Immunoperoxidase Quantitation of 4-Aminobiphenyl- and Polycyclic Aromatic Hydrocarbon-DNA Adducts in Exfoliated Oral and Urothelial Cells of Smokers and Nonsmokers

Ta-Ming Hsu, Yu-Jing Zhang, and Regina M. Santella

Division of Environmental Health Sciences, Columbia School of Public Health and Columbia Presbyterian Cancer Center, New York, New York 10032

Abstract

Immunoperoxidase methods using two antibodies were developed for detection and quantitation of DNA damage in single cells. A monoclonal antibody that recognizes 4-aminobiphenyl (4-ABP)-DNA adducts was initially tested on liver tissues of BALB/c mice treated with 4-ABP, then applied to the detection of adducts in oral mucosa and exfoliated urothelial cells of smokers and nonsmokers. Levels of 4-ABP-DNA in exfoliated urothelial cells were elevated in each of 20 smokers (mean relative staining intensity, 517 ± 137) compared with age-, race-, and sex-matched nonsmokers (313 ± 79; P < 0.0005). Significantly higher damage levels were also observed in oral mucosa cells of smokers compared with nonsmokers (552 ± 157 versus 326 ± 101; P < 0.0005). A polyclonal antiserum that recognizes benzo(a)pyrene and structurally related polycyclic aromatic hydrocarbon (PAH) diol epoxide-DNA adducts was also applied to the same study samples after validation by staining of 10T1/2 cells treated with (±)-trans-anti-benzo(a)pyrene diol epoxide. Smokers had higher levels of PAH-DNA damage in oral mucosa and exfoliated urothelial cells than nonsmokers (oral mucosa cells, 684 ± 107 versus 370 ± 83; P < 0.0005; urothelial cells, 689 ± 72 versus 495 ± 57; P < 0.0005). A similar 2-3-fold range in relative staining was found in smokers and nonsmokers for both 4-ABP- and PAH-DNA, suggesting the importance of individual differences in capacity to metabolize the carcinogens and/or repair damaged DNA. Significant correlations were found among the biomarkers in both cell types. This noninvasive method, requiring small numbers of cells and with a relatively low cost, will be useful for monitoring DNA damage in large-scale molecular epidemiology studies.

Introduction

Cigarette smoke contains several classes of compounds with demonstrated carcinogenic or cocarcinogenic activity, including nitro-

Received 7/12/96; revised 9/27/96; accepted 9/30/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by an award from the Lucille P. Markey Charitable Trust and National Institute of Environmental Health Sciences Grant 05116.

2 To whom requests for reprints should be addressed, at Columbia University, 701 West 168th Street, New York, NY 10032.

3 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; 4-ABP, 4-aminobiphenyl; GC/MS, gas chromatography/mass spectroscopy; BPDE-I, 7R,8S-dihydroxy-8,9,10-trihydroxybenz[a]pyrene; DAB, diaminobenzidine; ABC, avidin-biotin complex.
be readily and repeatedly collected by noninvasive methods and are ideal samples for biological monitoring of humans for the carcinogenic effects of cigarette smoking.

Materials and Methods

Human Study. Volunteers were recruited after approval by the Institutional Review Board by advertisement around Columbia Presbyterian Medical Center. Smokers were currently smoking one or more packs of cigarettes per day and had smoked for more than 5 years. Race-, sex-, and age (within 5 years)-matched nonsmokers were selected as a low-exposure group for a total of 20 pairs. After informed consent was obtained, oral mucosa cells were collected by gently rinsing the mouth with 1× PBS, and slides were prepared by cytocentrifugation. Exfoliated urothelial cells obtained from urine contain both transitional and squamous cells (25). Occasionally, a small number of granular and white blood cells were also observed. As in a previous report (26), more cells were obtained from the same volume of urine collected from females than from males. As a result, it was necessary to collect 30–50 ml of urine for females, and 90–250 ml for males, to ensure sufficient cells for analysis. After three washes with sucrose wash to dissolve contaminating crystals (27), slides were prepared by cytocentrifugation.

