Immunohistochemical Quantitation of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Human Lymphocytes

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Abstract

The formation of polycyclic aromatic hydrocarbon-DNA adducts was studied in peripheral blood lymphocytes obtained from men with occupational and environmental exposure. Subjects included coke factory workers, residents from the vicinity of the cokery, and rural region inhabitants (16 individuals in each exposure group). Adducts were determined by immunohistochemical analysis using a polyclonal antiserum recognizing benzo(a)pyrene and related polycyclic aromatic hydrocarbon diol epoxide-DNA adducts, a biotinylated secondary antiseraum, and streptavidin-conjugated FITC. Propidium iodide was used to quantitate nuclear DNA. Dual fluorescence intensities were simultaneously measured with a Zeiss Axiovert microscope and a Bio-Rad MRC-600 argon laser scanning confocal attachment. Adducts were significantly elevated ($P < 0.001$) in both occupational and environmental groups, as compared to the rural control group by Mann-Whitney $U$ test. The distribution of the data indicated the existence of cells with relatively higher adduct levels. The percentages of these so called "high-adduct-level cells" were $13.6$, $11.5$, and $3.7$ in cokery workers, environmentally exposed individuals, and rural controls, respectively. The immunohistochemical method allows visualization and relative quantitation of polycyclic aromatic hydrocarbon-DNA adducts in individual lymphocytes. It requires a much smaller amount of blood than the previously used $^{32}$P-postlabeling and ELISA methods, which used isolated bulk DNA. It can also be used for adduct quantitation in biopsy material. The results of this pilot study indicate that this technique is a promising addition to biomonitoring studies.

Introduction

PAH are common environmental pollutants, and their uptake has been causally associated with the induction of cancer. As a result of coal burning and/or processing, PAH are among the most prevalent mutagenic air pollutants in Silesia, Poland (1). The highest levels are found in the occupational environment, i.e., cok-oven plants ($0.12$--$89.58$ μg of B[a]P/m$^3$ of air). Chronic exposure to lower levels of PAH ($1.0$--$55.3$ ng B[a]P/m$^3$) occurs in the general environment of Silesia and mainly originates from coal-based heavy industry, power plants, and individual domestic heating, as well as automobile traffic.

Among the known molecular effects of PAH is the ability to bind covalently to DNA, giving rise to PAH-DNA adducts. A number of methods have been developed for quantitation of adducts, resulting from occupational or environmental exposure to PAH (reviewed in Refs. 2--5). They include $^{32}$P-postlabeling, gas chromatography/mass spectrometry, fluorescence, and immunosays (ELISA) using specific antisera. Most methods are not applicable to small amounts of blood or tissue, a major limitation in biomonitoring studies.

Here we report on an immunofluorescence technique for visualization and quantitation of PAH-DNA adducts in individual human lymphocytes. The formation of BPDE and related PAH-DNA adducts was studied in lymphocytes obtained from coke-factory workers in Silesia, Poland, residents from the vicinity of the cokery, and inhabitants of a rural region in Poland with PAH levels estimated to be 10-fold lower than in Silesia. This study builds upon our earlier results on aromatic adducts and PAH-DNA adducts measured on isolated blood DNA by $^{32}$P-postlabeling and ELISA (12). Both methods showed DNA adducts to be significantly elevated in the Silesian groups (cokery workers and subjects living in the surrounding area) compared to rural controls, with only a slight difference between cokery workers and local controls. In addition to DNA adducts, chromosomal aberrations and sister chromatid exchanges were significantly elevated in occupationally and environmentally exposed residents of Silesia compared to controls (13, 14).

Materials and Methods

Chemicals. BPDE (444 mCi/mmol) was supplied by Chemsyn Science Laboratories (Lenexa, KS). RNase, proteinase K, PI, Histopaque-1077, and 1,4-phenylenediamine were purchased from Sigma Chemical Co. (St. Louis, MO). Biotinylated goat anti-rabbit IgG and fluorescein-conjugated streptavidin was from Boehringer Mannheim (Indianapolis, IN). FCS was obtained from HyClone Laboratories, Inc. (Logan, UT), and DMEM medium was purchased from GIBCO-BRL Life Technologies (Gaithersburg, MD). Tissue culture chambers were supplied by NUNC (Naperville, IL).

