Evaluation of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Exfoliated Oral Cells by an Immunohistochemical Assay

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Abstract
Polycyclic aromatic hydrocarbon-DNA adducts were evaluated in oral cells from 98 healthy volunteers by an immunohistochemical method using a specific antiserum against benzo(a)pyrene-DNA adducts revealed by the immunoperoxidase reaction. Mean adduct content, determined as relative staining intensity by absorbance image analyzer, was significantly higher in the cells from tobacco smokers compared with nonsmokers (330 ± 98, n = 33 versus 286 ± 83, n = 64, respectively) with a P = 0.013 obtained by two-sample t test with equal variances. We found that in the smoker group, the PAH-DNA adduct content increases with the number of cigarettes. Thus, the relative staining intensity was 305 ± 105 in the group smoking 1–10 cigarettes/day (n = 16), 347 ± 77 in the 11–20 group (n = 14), and 386 ± 112 in the group smoking more than 20 cigarettes/day (n = 3; P = 0.03 by nonparametric test for trend). No significant association was detected between PAH-DNA adducts in oral cells and variables such as residential area, oral infections, alcohol or vitamin intake, grilled food consumption, and professional activity. This work confirms and extends previous data suggesting that this immunohistochemical method might be used as a valuable dosimeter of genotoxic damage in a carcinogen-exposed population, although further studies are needed to verify the applicability of the test in high-risk populations other than smokers.

Introduction
Environmental factors play an essential role in the development of several human tumors, including lung, bladder, and oral cavity cancers (1–4). PAHs are environmental carcinogens, products of incomplete combustion. They are ubiquitous pollutants encountered in the environment (air, water, and soil), in the food supply, and in mainstream and sidestream cigarette smoke (5). Some of these compounds (benzo(a)pyrene, benzanthracene, and chrysene) are procarcinogens and become carcinogens after metabolic conversion in electrophilic reactants (diol-epoxides), able to covalently interact with cellular macromolecules, including DNA, to form adducts. Such oxidative reactions are catalyzed by phase I cytochrome P-450 enzymes.

The ability of PAH-reactive metabolites to create specific covalent adducts with DNA correlates with their carcinogenic potential. In fact, the compounds of the PAH family (pyrene and anthracene), which are not able to form adducts, are not carcinogens (6, 7). This information about the carcinogenetic process has allowed the identification of biochemical markers reflecting molecular alterations, spanning from carcinogen exposure to clinical disease (8). Biochemical markers can be categorized as: (a) dose markers measuring carcinogen concentration in tissues and biological fluids; (b) response markers reflecting biological effects induced by exposure; and (c) susceptibility markers indicating the interindividual variations in response to carcinogenic exposure.

Quantitation of carcinogen-DNA adducts is a useful approach to screen individuals at risk in carcinogen-exposed populations because it allows the measurement of the biologically effective dose (9). Various methodological approaches have been developed for determination of DNA damage, such as immunoassay, gas chromatography-mass spectroscopy, fluorescence, and 32P-postlabeling (10). An immunohistochemical method for direct quantitation of DNA damage has also been developed. Such a method has many advantages: (a) it requires small amounts of biological material; (b) it allows a direct evaluation at the morphological level; (c) it has good selectivity and sensitivity for evaluating DNA adducts in human tissues and in culture cells; (d) it has relatively low cost; (e) it is easy to perform; and (f) it allows retrospective analyses in paraffin samples.

To evaluate DNA damage in single cells, in this study we have used a polyclonal antiserum raised against BPDE-DNA adducts. This antiserum cross-reacts with DNA modified by several other PAH diol epoxides, including those formed by benzanthracene and chrysene (11). A similar antiserum was used previously for measuring adducts by competitive ELISA in skin biopsies of coal-treated psoriasis patients and in WBCs of foundry workers (12, 13).

The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; BPDE, benzo(a)pyrene diol epoxide.

Received 7/16/98; revised 9/16/98; accepted 10/12/98.