A self-administered questionnaire was used to collect information on socioeconomic status, active and passive exposure to cigarette smoke, and dietary, occupational, medical, and residential history. A series of oral cells was also collected at different time points from a smoker who decreased smoking from 5 packs/day to 1 pack/week (days 0, 7, 17, and 26 since smoking level decreased).

Batches of 8–10 slides containing paired samples of smokers and nonsmokers were coded before staining and assayed together with controls. Controls for the 4-ABP-DNA assay consisted of frozen liver tissue sections from mice treated with 0 or 80 mg/kg 4-ABP as described (22). For the PAH-DNA assay, 10Ti/2 cells cultured in eight-chambered slides were treated with 0, 5, 50, and 40 μM BPDE-I as described (24).

Immunoperoxidase Staining of 4-ABP-DNA Adducts. Mouse liver tissues, human oral mucosa, and exfoliated urothelial cells were stained by the same protocol essentially as described previously (23), except that NCl3 was added to the DAB reagent as indicated by the manufacturer (Vector Laboratories, Burlingame, CA). Slides were first washed with PBS for 10 min and treated with RNase (100 μg/ml; Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h to degrade any RNA present. After another 10-min wash with PBS, slides were treated with 10 μg/ml proteinase K (Sigma) at room temperature for 10 min to remove histone and nonhistone proteins from the DNA and increase antibody accessibility, and were then washed. To denature the DNA, slides were incubated with 4N HCl for 7 min at room temperature to further increase antibody accessibility, then with 50 mm Tris base at room temperature for 5 min. A 15-min wash was conducted after this step. Non specific binding was blocked with 1.5% normal goat serum, and slides were incubated overnight at 4°C with anti-4-ABP-DNA monoclonal antibody 3C8 developed from a mouse immunized with 4-ABP-modified calf thymus DNA (22). To obtain optimal staining (clear nuclear and low background staining), antibody was diluted 1:10 in 1.5% normal goat serum for oral mucosa cells, 1:80 for bladder cells, and 1:100 for mouse liver sections. After washing, slides were incubated with the anti-mouse secondary antisera at 37°C for 30 min followed by another 15-min wash. Treatment with 0.3% H2O2 in methyl alcohol at room temperature for 30 min was used to quench endogenous peroxidase activity. Elite mouse ABC and DAB kits (Vector) were used for visualization of bound antisera as directed by the manufacturer. Slides were dehydrated and cleaned in serial ethyl alcohol and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Immunoperoxidase Staining of PAH-DNA Adducts. Staining for PAH-DNA was similar to the method described above for 4-ABP-DNA, except that a rabbit polyclonal antisera 1 (21) was used for PAH-DNA, except that a rabbit polyclonal antisera 1 (21) was used (1:800 diluted in 1.5% normal horse serum for 10Ti/2, oral, and urothelial cells). This anti-BPDE-I-DNA antisera cross-reacts with DNA modified by several other PAH-diol epoxides.4 Thus, the antisera recognizes a class of adducts rather than just those of BPDE-I. Because the antisera was derived from rabbit, an Elite anti-rabbit ABC kit was used.

Specificity of Staining. To demonstrate staining specificity, cells from smokers and nonsmokers were pretreated with DNase (100 μg/ml for 1 h at 37°C) before staining, stained with a nonspecific antibody (8G1; 1:10 dilution) recognizing DNA damage produced by the photoactivated drug 8-methoxypsoralen (28), or with antisera preabsorbed with specific antigen (4-ABP-DNA for monoclonal antibody 3C8 and BPDE-I-DNA for polyclonal antisera 1: 1 μg/μl for 20 min at room temperature) before use. An additional set of control experiments was carried out by preabsorbing antibody 3C8 with BPDE-I-DNA and antisera 1 with 4-ABP-DNA. Oral cells from a smoker were also stained with the combination of both antibodies at the same final concentration as used individually and with a combination of anti-mouse and rabbit ABC kit reagents.

