Applicability of Induced Sputum for Molecular Dosimetry of Exposure to Inhalatory Carcinogens: ^{32}P-Postlabeling of Lipophilic DNA Adducts in Smokers and Nonsmokers

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Abstract

The lung is a major target organ for smoking-associated cancer. We examined the applicability of induced sputum for molecular dosimetry of exposure to tobacco smoke-related carcinogens. Sputum induction was performed by inhalation of 4.5% saline delivered from an ultrasonic nebulizer for a period of up to 21 min in a group of smoking ($n = 20$) and nonsmoking ($n = 24$) healthy individuals. Samples were analyzed for total and differential cell counts and cell viability. Subsequently, DNA contents of the samples were isolated, and measurement of lipophilic DNA adducts was done by the ^{32}P-postlabeling assay using nuclease P1 (NP1) and butanol enrichment methods. All subjects tolerated the induction procedure without experiencing any troublesome symptoms, and 90% of smokers (18 of 20) and 88% of nonsmokers (21 of 24) succeeded in producing sufficient amounts of sputum. Total cell counts and percentages of viable cells in smokers were higher than those in nonsmokers (6.7 ± 6.0 *versus* $4.7 \pm 6.0 \times 10^6$, $P = 0.40$ and 80 ± 15 *versus* 63 ± 17, $P = 0.01$, respectively). In cell differentials, smokers had lower percentages of bronchoalveolar macrophages and higher percentages of neutrophils (69 ± 24 *versus* 92 ± 5, $P = 0.002$ and 26 ± 26 *versus* 4 ± 4, $P = 0.008$, respectively). Using the NP1 digestion method, all smokers and only one nonsmoker showed a diagonal radioactive zone in their adduct maps; adduct levels in smokers were higher than those in nonsmokers (3.1 ± 1.4 *versus* $0.6 \pm 0.8/10^8$ nucleotides; $P = 0.0007$), and also, adduct levels were significantly related to smoking indices. Applying the butanol extraction method, however, only half of the smokers and three nonsmokers showed the diagonal radioactive zone in their adduct maps; adduct levels in smokers were higher than those in nonsmokers (4.6 ± 3.7 *versus* $1.0 \pm 1.9/10^8$ nucleotides; $P = 0.02$), and the levels of adducts were significantly related to the smoking indices. There was a correlation between the levels of adducts determined by the two enrichment methods ($r = 0.7$; $P = 0.02$). Paired comparison showed no differences between the levels of adducts measured by the two methods ($P = 0.55$). We conclude that induced sputum can serve for molecular dosimetry of inhalatory exposure to carcinogens and that the NP1 version of the ^{32}P-postlabeling assay is a choice of preference for studying smoking-induced DNA adducts in the lower respiratory tract.

Introduction

Lung cancer is a leading cause of cancer-related death worldwide, and it is taking a toll of >3 million lives annually (1, 2). Epidemiological data have implied that tobacco smoking is a major risk factor for lung cancer (3–5); 80–85% of lung cancer cases are attributed to tobacco smoking (3–6) and 10–15% of lifetime smokers develop lung cancer (4, 6, 7). Because survival after diagnosis of lung cancer is quite poor and early detection is generally scarce (7), interventions can only be aimed at prevention, *e.g.*, by removing the risk factors and/or by identifying the individuals at high risk.

Two main classes of carcinogens present in tobacco smoke are polycyclic aromatic hydrocarbons and aromatic amines (3, 8, 9). The chemical carcinogenicity of these compounds is mainly ascribed to the capability of their reactive metabolites to covalently bind to cellular DNA and form DNA adducts (8, 10). Because formation of DNA adducts appears to be a crucial step in initiating the process of carcinogenesis, examination of DNA for the presence of adducts could serve as a valuable means for assessing exposure to carcinogens as well as risk for cancer (11). To date, the ^{32}P-postlabeling assay is the most widely used method for studying DNA adducts (12). Significant advantages of this assay for adduct analysis are its high sensitivity (1 adduct/10^9–10^{10} unmodified nucleotides), its requirement for only low amounts of DNA (1–5 μg), and its ability to detect DNA adducts formed by complex mixtures (13).

Thus far, measurement of DNA adducts to study smoking-induced lung cancer has often been performed in surrogate tissues, such as WBCs, and occasionally in biopsy or autopsy samples from the lung (14–25). However, uncertainty about the validity of WBCs as the surrogate for the lung consequent to the inconsistency in their DNA adduct analyses (26–30) and infeasibility of sampling lung tissue on a routine basis (7) have surfaced the need to explore other alternative tissues.

Macroscopic and/or microscopic examination of spontaneous sputum to investigate airway inflammation have been

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Table 1 Characteristics of study population and summary of DNA adducts analysis

	Smokers	Nonsmokers
No.	20	24
Sex (male/female)	7/13	6/18
Age[a]	39 ± 11	34 ± 8
Cigarettes/day[a]	17 ± 8	0
Tar/day (mg)[a]	345 ± 196	0
Smoking yr[a]	20 ± 10	0
Pack-yr[a]	18 ± 14	0
Sputum induction (success/failure)	18/2	21/3
Lipophilic DNA adducts in sputum (NP1 digestion method)[a,b]	3.1 ± 1.4[c]	0.6 ± 0.8
Lipophilic DNA adducts in sputum (butanol extraction method)[a,b]	4.6 ± 3.7[d]	1.0 ± 1.9

[a] Results expressed as mean ± SD.
[b] Adducts/10^8 nucleotides as determined by ^{32}P-postlabeling.
[c] Statistically significant compared to nonsmokers; $P = 0.0007$.
[d] Statistically significant compared to nonsmokers; $P = 0.02$.

common practice since the last half of the 19th century (31). However, recent innovations in sputum induction technique have made it possible to also obtain sputum from patients who are unable to produce it spontaneously. This, in turn, has enabled clinicians to deploy sputum induction as a noninvasive method of sampling cellular and biochemical constituents of the lower respiratory tract to study a variety of airway complications (32–35). Yet, applicability of sputum for molecular dosimetry studies of inhalatory carcinogens has not been tested.

