

Molecular Alterations and Lung Tumors in *p53* Mutant Mice Exposed to Cigarette Smoke¹

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ABSTRACT

Mutations and deletions in *p53* are the most common genetic lesions in human cancer, and an extraordinarily high incidence of lung cancer occurs in smokers suffering from Li-Fraumeni syndrome, which is characterized by germ-line inactivation of one *p53* allele. In contrast, *p53* mutations are infrequent in lung tumors formed in A/J mice. Moreover, despite the dominant role of cigarette smoke in the epidemiology of human lung cancer, it is very difficult to reproduce the lung tumorigenicity of this complex mixture in animal models. We used a transgenic mouse with a dominant-negative *p53* mutation to examine the effects of a mutant *p53* on smoke-induced lung carcinogenesis in mice. *p53* mutant (UL53–3 × A/J)F₁ mice of both genders and their wild-type (wt) littermate controls were exposed whole-body to environmental cigarette smoke (ECS) for up to 9.5 months. Untreated mutant mice of both genders underwent an early stimulus of bronchial cell proliferation, and an age-related formation of DNA adducts in lung and heart. In males, there was an age-related increase of micronucleated normochromatic erythrocytes in peripheral blood and an impairment of body weight gain. These findings underscore a physiological protective role of *p53* in wt A/J mice. The response of wt and mutant mice to ECS was similar in terms of oxidative DNA damage in lung and heart, proliferation of the bronchial epithelium, and levels of p53 oncoprotein, as assessed after exposure for 28 days. In contrast, ECS-exposed mutant mice underwent a lower induction of apoptosis in bronchial epithelium, a greater formation of DNA adducts in lung and heart, and a more intense cytogenetic damage, shown by a higher frequency of micronuclei in pulmonary alveolar macrophages and in peripheral blood normochromatic erythrocytes. Interestingly, at the end of the experiment, DNA adducts were not repaired in either wt or mutant mice after discontinuing exposure to ECS for 1 week. A weak but significant increase of lung tumor incidence and multiplicity was induced in *p53* mutant (UL53–3 × A/J)F₁ mice after exposure to ECS for either 5 months, followed by recovery in air for 4.5 months, or 9.5 continuative months. Conversely, no tumorigenic effect was observed in their wt littermate controls, carrying a 99.9% A/J background and 5% FVB genome. This contrasts with the weakly positive results obtained in previous studies using wt A/J mice. Thus, in agreement with the results of previous lung tumorigenicity studies performed with the smoke carcinogens benzo(a)pyrene and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone, (UL53–3 × A/J)F₁ mice carrying a mutant *p53* transgene appear to be more sensitive to ECS than the corresponding wt littermate controls. These findings provide evidence that *p53* mutations play a role in smoke-related carcinogenesis not only in humans but also in A/J mice.

INTRODUCTION

There is overwhelming evidence that tobacco smoke plays a major role in the pathogenesis of lung cancer, cancer at other sites, and a variety of chronic degenerative diseases (1–3). A number of experi-

mental studies have evaluated the genotoxicity of typical CS⁴ components, such as BP, as a prototype of polycyclic aromatic hydrocarbons, and NNK, as a prototype of tobacco-specific nitrosamines. Twenty CS components have been shown convincingly to cause lung tumors in laboratory animals or humans (4). Less attention has been paid to the overall effects of CS, MCS, SCS, or ECS. In fact, for a variety of reasons, it is difficult to reproduce the carcinogenicity of these complex mixtures in preclinical models (2, 5). This drawback limits our knowledge regarding the mechanisms of action of CS and hampers the development of prevention strategies toward tobacco use.

Most carcinogenicity studies with CS available in the literature have used murine models. There is no doubt that CS condensates are carcinogenic to the mouse skin (2, 6) and, interestingly, SCS condensates were found to be more potent than MCS in inducing local tumors (7). Inhalation studies were performed in different mouse strains. Despite a borderline result in (C57BL/Cum × C3H/AnfCum)F₁ mice exposed nose-only to MCS (8), C57BL mice are known to be resistant to induction of lung tumors by chemicals, also including CS (9). This was in part ascribed to the high immunological reactivity of these mice (9). The whole-body exposure of C57BL/6 mice to ECS for 6 months induced apoptosis-dependent alopecia but failed to induce lung tumors (10, 11). Similarly, no lung tumor occurred in SKH-1 hairless mice exposed whole-body to ECS for 6 months (11). However, after 28 days of exposure, ECS produced significant alterations in the respiratory tract of SKH-1 mice, including the formation of MN and PN PAMs, induction of proliferation and apoptosis in the bronchial epithelium, and enhancement of oxidative DNA damage and bulky DNA adducts in the lung (12). The whole-body exposure of female Swiss albino mice to ECS, for the pregnancy period, 5 months, or 9 months, was successful to produce a significant increase of lung tumor when the tumor yield was scored 9 months after the start of treatment (12). The ability of ECS to induce lung tumors in Swiss albino mice was also shown in another laboratory (13).

In general, A/J mice are known to be the most sensitive mouse strain to development of lung tumors, whose yield increases “spontaneously” with age (14). A/J mice carry the pulmonary adenoma susceptibility 1 (*Pas 1*) locus, which affects predisposition of mice to lung cancer (15). The *EcoR1*-generated 0.55-kb *K-ras* fragment associated with high susceptibility to lung tumor development is deleted in this strain (16). The possibility of inducing lung tumors in MCS-exposed A/J mice was reported 50 years ago (17), but these results were hardly reproducible even within the same laboratory (2). More recently, the whole-body exposure of A/J mice to either MCS for 6 months (18) or ECS for 9 months (11, 19) failed to induce lung tumors. Moreover, no tumorigenic effect was observed in the lung of A/J mice receiving weekly i.p. injections of MCS condensates for 5 months (11), or exposed whole-body either to MCS for 5 months (11)

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⁴The abbreviations used are: CS, cigarette smoke; wt, wild-type; MCS, mainstream cigarette smoke; SCS, sidestream cigarette smoke; ECS, environmental cigarette smoke; MN, micronucleated; PN, polynucleated; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte; PAM, pulmonary alveolar macrophage; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; BP, benzo(a)pyrene; PCNA, proliferating cell nuclear antigen; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone.

or to ECS for 2–2.5 months (11, 20). In all of these studies the mice were sacrificed 9 months after the start of treatment. An important discovery made by Witschi *et al.* (19, 20) was that the whole-body exposure of A/J mice to ECS for 5 months, followed by 4 months of recovery in filtered air, increased both lung tumor incidence (in 5 of 7 experiments) and multiplicity (in all 7 experiments). It was also shown that the gas phase of ECS is as tumorigenic as is full ECS. This suggests that tumorigenicity in the A/J mouse model is because of some as yet unidentified carcinogen(s) present in the ECS gas phase or because of free radical-mediated oxidative stress of the lung (21). We found that a significant increase of lung tumor yield occurs either in uninduced A/J mice exposed to ECS for 5 months or in Aroclor 1254-pretreated A/J mice exposed to ECS for 6 months, followed in both cases by 4 months of recovery in air (11). Induction of lung tumors by ECS under comparable experimental conditions was confirmed recently in a third laboratory (22). However, in all of the studies, the tumorigenic effect of ECS in A/J mice was weak.