Quantitation of DNA Adducts. A Cell Analysis System 200 microscope (Becton Dickinson, San Jose, CA) was used to measure the relative intensity of nuclear staining in 30 randomly selected cells with good morphology using the Cell Measurement Program software package. Data presented are the object average absorbance multiplied by 1000. Staining variability was determined by repeat analysis of 15% of smoker and nonsmoker samples.

The difference in mean value of relative staining intensity between smokers and nonsmokers was analyzed by paired t test. Spearman rank correlation, a nonparametric test, was used to examine the association between each biomarker because the relative staining intensity of 4-ABP- or PAH-DNA adducts among all subjects was not always normally distributed. Two-sided P values were calculated, and values <0.05 were considered significant.

Results

Immunoperoxidase Quantitation of 4-ABP-DNA Adducts. Immunoperoxidase staining of liver tissues from 4-ABP-treated mice indicated specific nuclear staining in treated liver tissues and weak background staining in control tissue (not shown). A dose-related increase in staining was observed with relative staining intensities of 189 ± 42, 247 ± 30, 329 ± 53, 368 ± 69, 459 ± 38, and 656 ± 76 in animals treated with 0, 4, 10, 20, 40, and 80 mg/kg, respectively. These tissues were analyzed previously by a quantitative immunofluorescence method (22). By that method, the difference in relative staining between liver tissue from animals treated with the highest (80 mg/kg) and lowest (4 mg/kg) dose of 4-ABP was much larger than that observed with the immunoperoxidase staining (13-fold versus

4 T-M. Hsu, Y-J. Zhang, and R. M. Santella, unpublished results.
Fig. 1. Immunoperoxidase staining of exfoliated urothelial cells from smoker 3 (A) and nonsmoker 50 (B) with monoclonal antibody 3C8 recognizing 4-ABP-DNA damage. Preabsorption of primary antibody with 4-ABP-DNA before use on cells from smoker 21 (C) and pretreatment of cells from smoker 10 with DNase before staining with primary antibody (D). ×400.

2.7-fold). Data on GC/MS analysis of 4-ABP-DNA adduct levels in DNA isolated from the same samples were also available (22). There were significant correlations between immunoperoxidase staining and either immunofluorescence staining \( r = 0.99, P < 0.001 \) or GC/MS analysis of DNA adducts \( r = 0.94, P < 0.01 \). To ensure the consistency of staining intensity in human samples on different days, tissues from the 0 mg/kg- and 80 mg/kg-treated mice were stained side by side with each batch of human samples. There was no significant difference in repeat staining of these tissues. The coefficient of variation ranged from 5 to 18% \( (v) = 5 \).

When the immunoperoxidase method was applied to the human samples, higher levels of nuclear staining for 4-ABP-DNA were observed in the exfoliated urothelial cells of every smoker compared with their matched nonsmoker, except for one pair. Representative staining in smoker 3 and nonsmoker 50 is illustrated in Fig. 1, A and B, respectively. Mean level of relative staining in all smokers \( (517 \pm 137) \) was 1.7-fold higher than in nonsmokers \( (313 \pm 79; P < 0.0005 \) by paired \( t \) test; Table 1). Similar differences between smokers and nonsmokers were observed in males and females. Mean values were significantly higher in females than males in urothelial cells of both smokers and nonsmokers \( (P < 0.01 \). The distribution of staining in smokers and nonsmokers is given in Fig. 2A. An approximately 3-fold variation in staining was observed both in nonsmokers \( (151 \pm 47 \) to \( 475 \pm 103 \) and smokers \( (200 \pm 64 \) to \( 720 \pm 185 \). Among smokers, no association was found between relative staining and number of cigarettes smoked per day.

