

# Pulmonary Alveolar Macrophages in Molecular Epidemiology and Chemoprevention of Cancer

by Silvio De Flora,<sup>1</sup> Alberto Izzotti,<sup>1</sup> Francesco D'Agostini,<sup>1</sup> Giovanni A. Rossi,<sup>2</sup> and Roumen M. Balansky<sup>1,3</sup>

*In addition to possessing an extraordinary sweeping activity, pulmonary alveolar macrophages (PAM) are equipped with the biochemical mechanisms involved in the metabolism of carcinogens, which were found to be inducible in humans by cigarette smoke. Moreover, several defense processes were stimulated in rat PAM after *in vivo* administration of the anticarcinogen *N*-acetylcysteine (NAC). Benzo[*a*]pyrene diol epoxide (BPDE)-DNA adducts, as revealed by synchronous fluorescence spectrophotometry, were selectively detected in PAM of smokers and persisted up to 6 months. The amount of adducts was significantly correlated with the number of currently smoked cigarettes but not with the cigarettes smoked in a lifetime (pack-years). Nevertheless, deviations from the regression line pointed out the role of interindividual variability factors in adduct formation. Probably due to the low mitotic rate of PAM in the respiratory lumen, the frequency of micronuclei was not enhanced in smokers. However, parallel assays in rats showed that micronuclei can be enhanced after massive intratracheal administration of benzo[*a*]pyrene or whole-body exposure to high amounts of mainstream cigarette smoke, which also induced BPDE-DNA adducts in lung cells and other organs, including the heart. All these adverse effects were markedly inhibited by the oral administration of NAC, which provides the premise and rationale for a future study on the protective effects of oral NAC in heavy smokers.*

## Introduction

Among the cell populations that may surrogate target cells for biomonitoring purposes, pulmonary alveolar macrophages (PAM) are of particular interest. These long-living cells provide a formidable bulwark protecting terminal airways, which by far represent the largest surface of communication between the organism and the environment. As many as  $24-120 \times 10^6$  PAM in humans (1) and  $0.75 \times 10^6$  PAM in rats (2) are removed daily from the alveolar spaces via the mucociliary escalator. This gives an idea of the extraordinary sweeping activity of these cells, which phagocytize inhaled particulate material, often containing carcinogens resulting from tobacco smoke and/or air pollution. In addition, PAM are equipped with the inducible metabolic machinery capable of activating benzo[*a*]pyrene (BaP), which may play a role in determining DNA damage in bronchial cells (3). However, carcinogen-detoxifying mechanisms are also present (4,5), which can be induced in humans by cigarette smoke (4) and in rats by anticarcinogenic thiols, such as *N*-acetylcysteine (NAC) (6).

We are using human PAM, which can be collected by broncho-

alveolar lavage (BAL), a commonly used clinical practice, to assess the occurrence of benzo[*a*]pyrene diol epoxide (BPDE)-DNA adducts and cytogenetic damage in humans and its relationship with cigarette smoke (CS) and possible air pollutants. Additionally, we are performing pilot studies in rats exposed to either BaP or CS to check the reliability of these models as biomarkers to be exploited in monitoring chemoprevention clinical trials.

## Materials and Methods

### Subjects, BAL Performance, and Recovery of PAM

BAL was performed according to a standardized technique (7) from 39 individuals in the study of BPDE-DNA adducts and, separately, from 31 individuals in the study on micronuclei. The characteristics of all these subjects, i.e., age, sex, occupation, current disease, and smoking habits, have already been presented in tabulated form (8,9). The cell pellet was recovered by centrifugation of the BAL fluid. The number of cells recovered from each subject ranged between 2.9 and  $31.6 \times 10^6$ , being more than twice in smokers compared to nonsmokers and ex-smokers. On an average, PAM accounted for 90% of BAL cellularity.

### Treatment of Animals

Groups of male Sprague-Dawley rats (Morini strain), each composed of six animals, were treated intratracheally for 3

<sup>1</sup>Institute of Hygiene and Preventive Medicine, University of Genoa, Genoa, Italy.

<sup>2</sup>Division of Pneumology, Istituto G. Gaslini, Genoa, Italy.

<sup>3</sup>Permanent address: National Centre of Oncology, Sofia, Bulgaria.

Address reprint requests to S. De Flora, Institute of Hygiene and Preventive Medicine, University of Genoa, I-16132 Genoa, Italy.

consecutive days with BaP (Sigma) (25 mg/kg body weight) in Tween 80 aqueous vehicle, or by gavage with NAC (Zambon) (1 g/kg body weight) in phosphate-buffered saline, pH 7.4, 5 hr before each BaP instillation. Suitable controls of Tween 80 intratracheally and NAC *per os* were also included. In other studies, groups of rats were either untreated or exposed for 3 or 8 consecutive days (60–120 min/day), in a chamber (capacity: 20 L) containing the mainstream smoke of commercially available cigarettes. Half of CS-exposed animals were pretreated with NAC *per os*. The animals were killed 24 hr after the last treatment, and BAL was immediately performed by means of five 10-mL infusions of saline. At the same time, bone marrow smears were prepared for cytogenetic analysis, and lungs and liver were removed for evaluation of BPDE–DNA adducts.