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1 This work was supported by funds from Consiglio Nazionale delle Ricerche, Ministero dell’Universita e Ricerca Scientifica e Tecnologica, and a fellowship to whom requests for reprints should be addressed, at Ricerche Oncologiche, Universita del Sacro Cuore, Largo Francesco Vito, 1, 00168 Rome, Italy. E-mail: ibipg@rm.unicatt.it.

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The aim of the present work is to evaluate PAH-DNA adduct levels in exfoliated oral mucosa cells from 98 volunteers and to relate this marker of biologically effective dose with the environmental exposure to voluntary (tobacco smoke and diet) and/or involuntary (pollution and work place) factors.

Materials and Methods

BPDE Treatment of MCF-7 Cells. To develop a standard dose-response curve, MCF-7 breast adenocarcinoma cells were treated with 0, 5, 10, 40, and 80 μM BPDE dissolved in DMSO in complete RPMI 1640. After 12 h, the medium was removed. After washing with PBS, cells were recovered by treatment with trypsin for 5 min at 37°C. Cells were collected and centrifuged at 1300 rpm for 10 min. The cell pellet was resuspended in sucrose buffer [0.25 M sucrose, 1.8 mM CaCl₂, 25 mM KCl, and 50 mM Trizma Base (pH 7.5)] to a final ratio of 1 × 10⁶ cells/ml. About 25 μl of cell suspension were added to 150 μl of carbowax-ethanol and cytospun at 300 rpm for 5 min on slides precoated with 0.2% poly-d-lysine. Slides were air dried, fixed with 95% ethanol at −20°C, and stored at −20°C until staining.

Human Subjects. Ninety-eight healthy volunteers (91 men and 7 women; ages 17–64; mean, 36.6) were enrolled from the group of subjects undergoing a medical check every 6 months. Among the volunteers, 18 lived in a countryside area, 42 in a suburban area, and 38 in an urban area. Most of these subjects were military and civilian flight pilots and flight crew members (n = 46) potentially exposed to toxic inhalants containing PAH derivatives likely present in a plane cabin or cockpit. The rest of the group was composed of military personnel not exposed to any particular occupational carcinogen.

Subjects were classified as smokers if they were currently smoking at the time of the analysis (n = 33) and nonsmokers if they had smoked <200 cigarettes during their lifetime or never used tobacco-related products (n = 64). For one of the subjects under study, smoking habit data were not available. A questionnaire was administered eliciting information on smoking habits, job activity, diet, area of residence, medications, alcohol, and grilled foods consumption. After informed consent was obtained, oral mucosa cells were collected in a single sample/subject by rinsing the mouth with 30 ml of saline solution. The samples, after washing in PBS, were treated with RNase (100 μg/ml Sigma; 100 μl of a 1:10 dilution of hybridoma supernatant, recognizing DNA damage produced by the photoactivated drug 8-methoxypsoralen (15) or with the specific polyclonal antiserum preabsorbed with BPDE-DNA (1 μg/ml for 20 min at room temperature). Because of the limited number of slides from each patient, these controls could not be run for all samples. An image analyzer (Optimas 3; Sistemi Avanzati, Rome, Italy) was used to measure the relative intensity of nuclear staining in 100 randomly selected cells, which has been shown to be sufficient for an accurate evaluation of the sample (14). The spot area was explored in terms of 25 fields, within each of which 4–5 cells were selected, excluding those carrying morphologically degraded and pyknotic nuclei. The image was obtained in black and white, and the absorbance was recorded. The image analyzer expressed the results for each sample as the average of relative absorbance ± SD multiplied by 1000.

Statistical Analysis. Adduct level in a single sample is expressed as the mean ± SD of relative absorbance multiplied by 1000. Normality of raw data was performed by the Shapiro-Wilk W test, and comparisons between means were assessed by Student’s t test or between medians by Mann-Whitney test, when appropriate. The differences for ordinal data were tested by a nonparametric test (np trend) for trend across ordered groups (16, 17). Multiple regression analysis was applied for evaluation of relationship between adduct levels and the variables under study. All statistical analyses were performed with the Stata 5.0 (Stata Corp.).