Preabsorption of primary antibody with 4-ABP-DNA before use decreased staining in urothelial cells of smoker 21 from \( 720 \pm 185 \) to \( 84 \pm 19 \) (Fig. 1C) and of nonsmoker 39 from \( 327 \pm 84 \) to \( 70 \pm 32 \) (not shown). Pretreatment of slides with DNase also decreased staining in smoker 10 from \( 659 \pm 151 \) to \( 129 \pm 30 \) (Fig. 1D) and to \( 97 \pm 31 \) in nonsmoker 39 (not shown). As an additional test of staining specificity, a later time point oral cells from one additional smoker were stained with antibody 3C8 with or without preabsorption with BPDE-I-DNA. There was little change in staining intensity with \( (314 \pm 101 \) or without \( (359 \pm 116 \) preabsorption with an alternate DNA adduct. Variability of staining was determined by repeat analysis \( (n = 3 \) ) of three smoker and three nonsmoker samples and ranged from 2 to 3% for smokers and from 5 to 9% for nonsmokers.

In oral cells, specific 4-ABP-DNA nuclear staining was also observed, with higher levels of staining detected in every smoker compared with their matched nonsmoker. The mean level of relative staining was elevated 1.7-fold in smokers \( (552 \pm 157) \) compared with nonsmokers \( (326 \pm 101; P < 0.0005 \) (Table 1). Similar differences were observed when male and female subjects were analyzed separately. Mean levels were higher in females than males in both smokers and nonsmokers, but this difference was not significant. In addition, there was a 3- to 4-fold variation in staining in nonsmokers \( (range, 115 \pm 57 \) to \( 539 \pm 157 \) and smokers \( range, 240 \pm 80 \) to \( 815 \pm 202, \) Fig. 2B). Again, no association was found in smokers between staining intensity and number of cigarettes smoked.

Staining specificity was confirmed as for the urothelial cells. Preabsorption of primary antibody with 4-ABP-DNA before use also decreased staining in oral cells of smoker 22 from \( 815 \pm 202 \) to \( 125 \pm 46 \) and of nonsmoker 48 from \( 305 \pm 153 \) to \( 110 \pm 32 \). Pretreatment of slides with DNase gave a value of \( 85 \pm 26 \) for smoker 22 and \( 63 \pm 19 \) for nonsmoker 48. Variability of staining was determined by repeat analysis \( (n = 3 \) ) of samples from three...
smokers and three nonsmokers and ranged from 2 to 4% for smokers and from 3 to 8% for nonsmokers.

**Immunoperoxidase Quantitation of PAH-DNA Adducts.** Treatment of cells with 0, 5, 10, 20, and 40 μM BPDE-I resulted in staining intensities of 198 ± 29, 274 ± 69, 318 ± 78, 423 ± 87, and 615 ± 115. 10T1/2 cells were stained side by side with each batch of human samples. The coefficient of variation for the different doses ranged from 6 to 23% (n = 5). One-way ANOVA indicated there was no significant difference in repeat staining of the 10T1/2 cells.

In oral cells, as in our previous study (23), higher levels of specific nuclear staining were observed in every smoker compared with their matched nonsmoker. The mean level of relative staining was elevated 1.8-fold in all smokers (mean, 684 ± 107) compared with nonsmokers (mean, 370 ± 83; P < 0.0005, Table 1). Similar differences were also observed in males and females. Mean values were higher in females than males in both smokers and nonsmokers, but this difference was not significant. In addition, there was an approximately 2-fold variation in staining in both nonsmokers (range, from 222 ± 71 to 491 ± 151) and smokers (range, from 531 ± 250 to 924 ± 215). The distribution of staining in smokers and nonsmokers is given in Fig. 3A. In agreement with the results of our previous study (23), no association was observed in smokers and nonsmokers is given in Fig. 3A. In agreement with the results of our previous study (23), no association was observed in smokers and nonsmokers.