Subjects and Blood Samples. Subjects included 16 healthy males from each exposure group as well as 16 rural controls. Donors were selected on the basis of their occupation; environmental and control groups did not include cokery workers or any other workers connected with the processing of coal. Additional information on cigarette smoking, diet, X-ray exposure, current medication, and family history of cancer was also collected. Samples were taken in September 1992 and 1993, which is considered the summer season. Therefore, the exposure was not influenced by the additional impact of PAH from domestic heating in the winter.

Treatment of Cells with BPDE. The immunostaining method was established, and the measurements were calibrated on cultured C3H 10T½ cells treated with BPDE. Cells were plated in eight-chambered slides and maintained in DMEM medium supplemented with 10% FCS. After 24 h, cells were
exposed to 0, 0.1, 1, 5, 10, and 20 μg/ml of BPDE (2 h in the dark), washed 2X with PBS, and fixed in 70% methanol at room temperature. BPDE was dissolved in DMSO with a final concentration of 0.05%. Two chambers were treated with DMSO alone and two with 10 μg/ml BPDE for immunostaining controls (see below).

**Immunohistochemical Detection of PAH-DNA Adducts.** Mononuclear cells were isolated from blood by centrifuging over Histopaque. Cell smears were prepared on microscope slides and, after air drying, fixed in methanol for 3 min (~20°C), followed by dipping in acetone (~20°C). Cells were then treated with RNase (100 μg/ml) for 1 h at 37°C and with proteinase K (10 μg/ml) for 10 min at room temperature. DNA was denatured with 4 N HCl (10 min at room temperature), and the pH was adjusted with 50 mM Tris-maleate for 5 min at room temperature. Nonspecific antisera binding was blocked by incubating slides with 10% normal goat serum at 37°C for 45 min. A newly developed primary rabbit polyclonal anti-BPDE-I-DNA antiserum (#1) was used at a 1:50 dilution, and slides were incubated at 37°C for 45 min. This antiserum was generated as described previously (15) and has cross-reactivity with structurally related PAH diol epoxide-DNA adducts similar to polyclonal antiserum #29, which was used in previous immunofluorescence studies (16). Primary antiserum was followed by biotinylated anti-rabbit IgG (1:200 dilution at 37°C for 45 min) and streptavidin-FITC (5 μg/ml at 37°C for 45 min). Nuclear DNA was counterstained with PI (1 μg/ml at room temperature for 45 s). To reduce fading of fluorescence, slides were mounted in PBS-glycerol (1:2) containing 0.1% of 1,4-phenylenediamine (pH 8.5).

For the 8-chambered slides, duplicate DMSO, and 10 μg BPDE/ml wells were incubated with or without the primary antiserum. Additional controls included preabsorption of the antiserum with BPDE-I-DNA (1 μg/ml; 20 min at room temperature) or treatment of slides with DNase (100 μg/ml for 1 h at 37°C) before staining.

**Measurement of Adducts.** Measurement of FITC and PI fluorescence intensities in 50 randomly selected cells per subject was performed with a Zeiss Axiovert microscope and a Bio-Rad MRC-600 argon laser scanning confocal attachment using A1 and A2 filter cubes. After defining the nuclear zone by PI pattern, fluorescence was quantified from a fixed area within the nucleus (boxes of 48 and 73 μm² for lymphocytes and C3H 10T½ cells, respectively), as well as from an adjacent area by the Bio-Rad image processing software. Background fluorescence was subtracted from the measured FITC and PI nuclear fluorescence, and the FITC:PI ratio calculated for each nucleus. To standardize the assay for possible day-to-day variations, each batch of seven human samples was assayed together with one eight-chambered slide of C3H 10T½ cells.