### Evaluation of BPDE–DNA Adducts

DNA was extracted from PAM as previously described (9), and BPDE–DNA adducts were evaluated by means of synchronous fluorescence spectrophotometry (SFS) (10). To obtain a standardized amount of 40  $\mu$ g DNA to be analyzed by SFS, in some cases, especially in nonsmokers, the DNA extracted from two to three individuals with homogeneous characteristics was pooled. In total, therefore, 27 samples were obtained as representative of the 39 subjects under study. Arbitrary fluorescence units (FU) were used for quantifying BPDE–DNA adducts (9).

### Evaluation of Micronucleated and Binucleated Cells

Cytocentrifuge-prepared slides were fixed and stained by means of Diff-Quik (Merz + Dade). Micronucleated (MN) and binucleated (BN) PAM were scored in blind-coded slides by two readers, each one examining 2–3000 PAM at a 1000 $\times$  magnification. As shown in Figure 1, MN can be easily distinguished from pigmented cytoplasmic granules by virtue of their structure and nucleuslike staining patterns.

## Results

### Micronuclei in Human PAM

The overall frequency of MN (mean  $\pm$  SD) in 31 subjects was  $3.9 \pm 1.8\%$ , without any significant difference between 11 smokers ( $3.4 \pm 2.5\%$ ) and 20 nonsmokers or ex-smokers ( $4.1 \pm 1.4\%$ ). No particular trend could be related to age (18–74 years) or to sex ( $4.1 \pm 2.1\%$  in 22 males, and  $3.3 \pm 0.7\%$  in 9 females), and no significant association could be established with occupation or pathological conditions.

### BPDE–DNA Adducts in Human PAM

Detection of BPDE–DNA adducts in 27 samples of PAM obtained from 39 individuals was selectively related to smoking habits. In fact, no adduct was detectable by SFS in samples from 13 nonsmokers and 6 ex-smokers, who had stopped smoking at least 2 years before BAL. In contrast, the 84.7% of samples obtained from 16 current smokers exhibited evident SFS peaks. Also positive were the two pools of PAM obtained from four individuals (former smokers) who had stopped smoking in the last 1–6 months preceding BAL. As shown in Figure 2, the amounts

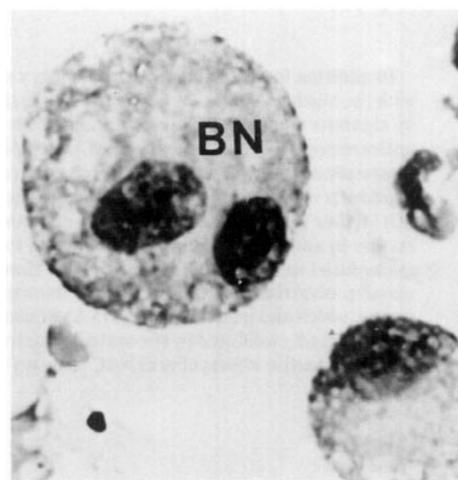
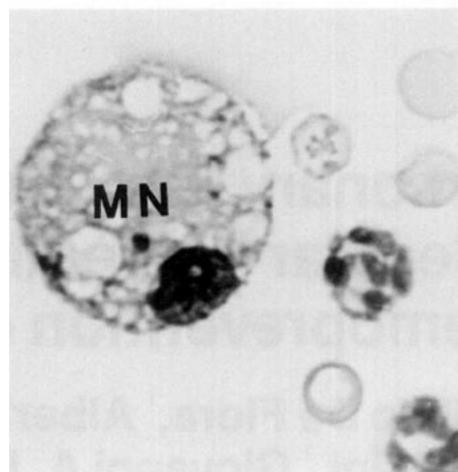


FIGURE 1. Appearance of human micronucleated (MN) or binucleated (BN) pulmonary alveolar macrophages, stained with Diff-Quik (original magnification 1000 $\times$ ).

of BPDE–DNA adducts were significantly related, in spite of important individual deviations from the regression line, to the number of currently smoked cigarettes. No significant relationships could be conversely observed with pack-years.

### Prevention by NAC of BaP- and Cigarette Smoke-Induced Cytogenetic Damage in Rat PAM

The intratracheal instillation of BaP induced a 5-fold increase in the frequency of MN as well as a considerable enhancement of BN PAM. Pretreatment with NAC prevented these effects (Table 1).

Whole-body exposure of rats to mainstream cigarette smoke for 3 consecutive days did not enhance the frequency of these end points, but after 8 days of treatment both MN and BN cell frequencies were significantly enhanced. Again, administration of NAC by gavage prevented the smoke-induced cytogenetic damage (Table 1).

