Immunoperoxidase Detection of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Oral Mucosa Cells of Smokers and Nonsmokers[1]

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Abstract

An immunoperoxidase method using a polyclonal antiserum which recognizes benzo(*a*)pyrene and structurally related polycyclic aromatic hydrocarbon diol epoxide-DNA adducts has been developed for the detection and quantitation of DNA damage in single cells. The method was used initially on 10T½ cells treated with [^{3}H]anti-benzo(*a*)pyrene diol epoxide then applied to the detection of adducts in oral mucosa cells of smokers and nonsmokers. Levels of DNA damage were elevated in each of 16 smokers (mean relative staining, 503 ± 104) compared to 16 age-, race- and sex-matched nonsmokers (251 ± 82; $P < 0.0001$). There was an approximately 3-fold range in relative staining in both smokers (252 ± 125 to 663 ± 189) and nonsmokers (157 ± 72 to 431 ± 269) suggesting the importance of individual differences in capacity to metabolize the carcinogens and/or repair damaged DNA. This noninvasive method, requiring small numbers of cells, will be useful for routine monitoring of DNA damage in intervention studies as well as for biofeedback in smoking cessation programs.

Introduction

A number of methods have been developed for quantitation of DNA damage resulting from environmental or occupational exposures to PAH[3] including immunoassays, gas chromatography-mass spectroscopy, fluorescence, and ^{32}P postlabeling (reviewed in Refs. 1–5). Most methods require the isolation of bulk DNA from tissue or blood samples and thus do not allow the detection of adducts in specific cell types. In addition, some methods require relatively large amounts of DNA for analysis, limiting their application.

We have used an ELISA to monitor PAH-DNA in foundry workers and coke oven workers (6, 7), smokers (8), and individuals with environmental exposure (7, 9). Antisera used in ELISA are sometimes applicable to immunohistochemical detection of DNA adducts in single cells. Major advantages include the detection of adducts in specific cells and the requirement for small numbers of cells making the method applicable to biopsy samples. Immunohistochemical methods have been used extensively to monitor DNA damage in animals, and limited studies have been carried out on humans exposed to PAH, aflatoxin, and 8-methoxypsoralen (reviewed in Refs. 1, 10). We have used the same polyclonal antiserum (Subject 29), raised against BPDE-I-DNA (11), used in the ELISA for immunofluorescence detection PAH-DNA in skin biopsies of coal tar-treated psoriasis patients (12). Application of a similar method to tissues and bronchial cells from smokers has also been reported (13, 14).

Here we report on the development of an immunohistochemical method for direct quantitation of DNA damage in oral mucosa cells, a target tissue for smoking-induced cancers and a tissue which can be readily and repeatedly collected by noninvasive methods. Although we initially attempted to use the immunofluorescence method to detect damage in oral cells, background autofluorescence interfered with the assay. Thus, an immunoperoxidase method using biotinylated secondary antisera and streptavidin-conjugated peroxidase was used in conjunction with direct quantitation of staining for detection of damage in oral cells. A similar method was used to monitor cisplatin modified DNA in oral cells of chemotherapy patients (15).

Materials and Methods

Treatment of 10T½ Cells. To develop the quantitative immunoperoxidase method, 10T½ cells cultured in 8-chambered slides (Nunc, Naperville, IL) were treated with 0, 5, 10, 20 and 40 μM [^{3}H] 7-*r*,8-*t*-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene [^{3}H]BPDE-I (444 mCi/mmol; Chemsyn Science Laboratories, Lenexa, KS) in DMEM (GIBCO-BRL Life Technologies, Gaithersburg, MD) for 1 h at 37°C. BPDE-I was dissolved in DMSO before addition to the media with a final DMSO concentration of 0.05%. Determination of cell toxicity by crystal violet dye exclusion indicated > 80% viability at all doses. After treatment, cells were washed with PBS twice and fixed in −20°C acetone for 20 min. At the same time, cells in 10-cm dishes were treated with similar concentrations of BPDE-I. After treatment and washing, cells were scraped from the dishes and DNA was extracted by standard RNase treatment and phenol/chloroform/isoamyl alcohol extraction methods. DNA concentration was determined from the absorbance at 260 nm and the modification levels determined from the specific activity.

