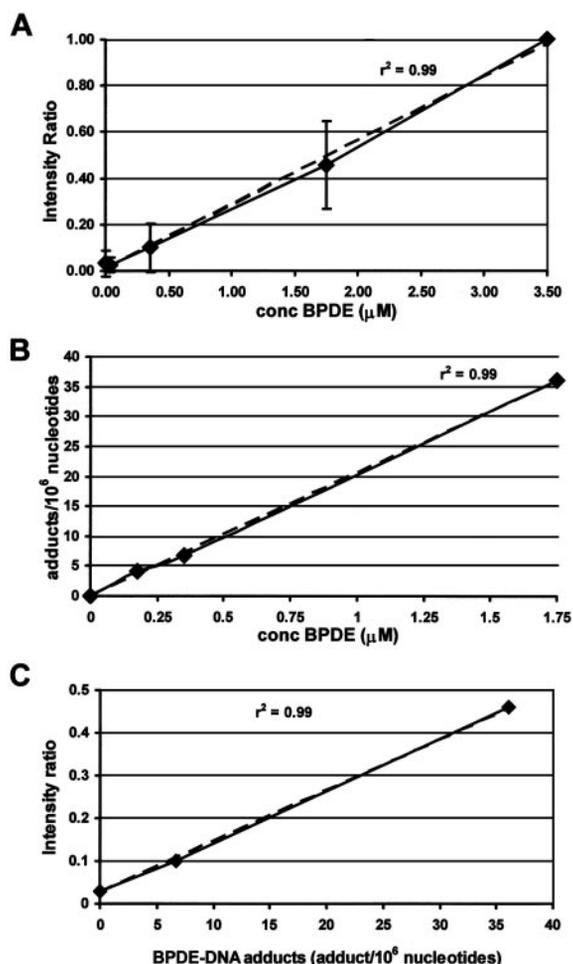


Fig. 2. A: ACIS determination of a dose-related increase in BPDE-DNA adducts in cultured human keratinocytes exposed to 0, 0.035, 0.35, 1.75, and 3.5  $\mu\text{M}$  BPDE for 1 h. The AU intensity in nuclei from cells exposed to the highest concentration of BPDE (3.5  $\mu\text{M}$ ) was designated 1 (100%), and AU intensity values for the lower doses were expressed as a fraction of the 100% value. Standard curves ( $\diamond$ — $\diamond$ ,  $n = 8$ ) are shown as mean of samples from two separate BPDE exposures stained four times each; bars,  $\pm$ SD. Linear regression line (---). B, values for BPDE-DNA adducts determined by quantitative immunoassay (CIA) in DNA isolated from cultured human keratinocytes exposed to 0.0, 0.18, 0.35, and 1.75  $\mu\text{M}$  BPDE for 1 h. Data are expressed as mean  $\pm$  SD for two to five assays ( $\diamond$ — $\diamond$ ), and error bars are too small to be visible. Linear regression line (---). C, correlation between BPDE-DNA adduct levels determined by CIA (from B) and intensity ratio of purple nuclear staining determined by ACIS (from A) for cultured human keratinocytes exposed to 0.0, 0.35, and 1.75  $\mu\text{M}$  BPDE ( $\diamond$ — $\diamond$ ). Linear regression line (---).

tinocytes were exposed to 0, 0.035, 0.35, 1.75, and 3.5  $\mu\text{M}$  BPDE for 1 h, paraffin embedded, sectioned, and incubated with BPDE-DNA antiserum. The nuclear staining intensity increased with increasing BPDE exposure as shown in Fig. 1B. The intensity of the nuclear staining determined by ACIS increased in a linear fashion from 0.05 AU in unexposed sections to 7.8 AU in sections exposed to the highest BPDE concentration (Fig. 1B). There was variability in ACIS values when the same standard curve slides were stained on different days, although the dose-response relationship was consistent. When the AU value for the highest exposure concentration, 3.5  $\mu\text{M}$  BPDE, was designated 1.0 (100%), the values for the lower concentrations could be expressed as a ratio (percentage) of that

value, and direct comparison of the curves generated on different days was made possible. When expressed in this fashion, there was a strong correlation ( $r^2 = 0.99$ ) between the dose-related intensity ratio of nuclear staining and the increase in BPDE concentration for eight standard curves using blocks from two different cell culture exposures, each stained four times (Fig. 2A). The coefficient of variation for the BPDE concentrations at an intensity ratio of 0.5 (50%) for eight standard curves 14.8%.