Results

The immunoperoxidase method was applied to detect the presence of PAH-DNA adducts in MCF-7 cells treated with BPDE-I. Quantitation of specific nuclear staining in 100 randomly selected cells treated with increasing amounts of BPDE indicated a dose-related increase in DNA damage. The mean nuclear staining level is reported in Fig. 1A. Representative staining for BPDE-DNA adducts in untreated controls and in cells treated with 10 and 80 μM BPDE is illustrated in Fig. 1, B, C, and D, respectively. Preabsorption of primary antiserum with BPDE-I-DNA (1 μg/ml) before use decreased relative staining intensity of cells treated with 40 μM BPDE from 494 ± 80 to 126 ± 53. Pretreatment of slides with DNase decreased staining of the same cells to 118 ± 55. Substitution of primary antiserum with a nonspecific antiserum recognizing DNA damage produced by 8-methoxypsoralen decreased the staining level of 40 μM BPDE-exposed cells to control cell levels (data not shown).

The immunoperoxidase method was then used to detect BPDE-DNA adducts in human oral mucosa cells. To minimize variations in the immunohistochemical assay, untreated control samples and 40 μM BPDE-treated MCF-7 cells were stained together with each batch of samples. The Shapiro-Wilk W test was applied to absorbance average values and showed that the raw data were normally distributed both in smoker, nonsmoker, and total groups.

As for the professional exposure to PAHs, these toxic substances can be delivered, even if inconsistently, from lubri-
cant-containing circuits or from an air compression system on board when flying in the trail of other planes, or in airport areas, or when performing acrobatics. The levels of PAH-DNA adducts in the flight group tended to be higher than those in the remaining group (median \(5305.5, \text{iqr} = 550, n = 46\) versus median \(5298, \text{iqr} = 552, n = 51\), but this difference was not statistically significant (\(P = 0.4\) by Mann-Whitney test).

Mean adduct levels were significantly higher in oral mucosa cells of smokers (mean \(\pm SD = 330 \pm 98, n = 33\)) with respect to nonsmokers (286 \(\pm 83, n = 64, P = 0.013\) by two-sample \(t\) test with equal variances). The staining distribution in smokers and nonsmokers is shown in Fig. 2. In the smoker group, adduct levels increased with number of cigarettes smoked: relative staining of 1–10 cigarettes/day group was 305 \(\pm 105 (n = 16)\), of 11–20 was 347 \(\pm 77 (n = 14)\), and of >20 was 386 \(\pm 112 (n = 3)\). The relationship between nuclear staining for PAH-DNA adducts and number of cigarettes smoked was statistically significant (\(P = 0.03\) by np trend test). Representative higher staining for BPDE-DNA adducts in oral cells of a smoker is illustrated in Fig. 3A; weaker nuclear staining in cells of a nonsmoker is shown in Fig. 3B. To demonstrate staining specificity, oral cells from a smoker (nuclear staining \(\pm SD = 484 \pm 98\)) were stained with primary antiserum preadsorbed with BPDE-I-DNA (1 \(\mu g/\mu l\); nuclear staining of this sample decreased to 144 \(\pm 51\) (Fig. 4). Pretreatment of the same cells with DNase decreased staining to 130 \(\pm 47\). Staining with a nonspecific antiserum recognizing DNA damage produced by 8-methoxypsoralen was 137 \(\pm 45\) (data not shown).

For assessing the influence of passive smoking on PAH adduct levels, we distinguished within the nonsmokers an exposed and a nonexposed group. The subjects were considered exposed if living and/or working with at least one heavy smoker. Information was available for 58 subjects, and a non-significant difference was observed (exposed: 282 \(\pm 83, n = 52\); nonexposed: 286 \(\pm 114, n = 6\)).