One of the mechanisms that hampers the formation of lung tumors in rodents exposed to CS may be represented by removal of damaged cells via apoptosis. In fact, apoptosis was induced strongly in bronchial/bronchiolar epithelium and in PAM of Sprague-Dawley rats exposed whole-body to MCS and ECS, respectively (23). A potent induction of apoptosis also occurred in the bronchial epithelium of SKH-1 mice exposed whole-body to ECS (12). Therefore, we deemed it interesting to evaluate CS-related lung tumors in mice having an impaired capacity to undergo apoptosis.

Use of *p53*-deficient mice for studying carcinogenesis and cancer prevention provides an interesting approach (24), also taking into account that the *p53* oncoprotein inhibits tumor growth by inducing apoptosis and/or by slowing down cell replication via G_1 arrest (25, 26). The relevance of these animal models to the situation in humans is highlighted by the fact that, whereas *p53* mutations are infrequent in lung tumors formed in A/J mice (27), point mutations or deletions in *p53* are the most common genetic lesions in human cancer (24–26, 28). Moreover, germ-line inactivation of one allele of *p53* is a hallmark of Li-Fraumeni syndrome, which is characterized by frequent occurrence of many cancers, including an extraordinarily high incidence of lung cancer in smokers (28).

Recently, a *p53* transgenic mouse model on an A/J background was obtained by crossing UL53–3 mice to A/J mice. The UL53–3 mice were developed by microinjection of oocytes of FVB/J mice (carrying three copies of the *p53* transgene) with a BALB/c mouse genomic clone of the *p53* gene, having the endogenous *p53* promoter region, but containing a point mutation at codon 135 (Ala → Val) in exon 5 of the *p53* gene (28). The (UL53–3 × A/J) F_1 *p53*-mutant mice retain both copies of the normal *p53* alleles, but introduction of a *p53* transgene expressing the mutant *p53* oncoprotein (Val₁₃₅) inactivates endogenous wt *p53* (29). The lung adenomas generated in this mouse model possess mutations in both the *p53* tumor suppressor gene and the *K-ras* proto-oncogene. A significant enhancement of lung tumor yield (~3-fold), compared with wt A/J mice, was observed in mutant A/J mice receiving i.p. injections either of *N*-methylnitrosourea or of the tobacco-related carcinogens BP or NNK (28). In addition, mutant mice were found to be more susceptible to the induction of colon carcinomas, adenomas, hepatomas, and especially uterine sarcomas by 1,2-dimethylhydrazine, whereas they exhibited an unchanged sensitivity to induction of bladder tumors by *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (30, 31). It is also noteworthy that *p53* mutant mice and wt A/J mice were equally susceptible to the therapeutic effects of Taxol and doxorubicin, as well as to the chemopreventive effects of green tea and dexamethasone plus *myo*-inositol in lung tumor models (28, 30) and to chemoprevention of colon cancer by piroxicam (31).

The goal of the present study was to compare *p53* mutant mice and

wt A/J mice in CS-related pulmonary carcinogenesis. To this purpose, (UL53–3 × A/J) F_1 *p53*-mutant mice of both genders and their littermate controls carrying 99.9% A/J background, which still contained 5% of FVB genome, were exposed whole-body to ECS for various time periods. A variety of biomarkers were evaluated after either 28 days or up to 9.5 months of exposure, whereas the lung tumor yield was assessed after exposure to ECS for either 5 months, followed by 4.5 months of recovery in filtered air, or 9.5 consecutive months. In parallel, the same biomarkers were evaluated in commercially available wt A/J mice. The results obtained indicate that sensitivity of mutant mice to ECS is not striking. Nevertheless, both susceptibility biomarkers in sham-exposed mice and the response to ECS, in terms of molecular biomarkers and lung tumors, were enhanced significantly in mutant mice compared with wt mice.

MATERIALS AND METHODS

Animals. Twenty female A/J wt mice were commercially available (Charles River, Calco, Lecco, Italy). A total of 222 mice, including 68 male and 40 female *p53* transgenic (UL53–3 × A/J) F_1 mutant mice, and 50 male and 64 female wt littermate controls carrying 99.9% A/J background, which contained 5% of FVB genome, were bred at the Ohio State University, Columbus, OH. The original *p53* transgenic mice carrying a 135 val *p53* mutation in exon 5 on a FVB/J mouse background were kindly provided by Dr. Roger W. Wiseman (National Institute of Environmental Health Sciences, NIH, Research Triangle Park, NC). These mice were rederived onto the A/J background after five generations of backcrossing. The *p53* transgenic mice were genotyped for the presence of *p53* transgene using the same procedure as described previously (28, 31). At the age of 4–5 weeks, the mice were shipped to the University of Genoa where the experiment was conducted. All of the mice were housed in a climatized environment at a temperature of $22 \pm 1^\circ\text{C}$, relative humidity of $50\% \pm 5\%$, ventilation accounting for 15 air renewal cycles/h, and with a 12-h light-dark cycle. The mice were maintained on standard rodent chow (MIL; Morini, S. Polo d'Enza, Reggio Emilia, Italy) and given drinking water *ad libitum*. The experiment was started after 20 days of acclimatization. Body weights were monitored monthly for the duration of the study. Animal care and treatments were in accordance with national and institutional guidelines.

Design of the Study. The mice were either kept in filtered air (sham-exposed) or exposed whole-body to ECS (ECS-exposed), according to the following scheme:

Group 1: Sham-exposed mutant (UL53–3 × A/J) F_1 mice (23 males and 14 females);

Group 2: ECS-exposed mutant (UL53–3 × A/J) F_1 mice (45 males and 26 females);

Group 3: Sham-exposed wt mice carrying 99.9% A/J background and 5% of FVB genome (17 males and 22 females);

Group 4: ECS-exposed wt mice carrying 99.9% A/J background and 5% of FVB genome (33 males and 42 females);

Group 5: Sham-exposed, commercially available wt A/J mice (10 females); and

Group 6: ECS-exposed, commercially available wt A/J mice (10 females).