In the present study, we examined whether sputum can be used for molecular dosimetry of exposure to carcinogens present in tobacco smoke. For this purpose, we measured the levels of lipophilic DNA adducts in induced sputum cells of smokers and nonsmokers by means of the ^{32}P-postlabeling assay using NP1[2] digestion and butanol extraction as enrichment methods.

Materials and Methods

Study Population. Volunteers were recruited by advertisements in local newspapers. Upon enrollment, every individual filled out a self-administered questionnaire and signed an informed consent. The volunteers were scrutinized thoroughly based on the information elicited from the questionnaires with regard to their age, sex, smoking behavior, alcohol consumption, medical history of disease, dietary/medicinal/occupational exposure to known carcinogens, and familial history of cancer. Accordingly, two groups of healthy individuals consisting of 20 smokers (age, 39 ± 11; smoking status, 17 ± 8 cigarettes/day) and 24 lifelong nonsmokers (age, 34 ± 8) with no occupational exposure to tobacco-related carcinogens were selected. Before sampling, all participants were interviewed in detail and briefed for sputum induction procedure. Table 1 summarizes the characteristics of the study population. The study was approved by the Medical Ethical Commission of Maastricht University.

Sputum Induction. Subjects were pretreated with 200 μg of salbutamol administered via an inhalatory chamber (medication was given prophylactically to reverse bronchoconstriction if any). Sputum induction was performed by inhalation of ultrasonically nebulized 4.5% saline delivered from an Ultra-Neb 2000 (De Vilbiss, Somerset, PA). Total inhalation time was 21 min with three 5-min intervals at the end of each 7-min period. During the intervals, subjects rinsed their mouths, gargled their throats, and then coughed up all produced expectorate into a 50-ml Greiner tube (Greiner Labortechnik, Frickenhausen, Germany) placed on ice. Additionally, they were advised to cough up the available expectorate at any moment regardless of the time of the induction. Induction was terminated as soon as sufficient amount of sputum (at least 5 ml) was obtained.

Sputum Processing. Processing of induced sputum was done within 2.5 h of sampling. The volume of the sample was measured, and four volumes of 0.1% Sputolysine (Calbiochem-Novabiochem Corp., La Jolla, CA) were added to produce mucolysis and rid sputum of its gelatinous form. The solution was gently aspirated with a 5-ml pipette for several times and placed in a shaking water bath for 15 min at 37°C. Intermittently, the sample was vortexed two to four times for 15 s each. To quench the activity of the Sputolysine, a further four volumes of PBS (pH 7.4) were added to the sample, and shaking continued for another 5 min. The resulting homogenate was centrifuged at 725 × *g* for 10 min at 4°C. Supernatant was discarded, the pellet was resuspended in 2 ml of PBS from which 100 μl were used for cytological examination, and the remaining was repelleted to be preserved at −80°C until DNA isolation. Determinations of cell viability according to trypan blue exclusion technique and total cell counts were carried out using 10 μl of the cell suspension in a standardized hemocytometer. From the remaining cell suspension, an aliquot of 30×10^3 cells (diluted in PBS to a final volume of 300 μl) was cytocentrifuged (Shandon Cytospin 3, Cheshire, United Kingdom) at 1500 rpm for 5 min onto Polysine microslides (E. Merck Nederland B.V., Amsterdam, the Netherlands). The slide was stained with May-Grünwald Giemsa, and cell differential was done through counting 500 nonsquamous cells.

DNA Isolation. Cell pellet was thawed and subsequently lysed with 400 μl of SET/SDS [100 mM NaCl, 20 mM EDTA, 50 mM Tris, 0.5% SDS (pH 8.0)] at 37°C, overnight. The resulting suspension was treated with 50 μl of RNase mixture (0.1 mg/ml RNase A and 1000 units/ml RNase T1) for 3 h at 37°C, followed by treatment with 75 μl Proteinase K (10 mg/ml) for 2 h at 37°C. DNA was isolated by repetitive extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) and then precipitated with two volumes of 100% cold ethanol and 1/30 volumes 3 M sodium acetate (pH 5.3). Precipitated DNA was rinsed with 70% ethanol and dissolved in 2 mM Tris (pH 7.4). Purity and concentration of the DNA were determined spectrophotometrically by absorbance at 230, 260, and 280 nm and ultimately, the concentration was adjusted to 2 mg/ml.

