Original article

Scand J Work Environ Health 1991;17(3):190-194

doi:10.5271/sjweh.1711

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This article in PubMed: www.ncbi.nlm.nih.gov/pubmed/2068558

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Immunologic measurement of polycyclic aromatic hydrocarbon-albumin adducts in foundry workers and roofers

by Byung Mu Lee, DrPH,[1,2] Yin Baoyun, MS,[2] Robin Herbert, MD,[3] Kari Hemminki, MD,[4] Frederica P Perera, DrPH,[2] Regina M Santella, PhD[2]

LEE BM, BAOYUN Y, HERBERT R, HEMMINKI K, PERERA FP, SANTELLA RM. Immunologic measurement of polycyclic aromatic hydrocarbon-albumin adducts in foundry workers and roofers. *Scand J Work Environ Health* 1991:17:190—4. Roofers and iron foundry workers with high exposure to polycyclic aromatic hydrocarbons (PAH) were monitored for levels of covalent PAH serum albumin adducts, quantitated in enzymatically digested samples by a sensitive competitive enzyme-linked immunosorbent assay. Albumin adducts were higher in the foundry workers (5.22 fmol/μg, 0.314 mmol/mol) than in the reference group (4.07 fmol/μg, 0.245 mmol/mol), but only of borderline significance probably due to the small sample size. In a subset of foundry workers, a significant difference in adduct levels was observed for samples collected immediately after vacation and after six weeks of workplace exposure. The roofers also showed higher levels of adducts (5.19 fmol/μg, 0.312 mmol/mol) than their reference group (3.28 fmol/μg, 0.197 mmol/mol). These results demonstrate the feasibility of PAH protein adduct measurement as a marker of human exposure to this class of chemicals.

Key terms: albumin adducts, benzo[a]pyrene, enzyme-linked immunosorbent assay, foundry workers, monoclonal antibodies, roofers.

Foundry workers and roofers exposed to high levels of benzo[a]pyrene (BaP) and several other polycyclic aromatic hydrocarbons (PAH) have served as model populations for validating adduct measurement as a marker of biologically effective dose. Sensitive enzyme-linked immunosorbent assay (ELISA) or ^{32}P-post-labeling has been used to determine PAH-deoxyribonucleic acid (DNA) adduct levels in these populations (1—4). PAH and other carcinogens also react, however, with proteins to form covalent adducts. Several methods have been developed for quantitating hemoglobin or albumin adduct levels as a surrogate for DNA adduct determination. These methods include immunoassays, gas chromatography-mass spectroscopy (GC-MS), and fluorescence spectroscopy. (See reference 5 for a review.) For example, immunoassays have detected elevated levels of aflatoxin-B1-albumin from individuals living in regions with high exposure (6—7). Ethylene oxide (8) and 4-aminobiphenyl (9) globin adducts, measured by GC-MS, have been shown to be higher in smokers than in nonsmokers.

We have previously developed a monoclonal antibody, 8E11, from animals immunized with benzo[a]pyrene diol epoxide I-modified guanosine (BaPDE-I-G) coupled to bovine serum albumin (10). This antibody recognizes deoxyguanosine, DNA, and protein modified by BaPDE-I, as well as BaPDE-I-tetrols and several other DNA modified by PAH diol epoxides (11). Sensitive quantitation of protein adducts could not be carried out directly due to the low antibody affinity for the adduct in intact protein. This low affinity is probably due to the burying of the adduct in hydrophobic regions of the protein. However, enzymatic digestion of protein to peptides and amino acids before ELISA resulted in a three- to fourfold increase in sensitivity and allowed the quantitative detection of adducts in globin isolated from animals treated with radiolabeled BaP (11). These animal studies also indicated that adduct levels were about tenfold higher in albumin than in globin. For this reason, our initial work on human samples has been on albumin isolated from workers occupationally exposed to PAH.

Materials and methods

Chemicals

BaPDE-I (7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene) and the other BaP metabolites were obtained from the Cancer Research Program of the National Cancer Institute, Division of Cancer Cause and Prevention, Bethesda, Maryland. BaPDE-I-tetrols were produced by the aqueous hydrolysis of racemic BaPDE-I and were a mixture of *cis* and *trans*

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opened tetrols. DNA modified by chrysene-1,2-diol-3,4-epoxide and benz[a]anthracene-3,4-diol-1,2 epoxide were obtained from Dr A Jeffrey, Columbia University, New York, New York. Pyrene, 1-nitropyrene, 1-aminopyrene, 7,12-dimethylbenz[a]anthracene, amino acids, goat antimouse immunoglobulin G-alkaline phosphatase, protease coupled to carboxymethylcellulose, and p-nitrophenyl phosphate (Sigma 104) were purchased from Sigma, St Louis, Missouri. 1-Hydroxypyrene was purchased from Molecular Probes, Eugene, Oregon. Fetal calf serum (FCS) was purchased from Hyclone Laboratories, Logan, Utah. A BCA (bicinchoninic acid) protein assay kit was purchased from Pierce Chemical Co, Rockford, Illinois.