At periodical intervals, the peripheral blood was collected from the tail lateral vein of either 15 mice/group/gender (at 0, 2, 15, and 28 days) or 10 mice/group/gender (at 7.5 and 9 months), for evaluating the frequency of MN NCEs. After 28 days of exposure, all of the mice in groups 5 and 6, and 10 mice/group/gender in groups 1–4 were anesthetized with diethyl ether and killed by cervical dislocation. Bronchoalveolar lavage was performed by lavaging the lungs of each mouse with two 5-ml aliquots of cold (4°C) 0.15 M NaCl infused via a cannula inserted into the trachea. The cells were washed twice with RPMI 1640, and then spun onto slides in a cytocentrifuge and fixed with methanol for cytogenetic analyses. The left femur of each animal was removed and dissected, and bone marrow cells were collected for evaluating the frequency of MN PCE. A lobe of left lung from each mouse was fixed in formalin and embedded in paraffin for evaluating *p53* oncoprotein, PCNA, and apoptosis in bronchial epithelium. The heart and lungs (the whole right lung and the remaining lobe of the left lung) were collected and stored at -80°C for the analysis of oxidative DNA damage and bulky DNA adducts.

All of the remaining ECS-exposed mice were either treated for 9.5 consecutive months or for 5 months followed by 4.5 months of recovery in filtered air. After 9.5 months, all of the surviving sham-exposed mice, all of the mice exposed to ECS for 5 months, and half of the mice exposed to ECS for 9.5 months were sacrificed. The remaining mice exposed to ECS for 9.5 months were sacrificed 1 week later. A small portion of lung from sham-exposed mice and from mice exposed to ECS for 9.5 months (± 1 week) was collected and stored at -80°C for the analysis of bulky DNA adducts. The remaining lung was fixed in formalin for evaluating the lung tumor yield.

Exposure to CS. All of the mice in groups 2, 4, and 6 were exposed whole-body to the smoke generated by 1R3 reference cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY), having a declared content of 27.1 mg total particulate matter, 22.8 mg tar, and 1.46 mg nicotine each. Before use, the cigarettes were kept for 48 h in a standardized atmosphere humidified with a mixture of 70% glycerol and 30% water. A mixture of SCS (89%) and MCS (11%), mimicking an exposure to ECS, was produced by using a smoking machine (model TE-10; Teague Enterprises, Davis, CA), where each smoldering cigarette was puffed for 2 s, once every min for a total of eight puffs, at a flow rate of 1.05 liters/min to provide a standard puff of 35 cm^3 (32). The machine was adjusted to burn five cigarettes at one time, 6 h a day divided into two 3-h rounds with a 3-h interval, for the time periods specified under "Design of the Study." SCS and MCS were aspirated in a mixing chamber before distribution to four exposure chambers. The total particulate matter in the exposure chambers was on an average 113 mg/m^3 , and CO concentration was 580 parts/million. The position of the cages in the exposure chambers was rotated daily.

Evaluation of p53 Oncoprotein. p53 oncoprotein was evaluated in the bronchial epithelium by an immunohistochemical technique using CM5 polyclonal antibody (Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom). Formalin-fixed, paraffin-embedded sections were pretreated with 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven at high temperature for 10 min. The sections were routinely processed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA), following the manufacturer's protocol. Slides were scored at a magnification of $\times 400$, and 1000 cells per mouse were examined. The results were expressed as percentage of p53-positive cells.

Evaluation of Cell Proliferation. PCNA was detected by immunohistochemistry in the bronchial epithelium by using the NCL-PCNA kit (Novocastra Laboratories) following the manufacturer's protocol. This detection kit is based on an anti-PCNA monoclonal antibody (clone PC10) and uses avidin/biotinylated horseradish peroxidase complex technology (avidin-biotin complex method). Slides were scored at a magnification of $\times 400$, and 1000 cells per mouse were examined. The results were expressed as percentage of PCNA-positive cells.

Evaluation of Apoptosis. Apoptotic cells in the bronchial epithelium were detected by terminal deoxynucleotidyl transferase-mediated nick end labeling method. The Tacs XL Blue Label *In Situ* Apoptosis kit (Trevigen, Gaithersburg, MD) was used according to the manufacturer's protocol. The slides were scored at a magnification $\times 400$, and 1000 cells per mouse were examined. The results were expressed as percentage of apoptotic cells.

DNA Purification. The hearts and lungs from each mouse, pooled within each group of mice, were thawed and homogenized in a Potter-Elvehjem apparatus at 4°C in 250 mM sucrose, 5 mM DTT, and 50 mM Tris-HCl (pH 7.6). DNA was isolated in a helium atmosphere by solvent extraction using an automatic extractor (Genepure 341; Applied BioSystems, Foster City, CA) according to the method of Gupta (33), with some minor modifications as described previously (34). Purity of DNA was checked by spectrophotometric analysis (34).

Detection of Oxidative DNA Damage. Oxidative damage was evaluated in lung and heart DNA samples pooled within each experimental group. A ^{32}P postlabeling procedure was used, as described previously (35). This procedure specifically detects 8-OH-dG, which is the only nucleotide retained in chromatograms developed in unbuffered formic acid. To avoid artifacts that may result from radiation-induced oxidation of guanine, a selective hydrolysis of unmodified dG was achieved by treating depolymerized DNA with 90% trifluoroacetic acid before the ^{32}P postlabeling reaction (35). The results were generated in four separate experiments and were expressed as 8-OH-dG/ 10^5 nucleotides.

Detection of DNA Adducts. Aliquots of 6 μg of mouse lung or heart DNA, pooled within each experimental group, were assayed for the presence of DNA adducts by butanol extraction, as described previously (36). Each sample was labeled with 64 μCi of carrier-free $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN Biochemicals, Irvine, CA), with a specific activity ≥ 7000 Ci/mmol. TLC was carried out on sheets of polyethylenimine (Macherey and Nagel, Duren, Germany) according to standard procedures (36). On the basis of the results of previous studies (35, 37), 7 M urea was used in D2 and D3. Autoradiography was performed by using a ^{32}P InstantImager Electronic Autoradiographic System equipped with InstantQuant software (model A2024; Packard, Meriden, CT). The relative adduct labeling index (cpm adduct/cpm normal nucleotides) was calculated, and DNA adduct levels in each sample were expressed as DNA adducts/ 10^8 nucleotides. The samples were assayed in three separate experiments. A 7R,8S,9S-trihydroxy-10R-(N_2 -deoxyguanosyl-3'-phosphate)-7,8,9,10-tetrahydrobenzo(a)pyrene reference standard (National Cancer Institute Chemical Carcinogen Reference Standard Repository, Midwest Research Institute, Kansas City, MO) was used as a positive control to check the labeling efficiency in each experiment.