To correlate nuclear staining intensity with BPDE-DNA adduct levels in extracted DNA, human keratinocytes were cultured and exposed to 0, 0.18, 0.35, and 1.75  $\mu\text{M}$  BPDE for 1 h. DNA was isolated, and BPDE-DNA adduct levels were measured by a quantitative competitive immunoassay, the BPDE-DNA CIA. The BPDE-DNA adduct levels (Fig. 2B) varied between 4.1 adducts/ $10^6$  nucleotides (0.18  $\mu\text{M}$  BPDE) and 36.0 adducts/ $10^6$  nucleotides (1.75  $\mu\text{M}$  BPDE), and a strong correlation ( $r^2 = 0.99$ ) was found between BPDE concentration and BPDE-DNA adduct levels. When BPDE-DNA adduct levels determined by CIA were plotted versus intensity ratio determined by ACIS (Fig. 2C) the correlation was also very strong ( $r^2 = 0.99$ ).

**PAH-DNA Adduct Staining in Human Esophageal Samples from Linxian.** Foci of histologically normal squamous epithelium obtained from individuals in Linxian, China, were stained for the presence of PAH-DNA adducts using the BPDE-DNA antiserum. Because the BPDE-DNA antiserum has cross-reactivity for DNA modified with several PAHs (5), a positive signal in human samples is considered to indicate the formation of multiple PAH-DNA adducts, including BPDE-DNA adducts. Purple nuclear staining, indicative of PAH-DNA adducts, was found in esophageal squamous epithelium from these patients. Fig. 1C shows data for 1 patient. There is PAH-DNA adduct staining in the nuclei, and faint pink staining in the cytoplasm (Fig. 1C, left panel; nuclear AU 1.6). The nuclear staining was reduced when sequentially sectioned slides were simultaneously incubated with immunogen (BPDE-DNA)-absorbed serum (Fig. 1C, middle panel; nuclear AU 1.0) and completely absent when the sections were incubated with normal rabbit serum (Fig. 1C, right panel; nuclear AU 0.0).

Esophageal sections from 5 Linxian patients were each stained on four different occasions with BPDE-DNA and immunogen-absorbed antiserum, and comparable results were obtained with all four of the experiments (Table 1). Values for the ACIS determinations from one of these experiments, along with control esophageal tissue from a laboratory-housed monkey, are shown in Fig. 3A. Staining for monkey control #2 was similar when the anti-BPDE-DNA and the immunogen-absorbed anti-BPDE-DNA antisera were used (Fig. 3A shows representative quantification), indicating a lack of specific PAH-related DNA staining. When all four of the experiments were taken into account (Table 1), the nuclear PAH-DNA adduct staining intensities found in the group of samples from Linxian were significantly different ( $P = 0.046$ ; mixed effects model) from the average staining intensity found in monkey esophageal tissue samples.

When sequentially sectioned slides were simultaneously incubated with the anti-BPDE-DNA antiserum and the immunogen (BPDE-DNA)-absorbed antiserum there was a reduction of between 20 and 57% of the anti-PAH-DNA signal for human samples 1, 2, and 5 (Table 1). The positive PAH-DNA signal is presumed because of DNA adducts of several PAHs, as the anti-BPDE-DNA serum cross-reacts with DNA adducts of multiple carcinogenic PAHs (5). However, as the absorption was carried out only with BP-modified DNA it is not surprising