For grilled or smoked foods consumption, the subjects were subdivided as follows: daily (median, 266; iqr, 164; \(n = 5\)); occasionally (having such foods once/twice a week; median, 316; iqr, 149; \(n = 61\)); and nonconsumers (median, 271; iqr, 110; \(n = 25\)). There was no association between consumption of charcoal-broiled or smoked foods and adducts (\(P = 0.09\) by np trend test).

Similarly, no significant association was seen between PAH-DNA adducts in oral cells and variables such as residential area, gender, oral infections, alcohol, or vitamin intake.

Multiple linear regression analysis was applied to evaluate linear coefficients of sex, smoke, oral infections, vitamin and alcohol intake, consumption of grilled foods, and job activity in the prediction of adduct level. Many possible models were tested, but in none was the proportion of explained variance >10%. Moreover, in some models the smoking status or the

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**Fig. 1.** A, dose-response curve of PHA-DNA adduct formation in MCF-7 cells detected by an immunoperoxidase method (see “Materials and Methods” for further technical details). Immunohistochemical staining for PAH-DNA adducts of MCF-7 cells treated with 0 (B), 10 (C), and 80 (D) \(\mu M\) BPDE \((X \times 400)\) is shown.

**Fig. 2.** Box-plot of relative staining intensity of BPDE-DNA adducts in oral cells by smoke class. The lower and upper edges of the box are the 25th and 75th percentiles, respectively. Median values are shown by the line within the box. The width of each box is proportional to sample number. Whiskers represent adjacent values. The mean value of nonsmokers is 285, of smokers 1–10 cigarettes/day is 320, of smokers >10 is 334 (not represented).
number of smoked cigarettes (never the both at the same times) showed coefficients with a difference from zero statistically significant.

Discussion
Many human tumors, such as lung, bladder, larynx, oral cavity, and colon cancers, are known to be related to environmental factors acting in conjunction with individual susceptibility. Cancer incidence might be diminished by $>80\%$, with prevention strategies able to reduce or exclude exposure to environmental carcinogens (such as tobacco smoke, air and water pollutants, radiation, and infectious agents; Ref. 18).

Chemical carcinogens, metabolically activated in vivo by phase I enzymes, covalently bind to DNA, forming DNA adducts. These adducts, if not repaired, can induce genetic alterations that contribute to cancer development. For example, in BPDE-treated HeLa and in bronchial epithelial cells, BPDE-DNA adducts occur in the $p53$ gene at codons 157, 448, and 243, which are the major mutational hotspots in human lung cancers (19).

In this study, we evaluated PAH-DNA adducts in oral cells, because the oral cavity is a target tissue for chemical carcinogens from the environment and cigarette smoke, and exfoliated cells are easily accessible and collectable in a non-invasive fashion, one limitation of this study being that smoking exposure is estimated from information collected on a questionnaire.

The immunoperoxidase assay, validated by analysis of 10T½ cells treated with $[^{3}H]$BPDE (20), allows us to satisfactorily evaluate adduct levels in a semiquantitative fashion, and the relatively low cost makes it suitable to screen large numbers of exposed subjects.

Our findings indicate that the immunoperoxidase assay using a polyclonal antiserum anti BPDE-DNA adducts (11) is sufficiently sensitive for evaluating PAH-modified nucleotides. With such an antiserum, in MCF-7 cells treated with BPDE, a dose-response curve for BPDE-DNA adducts was obtained. In oral cells, PAH-DNA adducts are associated with smoking status with a direct relationship between relative nuclear staining and number of cigarettes smoked daily. Similar results, demonstrating that smoking habit produces genotoxic damage in a dose-related fashion, have been reported previously in bladder, lung, and nasal tissue (21, 23). Oral cells from non-smokers show a weak nuclear staining, probably due to pollution exposure or to passive smoking. Although passive smoke does not seem to acquire an important significance in the present study, this is probably due to the disproportionate number of subjects exposed to passive smoke in the nonsmoker group.