**Statistical Analysis.** Both descriptive statistics and frequency histograms indicated that the data were not normally distributed. Therefore, the statistical significance of the difference between each exposure group was tested by
immunohistochemistry of PAH-DNA adducts

Results

To develop the immunofluorescence technique, mouse fibroblast C3H 10T½ cells were treated in vitro with increasing doses of BPDE. Adduct-specific nuclear staining (FITC) was observed in treated cells (Fig. 1a) but not untreated controls (Fig. 1c). Both treated and untreated cells were positively stained with PI (Fig. 1, b and d). As reported previously (16), nuclear FITC staining was negative when the primary antiserum was omitted, cells were treated with DNase before staining, or primary antiserum was preabsorbed with BPDE-I-DNA before use (data not shown).

To calibrate the immunofluorescence method, dose-response curves were generated by measurement of FITC and PI in 50 randomly selected cells at each dose. Fig. 2 shows the mean FITC:PI ratio of three independent stainings of cells treated at the same time with five doses of BPDE. A dose dependency was observed in each experiment; however, some variability between stainings was noted. A cytotoxic effect was observed only at the highest dose (20 μg/ml) of BPDE.

The immunofluorescence method was then applied to the detection of adducts in mononuclear cells of subjects with varying exposure to PAH. Characteristic FITC and PI fluorescence labeling of lymphocytes from a cokery worker are given in Fig. 3, a and b, respectively. Strong nuclear FITC staining was observed. Less intense FITC staining was observed for a nonoccupationally exposed Silesian inhabitant (Fig. 3c) and for a subject living in the control region (Fig. 3e). The intensity of FITC staining increased with exposure, while PI staining remained stable (Fig. 3, b, d, and f). FITC and PI fluorescence was quantitated in a total of 50 randomly selected cells/subject, and the FITC:PI ratio was calculated. The data on 16 subjects/group are summarized in Table 1. Adducts were significantly elevated in both the occupationally and environmentally exposed groups as compared to control subjects (Table 1). The difference between the two Silesian-exposed groups was not statistically significant. This is in agreement with our previous results in which only minor differences in aromatic and PAH-DNA adducts were detected by postlabeling or ELISA on DNA isolated from total WBC of cokery workers and environmental controls (12).

In this prior study, mean PAH-DNA adducts by ELISA were 15.2, 13.0, and 2.3/108 in occupational, environmental, and control groups, respectively. A similar trend was observed in mean FITC:PI ratio of lymphocytes. Although in a lower range of exposure than cokery workers, Silesian residents are under continuous, long-term exposure to PAH. The formation of similar amounts of adducts in those with occupational or environmental exposure may be due to their accumulation or to the impairment of DNA repair. Our preliminary results with the bleomycin sensitivity test support the suggestion that reduced DNA repair capacity is important.

Discussion

Adduct-specific nuclear FITC staining was dose dependent in 10T½ cells treated in vitro with 0.1–10 μg/ml BPDE-I (Fig. 2). There was an approximately 3-fold difference in the mean FITC:PI ratio between control cells and those treated with 10 μg/ml BPDE-I. The mean FITC:PI ratio was significantly elevated in lymphocytes from both the occupationally and environmentally exposed groups as compared to rural controls (Table 1). The difference between the two Silesian-exposed groups was not statistically significant. This is in agreement with our previous results in which only minor differences in aromatic and PAH-DNA adducts were detected by postlabeling or ELISA on DNA isolated from total WBC of cokery workers and environmental controls (12).

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F.png

Fig. 2. Dose-response curve obtained for C3H 10T½ cells treated with 0, 0.1, 1, 5, 10, and 20 μg/ml of BPDE. Mean FITC:PI ratio of three separate staining experiments in which 50 cells/sample were counted; bars, SD.

Nonparametric rank Mann-Whitney U test. Almost all subjects were smokers, therefore the effect of smoking and adduct level was not investigated. Cells with higher adduct-level were selected in a manner similar to the method of Moore and Carrano (17) in which high sister chromatid exchange frequency cells were determined. A threshold value of the 95th percentile of the distribution of pooled control data was defined (FITC:PI ratio, 2.2), and then the number of cells with a ratio ≥2.2 was calculated. A χ² test was used to evaluate the statistical significance of the difference in higher adduct-level cells between exposed and control groups.