Evaluation of Cytogenetic Damage. The cytogenetic damage was evaluated in PAM, bone marrow PCE, and peripheral blood NCE, as described previously (38). Briefly, slides of bronchoalveolar lavage cells were fixed with methanol and stained with a Giemsa 10% solution. A total of 2,000–4,000 PAM/mouse were scored for MN and PN cells. Bone marrow smears were air-dried and stained with May-Grunwald-Giemsa, and 5,000 PCE/mouse were scored for the presence of MN erythrocytes. Peripheral blood smears were air-dried and stained with May-Grunwald-Giemsa, and 25,000 to 45,000 NCE per mouse were scored for the presence of MN cells.

Evaluation of Lung Tumor Yield. The mice were sacrificed at the times indicated in the "Design of the Study" section. The lungs were removed and immersed in formalin. After 7 days of fixation, the number of surface tumors was scored by using a stereomicroscope. Chemically induced tumors are localized beneath the pleura, and there is no advantage in examining serial lung sections (14, 39). The lung tumors were classified according to three histopathological types including, in increasing progression toward malignancy, solid, mixed, and papillary adenomas (40). The results were expressed as lung tumor incidence (number of tumor-bearing mice/number of surviving mice) and multiplicity (number of tumors/mouse).

Statistical Analysis of Data. The data were expressed as mean \pm SE of the results obtained either among the mice belonging to each experimental group or in replicate analyses carried out by testing organs pooled within each experimental group, as indicated in Tables 1–4. Differences between groups in body weight, measured at monthly intervals, were evaluated by using ANOVA with Fisher's Protected Least Significant Difference *post hoc* test. Differences between groups in the incidences of lung tumors were evaluated by Fisher's exact test. Those relative to the multiplicity of lung tumors and to the mean values of each biomarker were evaluated by Student's *t* test for unpaired data.

RESULTS

Body Weight Gain. The weights at time 0 in the 12 experimental groups ranged between 19.9 ± 0.33 (mean \pm SE) and 21.3 ± 0.27 g in males, and between 16.9 ± 0.42 and 17.8 ± 0.34 g in females. In sham-exposed mice, the initial body weights were not significantly affected by *p53* status. However, from 3 months onwards there was a progressive trend to higher body weights in male wt mice than in male mutant mice. At 9 months, this difference was statistically significant ($P = 0.05$). Irrespective of gender and *p53* status, exposure to ECS produced a moderate but consistent and statistically significant decrease of body weight as compared with sham-exposed mice. This effect started from the first month after exposure and continued until the end of the experiment in the mice exposed to ECS for 9.5 months. In contrast, the mice exposed to ECS for 5 months recovered and gained normal body weights after discontinuation of treatment (data not shown).

Accumulation of p53 Oncoprotein, Proliferation, and Apoptosis in the Bronchial Epithelium. After 28 days of treatment, a very low proportion of cells in the bronchial epithelium were found to be

Table 1 *p53* oncoprotein, PCNA, and apoptosis in mouse bronchial epithelium

Line of mice	<i>p53</i> status	Gender	Treatment (28 days)	<i>p53</i> -positive cells (%)	PCNA-positive cells (%)	Apoptotic cells (%)
(UL53-3 × A/J)F ₁	Mutant	M	Sham	0.02 ± 0.02 ^a	2.6 ± 0.40	0.4 ± 0.25
(UL53-3 × A/J)F ₁	Mutant	M	ECS	0.04 ± 0.04	4.2 ± 0.58 ^b	1.0 ± 0.45
(UL53-3 × A/J)F ₁	Mutant	F	Sham	0.04 ± 0.02	2.4 ± 0.75	0.2 ± 0.20
(UL53-3 × A/J)F ₁	Mutant	F	ECS	0.06 ± 0.06	3.4 ± 0.51	0.8 ± 0.37
A/J ^c	Wt	M	Sham	0.01 ± 0.01	1.2 ± 0.58	0.6 ± 0.25
A/J ^c	Wt	M	ECS	0.04 ± 0.02	3.4 ± 0.68 ^b	2.0 ± 0.55 ^c
A/J ^c	Wt	F	Sham	0.02 ± 0.02	1.4 ± 0.25	0.6 ± 0.40
A/J ^c	Wt	F	ECS	0.04 ± 0.04	2.4 ± 0.51	2.2 ± 0.37 ^c
A/J	Wt	F	Sham	0.01 ± 0.01	1.5 ± 0.27	0.5 ± 0.22
A/J	Wt	F	ECS	0.02 ± 0.02	2.8 ± 0.44 ^b	2.4 ± 0.50 ^d

^a The data are mean ± SE of the results obtained within each experimental group.

^b $P < 0.05$, compared with the corresponding sham.

^c 99.9% A/J background, containing 5% FVB genome.

^d $P < 0.01$, compared with the corresponding sham.

positive by immunohistochemistry for *p53* oncoprotein (Table 1). In general, the values were slightly higher in mutant mice than in wt mice, and in ECS-exposed mice than in sham-exposed mice. However, these differences were not statistically significant.

In parallel, proliferation of the same cells was evaluated by measuring the percentage of PCNA-positive cells. In sham-exposed mice this index was not affected by gender, whereas it was higher in mutant mice than in wt mice. This difference was statistically significant ($P < 0.05$) when combining males and females. Exposure to ECS enhanced the proportion of PCNA-positive cells, especially in males (Table 1). By combining males and females, stimulation of proliferation by ECS was significant in both mutant mice ($P < 0.05$) and wt mice ($P < 0.01$).

As shown in Table 1, evaluation by terminal deoxynucleotidyl transferase-mediated nick end labeling method of the apoptotic index in the bronchial epithelium of sham-exposed mice did not show any significant difference related to gender. The higher apoptotic index recorded in both male and female wt mice was not statistically significant. The *p53* status influenced the response to ECS after 28 days of exposure. In fact, irrespective of gender, apoptosis was significantly stimulated by ECS in wt mice but not in mutant mice.

Oxidative DNA Damage in Lung and Heart. Oxidative DNA damage was evaluated by measuring 8-OH-dG levels in lung and heart of mice after 28 days of exposure (Table 2). 8-OH-dG levels in sham-exposed mice were neither affected by gender nor by *p53* status. Exposure to ECS resulted in a significant increase of 8-OH-dG levels, which were ~2-fold higher in both lung and heart, irrespective of gender and *p53* status.