We found about a 4-fold range in adduct level variation both in the nonsmoker (from 114 ± 61 to 461 ± 82) and smoker group (from 123 ± 55 to 479 ± 113). This large interindividual difference may be due to a number of variables such as: (a) the ubiquitous presence of PAH pollutants so that nonsmokers may be exposed through environmental pollution, diet, and passive smoke; (b) variation in the expression of phase I cytochrome P-450 activating enzymes, which catalyze the oxidative metabolism of PAHs into electrophilic intermediates. Many studies suggest that CYP1A1-MSP1 polymorphism increases cancer risk (24, 25), and that adduct levels in lung tissue correlate with CYP1A1 expression or enzyme activity (26); (c) polymorphism of phase II enzymes, which detoxify PAH metabolites, producing excretable hydrophilic compounds. Some studies suggest that individuals with GSTM1 null genotype have higher PAH-DNA adduct levels and $p53$ mutations in lung tissue (24, 27); and (d) variation in repair capacity. It has been demonstrated that patients with lung cancer have reduced ca-
pacity to repair genotoxic damage induced by PAHs with respect to healthy controls (28).

Our data suggest an interindividual variation in relative staining in the >10 cigarettes/day smokers group (range, 204–489; mean, 355; SD, 82; n = 17) smaller than in light smokers (1–10 cigarettes/day; range, 123–479; mean, 305; SD, 106; n = 16). This observation agrees with the hypothesis that polymorphisms in genes encoding activation/detoxification enzymes have a great impact on DNA adduct formation at low carcinogen exposure. At high carcinogen doses, the effect of susceptibility factors appears to be less evident than at low doses (25, 29, 30).

Many reports have investigated DNA adducts in oral cells by the 32P-postlabeling assay, producing conflicting results about the relation with smoke exposure (31–34). This inconsistency could be attributed to differences in extraction and enhancement procedures and to the small number of samples examined. Two other studies have previously investigated genotoxic damage in oral exfoliated cells by an immunohistochemical method and found an increase of PAH and 4-aminonobiphenyl-DNA adducts in smokers compared with nonsmokers. In both studies, however, only a small number of subjects were examined. The present study confirms, for the first time, the validity of this method on a large series of subjects, although the differences observed between smokers and non-smokers are weaker than those reported previously in smaller selected groups of donors (20, 35, 36).

Consumption of foods with high levels of PAHs, such as broiled or grilled products, was not associated with a significant increase in DNA adducts in oral cells. Foods pass quickly through the oral cavity but stay longer in the colorectal tract, which may represent the principal target tissue for this exposure. In fact, consumption of charcoal-broiled or smoked meats is more connected to colorectal than oral cancers (37).

Most of the military and civilian volunteers enrolled in this study are not exposed to carcinogenic agents in their workplace. Only pilots and flight crew can be in contact with toxic inhalants delivered from board instruments, but we did not find any relation between PAH-DNA adducts and this activity.

There are limited experimental data indicating that women are more susceptible to carcinogens than men (38, 39). In our study, we could not address this point because of the small number of women (a total of seven) enrolled in the study. Our results only confirm the validity of this assay in recognizing genotoxic damage in the active smoke-exposed group. Further studies are needed to validate the use of this test to identify DNA adducts from exposure to passive smoke. Moreover, the results of this study suggest that this method may not be adequate to identify occupational PAH exposure, at least at low carcinogen doses. More careful studies are ongoing to verify whether the method might be useful for identifying DNA adducts in other exposed workers, as demonstrated previously with an ELISA using the same antibody (10, 13). The reduced sensitivity of the immunohistochemical method (1 adduct/10^7 nucleotides) might, in any case, be a limitation (14).

In the same way, our results suggest that evaluation of PAH-DNA adducts in oral cells cannot be considered a useful indicator of high-risk food consumption.

In conclusion, this study performed on healthy subjects confirms and extends on a larger series previous data suggesting the usefulness of the immunoperoxidase method as a genotoxic damage dosimeter and, potentially, as an indicator of risk for cancer development, but further studies are needed to verify the applicability of the test in other types of populations at risk.

References

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Cancer Epidemiol Biomarkers Prev 1999;8:91-96.

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