^{32}P-Postlabeling Assay. The ^{32}P-postlabeling assay was performed as described earlier (36). Briefly, 5 μg of DNA were digested into deoxyribonucleoside 3′-monophosphates using micrococcal endonuclease (0.25 units/μl) and spleen phosphodiesterase (2 μg/μl). Half of the digest was treated with NP1 (2.5 g/μl) or alternatively, extracted with 1-butanol according to the method of Gupta (37). Subsequently, the modified nucleotides were labeled with [γ-^{32}P]-ATP in the presence of T4-polynucleotide kinase. Radiolabeled adducted nucleotide biphosphates were separated by two-dimensional chromatography on PEI-cellulose sheets (Machery Nagel, Düren, Germany) using the following solvent systems: D1, 1 M NaH_2PO_4 (pH 6.5); D2, 8.5 M Urea, 5.3 M lithiumformate (pH 3.5); D3, 1.2 M lithiumchloride, 0.5 M Tris, 8.5 Urea (pH 8.0); and D4, 1.7 M NaH_2PO_4 (pH 6.0). To ensure the efficiency of NP1 treatment and ATP excess, an aliquot of the digest was one-

[2] The abbreviations used are: NP1, nuclease P1; BAL, bronchoalveolar lavage; BAM, bronchoalveolar macrophage; DRZ, diagonal radioactive zone; PEI, polyethyleneimine; BPDE, benzo(a)pyrene diol-epoxide.

Table 2 Cellular characteristics and DNA yield of induced sputum

	Smokers (mean ± SD)	Nonsmokers (mean ± SD)
Total cell count ($\times 10^6$)	6.7 ± 6.0	4.7 ± 6.0
Viability (%)	80 ± 15[a]	63 ± 17
Squamous cells (%)	17 ± 13	16 ± 12
Nonsquamous cells (%)	83 ± 13	84 ± 12
BAMs (%)	69 ± 24[b]	92 ± 5
Neutrophils (%)	26 ± 26[c]	4 ± 4
Bronchoepithelial cells (%)	5 ± 5	3 ± 4
Lymphocytes (%)	0.3 ± 0.5	0.3 ± 0.5
Methachromatic cells (%)	0.2 ± 0.6	0.2 ± 0.6
DNA yield (μg)	56 ± 55	30 ± 35

[a] Statistically significant compared to nonsmokers; $P = 0.01$.
[b] Statistically significant compared to nonsmokers; $P = 0.002$.
[c] Statistically significant compared to nonsmokers; $P = 0.008$.

dimensionally chromatographed on PEI-cellulose sheet (Merck, Darmstadt, Germany) using a solvent system of 0.12 M NaH_2PO_4 (pH 6.8). For quantification purposes, two standards of [^{3}H]BPDE-modified DNA with known modification levels of 1 adduct/10^7 and 10^8 unmodified nucleotides were run in parallel in all experiments. Quantification was performed using phosphor imaging technology (Molecular Dynamics, Sunnyvale, CA), which renders a detection limit of 1 adduct/10^9 nucleotides. Quantitatively, half of the detection limit for the DRZ (0.25 adducts/10^8 nucleotides) was considered as the determined level of adducts for samples that showed neither a DRZ nor an adduct spot in their adduct maps. To assess the efficiency of labeling in NP1 digestion and butanol extraction methods, standards of [^{3}H]BPDE-modified DNA with known modification levels were enriched by the respective methods and simultaneously assayed ($n = 4$). Adduct recoveries in the NP1 digestion and butanol extraction methods were 69 ± 2.6% and 39 ± 4.6%, respectively. Nucleotide quantifications were done by labeling the remaining half of the digested DNA with [γ-^{32}P]-ATP in the presence of T4-polynucleotide kinase and by subsequent separation of the nucleotides with one-dimensional chromatography on a PEI-cellulose sheet using the solvent system of 0.12 M NaH_2PO_4 (pH 6.8). Samples with evident protein and/or RNA contaminations were excluded from the analysis.

Statistical Analysis. Results were expressed as mean ± SD. In all cases, comparisons were made using the unpaired Student's *t* test unless otherwise indicated. Simple linear regression analysis was performed to study the relationships between different variables. Statistical significance was considered at $P < 0.05$.

Results

Success Rate for Sputum Induction. Sputum induction was well-tolerated by all subjects, and no troublesome symptoms occurred throughout the procedure. Eighteen of 20 smokers (90%) and 21 of 24 nonsmokers (88%) could produce sufficient amount of sputum (Table 1).

Cytological Examinations and DNA Yield. Cellular characteristics of the induced sputum of smokers and nonsmokers are presented in Table 2. Total cell counts and percentages of viabilities in smokers were higher than those in nonsmokers ($P = 0.40$ and $P = 0.01$, respectively). Fig. 1 illustrates the representative cell differentials of an induced sputum sample from a smoker. Smokers had lower percentages of BAMs ($P = 0.002$) and higher percentages of neutrophils ($P = 0.008$) as compared to nonsmokers. Percentages of neutrophils were related to smoking indices as follows: number of cigarettes smoked per day ($r = 0.4$; $P = 0.04$), amount of tar consumed per day (tar content of the cigarette × number of cigarettes smoked per day: $r = 0.5$, $P = 0.02$), and pack-years (number of cigarettes smoked per day/20 × smoking years: $r = 0.4$, $P = 0.03$). DNA yields were related to total cell counts ($r = 0.7$; $P = 0.0001$), and smokers had higher DNA yields (adjusted for total cell counts) compared to nonsmokers (56 ± 55 *versus* 30 ± 35 μg; $P = 0.14$). Samples with high percentages of squamous cells (>40%) and low total cell counts ($<1 \times 10^6$) were excluded from the ^{32}P-postlabeling analysis due to the facts that the presence of squamous cells in induced sputum is indicative of salivary contamination (38, 39) and that the DNA yield of 1×10^6 cells (~5 μg) is just sufficient for one ^{32}P-postlabeling assay.