Because control stainings with nonspecific antibody, antibody 1 preabsorbed with BPDE-I-DNA, or cells pretreated with DNase were carried out in our previous study (23), they were not repeated here. As a further test of specificity, oral cells from an additional smoker were stained with antiserum 1 preabsorbed with 4-ABP-DNA. Staining was slightly higher (429 ± 127) than for cells stained without preabsorption (396 ± 108), further confirming the specificity of staining. Oral cells from this smoker were also stained with a combination of both antibody 3C8 and antiserum 1. Intensity of staining (711 ± 250) was just below that expected from addition of intensity of staining for 4-ABP-DNA (359 ± 116) and PAH-DNA (396 ± 108) alone. The variability of staining was determined by repeat analysis (n = 3) of three smoker and three nonsmoker samples and ranged from 1 to 3% for smokers and from 1 to 7% for nonsmokers.

**Fig. 2.** Comparison of relative intensity of immunoperoxidase staining for 4-ABP-DNA damage in urothelial (A) and oral mucosa (B) cells in smokers and nonsmokers. Bar, mean value.

Because fewer cells were obtained from the urine samples of males than females, although larger amounts (3–5-fold) of urine were collected, only exfoliated bladder cells from the 11 pairs of female subjects could be assayed for PAH-DNA. Higher levels of specific nuclear staining were observed in each smoker compared

---

**Table 1** Differences in DNA damage in exfoliated oral and urothelial cells between smokers and nonsmokers

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Smoking status</th>
<th>Female subjects</th>
<th>Male subjects</th>
<th>Total subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD (min-max)</td>
<td>two-tailed significance</td>
<td>Mean ± SD (min-max)</td>
</tr>
<tr>
<td>Oral PAH-DNA</td>
<td>Smoker</td>
<td>713 ± 111 (547-924)</td>
<td>P &lt; 0.0005</td>
<td>657 ± 102 (531-818)</td>
</tr>
<tr>
<td></td>
<td>Nonsmoker</td>
<td>379 ± 95 (222-466)</td>
<td></td>
<td>360 ± 69 (285-491)</td>
</tr>
<tr>
<td>Oral 4-ABP-DNA</td>
<td>Smoker</td>
<td>580 ± 171 (293-817)</td>
<td>P &lt; 0.0005</td>
<td>518 ± 141 (240-727)</td>
</tr>
<tr>
<td></td>
<td>Nonsmoker</td>
<td>355 ± 111 (230-539)</td>
<td></td>
<td>301 ± 110 (115-442)</td>
</tr>
<tr>
<td>Bladder PAH-DNA</td>
<td>Smoker</td>
<td>594 ± 99 (423-720)</td>
<td>P &lt; 0.0005</td>
<td>22 ± 118 (200-555)</td>
</tr>
<tr>
<td></td>
<td>Nonsmoker</td>
<td>342 ± 67 (247-475)</td>
<td></td>
<td>263 ± 64 (151-373)</td>
</tr>
<tr>
<td>Bladder PAH-DNA</td>
<td>Smoker</td>
<td>689 ± 72 (554-759)</td>
<td>P &lt; 0.0005</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonsmoker</td>
<td>495 ± 57 (363-577)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
with her matched nonsmoker control (not shown). The mean level of relative staining was elevated 1.4-fold in female smokers (mean, 495 ± 57; P < 0.0005; Table 1). The staining variation in nonsmokers was slightly higher than in smokers. It ranged from 363 ± 105 to 577 ± 143 in nonsmokers and from 554 ± 184 to 759 ± 189 in smokers (Fig. 3B). Again, no association was found between relative staining intensity and number of cigarettes smoked in smokers and nonsmokers.

Preabsorption of primary antiserum with BPDE-I-DNA before use on urothelial cells decreased staining of smoker 20 from 759 ± 189 to 249 ± 87 and of nonsmoker 31 from 363 ± 105 to 221 ± 35. Staining with a nonspecific antiserum resulted in 90% decreased relative staining in smoker 21 from 759 ± 189 to 246 ± 93 and in nonsmoker 31 from 363 ± 105 to 191 ± 59. Pretreatment of slides with DNase gave a value of 190 ± 60 in smoker 20 and 197 ± 39 in nonsmoker 31. Variability of staining was determined by repeat analysis (n = 3) of two smoker and two nonsmoker samples and ranged from 2 to 3% for smokers and from 4 to 6% for nonsmokers.