Human Subjects. Volunteers were recruited, after approval by the Institutional Review Board by advertisements, around the Columbia Presbyterian Medical Center. After informed consent was obtained, oral mucosa cells were collected by gently scraping the inside of the cheek with a

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[3] The abbreviations used are: PAH, polycyclic aromatic hydrocarbons; BPDE-I, 7-*r*,8-*t*-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene.

wooden tongue depressor. Samples were obtained from 16 smokers of at least 1 pack/day (range, 20–30 cigarettes/day) and 16 race-, sex-, and age-matched (within 5 years) nonsmokers and were coded for analysis. A questionnaire was administered collecting information on smoking history as well as dietary consumption of charcoal broiled or smoked foods containing high levels of PAH over the previous 2 weeks. A subset of 6 smokers provided repeat samples 3 months after original sample collection. Cells were smeared on slides precoated with 0.2% poly-D-lysine, air dried, fixed in −20°C acetone for 20 min, and stored at −80°C until staining.

Immunoperoxidase Staining. Slides were washed with PBS, treated with RNase (100 μg/ml; Sigma Chemical Co., St Louis, MO) at 37°C for 1 h, washed with PBS, treated with proteinase K (10 μg/ml; Sigma) at room temperature for 10 min and washed. To denature the DNA, slides were incubated with 4N HCl for 10 min at room temperature and then with 50 mM Tris base for 5 min at room temperature. After washing with PBS, slides were incubated with 0.3% H_2O_2 in methyl alcohol at room temperature for 30 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with 1.5% normal horse serum and then slides were incubated with the anti BPDE-I-DNA polyclonal antiserum #29 (11) (1:800 dilution in 1.5% horse serum) overnight at 4°C. This antiserum was obtained from animals immunized with BPDE-I-DNA, but cross-reacts with DNA modified by several other PAH-diol epoxides (16). Thus, the antiserum recognizes a class of adducts rather than just those of BPDE-I. Elite rabbit or mouse ABC and DAB kits (Vector Laboratories, Burlingame CA) 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 Premount (Fisher Scientific, Pittsburgh PA). To demonstrate the specificity of the staining in the human oral cells, cells from a smoker (subject 12) and nonsmoker (subject 32) were pretreated with DNase (100 μg/ml for 1 h at 37°C) before staining, stained with a nonspecific antiserum (8G1; 1:10 dilution of hybridoma supernatant) recognizing DNA damage produced by the photoactivated drug 8-methoxypsoralen (17) or with antiserum preabsorbed with BPDE-I-DNA (1 μg/μl; for 20 min at room temperature) before use. A Cell Analysis System 200 microscope (Becton Dickinson, Elmhurst, IL) was used to measure the relative intensity of nuclear staining in 50 randomly selected cells using the Cell Measurement Program software package. Data presented are the object average optical density multiplied by 1000.

For analysis of the human samples, paired samples of nonsmokers and smokers (single or repeat samples) were assayed together in batches of 8–10 samples with an 8-chambered slide of 10T½ cells treated *in vitro* with BPDE-I.

Results

Specific nuclear staining was observed in 10T½ cells treated with [^{3}H]BPDE-I but not in control cells (data not shown). Quantitation of staining, obtained on a total of 50 randomly selected cells, indicated a dose-related increase in relative staining intensity (Table 1). DNA adduct levels, determined from the specific activity of DNA isolated from treated cells, also increased with increasing dose of BPDE-I (Table 1). While a linear relationship was observed between immunoperoxidase staining and BPDE-I-DNA adduct levels, the slope was less than 1 (Fig. 1).

Table 1 Comparison of [^{3}H]BPDE-I dose, DNA adduct level, and quantitative immunoperoxidase staining in 10T½ treated in culture

[^{3}H]BPDE-I dose (μM)	DNA adducts/10^5 nucleotides	Relative staining[a]
0	0	116 ± 8
5	2.8[b]	134 ± 9
10	4.4[b]	143 ± 25
20	11.1[b]	176 ± 12
40	18.4[c]	198 ± 10

[a] Mean of four separate staining experiments in which 50 cells/sample were counted.
[b] Mean adduct level from treatment of cells with [^{3}H]BPDE-I at two different time points.
[c] Adduct level from a single treatment of cells with [^{3}H]BPDE-I.