Bulky DNA Adducts in Lung and Heart. As shown in Table 2, neither gender nor *p53* status affected DNA adduct levels in lung or heart of sham-exposed mice. Exposure of A/J mice to ECS for 28 days resulted in a significant increase of bulky DNA adducts. The autora-

diographic patterns showed the occurrence of a diagonal radioactive zone and of an individual spot (data not shown). The increase of DNA adducts after exposure to ECS was similar in lung and heart. The effect of ECS was more pronounced in males than in females. Moreover, ECS-related DNA adduct levels were consistently higher in mutant mice than in wt mice to a significant extent ($P < 0.05$) in male lung and female heart.

To evaluate accumulation with time of DNA adducts and to pinpoint possible differences related to the *p53* status in removing these molecular lesions, additional analyses were carried out after 9.5 months, at the time of sacrifice of mice. Half of the ECS-exposed mice were sacrificed the day after the last exposure to ECS, whereas the remaining mice were sacrificed 1 week later. Lung and heart aliquots were pooled within males and females of each experimental group. The first finding emerging from these analyses was that, compared with the evaluation made after 28 days, the levels of spontaneous DNA adducts were not changed significantly in wt mice, being 1.8 ± 0.37 adducts/ 10^8 nucleotides (mean ± SE of triplicate analyses) in lung and 1.9 ± 0.42 in heart at the end of the experiment. These values are similar to those recorded almost 9 months earlier (Table 2). In contrast, in untreated mutant mice there was an appreciable age-related increase of endogenous DNA adducts in both lung (3.9 ± 0.31 , $P < 0.001$) and heart (3.9 ± 0.31 , $P < 0.001$). In addition, ECS-related DNA adduct levels were significantly and remarkably higher after 9.5 months than after 28 days in both wt mice (6.6 ± 1.03 in lung and 11.9 ± 1.22 in heart) and mutant mice (9.5 ± 1.17 in lung and 14.8 ± 1.25 in heart). After 9.5 months, DNA adduct levels were significantly higher in mutant mice than in wt mice both in lung ($P < 0.001$) and heart ($P < 0.01$). Irrespective of *p53* status, these DNA modifications were unchanged when the mice were sacrificed 1 week after discontinuation of exposure to ECS. In fact, at that time the levels of DNA adducts

Table 2 Oxidative DNA damage (8-OH-dG) and bulky DNA adducts in mouse lung and heart

Line of mice	<i>p53</i> status	Gender	Treatment (28 days)	8-OH-dG/ 10^5 nucleotides		DNA adducts/ 10^8 nucleotides	
				Lung	Heart	Lung	Heart
(UL53-3 × A/J)F ₁	Mutant	M	Sham	8.2 ± 2.83 ^a	10.7 ± 0.94	1.1 ± 0.32	1.2 ± 0.23
(UL53-3 × A/J)F ₁	Mutant	M	ECS	17.9 ± 2.14 ^b	25.7 ± 4.61 ^b	7.8 ± 1.12 ^c	6.4 ± 1.32 ^b
(UL53-3 × A/J)F ₁	Mutant	F	Sham	8.0 ± 1.61	10.2 ± 0.83	1.2 ± 0.24	1.4 ± 0.37
(UL53-3 × A/J)F ₁	Mutant	F	ECS	16.1 ± 2.20 ^b	20.2 ± 4.03 ^b	3.5 ± 0.73 ^b	4.9 ± 0.64 ^b
A/J ^d	Wt	M	Sham	7.9 ± 2.68	10.9 ± 0.51	1.2 ± 0.19	1.8 ± 0.23
A/J ^d	Wt	M	ECS	17.1 ± 2.62 ^b	18.7 ± 2.11 ^b	4.0 ± 0.48 ^c	5.1 ± 0.91 ^b
A/J ^d	Wt	F	Sham	8.9 ± 2.73	10.7 ± 1.02	1.3 ± 0.22	1.6 ± 0.30
A/J ^d	Wt	F	ECS	16.9 ± 1.84 ^b	24.4 ± 3.92 ^b	2.5 ± 0.21 ^b	3.0 ± 0.37 ^b
A/J	Wt	F	Sham	8.7 ± 1.76	7.0 ± 1.90	1.1 ± 0.41	1.6 ± 0.32
A/J	Wt	F	ECS	17.4 ± 3.18 ^b	23.3 ± 3.27 ^b	2.9 ± 0.58 ^b	5.1 ± 1.50 ^b

^a The results are mean ± SE of either 3 (DNA adducts) or 4 (8-OH-dG) replicate analyses carried out by testing organs pooled within each experimental group.

^b $P < 0.05$, compared with the corresponding sham.

^c $P < 0.01$, compared with the corresponding sham.

^d 99.9% A/J background, containing 5% FVB genome.

Table 3 Cytogenetic analyses in mouse respiratory tract (MN and PN PAM), bone marrow (MN PCE), and peripheral blood (MN NCE)