**Correlations between 4-ABP- and PAH-DNA in Exfoliated Oral and Bladder Cells.** Correlations between the two types of DNA damage in the different cells in all subjects were significant (P < 0.001; Spearman rank correlation; Table 2). Among the three combinations, the highest association was seen between PAH- and 4-ABP-DNA in oral mucosa cells, followed by the correlation between PAH-DNA in oral cells and 4-ABP-DNA in exfoliated urothelial cells and that between 4-ABP-DNA in oral and exfoliated urothelial cells. For female subjects in which data on PAH-DNA in urothelial cells was available, correlations for all six combinations of biomarkers were determined. Similar to the results in total subjects, significant correlations were observed for all combinations (P < 0.001).

**Detection of 4-ABP- and PAH-DNA Damage in Oral Mucosa Cells of a Smoker Who Decreased Smoking.** Oral cells were obtained at different time points from a smoker who dramatically decreased, but did not stop, smoking (5 packs/day to 1 pack/week). Decreased relative staining was observed in cells stained for either PAH-DNA or 4-ABP-DNA (Table 3).

### Discussion

**Immunoperoxidase Quantitation of 4-ABP-DNA Damage.** Previously, we had developed an immunofluorescence method for detection of 4-ABP-DNA, demonstrated a dose-response relationship between administered dose and relative fluorescence staining intensity in mouse liver tissues, and found an excellent correlation with GC/MS detection of DNA damage (22). A similar dose-response was found in the present study for immunoperoxidase staining. The specificity of staining was further confirmed by preabsorbing antibody with an alternate modified DNA, BPDE-I-DNA, and by demonstrating no change in staining intensity. Significant correlations were observed between quantitative immunoperoxidase intensity and either immunofluorescence staining intensity (r = 0.99) or level of DNA adduct determined by GC/MS (r = 0.94). However, there was much smaller increase in immunoperoxidase (2.7-fold) than immunofluorescence staining (13-fold) for animals treated with 4 mg/kg 4-ABP (20-fold difference in dose). This may be due to the detection limits and/or dynamic range of each method. High background

---

**Table 2** Correlations between 4-ABP and PAH-DNA in exfoliated oral and bladder cells in females only (n = 22) and in all subjects (n = 40)

<table>
<thead>
<tr>
<th></th>
<th>Oral cells</th>
<th>Oral cells</th>
<th>Bladder cells</th>
<th>Bladder cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-ABP-DNA</td>
<td>1.000</td>
<td>0.738b</td>
<td>0.592b</td>
<td>0.536b</td>
</tr>
<tr>
<td>PAH-DNA</td>
<td>1.000</td>
<td>0.723c</td>
<td>0.714b</td>
<td>0.690b</td>
</tr>
</tbody>
</table>

*P < 0.001.

*a* Correlation for female subjects only.

*b* Correlation for all subjects.

---

**Table 3** Immunoperoxidase detection of 4-ABP-DNA and PAH-DNA in oral cells of a smoker who reported decreasing smoking intensity from 5 packs per day to 1 pack per week

<table>
<thead>
<tr>
<th>Days since reduced smoking level</th>
<th>Relative staining intensity for 4-ABP-DNA</th>
<th>Relative staining intensity for PAH-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>262 ± 77</td>
<td>573 ± 43</td>
</tr>
<tr>
<td>7</td>
<td>202 ± 78</td>
<td>369 ± 55</td>
</tr>
<tr>
<td>16</td>
<td>134 ± 36</td>
<td>330 ± 35</td>
</tr>
<tr>
<td>26</td>
<td>89 ± 33</td>
<td>310 ± 42</td>
</tr>
</tbody>
</table>

*a* Mean staining intensity in 30 randomly selected cells ± SD.