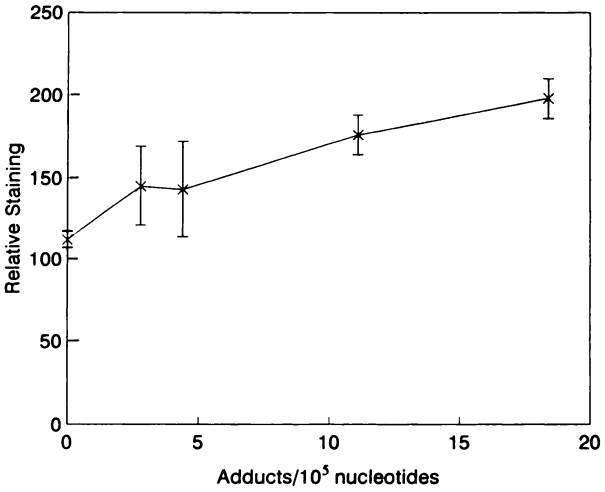

Fig. 1. Relationship between relative intensity of immunoperoxidase staining with an antiserum recognizing PAH-DNA adducts in 10T½ cells treated with [^{3}H]BPDE-I and DNA adduct levels determined by measurement of specific activity. *Points,* mean; *bars,* SD.

The immunoperoxidase method was then applied to the detection of damage in oral mucosa cells. To standardize the assay for possible day to day variations, an 8-chambered slide of treated 10T½ cells was stained with each batch of human samples. The coefficient of variation for the different doses ranged from 9–25% (n = 4). One-way analysis of variance indicated there was no significant difference in repeat staining of the 10T½ cells.

Representative staining in a smoker (subject 12; Table 2) and nonsmoker (subject 32) are illustrated in Fig. 2, *A* and *B*, respectively. Quantitative staining intensity data for each subject are given in Table 2. Preabsorption of primary antiserum with BPDE-I-DNA before use decreased staining in smoker 12 from 520 ± 146 to 109 ± 49 (Fig. 2*C*) and from 237 ± 58 to 125 ± 41 for nonsmoker 32 (Fig. 2*D*). Staining with a nonspecific antiserum recognizing DNA damage produced by 8-methoxypsoralen gave a value of 118 ± 95 in smoker 12 (Fig. 2*E*) and 68 ± 42 in nonsmoker 32 (Fig. 2*F*). Pretreatment of slides from smoker 12 with DNase also decreased relative staining (139 ± 70) as did omission of primary antiserum (94 ± 26; not shown). Background staining with these control conditions demonstrate the specific-

Table 2 Immunoperoxidase staining of oral mucosa cells from smokers and nonsmokers for polycyclic aromatic hydrocarbon-DNA damage

		Nonsmokers			Smokers				
Race[a]	Sex[b]	ID[c]	Age	Mean ± SD[d]	ID	Age	Cigarettes/day	1st sample Mean ± SD	2nd sample[e] Mean ± SD
C	F	22	44	377 ± 119	3	43	20	628 ± 154	673 ± 196
H	M	30	43	180 ± 77	4	40	20	422 ± 117	584 ± 174
C	M	24	36	210 ± 117	5	31	25	518 ± 144	644 ± 215
A	F	27	34	329 ± 189	8	34	20	516 ± 192	674 ± 206
A	F	23	46	274 ± 88	9	48	20	543 ± 121	615 ± 188[f]
C	M	26	35	278 ± 71	10	29	25	469 ± 183	433 ± 155
C	M	21	45	196 ± 112	1	45	25	436 ± 145	
C	F	34	26	323 ± 126	2	28	30	383 ± 183	
C	F	31	48	174 ± 71	6	46	25	252 ± 125	
C	F	36	32	157 ± 72	7	27	20	551 ± 258	
C	F	35	34	193 ± 83	11	35	25	642 ± 181	
A	M	28	40	431 ± 269	12	45	20	520 ± 146	
A	M	37	25	157 ± 78	13	25	20	527 ± 248	
C	F	32	44	237 ± 58	14	48	20	663 ± 189	
C	F	33	47	211 ± 86	15	42	22	560 ± 258	
C	M	25	32	298 ± 146	16	27	30	426 ± 126	