^{32}P-Postlabeling of Lipophilic DNA Adducts. Fig. 2, *A* and *B* depicts the representative chromatograms of ^{32}P-postlabeled DNA adducts from a smoker and a nonsmoker, respectively, by the NP1 digestion method. In this version of the ^{32}P-postlabeling assay, all smokers and only one nonsmoker showed a DRZ in their adduct maps. Adduct levels in smokers were higher than those in nonsmokers ($P = 0.0007$; Table 1). Levels of adducts in smokers and nonsmokers ranged from 1.8 to 5.6 and from 0.3 to 1.9 /10^8 nucleotides, respectively. Distribution of the levels of adducts in smokers and nonsmokers are shown in Fig. 3. Adduct levels were related to the number of cigarettes smoked per day ($r = 0.6$; $P = 0.007$), the amount of tar consumed per day ($r = 0.7$; $P = 0.006$), and the pack-years ($r = 0.6$; $P = 0.01$).

By butanol extraction method of the ^{32}P-postlabeling assay, however, only half of the smokers and three nonsmokers showed the DRZ in their adduct maps (Fig. 2, *D* and *E*). Adduct levels in smokers were higher than those in nonsmokers ($P = 0.02$; Table 1). Levels of adducts in smokers and nonsmokers varied in the range of 0.3–10.0 and 0.3–4.1/10^8 nucleotides, respectively. Adduct levels were also related to the smoking indices as follows: number of cigarettes smoked per day ($r = 0.6$; $P = 0.01$), amount of tar consumed per day ($r = 0.5$; $P = 0.06$), and pack-years ($r = 0.6$; $P = 0.01$). There was a correlation between the levels of adducts measured by the butanol extraction method and those quantified by the NP1 digestion method ($r = 0.7$; $P = 0.02$). Paired comparison showed no differences between the levels of adducts determined by the two methods (paired Student's *t* test; $P = 0.55$).

Discussion

The high success rate for sputum induction shown in the present study is in favor of the feasibility and reproducibility of this technique for effectively sampling of healthy individuals. This is of importance because the applicability of the technique has mainly been demonstrated in patients with respiratory disorders who are likely to produce sputum even spontaneously (34). Apart from a few clinical studies (39–46) in which sputum induction has been performed in a small number of nondiseased control subjects, our study is the first one that shows the plausibility of this technique in a relatively large number of healthy individuals.

The results of cytological examinations are in good agreement with the findings of other clinical studies (39, 40, 44); however, our cell differential data are partially different from those of one recent study (46). Similar to that study, we observed interrelated percentages of BAMs and neutrophils in both smokers and nonsmokers. In contrast, we found higher percentages of neutrophils and lower percentages of BAMs in

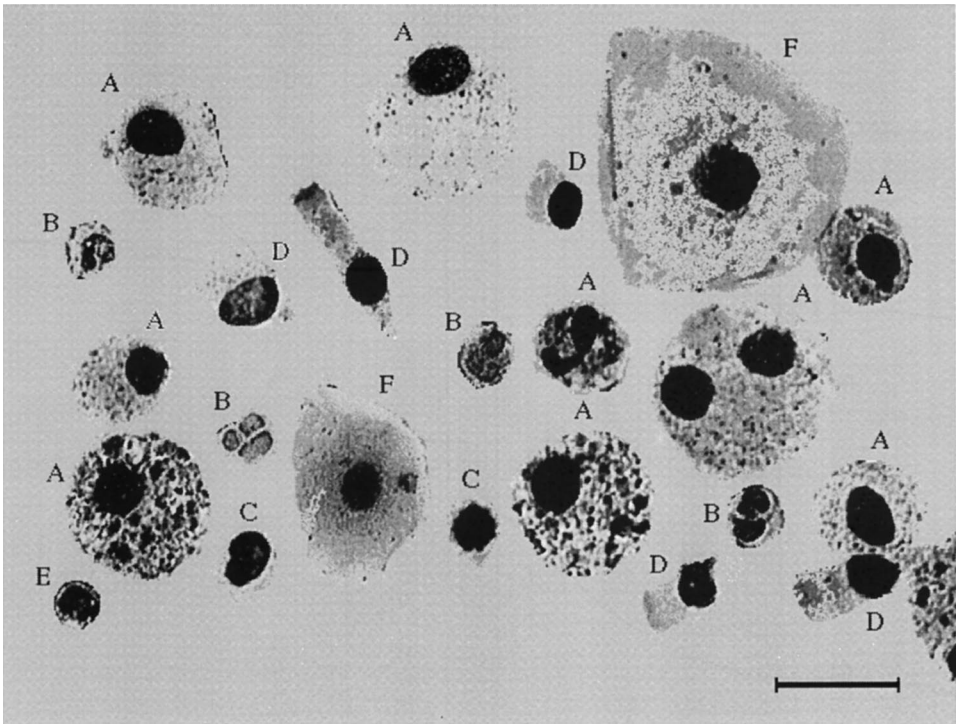

Fig. 1. Representative cell composition of induced sputum sample from a smoker [for visualization, three sections of the slide are assembled together by Image Processing and Analysis System (Quantimet 500, Leica, Cambridge, United Kingdom)]. A, BAM; B, neutrophil; C, bronchoepithelial cell; D, ciliated cell; E, lymphocyte; F, squamous cell. May Grünwald-Giemsa staining. Magnification, ×400. *Bar*, 30 μm.