---

**Fig. 3.** Comparison of relative intensity of immunoperoxidase staining for PAH-DNA damage in oral mucosa (A) and urothelial (B) cells in smokers and nonsmokers. Bar, mean value.
staining related to nonspecific binding of reagents in the immunoperoxidase method may be responsible for the shallow slope of the dose-response curve. An 8-fold difference in immunoperoxidase staining between liver tissue of mice treated with 80 or 4 mg/kg 4-ABP was found after subtracting the value for intensity of staining of control tissue (mean = 189) from that for the treated animal tissues. Although background staining in the immunofluorescence method is much lower, subtraction of the staining intensity of control tissue also increased the difference in staining between the highest and lowest dose-treated animals (from 13-fold to 21-fold). Inefficient digestion with proteinase K or incomplete denaturation of DNA during the staining procedure, leading to lower antibody binding to adduct, and/or nonlinear enzyme amplification of signal may also reduce the ability of the immunoperoxidase method to efficiently quantitate adduct levels.

In this study, higher levels of specific nuclear staining were observed in exfoliated urothelial cells in every smoker compared with their matched nonsmoker, except for one pair. A 1.7-fold higher level of relative staining intensity was observed in smokers than in nonsmokers (P < 0.0005). We previously observed a 2.4-fold higher staining intensity in bladder biopsies of smoking than nonsmoking bladder cancer patients (24). Comparable results were found by 32P-postlabeling of DNA isolated from human exfoliated urothelial cells (29). A smoking-related adduct was found to be 1.7-fold higher in smokers compared with nonsmokers, and correlated significantly with the amount of tobacco the individual smoked and the level of 4-ABP-hemoglobin adducts in the red blood cells. Fivefold higher adduct levels in smokers compared with nonsmokers were observed in another study of human bladder biopsy samples using butanol enrichment before postlabeling (5). Among these studies, measured 4-ABP-DNA adduct levels ranged from 1 adduct/107 nucleotides to 1 adduct/109 nucleotides (5, 16, 29, 30). After dosing for 6 weeks, 4-ABP-DNA adducts in exfoliated urothelial cells of dogs were identical and equal in level to adducts found in intact urinary bladder (27). This further strengthens the applicability of monitoring carcinogen-DNA adduct levels in the target organ using exfoliated urothelial cells.

Specific nuclear staining of 4-ABP-DNA in two cell types (oral mucosa and exfoliated urothelial cells) was observed in nonsmokers as well as smokers. It is not surprising that the immunohistochemical assay can also detect DNA damage in nonsmokers. Although, in this study, only 1 of 20 nonsmoking subjects reported environmental tobacco smoke exposure, this is a potential source of exposure. Structurally related compounds (e.g., 4-nitrophenyl in diesel exhaust; Ref. 31), are other possible sources of 4-ABP-DNA adducts in nonsmokers. In addition, the background staining of 4-ABP-DNA in nonsmokers may also be partially due to the low cross-reactivity of antibody 3C8 with unadducted DNA (22).

**Immunoperoxidase Quantitation of PAH-DNA Adducts.**

We reported previously specific detection of adducts in BPDE-I-treated 10T1/2 cells (23). The addition of nickel chloride to the DAB solution resulted in darker staining and a larger difference in staining intensity of cells treated with the highest dose compared with control cells than in our previous report (3.1-fold versus 1.7-fold). Specific nuclear staining was observed in oral mucosa cells of both smokers and nonsmokers. The 1.7-fold increase in damage levels observed in this study is similar to that of our previous study (2-fold; Ref. 23). It is also comparable to the 3-fold difference in PAH-DNA in mononuclear cells of smokers and nonsmokers using an ELISA with a similar antiserum (32). In addition, oral mucosa cells have been used in several studies to monitor exposure to PAH by 32P-postlabeling with adduct levels ranging from 1.6/106 to 1/109 (8, 10, 33–35). Significant 2–3-fold differences were found between smokers, and either nonsmokers or ex-smokers in some studies (33–35), although no association between smoking and adduct level was observed in others (8, 10). We also observed an almost additive effect when simultaneously staining oral cells for both 4-ABP- and PAH-DNA, suggesting that multiple antigens can be detected in the same sample.