[a] A, African-American; C, Caucasian; H, Hispanic.
[b] M, male; F, female.
[c] ID, subject number.
[d] Relative staining intensity measured in 50 cells/subject.
[e] Subjects resampled 3 months after initial sample collection.
[f] This subject decreased the number of cigarettes smoked to 5–7/day 1 month before sample collection.

ity of the method. Variability of staining was determined by repeat analysis ($n = 3$) of 3 smoker and 2 nonsmoker samples. Variability ranged from 4 to 6% for the smoker samples and from 16 to 28% for the nonsmokers.

Higher levels of specific nuclear staining were observed in every smoker compared to their matched nonsmoker. The distribution of staining in smokers and nonsmokers is given in Fig. 3. There was an approximately 3-fold variation in staining in both nonsmokers (range, 157 ± 72 to 431 ± 269) and smokers (range, 252 ± 125 to 663 ± 189). Mean level of relative staining was elevated 2-fold in smokers (mean, 503 ± 104) compared to nonsmokers (mean, 251 ± 82; $P < 0.0001$ by paired student's t test). In this group of heavy smokers (≥ 20 cigarettes/day; range, 20–30), there was no association between staining intensity and the number of cigarettes smoked/day, nor was there an association with consumption of charcoal broiled or smoked foods during the prior 2 weeks among the smokers or nonsmokers ($P > 0.3$; Spearman rank correlation). However, only 8 subjects reported consumption of these foods over the previous 2 weeks and only 6 subjects reported this within the past week. Analysis of repeat samples from 6 smokers 3 months after initial sample collection indicated levels of DNA damage remained high.

Discussion

A simple, noninvasive method for monitoring humans for the carcinogenic effects of cigarette smoking in a target tissue for smoking-induced cancers has been developed. The 2-fold increase in damage levels is comparable to the 3-fold difference observed in DNA from mononuclear cells of smokers and nonsmokers using an ELISA with the same antiserum (8). Repeat sampling of smokers 3 months after initial sampling indicated similar damage levels. It would be of interest to analyze a smoker who quit smoking. The time frame of cell migration from the basal layer to exfoliation has been estimated to be 5–7 days based on the disappearance of micronuclei in radiation therapy patients (18). The immunohistochemical assay can also detect damage in nonsmokers which may be the result of dietary or environmental exposures. While no relationship was observed between consumption of charcoal broiled or smoked foods and intensity of staining, other dietary sources of PAH exposure may be relevant. For example, high levels of PAH have been found in green leafy vegetables (19). The exact source of elevated PAH-DNA adducts in the nonsmokers is unknown.

Approximately a 3-fold range in DNA adducts was observed in both the smokers and nonsmokers. Previous studies on WBC DNA demonstrated similar interindividual differences in DNA damage level for the same exposure level (6, 8, 20–22). These differences may be due to individual genetic variation in metabolism and detoxification of carcinogens and/or repair of DNA damage when formed (23, 24).

We intended to use the relationship between staining intensity and BPDE-I-DNA adducts in 10T½ cells (Fig. 1) for conversion of relative staining intensity to absolute adduct levels in the human samples. However, most values for the human oral cells were higher than that for the highest dose treatment of 10T½ cells. Only 6 nonsmokers had values below that of the highest dose treated 10T½ cells. Conversion of relative staining in human samples to modification level using an extrapolated standard curve (Fig. 1) would result in unrealistically high values. The high relative staining intensity in the oral cells may be a result of the different cell types or the presence of mucus in the oral samples. In addition, the 10T½ cells contain only BPDE-I-DNA adducts while the human samples may contain a range of PAH adducts with different affinity for the antiserum. Nevertheless, although absolute adduct levels cannot be determined,