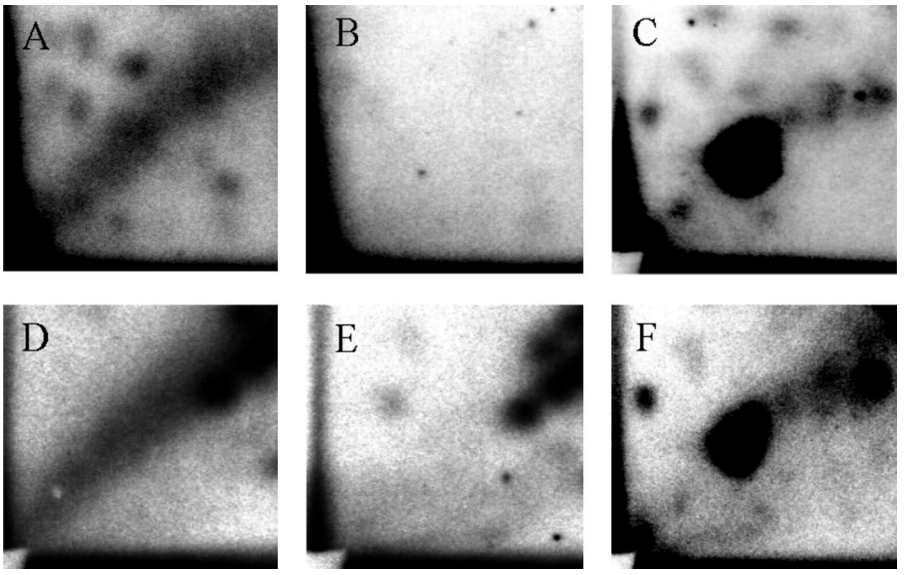

Fig. 2. Representative chromatograms of the ^{32}P-postlabeled DNA adducts in induced sputum of a smoker and a nonsmoker by the NP1 digestion method (*A* and *B*, respectively) and by the butanol extraction method (*D* and *E*, respectively; the radioactivity in the *upper left-hand corner* was also observed at the same location in standard DNA and therefore considered as background). *C* and *F*, BPDE-DNA adduct standards (1 adduct/10^7 nucleotides by NP1 and butanol enrichment methods, respectively).

smokers compared to nonsmokers (46). This discrepancy could be due to the different group sizes because the study population in that report is quite small (46). Moreover, smokers are more likely to have higher percentages of neutrophils as compared to nonsmokers because inhaled materials of smoking may induce inflammation in the airway where neutrophils are functioning as the first line of the inflammatory cells (47). Interestingly, the significant dose-response relationship found between percentages of neutrophils and the smoking indices supports this idea.

Applying the NP1 and the butanol enrichment methods of the ^{32}P-postlabeling, we found significant differences between DNA adducts of smokers and nonsmokers; the differences were much more distinguishable when the former method was applied. Using the NP1 digestion method, we observed the DRZ as the indicator of exposure to complex mixtures, *e.g.*, tobacco smoke (48, 49) in the adduct maps of all smokers and only one nonsmoker. However, this difference was less striking when the butanol extraction method was applied. This might be explained by the fact that the butanol extraction method enriches a wide range of adducts, some of which are nonspecific for tobacco smoke (50, 51). Lower recovery of BPDE-DNA adducts by this method, which was shown in our study, can also

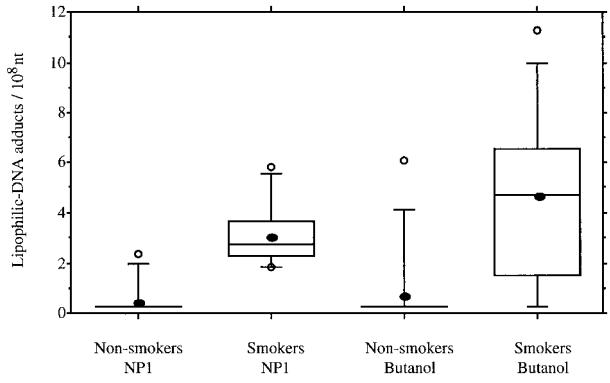

Fig. 3. Levels of lipophilic DNA adducts in induced sputum of smokers and nonsmokers as determined by NP1 digestion and butanol extraction versions of the ^{32}P-postlabeling assay. *Lower* and *upper edges of the boxes*, the 25th and the 75th percentiles, respectively. ○ and —— *within the boxes*, the means and the medians, respectively. *Lower* and *upper bars*, the 10th and the 90th percentiles, respectively. ○, individual values <10th or >90th percentiles.

confirm the less specificity of butanol extraction method for enriching lipophilic DNA adducts. Using the NP1 digestion method as well as the butanol extraction method, we found significantly higher levels of adducts in smokers compared to nonsmokers; much clearer difference was observed by the NP1 method. Further, by both methods, we could establish dose-response relationships between the levels of adducts and the smoking indices. These findings imply that not only are the levels of lipophilic DNA adducts in induced sputum cells related to exposure to tobacco smoke, but they also mirror the magnitude of such exposure.