Line of mice	<i>p53</i> status	Gender	Treatment	MN PAM (%)	PN PAM (%)	MN PCE (%)	MN NCE (%)					
				28 days	28 days	28 days	Time 0	2 days	15 days	28 days	7 months	9.5 months
(UL53-3 × A/J)F ₁	Mutant	M	Sham	2.3 ± 0.37	31.0 ± 2.82	8.1 ± 0.82 ^a	1.6 ± 0.08	1.4 ± 0.12	1.9 ± 0.11	1.9 ± 0.07	2.2 ± 0.20	2.3 ± 0.09
(UL53-3 × A/J)F ₁	Mutant	M	ECS ^b	3.5 ± 0.29 ^c	29.7 ± 2.93	9.7 ± 1.03	1.4 ± 0.23	3.6 ± 0.16 ^d	3.3 ± 0.17 ^d	3.1 ± 0.19 ^d	2.7 ± 0.19	2.4 ± 0.13
(UL53-3 × A/J)F ₁	Mutant	M	ECS ^e	NA	NA	NA	NA	NA	NA	NA	3.7 ± 0.22 ^d	3.0 ± 0.14 ^d
(UL53-3 × A/J)F ₁	Mutant	F	Sham	2.3 ± 0.14	37.5 ± 4.37	3.1 ± 0.62	1.0 ± 0.09	1.0 ± 0.14	1.4 ± 0.12	1.3 ± 0.10	1.6 ± 0.04	1.3 ± 0.07
(UL53-3 × A/J)F ₁	Mutant	F	ECS ^b	4.1 ± 0.51 ^c	36.5 ± 2.43	4.4 ± 0.44	1.1 ± 0.24	2.1 ± 0.14 ^d	2.1 ± 0.12 ^d	1.9 ± 0.15 ^d	1.5 ± 0.16	1.3 ± 0.11
(UL53-3 × A/J)F ₁	Mutant	F	ECS ^e	NA	NA	NA	NA	NA	NA	NA	2.3 ± 0.07 ^f	2.0 ± 0.10 ^d
A/J ^g	Wt	M	Sham	1.5 ± 0.22	38.7 ± 4.73	7.4 ± 0.99	1.5 ± 0.12	1.9 ± 0.09	1.9 ± 0.09	1.8 ± 0.11	2.3 ± 0.09	2.1 ± 0.14
A/J ^g	Wt	M	ECS ^b	2.1 ± 0.63	32.9 ± 3.10	9.1 ± 0.98	1.4 ± 0.16	3.0 ± 0.26 ^d	2.8 ± 0.13 ^d	2.4 ± 0.14 ^d	2.4 ± 0.12	1.9 ± 0.12
A/J ^g	Wt	M	ECS ^e	NA	NA	NA	NA	NA	NA	NA	3.3 ± 0.21 ^d	2.7 ± 0.13 ^f
A/J ^g	Wt	F	Sham	2.4 ± 0.58	35.8 ± 1.38	3.7 ± 0.84	1.2 ± 0.07	1.0 ± 0.10	1.4 ± 0.09	1.3 ± 0.08	1.4 ± 0.09	1.3 ± 0.11
A/J ^g	Wt	F	ECS ^b	2.5 ± 0.57	35.8 ± 1.38	4.8 ± 0.30	1.0 ± 0.21	1.5 ± 0.13 ^f	2.2 ± 0.10 ^d	2.1 ± 0.07 ^d	1.4 ± 0.07	1.1 ± 0.07
A/J ^g	Wt	F	ECS ^e	NA	NA	NA	NA	NA	NA	NA	2.2 ± 0.05 ^d	1.8 ± 0.07 ^f
A/J	Wt	F	Sham	2.7 ± 0.27	42.2 ± 4.24	5.1 ± 0.61	1.3 ± 0.12	1.2 ± 0.12	1.3 ± 0.07	1.0 ± 0.06	NA	NA
A/J	Wt	F	ECS ^h	3.4 ± 0.78	45.4 ± 2.68	8.1 ± 0.54 ^c	1.1 ± 0.21	1.5 ± 0.06 ^c	2.1 ± 0.06 ^d	1.8 ± 0.09 ^d	NA	NA

^a The data are mean ± SE of the results obtained within each experimental group. NA, not available. See text for additional statistical analysis.

^b ECS for 5 months, followed by recovery in filtered air for 4.5 months.

^c $P < 0.05$, compared with the corresponding sham.

^d $P < 0.001$, compared with the corresponding sham.

^e ECS for 9.5 months.

^f $P < 0.01$, compared with the corresponding sham.

^g 99.9% A/J background, containing 5% FVB genome.

^h ECS for 28 days.

were 7.0 ± 0.62 in lung and 12.4 ± 0.62 in heart of wt mice, and 9.3 ± 0.91 in lung and 15.5 ± 1.34 in heart of mutant mice.

Cytogenetic Damage. The cytogenetic damage was evaluated in PAM and bone marrow PCE analyzed after 28 days of treatment, and in peripheral blood NCE analyzed at the start and at various time intervals until the end of the experiment (Table 3).

The bronchoalveolar lavage cellularity was not affected by *p53* status, gender, or treatment. Neither gender nor *p53* status affected the frequency of MN PAM in sham-exposed mice. A significant increase of MN PAM was induced by ECS in mutant mice, both males and females. The increase was more modest and not statistically significant in wt mice. There was no treatment-related difference between wt and mutant mice regarding the frequency of PN PAM.

The frequency of MN PCE in the bone marrow of sham-exposed mice was significantly higher ($P < 0.001$) in males than in females, both mutant and wt. Exposure to ECS increased this index, but the effect was statistically significant only in the female wt mice of commercial source. The PCE:NCE ratio was in the 1.5–1.7 range in sham-exposed mice. Exposure to ECS resulted in a nonsignificant increase of this ratio in both mutant and wt mice (data not shown). The ECS-related increase was remarkable (from 1.6 ± 0.10 to 3.0 ± 0.54) and statistically significant ($P < 0.05$) only in wt female A/J mice of commercial source.

Regardless of *p53* status, the frequency of spontaneous MN NCE was consistently and significantly higher in males than in females ($P < 0.001$ at all of the monitored times). Moreover, this cytogenetic index tended to increase with time, especially in male *p53* mutant mice. In fact, by combining the data obtained after 2, 15, and 28 days, the MN NCE frequencies were similar in wt and *p53* mutant mice. After 7–9.5 months, the overall MN NCE frequencies in female mice were marginally and nonsignificantly increased. Conversely, the increase was statistically significant in males, both wt and mutant. Interestingly, the damage observed after 7–9.5 months was significantly higher in mutant mice than in wt mice ($P < 0.05$).

Since the first monitoring time after starting treatments (2 days), exposure to ECS induced a significant increase of MN NCE, which was slightly more pronounced in *p53* mutant mice than in wt mice. In particular, by combining the data recorded at all times, from 2 days to 9.5 months, the overall frequencies of MN NCE in ECS-exposed wt mice were 2.8 ± 0.08 in males and 1.8 ± 0.06 in females versus

corresponding values of 3.3 ± 0.08 ($P < 0.05$) and 2.1 ± 0.05 ($P < 0.01$) in mutant mice. In both mutant and wt mice, the frequencies of ECS-induced MN NCE reached a plateau immediately, which persisted until the end of the experiment in mice exposed for 9.5 months. Conversely, in the mice in which exposure to ECS had been discontinued after 5 months there was a progressive decrease of cytogenetic damage after 7 and 9.5 months (Table 3).

Survival and Lung Tumor Yield. Survival of mice after 9.5 months of treatment was in the 61.5–93.8% range in the 12 experimental groups, without any significant difference attributable to *p53* status, gender, or treatment (Table 4).