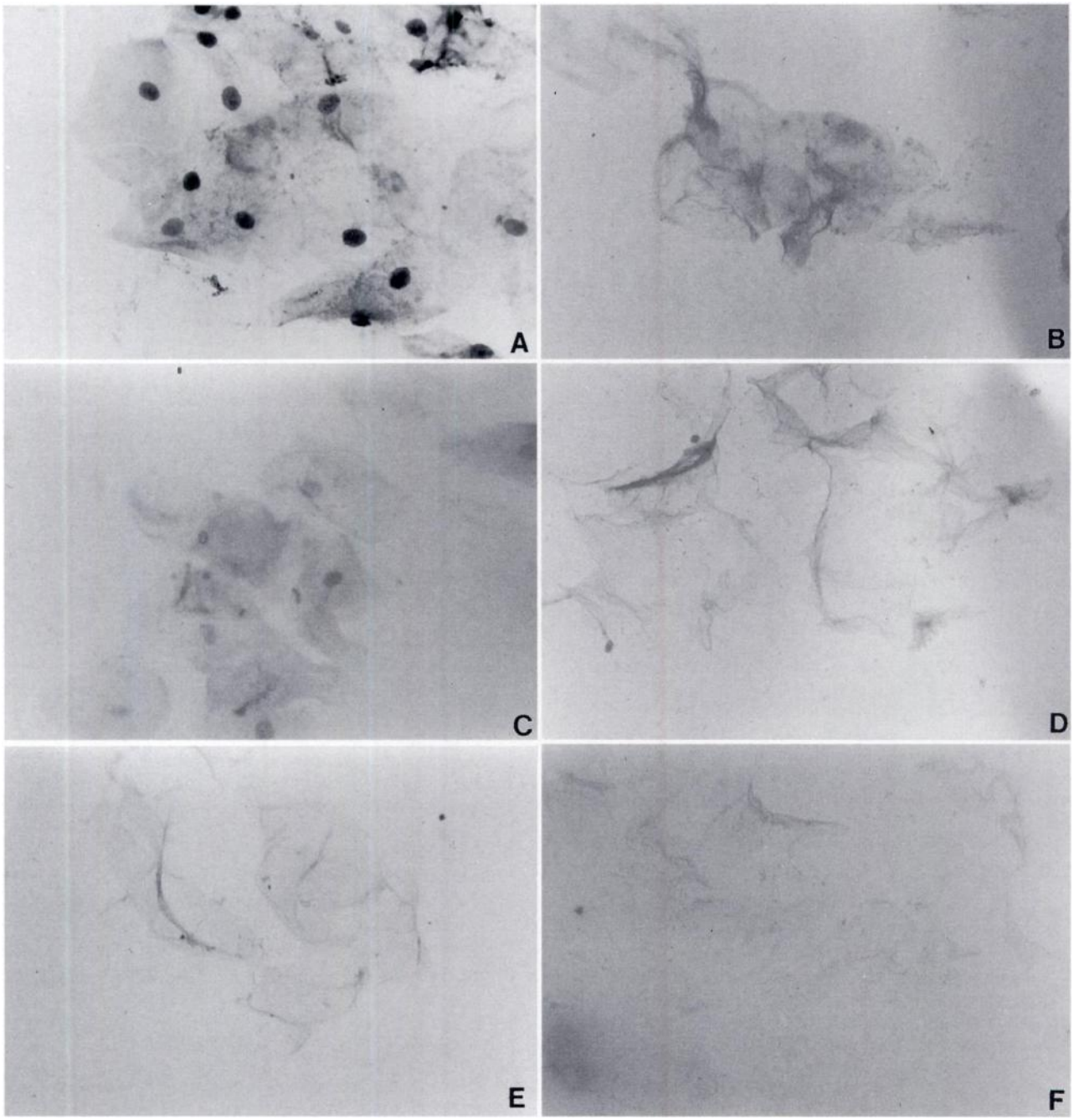

Fig. 2. Immunohistochemical staining of oral mucosa cells from a smoker and nonsmoker with a polyclonal antiserum recognizing PAH-DNA adducts. Staining of cells from smoker 12 (*A*) and nonsmoker 32 (*B*); cells from smoker 12 (*C*) and nonsmoker 32 (*D*) stained with antiserum 29 that was preabsorbed with BPDE-I-DNA before use; cells from smoker 12 (*E*) and nonsmoker 32 (*F*) stained with an antiserum recognizing 8-methoxypsoralen-DNA (× 400).

the immunohistochemical method provides useful information on relative levels of DNA damage in specific cell types.