It has already been shown that DNA adduct analysis in cells present in BAL is an informative way of assessing inhalatory exposure to carcinogens (30, 36, 52, 53). However, invasiveness of the method of sampling has hampered routine application of the BAL cells for molecular dosimetry study of the airway. Comparing the results of the BAL studies with those of ours, we observed that in both samples, adduct levels were dose-dependently related to exposure; however, mean adduct levels in the BAL cells, *e.g.*, in a smoking group from one of these studies (36), was 2-folds higher than that in the induced sputum cells from our study. This could be due to the different levels of exposure because the exposed group in the BAL study was composed of heavy smokers (smoking status, 27 ± 13 cigarettes/day; Ref. 36), whereas the smokers in our study had a moderate smoking status. Besides, different cell compositions in the two samples might also reason for such findings because the BAL contains higher percentages of BAMs as compared to induced sputum (>90 *versus* <70), and it has been shown that BAMs have a higher DNA adduct content than other cell types (30). Nonetheless, because the information obtained through the analysis of these two samples is to a great extent identical and because the sputum induction has the merit of being noninvasive, it can be concluded that induced sputum cells have the potential to substitute the BAL cells for molecular dosimetry study of the airway.

Prospectively, we plan to validate our results and examine their consistency as compared to the existing inconsistency in the results of DNA adduct analysis in surrogate tissues, *e.g.*, WBCs (26–30). Furthermore, we intend to get insight into the exact nature of the detected adducts by means of the immunoassays applying antibodies raised against specific DNA adducts by means of the ^{32}P-postlabeling assay employing reference adducts and using nonurea solvent systems (54). By accomplishing these tasks, we hope to introduce induced sputum as a noninvasive derivative from the airway to be used in future human biomonitoring and cancer chemoprevention studies.

In summary, we conclude that induced sputum can be used for molecular dosimetry of inhalatory exposure to carcinogens and that the NP1 version of the ^{32}P-postlabeling assay is a preferable choice for the study of smoking-related DNA adducts in the lower respiratory tract.

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References

1. Pisani, P., Parkin, D. M., and Ferlay, J. Estimates of the worldwide mortality for eighteen major cancers in 1985: implications for prevention and projections of future burden. Int. J. Cancer, *55:* 891–903, 1993.

2. Boyle, P., and Maisonneuve, P. Lung cancer and tobacco smoking. Lung Cancer, *12:* 167–181, 1995.

3. IARC. Tobacco smoking. *In:* Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 38. Lyon, France: IARC, 1986.

4. Loeb, L. A., Ernster, V. L., Warner, K. E., Abbots, J., and Laszlo, J. Smoking and lung cancer: an overview. Cancer Res., *44:* 5940–5958, 1984.

5. Hoffmann, D., and Hecht, S. S. Nicotine-derived *N*-nitrosamines, and tobacco-related cancer: current status and future directions. Cancer Res., *45:* 935–944, 1985.

6. Surgeon General. The health consequences of involuntary smoking. *In:* A Report of Surgeon General. Rockville, Maryland: Office of Smoking and Health, 1986.

7. Spivack, S. D., Fasco, M. J., Walker, V. E., and Kaminsky, L. S. The molecular epidemiology of lung cancer. Crit. Rev. Toxicol., *27:* 319–365, 1997.

8. Patriakanos, C., and Hoffmann, D. Chemical studies on tobacco smoke. J. Anal. Toxicol., *3:* 184–197, 1979.

9. Hoffmann, D., and Hoffmann, I. The changing cigarette, 1950–1995. J. Toxicol. Environ. Health, *50:* 307–364, 1997.

10. Miller, E. C., and Miller, J. A. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer (Phila.), *47:* 2327–2345, 1981.

11. Hemminki, K. DNA adducts, mutations and cancer. Carcinogenesis (Lond.), *14:* 2007–2012, 1993.

12. Beach, A. C., and Gupta, R. C. Human biomonitoring and the ^{32}P-postlabeling assay. Carcinogenesis (Lond.), *13:* 1053–1074, 1992.

13. Poirier, M. C., and Weston, A. Human DNA adduct measurements: state of the art. Environ. Health Perspect., *104:* 883–893, 1996.

14. Phillips, D. H., Hewer, A., Martin, C. N., Garner, R. C., and King, M. M. Correlation of DNA adduct levels in human lung with cigarette smoking. Nature (Lond.), *336:* 790–792, 1988.

15. Perera, F., Mayer, J., Jaretzki, A., Hearne, S., Brenner, D., Young, T. L., Fischman, H. K., Grimes, M., Grantham, S., Tang, M. X., Tsai, W. Y., and Santella, R. M. Comparison of DNA adducts and sister chromatid exchange in lung cancer cases and controls. Cancer Res., *49:* 4446–4451, 1989.

16. Cuzick, J., Routledge, M. N., Jenkins, D., and Colin Garner, R. DNA adducts in different tissues of smokers and non-smokers. Int. J. Cancer, *45:* 673–678, 1990.

17. Van Schooten, F. J., Hillebrand, M. J. X., Van Leeuwen, F. E., Lutgerink, J. T., Van Zandwijk, N., Jansen, H. M., and Kriek, E. Polycyclic aromatic hydrocarbon-DNA adducts in lung tissue from lung cancer patients. Carcinogenesis (Lond.), *11:* 1677–1681, 1990.