Human samples

Stored plasma samples from previous studies on DNA adduct levels in white blood cells were used for albumin isolation. One group comprised Finnish iron foundry workers and another was a reference group from different parts of Finland with no occupational exposure to PAH. The workers were classified as having high (>0.2 $\mu g/m^3$), medium (0.05—0.2 $\mu g/m^3$), or low (<0.05 $\mu g/m^3$) exposure to BaP according to historical data and an industrial hygienist evaluation of job description. From a subset of workers, blood was collected immediately after their one-month vacation and again after six weeks of exposure. Details of this population have been reported earlier (4). Because of the small number of samples available, all the workers were grouped together for the statistical analysis. The second group was American roofers and nonoccupationally exposed referents matched for age, gender, and smoking status (1). The exposure to BaP in the air of the exposed subjects ranged from 0.60 to 1.30 $\mu g/m^3$.

Serum albumin was prepared from plasma by affinity chromatography on Reactive Blue 2-Sepharose CL-4B and dialyzed against phosphate buffered saline (PBS) (12). Protein concentrations were determined with a BCA protein assay kit. Samples (10 mg/ml) were enzymatically digested with insoluble protease coupled to carboxymethylcellulose at 37°C for 24 h with rotation, then centrifuged to remove enzyme, and assayed directly with ELISA.

Competitive enzyme-linked immunosorbent assay

Competitive ELISA with monoclonal antibody 8E11 was carried out essentially as described earlier (13). Polystyrene 96 microwell plates (Immulon 2, Dynatech Laboratory, Alexandria, Virginia) were coated with 5 ng of BaPDE-I-DNA in 100 µl of PBS by drying. The plates were washed with PBS containing 0.05 % Tween 20 (polyoxyethylenesorbitan monolaurate) by an automatic plate washer (Flow Multiwash, Mclean, Virginia) set for three 200-µl washes. This wash step followed each incubation at 37°C. Wells were incubated with 200 µl of 1 % FCS in PBS-Tween for 1 h to minimize nonspecific binding of protein to the plate. To test antibody cross-reactivity, competitors were dissolved in methanol for concentration determination by absorbance and then diluted with methanol and PBS such that the final concentration of methanol per well was <5—10 %. For the standard curve, serial dilutions of tetrols from 25—2500 fmol/50 µl were mixed with 50 µl of antibody 8E11 (diluted 1:30 000) before addition to the wells. Digested albumin samples were assayed at 500 µg/50 µl. After incubation for 1.5 h and washing of the plates with 0.05 % Tween-PBS, 100 µl of goat antimouse immunoglobulin G-alkaline phosphatase (diluted 1:500) was added, and the plate was incubated for 1.5 h. Finally, 100 µl of p-nitrophenyl phosphate in 1 M diethanolamine (pH 8.6) was added. Absorbance at 405 nm was measured with a Flow Multiscan MC microplate reader. Samples were run in duplicate or triplicate assays with duplicate wells. The values are the mean of all assays and expressed as femtomole equivalents of BaPDE-I adducts. Samples with less than 20 % inhibition in the ELISA were considered nondetectable and given a value of 1 fmol/µg for purposes of the statistical analysis. For the comparison of the data between the foundry workers and their referents the t-test was used. Data for the matched roofers and referents, as well as those for the foundry workers at work and after vacation, were analyzed by the paired t-test.

Results

Since humans are exposed to BaP in complex mixtures of multiple PAH, albumin samples may contain a number of PAH adducts. The cross-reactivity of antibody 8E11 with a range of other aromatic compounds was therefore tested. As reported previously (11), 8E11 recognizes BaPDE-I-DNA adducts and BaPDE-I-protein adducts in enzymatically digested samples and BaPDE-I-tetrols with about equal sensitivity (table 1). There is also significant cross-reactivity with a number of BaP metabolites with the greatest sensitivity for metabolites containing multiple hydroxide substituents. The DNA adducts of other PAH diol epoxides are also recognized, and this occurrence suggests that the protein adducts of these compounds will also be recognized. While significant cross-reactivity was seen with several other PAH, including pyrene, 1-hydroxypyrene and 1-nitropyrene, there was no cross-reactivity at the highest concentration of 7,12-dimethylbenz[a]anthracene, anthracene, benzene, phenol, or naphthalene tested. Since protein digests were to be assayed directly, cross-reactivity of 8E11 with several aromatic amino acids was tested. There was no cross-reactivity with phenylalanine, tyrosine, or tryptophan at the highest concentration tested.