Oral mucosa cells have been used previously to monitor exposure to PAH by ^{32}P postlabeling (25–28). An early study (25), using the carrier-free [^{32}P]ATP method, in groups at high risk for oral cancer, (including betel nut chewers, inverted smokers, and tobacco chewers) and Canadian controls found similar adducts in exposed and unexposed individuals. Estimated levels of adducts ranged from nondetectable to > 1 / 10^7. The butyl alcohol extraction method has also been used to demonstrate similar adducts in both smokers and nonsmokers in the range of $1/10^9$ to $6/10^8$ (26). No adduct was detected in the samples of tobacco users that was not present in the controls. Another study using the butyl alcohol extraction method also found no adduct spots consistently associated with exposure to alcohol or tobacco (27). Relative adduct labeling values ranged from $7.7/10^{11}$ to $1.6/10^6$. Adduct levels in

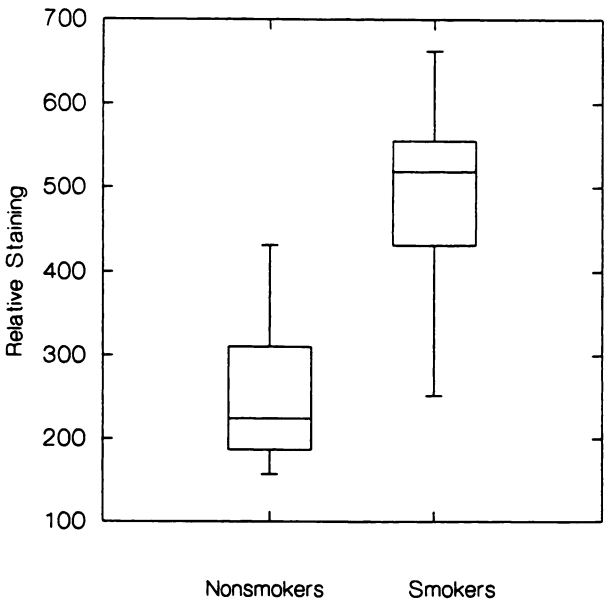

Fig. 3. Comparison of relative intensity of staining of oral mucosa cells of smokers and nonsmokers for PAH-DNA damage. The *lower* and *upper* edges of the box are the 25 and 75 percentile values. Median values are shown by the *line* within the box.

smokers (median $4.8/10^8$) were 1.71-fold higher ($P < 0.001$) than levels in nonsmokers ($2.9/10^9$). Oral biopsies of clinically normal tissue from smoking and nonsmoking patients undergoing surgery for intraoral squamous cell carcinoma have been assayed by the butyl alcohol extraction and nuclease P1 enrichment methods (28). The butyl alcohol extraction method revealed a wider range and higher level of adducts than the nuclease P1 method. Adduct levels in smokers, exsmokers, and nonsmokers were 3.75, 2.59, and $2.18/10^7$, respectively. The differential results with the butyl alcohol and nuclease P1 methods suggest that aromatic amines and nitroaromatics may be a source of some of the DNA adducts detected.

A single study has used immunohistochemical methods to detect DNA damage in oral mucosa cells (15). Cells from cancer patients receiving carboplatin and cisplatin combination chemotherapy were analyzed with an antiserum recognizing cisplatin modified DNA and peroxidase-(rabbit) antiperoxidase staining. All patients demonstrated increased nuclear staining but large interindividual differences in intensity were observed, suggesting differences in adduct formation and/or repair.

The immunoperoxidase method developed here will be useful to further investigate interindividual differences in damage and, potentially, of risk for cancer development. Because samples can be so easily collected it will be useful for repeated analysis of subjects. It can also be used as an intermediate biomarker in intervention studies in which modulation of DNA damage is an endpoint. Finally, feedback of specific individual DNA damage information to smokers may provide additional motivation for smoking cessation.

Acknowledgments

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