18. Geneste, O., Camus, A. M., Castegnaro, M, Petrusselli, S., Macchiarini, P., Angeletti, C. A., Giuntini, C., and Bartsch, H. Comparison of pulmonary DNA adduct levels, measured by ^{32}P-postlabeling and aryl hydrocarbon hydroxylase activity in lung parenchyma of smokers and ex-smokers. Carcinogenesis (Lond.), *12:* 1301–1305, 1991.

19. Nowak, D., Meyer, A., Schmidt Preuss, U., Gatzemeier, U., Magnussen, H., and Rüdiger, H. W. Formation of benzo[a]pyrene-DNA adducts in blood monocytes from lung cancer patients with a familial history of lung cancer. J. Cancer Res. Clin. Oncol., *118:* 67–71, 1992.

20. Alexandrov, K., Rojas, M., Geneste, O., Castegnaro, M., Camus, A. M., Petruzzelli, S., Ciuntini, C., and Bartsch, H. An improved fluorometric assay for dosimetry of benzo[a]pyrene diol-epoxide-DNA adducts in smokers' lung: comparisons with total bulky adducts and aryl hydroxylase activity. Cancer Res., *52:* 6248–6253, 1992.

21. Dennis, N., Meyer, A., Schmidt Preuss, U., Gatzemeier, U., Magnussen, H., and Rüdiger, H. W. Formation of benzo[a]pyrene-DNA adducts in blood monocytes from lung cancer patients with a familial history of lung cancer. J. Cancer Res. Clin. Oncol., *118:* 67–71, 1992.

22. Weston, A., Bowman, E. D., Schields, P. G., Trivers, G., E., Poirier, M., C., Santella, R. M., and Manchester, D. K. Detection of polycyclic aromatic hydrocarbon-DNA adducts in human lung. Environ. Health Perspect., *99:* 257–259, 1993.

23. Andreassen, A., Kure, E. H., Nielsen, P. S., Autrup, H., and Haugen, A. Comparative synchronous fluorescence spectrophotometry and ^{32}P-postlabeling analysis of PAH-DNA adducts in human lung and the relationship to *TP53* mutations. Mutat. Res., *368:* 275–282, 1996.

24. Ryberg, D., Skaug, V., Hewer, A., Phillips, D. H., Harries, L. W., Ronald Wolf, C., Øgreid, D., Ulvik, A., Vu, Ph., and Haugen, A. Genotypes of glutathione transferase M1 and P1 and their significance for lung DNA adduct levels and cancer risk. Carcinogenesis (Lond.), *18:* 1285–1289, 1997.

25. Tang, D. L., Rundle, A., Warburton, D., Santella, R. M., Tsai, W. Y., Chiamprasert, S., Hsu, Y. Z., and Perera, F. P. Association between both genetic and environmental biomarkers and lung cancer: evidence of a greater risk of lung cancer in women smokers. Carcinogenesis (Lond.), *19:* 1949–1953, 1998.

26. Phillips, D. H., Schoket, B., Hewer, A., Bailey, E., Kostic, S., and Vincze, I. Influence of cigarette smoking on the levels of DNA adducts in human bronchial epithelium and white blood cells. Int. J. Cancer, *46:* 569–575, 1990.

27. Savala, K., and Hemminki, K. DNA adducts in lymphocytes and granulocytes of smokers and non-smokers detected by ^{32}P-postlabelling assay. Carcinogenesis (Lond.), *12:* 503–508, 1990.

28. Van Schooten, F. J., Hillebrand, M. J. X., Van Leeuwen, F. E., Van Zandwijk, N., Jansen, H. M., Den Engelse, L., and Kriek, E. Polycyclic aromatic hydrocarbon-DNA adducts in white blood cells from lung cancer patients: no correlation with adduct levels in lung. Carcinogenesis (Lond.), *13:* 987–993, 1992.

29. Wiencke, J. K., Kelsey, K. T., Varkonyi, A., Semey, K., Wain, J. C., Mark, E., and Christini, D. C. Correlation of DNA adducts in blood mononuclear cells with tobacco carcinogen-induced damage in human lung. Cancer Res., *55:* 4910–4914, 1995.

30. Godschalk, R. W. L., Maas, L. M., Van Zandwijk, N., Van't veer, L. J., Breeedijk, A., Borm, P. J. A., Verhaert, J., Kleinjans, J. C. S., and Van Schooten, F. J. Differences in aromatic-DNA adduct levels between alveolar macrophages and subpopulations of white blood cells from smokers. Carcinogenesis (Lond.), *19:* 819–825, 1998.

31. Sakula, A. Charcot-leyden crystals and cruschmann spirals in asthmatic sputum. Thorax, *41:* 503–507, 1986.

32. Shata, A. M. A., Coulter, J. B. S., Parry, C. M., Ching'ani, G., Broadhead, R. L., and Hart, C. A. Sputum induction for the diagnosis of tuberculosis. Arch. Dis. Child., *74:* 535–537, 1996.

33. Keatings, V. M., Collins, P. D., Scott, D. M., and Barnes, P. J. Differences in interleukin-8 and tumor necrosis factor-α in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am. J. Respir. Crit. Care Med., *153:* 530–534, 1996.

34. Pavord, I. D., Pizzichini, M. M. M., Pizzichini, E., and Hargreave, F. E. The use of induced sputum to investigate airway inflammation. Thorax, *52:* 498–501, 1997.