**Table 1** Values (mean  $\pm$  SE in AU) for nuclear PAH-DNA staining in human esophagus from Linxian, China, and the United States, as well as monkey esophagus

Source	Sample	Immunohistochemical staining—AU	
		BPDE-DNA antiserum	Absorbed BPDE-DNA antiserum
Linxian <sup>a</sup>	1	2.2 $\pm$ 0.4	1.8 $\pm$ 0.6
	2	2.3 $\pm$ 0.6	1.4 $\pm$ 0.5
	3	1.3 $\pm$ 0.4	0.8 $\pm$ 0.3
	4	0.7 $\pm$ 0.2	0.7 $\pm$ 0.2
	5	2.3 $\pm$ 0.6	1.4 $\pm$ 0.3
United States <sup>b</sup>	1	0.6	0.5
	2	0.1	0.1
	3	0.4	0.1
	4	0.1	0.1
	5	0.3	0.1
	6	0.1	0.1
Monkey <sup>c</sup>	1	1.0	No sample
	2	1.0 $\pm$ 0.2	0.6
	3	0.8	0.01
	4	0.2	0.2

<sup>a</sup> The complete set of samples was stained and counted on four separate occasions. Mean  $\pm$  SE for 5 samples stained with BPDE-DNA antiserum was 1.8  $\pm$  0.3 AU. Linxian mean value was statistically significant ( $P = 0.052$ ) by mixed effects model compared to the mean value for 4 unexposed monkeys<sup>(c)</sup> stained with BPDE-DNA antiserum.

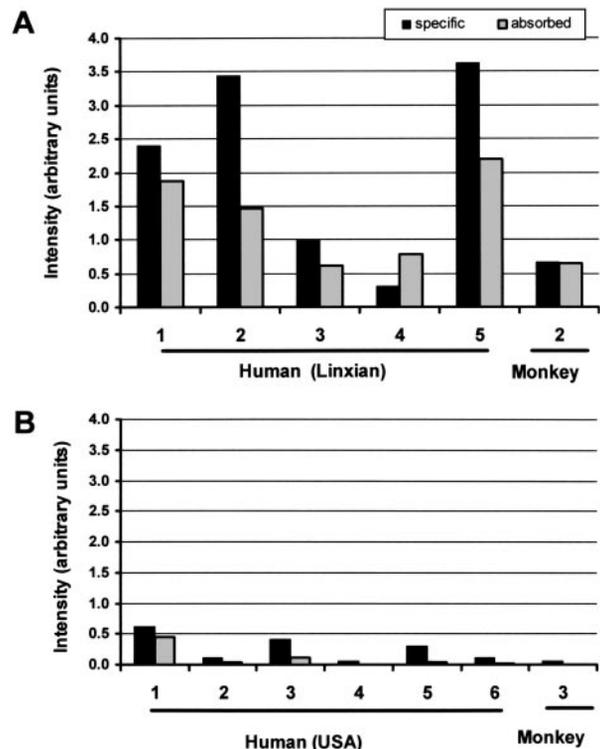
<sup>b</sup> Samples were stained and counted once. Mean  $\pm$  SE for 6 samples was 0.3  $\pm$  0.1 AU.

<sup>c</sup> Monkeys 1 and 4 were stained with BPDE-DNA antiserum and counted once with Linxian samples. Monkey 2 was stained with BPDE-DNA antiserum and counted three times with Linxian samples. Monkey 3 was stained with BPDE-DNA serum and counted twice with the Linxian samples. Monkey 3 was counted on two additional separate independent occasions with values of 0.05 and 1.08. This additional data was included in the statistical analysis. Mean  $\pm$  SE for 4 monkeys was 0.7  $\pm$  0.2 AU.

that the immunogen-absorbed serum did not reduce the signal to background levels. The data indicate that the 20–57% of nuclear staining signal in the Linxian samples is because of the presence of DNA adducts of BP.