Table 4 reports incidence and multiplicity of lung tumors, as well as their histopathological type and size, as assessed 9.5 months after the start of the experiment. Interestingly, the yield of tumors in sham-exposed mice was similar in mice with or without a *p53* mutation. Irrespective of the exposure schedule, exposure of wt mice to ECS did not affect the lung tumor yield. In contrast, mutant mice underwent a significant increase of both lung tumor incidence and multiplicity, without any significant difference between mice exposed for 9.5 continuative months and mice exposed for 5 months, followed by 4.5 months of recovery in air. These effects were statistically significant in both males and females, and became even more evident by combining the two genders. In fact, the overall (males plus females) incidences were 5 of 26 (19.2%) in sham-exposed mice, 15 of 23 (65.2%; $P < 0.01$) in mice exposed to ECS for 5 months, followed by 4.5 months of recovery in air, and 17 of 30 (56.7%; $P < 0.01$) in mice exposed to ECS for 9.5 continuative months. The overall multiplicities were 0.27 ± 0.10 , 0.74 ± 0.11 ($P < 0.01$), and 0.93 ± 0.18 ($P < 0.01$), respectively. Besides the increase in the number of lung tumors, exposure to ECS of mutant mice determined the appearance of mixed and papillary adenomas. There was also a trend to an increase in the diameter of tumors in ECS-exposed mice (see Table 2), which, however, was not statistically significant.

DISCUSSION

The results of the present study provide evidence that *p53* mutant A/J mice differ significantly from their wt littermate controls in certain background characteristics, as well as in susceptibility to molecular alterations and induction of lung tumors after exposure to

Table 4 Survival and lung tumor yield in mutant (*UL53-3* × *A/J*)*F*₁ mice and wt *A/J* mice carrying 99.9% *A/J* background and containing 5% *FVB* genome, as related to gender, *p53* status, and treatment

<i>p53</i> status	Gender	Treatment (months of exposure)	Survival (%)	Incidence (%)	Multiplicity (mean ± SE)	Histopathology of tumors			Diameter (mm) (mean ± SE)
						Solid	Mixed	Papillary	
Mutant	M	Sham	16/23 (69.6)	3/16 (18.8)	0.2 ± 0.16	3	1	0	1.6 ± 0.48
Mutant	F	Sham	10/14 (71.4)	2/10 (20.0)	0.2 ± 0.14	2	0	0	1.3 ± 0.05
Mutant	M	ECS (5 + 4.5)	15/23 (65.2)	9/15 (60.0) ^a	0.7 ± 0.15 ^a	6	3	1	2.1 ± 0.16
Mutant	F	ECS (5 + 4.5)	8/13 (61.5)	6/8 (75.0) ^a	0.8 ± 0.16 ^a	4	1	1	2.5 ± 0.40
Mutant	M	ECS (9.5)	19/22 (86.4)	10/19 (52.6) ^a	0.9 ± 0.23 ^a	15	2	0	1.8 ± 0.23
Mutant	F	ECS (9.5)	11/13 (84.6)	7/11 (63.6) ^a	1.0 ± 0.30 ^a	9	2	0	1.7 ± 0.37
Wt	M	Sham	12/17 (70.6)	3/12 (25.0)	0.4 ± 0.18	4	0	0	1.2 ± 0.36
Wt	F	Sham	14/22 (63.6)	4/14 (28.6)	0.3 ± 0.12	2	2	0	1.8 ± 0.31
Wt	M	ECS (5 + 4.5)	12/17 (70.6)	4/12 (33.3)	0.3 ± 0.13	4	0	0	0.9 ± 0.11
Wt	F	ECS (5 + 4.5)	16/21 (76.2)	5/16 (31.2)	0.3 ± 0.12	5	0	0	1.3 ± 0.16
Wt	M	ECS (9)	15/16 (93.8)	4/15 (26.7)	0.3 ± 0.12	4	0	0	1.2 ± 0.37
Wt	F	ECS (9)	18/21 (85.7)	6/18 (33.3)	0.4 ± 0.15	6	1	0	1.3 ± 0.21

^a *P* < 0.05, compared with the corresponding sham.

ECS. First of all, in unexposed mutant mice there was an early (28 days) enhancement in proliferation of bronchial cells compared with unexposed wt mice. Regulation of cell proliferation is one of the crucial mechanisms of *p53* (24, 25). PCNA is a replication-associated factor also known as the polymerase processivity factor, and is required for DNA replication as a component of the DNA replicative machinery (41). In addition, PCNA is required for DNA repair, both mismatch (42) and nucleotide excision repair (43), which eliminates the bulky DNA adducts. Dysregulation of cell growth is a hallmark of epithelial carcinogenesis, and PCNA is a marker of altered proliferation that occurs in the bronchial epithelium of smokers (44). Therefore, the enhancement of PCNA-positive bronchial cells in untreated mutant mice is likely to represent a predisposing factor, which may favor the action of carcinogens. Another selective effect observed in untreated mutant mice was a significant age-related increase of DNA adduct levels in both lung and heart, as shown by a 3–4-fold increase of these promutagenic lesions from 28 days to 9.5 months after starting the experiment. These lesions are age-related bulky DNA adducts of endogenous origin. Therefore, they can be classified, according to Randerath *et al.* (45), as indigenous compounds. A higher level of liver indigenous compounds was also reported in *p53*^{+/-} hemizygous knockout C57BL/6J mice than in wt mice (46). As discussed elsewhere extensively (47), unrepaired DNA adducts in the respiratory tract represent a likely step in the evolution toward lung cancer. In contrast, DNA adducts in the heart do not predict any evolution toward cancer, because cardiac myocytes are perennial cells. Proliferation is a *sine qua non* condition for development of tumors. However, for the same reason, these molecular alterations tend to accumulate with age in heart, where they might be responsible for degenerative diseases other than cancer (47). It was shown previously that in untreated C57BL/6Nia mice there was a 9–10-fold increase with aging of DNA adducts in heart (48). The findings of the present study suggest that *p53* mutations favor spontaneous nucleotide alterations in both lung and heart of *A/J* mice. In parallel, in wt male mice there was an age-related increase of spontaneous systemic genotoxic damage, evaluated in terms of MN NCE in peripheral blood. Furthermore, it is noteworthy that the body weight gain in mutant male mice was lower than in wt male mice. Taken together, the findings that untreated *p53* mutant (*UL53-3* × *A/J*)*F*₁ mice underwent: (a) an early stimulus of bronchial cell proliferation; (b) an age-related increase of DNA adduct levels in lung and heart; (c) an age-related increase of MN NCE frequency in peripheral blood; and (d) an impairment of body weight gain, at least in males, highlight a physiological protective role of *p53* in wt mice.

In addition, *p53* mutant mice were more susceptible than wt mice to molecular and cytogenetical alterations produced by exposure to ECS. In fact, whereas mutant mice responded weakly and not signif-

icantly to ECS-induced apoptosis, both male and female wt mice underwent a significant increase of apoptotic cells in the bronchial epithelium. This finding indicates that the loss of *p53* contributes to genomic instability by permitting inappropriate survival of cells that would normally undergo apoptosis in response to DNA damage. Thus, the higher background proliferation rate of the bronchial epithelium and its lower sensitivity to ECS-induced apoptosis in mutant mice are in line with the known mechanisms of *p53* (24, 25) and with the expectations of this study reported previously.