Albumin was isolated from the plasma of Finnish foundry workers, American roofers, and referents and

digested enzymatically with insoluble protease to peptides and amino acids before analysis by competitive ELISA. The mean adduct levels increased for the foundry workers with estimated exposure [5.08 (low), 5.14 (medium), and 5.80 (high) fmol BaPDE-I-tetrol/μg]. However, because of the small number of samples in each group, the values for all of the exposure groups were combined for the statistical analysis. As shown in table 2, the mean adduct levels for the foundry workers (5.22 fmol/μg, 0.314 mmol/mol) were elevated compared with those of the referents (4.07 fmol/μg, 0.245 mmol/mol, $0.05 < P < 0.1$). From a subset of workers samples were obtained after a one-month vacation and again after six weeks on the job. A significant increase in the adduct levels was found in workers at work (5.22 fmol/μg, 0.314 mmol/mol) compared with the corresponding values after vacation (4.18 fmol/μg, 0.251 mmol/mol, $0.025 < P < 0.05$). The DNA adducts in the white blood cells of these subjects had been analyzed previously by competitive ELISA with a polyclonal antibody recognizing BaPDE and related diol epoxide DNA adducts (4). There was a weak correlation ($r = 0.32$, $0.05 < P < 0.1$) between the level of PAH protein and DNA adducts determined by the two ELISA determinations (4). Roofers also showed elevated adduct levels (5.19 fmol/μg, 0.312 mmol/mol) compared with those of the referents (3.28 fmol/μg, 0.197 mmol/mol, $0.05 < P < 0.1$), but the difference was of borderline significance. DNA adducts, previously measured for these subjects by ^{32}P-postlabeling (1), had a weak correlation ($r = 0.42$, $0.05 < P < 0.1$) with the PAH protein adducts.

Table 1. Competitive inhibition of antibody 8E11 binding to BaPDE-I-BSA. (BaP = benzo[a]pyrene, BaPDE-I = 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, BSA = bovine serum albumin, DNA = deoxyribonucleic acid)

Competitors	Femtomole causing 50 % inhibition
BaP-9,10-diol	150
Chrysene diol epoxide-DNA	160
BaP-7,8-diol	250
BaPDE-I-tetrol	350
BaPDE-I-DNA	350
BaPDE-I-BSA digested	400
Benz[a]anthracene diol epoxide-DNA	1350
BaPDE-I-BSA nondigested	1450
1-OH-pyrene	3400
BaP	6000
1-Nitropyrene	16000
Pyrene	16200
4-OH-BaP	42700
1-Aminopyrene	70000
5-OH-BaP	$>1 \times 10^5$
Dimethylbenz[a]anthracene	$>1 \times 10^6$
Anthracene	$>1 \times 10^6$
Naphthalene	$>1 \times 10^6$
Benzene	$>1 \times 10^6$
Phenol	$>1 \times 10^6$
Phenylalanine	$>3.0 \times 10^8$
Tyrosine	$>2.8 \times 10^8$
Tryptophan	$>2.5 \times 10^8$

Discussion

Monoclonal antibody 8E11 recognizes the DNA and protein adducts of BaPDE-I and a number of BaP metabolites, including 7,8,9,10-tetrol and 7,8- and 9,10-diols (table 1). In addition, it cross-reacts with several other PAH and thus will probably recognize a number of PAH protein adducts but with differing affinities. Studies on animals treated with [^{3}H] BaP demonstrated that the ELISA gave quantitative recovery of levels determined by radioactivity measurements (11). However, humans are exposed to BaP as part of a complex mixture of PAH, and multiple adducts may be present. Since the identity of the adducts being measured in the human samples cannot be determined, the selection of an appropriate standard for absolute quantitation is not possible. Data are therefore expressed as femtomole equivalents of BaPDE-I tetrols which would cause a similar inhibition in the assay. These studies demonstrated the feasibility of measuring PAH-albumin adducts in plasma isolated from individuals with various types of occupational and environmental exposure. Adducts in the foundry workers and roofers were elevated compared with the corresponding values of referents and increased in foundry workers six weeks after they returned to work from a one-month vacation. As in studies on DNA, adducts were also detectable in the referents and probably reflect ubiquitous exposure to PAH. Although there was no cross-reactivity with individual amino acids, it is still possible that some of the high background level of antigenicity in the referents may be due to some nonspecific binding.

Table 2. Mean PAH-albumin adduct levels in roofers, foundry workers, and their respective referents (PAH = polycyclic aromatic hydrocarbons, BaPDE-I = 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene)

	N	fmol/μg[a]		mmol/mol[a]	
		Mean	SD	Mean	SD
Foundry workers at work	13	5.22	1.49	0.314	0.089
Foundry workers after one month's vacation	12	4.18	1.43	0.251	0.086
Referents	10	4.07	2.14	0.245	0.128
Roofers	12	5.19	4.02	0.312	0.242
Referents	12	3.28	2.07	0.197	0.124

[a] Values are expressed as mole equivalents of BaPDE-I-tetrols per microgram or mole of albumin.