Variance components models were fit to the PAH-DNA staining intensities from the repeated experiments with the Linxian samples to estimate the distinct sources of variability because of sample, experiment, and error. The sample, experiment, and error variance estimates for the BPDE-DNA antiserum intensities were 0.40, 0.42, and 0.57. The corresponding values for the absorbed BPDE-DNA antiserum intensities were 0.15, 0.40, and 0.22. The large experiment and error variances suggest that, in future studies, the assay should be repeated multiple (three or four) times, and the resulting staining intensities averaged to minimize this variation.

**PAH-DNA Adduct Staining in Human Esophageal Samples from the United States.** Sections of esophageal tissue obtained at autopsy in the United States from 6 individuals were incubated on one occasion with BPDE-DNA antiserum or immunogen-absorbed BPDE-DNA antiserum (Table 1; Fig. 3B shows representative quantification). None of the samples showed an increase in purple nuclear staining in the squamous cell layer compared with the unexposed monkey controls (Table 1). The mean  $\pm$  SE staining value for the 6 samples was 0.3  $\pm$  0.1 AU, and all were similar to the representative photos shown for 2 individuals in Fig. 1D (AU 0.6 and 0.1). The staining observed with these samples was similar to that seen with esophageal tissue from the 4 laboratory-housed monkeys (0.6  $\pm$  0.2 AU), 1 of which is shown in Fig. 1E (AU 0.8).



**Fig. 3.** A, representative quantitation by ACIS of the intensity of purple nuclear staining in the squamous cell layer of human esophageal tissue from Linxian and esophageal tissue from patas monkey number 2 after incubation with BPDE-DNA antiserum (■) or with BPDE-DNA-absorbed BPDE-DNA antiserum (□). B, representative quantitation by ACIS of the intensity of purple nuclear staining in the squamous cell layer of human esophageal tissue obtained at autopsy in the United States, and esophageal tissue from monkey number 3, stained as in A.

Among the tissues taken at autopsy, samples 4 and 5 were from smokers, but there was no indication that these individuals had increased levels of PAH-DNA adducts compared with the 4 nonsmokers.

## Discussion

In human esophagus from Linxian, China, a high-risk region for esophageal carcinoma, nuclear staining for PAH-DNA adducts was detectable in the squamous epithelium of esophageal sections incubated with BPDE-DNA antiserum. The nuclear localization was very clear, and quantitation by the ACIS imaging system showed that the human samples from Linxian had significantly increased staining compared with esophageal samples from laboratory-housed unexposed monkey controls. In addition, there was an absence of nuclear staining when sequential human sections were incubated with normal rabbit serum. Analysis of the sources of variability in these experiments suggests that, in future studies, staining should be repeated three or four times to minimize variation.

An additional indication of PAH-DNA adduct formation in the samples from Linxian was obtained through the use of immunogen-absorbed antiserum. This critical control indicates that staining is specific by removing the antibodies elicited against the immunogen in question and demonstrating that the staining disappears. In the case of cultured cells exposed only to BP, the BPDE-DNA signal was removed completely when

the immunogen-absorbed samples were used. In the case of the Linxian samples, the story is more complex. Whereas the original immunogen was BPDE-modified DNA, the anti-BPDE-DNA antibody cross-reacts with DNA samples modified by several different structurally similar carcinogenic PAHs (5). Thus, the signal recognized in the human samples may reflect multiple different PAH-DNA adducts. When the BPDE-DNA antiserum was absorbed only with BPDE-DNA, the signal in the Linxian samples was reduced 20–57%, suggesting that a fraction of the positive staining was because of BPDE-DNA adducts and that the remainder might be because of other PAH-DNA adducts (5), incomplete absorption, or nonspecific background. It has not been possible to use the immunoassay to determine the affinities of the residual antibodies recognizing PAH-DNA adducts that are not absorbed when BPDE-modified DNA is used to absorb the specific antiserum.