Specific nuclear staining for PAH-DNA in both cells types was observed in nonsmokers. Because PAHs are ubiquitous, these adducts may come from exposure to environmental air pollution, including tobacco smoke. Exposure to PAHs from other sources such as water, soil, or green leafy vegetables may also contribute to adduct formation (36). Twofold to 3-fold variation in staining in the two cell types was also observed in both smokers and nonsmokers, which may be due to individual genetic variation in metabolism of carcinogens, as well as differences in exposure levels. However, larger interindividual differences were found in previous studies on white blood cell DNA from individuals exposed to similar levels (37–40).

**Correlations between Biomarkers and Other Factors.**

No association between relative staining and consumption of charcoal-broiled and smoked foods was observed; however, we still cannot rule out this means of exposure. Accurate dietary data are difficult to obtain, and study subjects reported low frequency of consumption of these foods. The small sample size also reduced our ability to detect differences between charcoal-broiled/smoked food consumption and adduct levels. The lack of association between the number of cigarettes smoked per day and relative staining intensity of each biomarker in the smokers was not unexpected, because all smokers in this study smoked 20–40 cigarettes/day. A linear, dose-related increase in staining intensity for 4-ABP-DNA was observed in our previous study of bladder biopsies of bladder cancer patients classified into four groups: nonsmokers, and smokers of 1–19, 20–40, and >40 cigarettes/day (24). In an endeavor to validate markers in a highly exposed group, the heavy exposure of the subjects in this study may have reduced our ability to see dose-response relationships. Differences in smoking habits, types of cigarettes (air- or flue-cured), and genetic factors that influence carcinogen metabolism and DNA repair may also affect adduct formation in individuals. Other environmental exposures or differences in diet or lifestyle of study subjects are other possible explanations for the lack of association with number of cigarettes smoked. Significant correlations among the biomarkers demonstrated that they are good indicators of exposure to cigarette smoke and a good measure of biologically effective dose in the body. Analysis of samples collected at four different time points from a smoker who reported decreased smoking demonstrated lower relative staining for both 4-ABP- and PAH-DNA between days 0 and 7. This is comparable to the estimated turnover rate of oral mucosa cells. A study of the disappearance of micronuclei in radiation therapy patients estimated as 5–7 days the time frame of cell migration from the basal layer to exfoliation (41). For urothelial cells, the estimated cell turnover rate is much longer (30–200 days) than oral cells (5 days) (42). However, with chronic exposure, measurement of DNA damage in these cell types may reflect current, as well as previous, exposure to carcinogens.

The immunohistochemical method developed here allows investigation of adduct formation at the individual cell level, with high specificity and relatively low cost. The major limitation is that it is semiquantitative and provides information only on relative levels of carcinogen-DNA adducts. The re-
requirement for small numbers of cells allows application to biopsy samples and exfoliated cells that are easily collected, and represents a simple way to obtain human tissue samples by noninvasive methods. As target tissues for smoking-induced cancers, DNA damage observed in these cells may also reflect the potential risk of an individual to develop cancer. In addition to the study of the mechanisms of oral and bladder carcinogenesis, these methods may also be valuable in more general studies of exposure to environmental carcinogens. We are currently using these biomarkers as intermediate end points in an antioxidant vitamin intervention study in heavy smokers.

References

Immunoperoxidase quantitation of 4-aminobiphenyl- and polycyclic aromatic hydrocarbon-DNA adducts in exfoliated oral and urothelial cells of smokers and nonsmokers.

T M Hsu, Y J Zhang and R M Santella