The distribution of the combined FITC:PI ratios for each exposure group (Fig. 4) indicated the existence of cells with relatively higher adduct levels in the more highly exposed groups. A cutoff of 2.2 for the FITC:PI ratio, based on the 95 percentile of control cells, was selected for determination of cells containing higher adduct levels. The percentage of the cells with ratios ≥2.2 increased with exposure from 3.7, 11.5, and 13.6 for control, environmental, and occupational exposure groups (Table 2).

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The anti-BPDE-I-DNA antiserum is suitable for in situ immunofluorescence detection of PAH-DNA adducts in human lymphocytes. The method can be applied to lymphocytes from as little as 1–2 ml of blood, which makes it convenient for biomonitoring studies. It can also be used for adduct quantitation in biopsy material. A major advantage of the present method is the simultaneous measurement of FITC and PI, which allows for the correction of quantitation of individual-cell adduct levels for the amount of nuclear DNA. A similar immunocytochemical assay was developed by Müller et al. (18) for quantitation of O⁶-ethylguanine in human leukemic cells. In that study, adduct-specific antiserum binding was visualized with a rhodamine isothiocyanate conjugated secondary antiserum, and data was corrected for DNA content based upon 4,6-diamidino-2-phenylindole staining.

Human WBC are easily accessible and have been used successfully for studying PAH-DNA adducts in occupationally and environmentally exposed individuals (reviewed in Refs. 2 and 4). However, the relationship between blood adduct levels and target tissue levels is still uncertain. No correlation was observed between DNA adduct levels in total WBC and normal lung tissue of lung cancer patients in a prior study by ³²P-postlabeling (19). In a more recent study using ELISA to measure PAH-DNA, a modest correlation was seen between WBC DNA adduct levels and lung tumor DNA (r = 0.31; P = 0.096) but not nontumor DNA (r = 0.18; P = 0.32) (20). The heterogeneity of the white cell population may be one of the reasons for the large interindividual variability in adduct levels which has been observed. Most blood cells (40–75%) are short-lived granulocytes, which can only indicate the effects of recent exposure. The remainder are the...
Fig. 3. Immunofluorescence staining with anti-BPDE-I-DNA antiserum #1 of PAH-DNA adducts in human lymphocytes. Examples of immunofluorescence stainings of lymphocytes of cokery worker (a and b); Silesian inhabitant (c and d) and control region donor (e and f). Left (a, c, and e) and right (b, d, and f) sides of the photographs present images of the same cells stained with FITC and PI, respectively. × 60.
Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Lower quartile</th>
<th>Upper quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupational</td>
<td>763</td>
<td>1.35 ± 1.11</td>
<td>1.25</td>
<td>0.38</td>
<td>5.45</td>
<td>0.68</td>
<td>1.66</td>
</tr>
<tr>
<td>Environmental</td>
<td>744</td>
<td>1.25 ± 0.85</td>
<td>1.2</td>
<td>0.13</td>
<td>4.39</td>
<td>0.55</td>
<td>1.76</td>
</tr>
<tr>
<td>Control</td>
<td>732</td>
<td>0.81 ± 0.62</td>
<td>0.49</td>
<td>0.17</td>
<td>3.14</td>
<td>0.31</td>
<td>1.26</td>
</tr>
</tbody>
</table>

*Occupational/Control, P < 0.01; Environmental/Control, P < 0.001; Occupational/Environmental, P = 0.40 by Mann-Whitney U test.*

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells with FITC:PI ratio ≥2.2/total cells</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupational</td>
<td>104/763</td>
<td>13.6</td>
</tr>
<tr>
<td>Environmental</td>
<td>86/746</td>
<td>11.5</td>
</tr>
<tr>
<td>Control</td>
<td>28/732</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Significantly different from the controls by χ² test (P < 0.05).*

In analyzing human samples, an obvious question is the sensitivity of the immunocytochemical staining technique. In our study, in order to increase sensitivity of a previously used method (16), a biotin-streptavidin signal amplification system was used. This modified technique was sufficiently sensitive to detect PAH-DNA adducts in humans environmentally exposed to aromatic compounds. However, to quantify the absolute amount of adducts, additional validation of the method is necessary. The results of this pilot study indicate that this technique is a promising addition to biomonitoring studies.

Acknowledgments

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References


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