35. Mathis, A., Rainer, W., Kuster, H., and Speich, R. Simplified sample processing combined with sensitive one-tube nested PCR assay for detection of *Pneumocystis carinii* in respiratory specimens. J. Clin. Microbiol., *35:* 1691–1695, 1997.

36. Van Schooten, F. J., Godschalk, R. W. L., Breedijk, A., Maas, L. M., Kriek, E., Sakai, H., Wigbout, G., Baas, P., Van't Veer, L., and Van Zandwijk, N. ^{32}P-postlabeling of aromatic DNA adducts in white blood cells and alveolar macrophages of smokers: saturation at high exposures. Mutat. Res., *378:* 65–75, 1997.

37. Gupta, R. C. Enhanced sensitivity of ^{32}P-postlabeling analysis of aromatic carcinogen: DNA adducts. Cancer Res., *45:* 5656–5662, 1985.

38. Gershman, N. H., Wong, H. H., Liu, J. T., Mahlmeister, M. J., and Fahy, J. V. Comparison of two methods of collecting induced sputum in asthmatic subjects. Eur. Respir. J., *9:* 2448–2453, 1996.

39. Pizzichini, E., Pizzichini, M. M. M., Efthimiadis, A., Hargreave, F. E., and Dolovich, J. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. Eur. Respir. J., *9:* 1174–1180, 1996.

40. Popov, T. A., Pizzichini, M. M. M., Pizzichini, E., Kolendowicz, R., Punthakee, Z., Dolovich, J., and Hargreave, F. E. Some technical factors influencing the induction of sputum for cell analysis. Eur. Respir. J., *8:* 559–565, 1995.

41. Fahy, J. V., Wong, H., Liu, J., and Boushey, H. A. Comparison of samples collected by sputum induction and bronchoscopy from asthmatic and healthy subjects. Am. Respir. Crit. Care Med., *152:* 53–58, 1995.

42. Vagaggini, B., Paggiaro, P. L., Giannini, D., Di Franco, A., Cianchetti, S., Carnevali, S., Taccola, M., Bacci, E., Bancalari, L., Dente, F. L., and Giuntini, C. Effect of short-term NO_2 exposure on induced sputum in normal, asthmatic and COPD subjects. Eur. Respir. J., *9:* 1852–1857, 1996.

43. Kidney, J. C., Wong, A. G., Efthimiadis, A., Morris, M. M., Sears, M. R., Dolovich, J., and Hargreave, F. Elevated B cells in sputum of asthmatics: close correlation with eosinophils. Am. J. Respir. Crit. Care Med., *153:* 540–544, 1996.

44. Keatings, V. M., and Barnes, P. J. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. Am. J. Respir. Crit. Care Med., *155:* 449–453, 1997.

45. Chalmers, G. W., Thomson, L., Macleod, K. J., Dagg, K. D., McGinn, B. J., McSharry, Ch., Patel, K. R., and Thomson, N. C. Endothelin-1 levels in induced sputum samples from asthmatic and normal subjects. Thorax, *52:* 625–627, 1997.

46. Lensmar, C., Elmberger, G., Skold, M., and Eklund, A. Smoking alters the phenotype of macrophages in induced sputum. Respir. Med., *92:* 415–420, 1998.

47. Smith, C., and Wood, E. Immunological defense. *In:* C. Smith and E. Wood (eds.), Cell Biology, pp. 430. London: Chapman & Hall, 1996.

48. Øvrebø, S., Haugen, A., Phillips, D. H., and Hewer, A. Detection of polycyclic aromatic hydrocarbon-DNA adducts in white blood cells from coke oven workers: correlation with job categories. Cancer Res., *52:* 1510–1514, 1992.

49. Simons, A. M., Van Herckenrode, C. M., Rodriguez, J. A., Maitland, N., Anderson, M., Phillips, D. H., and Coleman, D. V. Demonstration of smoking-related DNA damage in cervical epithelium and correlation with human papillomavirus type 16, using exfoliated cervical cells. Br. J. Cancer, *71:* 246–249, 1995.

50. Gupta, R. C., and Early, K. ^{32}P-adduct assay: comparative recoveries of structurally diverse DNA adducts in the various enhancement procedures. Carcinogenesis (Lond.), *9:* 29–36, 1988.

51. Gallagher, J. E., Jackson, M. J., George, M. H., Lewtas, J., and Robertson, I. C. G. Differences in detection of DNA adducts in the ^{32}P-postlabeling assay after either 1-butanol extraction or the nuclease P1 treatment. Cancer Lett., *45:* 7–12, 1989.

52. Izzotti, A., Rossi, G. A., Bagnasco, M., and De Flora, S. Benzo[a]pyrene diolepoxide-DNA adducts in alveolar macrophages of smokers. Carcinogenesis (Lond.), *12:* 1281–1285, 1991.

53. De Flora, S., Izzotti, A., D'Agostini, F., Rossi, G. A., and Balansky, R. M. Pulmonary alveolar macrophages in molecular epidemiology and chemoprevention of cancer. Environ. Health Perspect., *99:* 249–252, 1993.

54. Spencer-Beach, G. G., Beach, A. C., and Gupta, R. C. High-resolution anion-exchange and partition thin-layer chromatography for complex mixtures of ^{32}P-postlabeled DNA adducts. J. Chromatogr. B. Biomed. App., *677:* 265–273, 1996.