Previous studies in the literature have described measurement of DNA adducts in human samples by semiquantitation of nuclear staining intensity (10–13). Most of these investigations have involved detection of gray to black color produced by 3,3'-diaminobenzidine, and examination of 50–100 nuclei. Often the background levels have been high, and it has been difficult to demonstrate linearity with dose over a broad concentration range. The ACIS methodology described here has several aspects that make it particularly advantageous for determining the extent of DNA adduct formation in tissue nuclei. The system can count several thousand nuclei within minutes, which increases the statistical power, and the analysis of color by wavelength allows the instrument to differentiate the color of interest resulting in a more specific signal and lower backgrounds. With the ACIS, the histological reconstruction feature of the software makes it possible to outline specific areas of the section, allowing one to count only nuclei in the squamous epithelium. Using cultured cells exposed to BPDE we found nuclear BPDE-DNA adduct linearity over a 20-fold concentration range, and the value for unexposed cells was 156-times lower than the value for the highest exposed sample (Fig. 1B). An additional strength is the high correlation between dosimetry for BPDE-DNA adducts measured by ACIS and by the quantitative immunoassay, the BPDE-DNA CIA.

Arguably the greatest challenge and achievement of the current application of the ACIS system has been the determination of PAH-DNA adducts in archived human esophageal tissue from Linxian, China. The fact that 4 of 5 samples from China gave a positive signal, whereas autopsy material from 6 smokers and nonsmokers in the United States did not, suggests that the Chinese may be exposed to higher overall levels of PAHs, that they may metabolize these agents differently than Caucasians, and/or that they are less efficient in repairing PAH-DNA damage once it is formed. Roth *et al.* (2) showed that the BP content of cooked and uncooked food (3.1–13.8 ng BP/g staple food) taken from homes in Linxian are comparable with or higher than char-broiled meat in the United States that has been cooked to an excessive degree (14). Whereas most individuals in the United States consume such meat infrequently, the individuals in Linxian are more consistently ingesting food containing high levels of PAHs. Taken together, this would support the role of higher PAH intake in individuals in Linxian.

The esophageal samples obtained at autopsy in the United States came from smokers and nonsmokers. We also stained livers and lungs from the same individuals (results not shown), and were able to identify correctly the smoking status of each

individual using the other tissues, as the smokers gave positive staining in both liver and lung tissues. The data will be presented elsewhere.

DNA adduct formation in human tissues is an indicator of chemical exposure and a presumed indicator of cancer risk. Whether or not cancer risk can be predicted by DNA adduct formation occurring years before tumor diagnosis is a central question in molecular cancer epidemiology. For exposure to aflatoxin B1 and liver cancer, adduct excretion in urine is associated with a 9-fold increased risk of liver cancer (15, 16). The Linxian samples provide a unique opportunity to address this question in an epidemiological study where DNA adduct measurements can be performed in the target tissue for tumorigenicity. Our finding of PAH-DNA adducts in esophageal samples taken 7 years ago in Linxian, China, support the hypothesis that chronic ingestion of foods with a high PAH content may induce potentially mutagenic DNA damage that may be associated with the elevated esophageal cancer risk observed in this region. Recently we have been successful in finding positive PAH-DNA staining in esophageal biopsies from Linxian taken in 1985, suggesting that PAH-DNA adducts are a sufficiently persistent indicator of PAH exposure to be applicable in large studies exploring an association between esophageal PAH-DNA adduct formation and esophageal cancer risk.