There was a weak correlation between albumin adducts and DNA adducts in white blood cells previously analyzed for both roofers and foundry workers. For the Finnish foundry workers, the DNA adduct levels were determined by competitive ELISA with a polyclonal antibody which recognizes both BaPDE-I-DNA and the DNA adducts of several other PAH diol epoxides. The foundry workers had significantly elevated adduct levels compared with the referents (4). The mean adduct level of the referents was $0.22/10^7$ nucleotides, while that for the high, medium, and low exposure was 5.0, 2.1, and $0.80/10^7$, respectively. Thus there was an approximately four- to twentyfold difference between the workers and the referents. This difference can be contrasted with the 1.3-fold increase seen in the albumin adduct levels of workers in comparison with the levels of the referents (table 2). Similarly, in the roofers' studies there was an approximately tenfold difference between the workers and the referents for the DNA adducts in white blood cells, as measured by ^{32}P-postlabeling (1), but only a 1.6-fold difference in albumin adducts (table 2).

The recency of exposure measurable by protein and DNA adduct quantitation may be different. Albumin has a half-time of 21 d, while white blood cells have life-spans ranging from hours to months to years. In addition, there is an active repair system in white blood cells but no repair of proteins. If we assume that the adduct levels before the one-month vacation were similar to those determined after six weeks of reexposure, the decrease in adduct levels in albumin after the vacation (table 2) was probably due to protein turnover, while that determined previously in DNA of white blood cells (4) may have resulted from a combination of repair and cell turnover. Previous studies on mice treated with radiolabeled BaP indicated a strong correlation between lung DNA and serum albumin adduct levels over several doses (11). These studies were carried out after a single exposure to BaP, while workers are chronically exposed, and adducts may accumulate over the life-span of the protein. The relationship between albumin and DNA adduct levels needs further investigation before it can be determined which will be a better marker of risk for cancer development. However, smaller amounts of blood are required by ELISA for the analysis of adducts on albumin (1—2 ml) than DNA (35 ml). For this reason, measurement of albumin adducts may still be useful as a marker of exposure and provide information complimentary to that obtained from DNA adduct measurement. BaPDE-I-protein adducts have also been measured on human hemoglobin (14—15). BaPDE-I-tetrols were released from hemoglobin by either acid treatment (14) or enzyme digestion (15) and isolated by high-pressure liquid chromatography (14) or immunoaffinity chromatography plus high-pressure liquid chromatography (15). Synchronous fluorescence spectroscopy was utilized in one study (14) for the quantitation of BaPDE-I-tetrol levels in the range of 1 ng BaPDE/gm hemoglobin (3 amol/μg or 0.18 μmol/mol). GC-MS was utilized in the other study and gave values between 5 and 17 amol/μg (15). In addition, another group has recently utilized our antibody 8E11 to analyze PAH albumin adducts after acid treatment of the protein followed by C18 SepPak solid phase extraction (16). They reported values of 240 amol/μg for foundry workers compared with 72.3 amol/μg for the referents.

There are large differences in the absolute levels of adducts reported by the various studies, and these differences may be due to differences between hemoglobin and albumin and to differences in the methods used for quantitation. Only BaPDE-I adducts were measured in those studies using material isolated by high-pressure liquid chromatography, while ELISA may be measuring multiple PAH adducts. There are also quantitative differences between the two studies utilizing antibody 8E11 (this report and reference 16). The lower level of the adducts determined by immunoassay after acid treatment and SepPak isolation may be due to the incomplete release of PAH by acid treatment and/or incomplete recovery by the extraction procedure. In contrast, the method reported in the present study, direct analysis after enzyme digestion, may give higher values because of nonspecific antibody binding to the protein digest. In addition, since the adducts being measured are unknown, utilization of BaPDE-I-tetrols in the standard curve in both immunologic studies could lead to overestimation or underestimation, depending on whether the actual adduct(s) have better or poorer affinity for the antibody than BaPDE-I-tetrols. In summary, these studies demonstrate the feasibility of albumin adduct detection by simplified ELISA, which can easily be carried out on large numbers of samples. No matter what the absolute level of adducts, this type of study can provide important information on human exposure to environmental and occupational carcinogens. Additional studies will be necessary to understand the relationship between albumin, hemoglobin, and DNA adducts.

Acknowledgments

This work was supported by grants from the National Institute of Health (CA21111, OH0262)2 and a gift from the Lucille P Markey Charitable Trust.

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Received for publication: 3 July 1990