Moreover, ECS induced higher levels of DNA adducts in lung and heart of mutant mice compared with wt mice. Interestingly, the DNA adduct levels in lung and heart of both mutant mice and wt mice were unvaried 1 week after discontinuation of exposure to ECS at the end of the experiment. This type of analysis had been designed to pinpoint possible differences in removal of DNA adducts because of *p53* status. In fact, the results of a previous study in Sprague-Dawley rats had shown that, 1 week after discontinuation of exposure to ECS for 4 weeks, there is a significant loss of DNA adducts in lung, tracheal epithelium, and bronchoalveolar lavage cells but not in heart (37). The observed persistence of DNA adducts indicates that, regardless of *p53* status, these molecular lesions accumulate after a long-lasting exposure to ECS (9.5 months) without being repaired in at least 1 week. This may reflect a different behavior of (*UL53-3* × *A/J*)*F*₁ mice compared with Sprague-Dawley rats. Alternatively, it is possible that the DNA adducts that have accumulated after long-term exposure are more difficult to repair than the DNA adducts that are observed after short-term exposure to ECS. Thus, the long-term exposure may reflect adducts that are innately more difficult to repair. For example, accumulation in nontranscribed regions has been shown to be repaired less efficiently, as well as DNA damage in cells that have lower turnover rate.

The greater genotoxicity of ECS in mutant mice was additionally supported by the findings that in both males and females the overall frequencies of ECS-induced MN NCE in peripheral blood were higher in mutant mice than in wt mice. In addition, after 28 days of exposure, ECS induced a significant increase of MN PAM only in mutant mice. Because of their function as sweeping cells and their ability to metabolize mutagens, PAM represent ideal cells for the cytogenetic monitoring of exposure to inhaled carcinogens such as CS (38). To investigate the molecular nature of all of the aforementioned lesions, we have in progress now the analysis, by cDNA array, of the expression of 1200 genes related to the carcinogenesis process in the lung of *A/J* mice, as related to gender, *p53* status, and exposure to ECS for 28 days.

All of the indications emerging from the analysis of molecular and cytogenetical end points are consistent with the results of the tumorigenicity study. In fact, ECS induced a significant increase of lung

tumor incidence and multiplicity only in *p53* mutant (UL53-3 × A/J)_{F1} mice, both males and females. Moreover, in mutant mice there was an evident trend to formation of lung adenomas of increasing progression toward malignancy. The loss of one copy of *p53* might result in genomic instability leading to additional genetic alterations and acceleration of the tumor progression, as shown in the case of bladder tumors (49). Induction of lung tumors in mutant mice occurred both after exposure for 5 months, followed by recovery in filtered air for 4.5 months, and after continuative exposure for 9.5 months. This type of response is similar to that observed in Swiss albino mice (12), whereas in previous studies with commercially available wt A/J mice a tumorigenic effect only occurred after exposure to ECS for 5–6 months, followed by 4 months of recovery in air, and not after continuative exposure to ECS for 9 months (11, 19). The study of tumors at other sites in the same animals, based on the histopathological analysis of a number of organs, is now in progress.

In general, the response of wt A/J mice to ECS was lower than expected, and even in *p53* mutant mice the response was not striking, in terms of molecular alterations, cytogenetic damage, and lung tumors. For instance, irrespective of *p53* status, the frequency of bronchial cells undergoing apoptosis after exposure to ECS was much lower than in Sprague-Dawley rats (23) or in SKH-1 hairless mice (12).⁵ Moreover, the levels of DNA adducts in lung and heart after 28 days of exposure to ECS were not as high as those observed either in Sprague-Dawley rats (35, 37) or SKH-1 hairless mice (12).⁵ The tumorigenic response to ECS in mutant A/J mice was significant but rather weak, of the same order of magnitude as the one observed in previous studies using commercially available wt A/J mice (11, 19, 22). The lack of sensitivity to ECS tumorigenicity of the wt mice carrying 99.9% A/J background and 5% FVB genome, used in the present study, contrasts with the results obtained previously with wt A/J mice in seven experiments carried out at the University of Davis, Davis, CA (19), two experiments carried out at the University of Genoa (11), and one experiment carried out at INFIBO Institute, Cologne, Germany (22). It remains to be established whether the presence of 5% FVB genome may affect tumor susceptibility, *e.g.*, in case it contains susceptibility modifier locus or loci. In this respect, it should be noted that the commercially available wt A/J mice responded to ECS better than mice carrying 99.9% A/J background and 5% FVB genome in terms of cytogenetic alterations, including induction of PN PAM and MN PCE, and increase of the PCE:NCE ratio in bone marrow.

The only methodological difference between the present study and the prior studies is the type of reference cigarette used for generating ECS in an identical smoking machine. In fact, 1R4F cigarettes were used in tumorigenicity studies at the University of Davis (19), and 2R1 cigarettes were used in previous studies at the University of Genoa (10–12, 23, 35, 37). In the present study, because of unavailability of 2R1, we used 1R3 cigarettes, which, among Kentucky reference cigarettes, are second to 2R1 in total particulate matter, tar, and nicotine content. The analysis of mutagenicity in *Salmonella typhimurium* strain YG1024, in the presence of S9 mix, of MCS collected from 2R1, 1R3, and 1R4F (supplied by Dr. G. Gairola, Tobacco and Health Research Institute, University of Kentucky, KY) provided evidence that these cigarettes generate 3.4, 3.8, and 5.1 revertants/mg particulate, corresponding to 128,000, 73,000, and 52,000 revertants per cigarette equivalent, respectively.⁶ Thus, the differences in mutagenic potency are not dramatic. In any case, it should be noted that mutagenicity of MCS to *S. typhimurium* is mainly because of aromatic amines (50), whereas tumorigenicity of ECS to

A/J mice is because of its gas phase (21). Although the mutagenicity of these different cigarettes type is similar, there may still be significant differences in the relative ability of the cigarettes to induce cancer in this specific model.

In conclusion, in agreement with the lung tumorigenicity data obtained with typical carcinogens, such as BP and NNK (28), the results of the herein reported molecular and tumorigenicity studies show that (UL53-3 × A/J)_{F1} mice carrying a mutant *p53* transgene are more sensitive to ECS than their wt littermate controls. These findings provide evidence that *p53* mutations play a role in CS-related carcinogenesis not only in humans but also in mice.

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