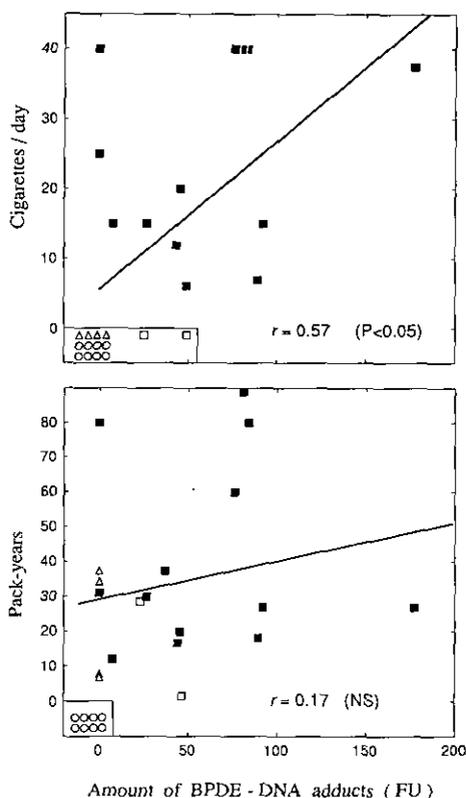


FIGURE 2. Correlation between the amount of BPDE-DNA adducts in pulmonary alveolar macrophages and the number of currently smoked cigarettes/day or pack-years. Correlation coefficients ( $r$ ) were calculated among current smokers (■) only in the case of cigarettes/day, and among current smokers plus former smokers (□) plus ex-smokers (△) in the case of pack-years: (○) nonsmokers.

Table 1. Frequency (mean  $\pm$  SD) of micronucleated and binucleated PAM in variously treated Sprague-Dawley rats.

Treatment of rats	MN PAM, %	BN PAM, %
Controls	2.0 $\pm$ 0.9	16.3 $\pm$ 3.5
BaP intratracheally	9.7 $\pm$ 1.1**	42.0 $\pm$ 11.1*
BaP intratracheally + NAC per os	4.2 $\pm$ 0.9 <sup>†</sup>	15.0 $\pm$ 4.2 <sup>†</sup>
Controls	3.2 $\pm$ 1.8	19.4 $\pm$ 9.2
Smoke (3 days)	4.1 $\pm$ 2.0	23.3 $\pm$ 9.5
Smoke (3 days) + NAC per os	4.5 $\pm$ 1.3	29.2 $\pm$ 7.2
Smoke (8 days)	6.4 $\pm$ 2.1*	35.9 $\pm$ 11.8*
Smoke (8 days) + NAC per os	3.5 $\pm$ 1.6 <sup>†</sup>	30.0 $\pm$ 8.6

Abbreviations: MN, micronucleated; BN, binucleated; PAM, pulmonary alveolar macrophages; BaP, benzo[*a*]pyrene; NAC, *N*-acetylcysteine.

\* $p$  < 0.05 as compared to controls.

\*\* $p$  < 0.001 as compared to controls.

<sup>†</sup> $p$  < 0.05 as compared to BaP-treated rats.

<sup>‡</sup> $p$  < 0.001 as compared to BaP-treated rats.

## Discussion

The results herein reported provide evidence that human PAM represent an ideal target for assessing carcinogen-DNA adducts resulting from cigarette smoke. At variance with other surrogate cells, such as peripheral blood leukocytes, in which adducts are poorly related to smoking habits (11,12) and strongly affected by

dietary carcinogens (13), PAM analysis could selectively discriminate smokers from nonsmokers. Detection of BPDE-DNA adducts in these cells therefore provides a sensitive and specific tool in human biomonitoring. Clearly, this does not rule out the possibility that application of even more sensitive techniques, such as the <sup>32</sup>P-postlabeling technique, may detect carcinogen-DNA adducts in PAM due to other sources, e.g., air pollution or passive smoke. We are currently exploring this possibility.

In spite of the significant correlation observed between the amount of BPDE-DNA adducts and the number of currently smoked cigarettes, deviations from the regression line pointed out the role of smoking style and of interindividual variability in the local metabolism of carcinogens, which was previously investigated in human PAM (4) and lung peripheral parenchyma (14,15). These considerations emphasize the usefulness of assessing the internal dosimetry in exposed individuals.

Probably because of the low mitotic index of PAM in the respiratory lumen, the frequency of MN was poorly affected by the variability factors under study, including smoking habits. Therefore, this cytogenetic index is not a sensitive biomarker of human exposure to the amounts of clastogens normally inhaled with cigarette smoke. However, the parallel experiments in rats showed that a whole-body exposure to high doses of mainstream cigarette smoke, which markedly enhanced the frequency of MN in bone marrow polychromatic erythrocytes in both mice (16) and rats (this study), significantly increased MN in PAM. The intratracheal administration of high amounts of BaP, which conversely were not clastogenic in bone marrow cells, considerably enhanced MN in PAM and also produced the formation of BPDE adducts both to lung and liver DNA (17). As shown by preliminary results, exposure of rats to cigarette smoke also causes the formation of BPDE-DNA adducts detectable by SFS in the lung and other organs, including the heart (unpublished data).

It is of interest that cytogenetic changes and adducts produced by either BaP or cigarette smoke were efficiently prevented by the oral administration of the thiol NAC, an anticarcinogen working through multiple mechanisms (18,19). We are now planning to monitor several end points, including those described in this article, to evaluate the protective effects of oral NAC in heavy smokers.

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