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#### References

1. Yang, C. S. Research on esophageal cancer in China: a review. *Cancer Res.*, 40: 2633–2644, 1980.
2. Roth, M. J., Strickland, K. L., Wang, G. Q., Rothman, N., Greenberg, A., and Dawsey, S. M. 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, 1998.
3. Roth, M. J., Qiao, Y.-L., Rothman, N., Tangrea, J. A., Dawsey, S. M., Wang, G. Q., Cho, S.-H., Kang, D. H., Taylor, P. R., and Strickland, P. T. High urine 1-hydroxypyrene glucuronide concentrations in Linxian, China, an area of high risk for squamous oesophageal cancer. *Biomarkers*, 6: 381–386, 2001.
4. Poirier, M. C., Santella, R., Weinstein, I. B., Grunberger, D., and Yuspa, S. H. Quantitation of benzo(a)pyrene-deoxyguanosine adducts by radioimmunoassay. *Cancer Res.*, 40: 412–416, 1980.
5. Weston, A., Manchester, D. K., Poirier, M. C., Choi, J. S., Trivers, G. E., Mann, D. L., and Harris, C. C. Derivative fluorescence spectral analysis of polycyclic aromatic hydrocarbon-DNA adducts in human placenta. *Chem. Res. Toxicol.*, 2: 104–108, 1989.
6. Woodworth, C. D., Bowden, P. E., Doniger, J., Pirisi, L., Barnes, W., Lancaster, W. D., and DiPaolo, J. A. Characterization of normal human exocervical epithelial cells immortalized *in vitro* by papillomavirus types 16 and 18 DNA. *Cancer Res.*, 48: 4620–4628, 1988.
7. Divi, R. L., Dragan, Y. P., Pitot, H. C., and Poirier, M. C. Immunohistochemical localization and semi-quantitation of hepatic tamoxifen-DNA adducts in rats exposed orally to tamoxifen. *Carcinogenesis (Lond.)*, 22: 1693–1699, 2001.
8. Divi, R. L., Osborne, M. R., Hewer, A., Phillips, D. H., and Poirier, M. C. Tamoxifen-DNA adduct formation in rat liver determined by immunoassay and 32P-postlabeling. *Cancer Res.*, 59: 4829–4833, 1999.
9. Pinheiro, J. C., and Bates, D. M. *Mixed-Effects Models in S and S-plus*. New York: Springer Verlag, 2000.
10. Blommaert, F. A., Michael, C., Terheggen, P. M., Muggia, F. M., Kortes, V., Schornagel, J. H., Hart, A. A., and den Engelse, L. Drug-induced DNA modification in buccal cells of cancer patients receiving carboplatin and cisplatin combination chemotherapy, as determined by an immunocytochemical method: interindividual variation and correlation with disease response. *Cancer Res.*, 53: 5669–5675, 1993.

11. Motykiewicz, G., Malusecka, E., Grzybowska, E., Chorazy, M., Zhang, Y. J., Perera, F. P., and Santella, R. M. Immunohistochemical quantitation of polycyclic aromatic hydrocarbon-DNA adducts in human lymphocytes. *Cancer Res.*, 55: 1417-1422, 1995.
12. Zhang, Y. J., Hsu, T. M., and Santella, R. M. Immunoperoxidase detection of polycyclic aromatic hydrocarbon-DNA adducts in oral mucosa cells of smokers and nonsmokers. *Cancer Epidemiol. Biomark. Prev.*, 4: 133-138, 1995.
13. Zhang, Y. J., Weksler, B. B., Wang, L., Schwartz, J., and Santella, R. M. Immunohistochemical detection of polycyclic aromatic hydrocarbon-DNA damage in human blood vessels of smokers and non-smokers. *Atherosclerosis*, 140: 325-331, 1998.
14. Rothman, N., Poirier, M. C., Baser, M. E., Hansen, J. A., Gentile, C., Bowman, E. D., and Strickland, P. T. Formation of polycyclic aromatic hydrocarbon-DNA adducts in peripheral white blood cells during consumption of charcoal-broiled beef. *Carcinogenesis (Lond.)*, 11: 1241-1243, 1990.
15. Ross, R. K., Yuan, J. M., Yu, M. C., Wogan, G. N., Qian, G. S., Tu, J. T., Groopman, J. D., Gao, Y. T., and Henderson, B. E. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet*, 339: 943-946, 1992.
16. Qian, G. S., Ross, R. K., Yu, M. C., Yuan, J. M., Gao, Y. T., Henderson, B. E., Wogan, G. N., and Groopman, J. D. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai. People's Republic of China. *Cancer Epidemiol. Biomark. Prev.*, 3: 3-10, 1994.