



HEALTH EFFECTS INSTITUTE

## **Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats**

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### **Part III: Examination of Possible Target Genes**

Steven A. Belinsky, Charles E. Mitchell, Kristen J. Nikula,  
and Deborah S. Swafford

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Environmental Research Institute, Albuquerque, NM*

**Includes the Commentary of the Institute's  
Health Review Committee**

**Research Report Number 68  
December 1995**

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The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI supports research on all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate materials) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 120 projects at institutions in North America and Europe. Consistent with its mission to serve as an independent source of information on the health effects of motor vehicle pollutants, the Institute also engages in special review and evaluation activities.

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# HEI Statement

HEALTH EFFECTS INSTITUTE

## *Synopsis of Research Report Number 68 Part III*

# No Evidence For Genetic Mutations Found In Lung Tumors From Rats Exposed To Diesel Exhaust or Carbon Black

### **Background**

Diesel engine exhaust contains gases and carbon particles that have many mutagenic or carcinogenic chemicals adsorbed onto them. Some epidemiologic studies suggest that workers exposed occupationally to diesel exhaust have an increased risk of lung cancer. Inhaling high concentrations of diesel exhaust causes lung cancer in rats when the particles accumulate in their lungs. Recent inhalation studies comparing diesel exhaust with carbon black particles (which contain little adsorbed organic material) indicate no differences in the kinds and numbers of lung tumors found in rats. Thus, in rats, lung cancer induced by diesel exhaust appears to be due to the particles themselves, and not to the adsorbed organic compounds.

Substantial evidence indicates that mutations in certain genes that control cell proliferation (such as protooncogenes and tumor suppressor genes) are strongly associated with lung cancer development in humans and laboratory animals. The frequency and pattern of these mutations in tumor DNA may be unique for each causative agent. Such information would be valuable for understanding the mechanisms by which tumors develop, and could possibly serve as an indicator of exposure to environmental carcinogens.

### **Approach**

Part I of this report describes the work of Dr. Joe L. Mauderly and his colleagues, who conducted a carcinogenesis study in which rats inhaled high concentrations of diesel engine exhaust or carbon black particles (see HEI Research Report Number 68 Part I). Dr. Belinsky and his associates examined lung tumors from the rats in Dr. Mauderly's study and applied molecular biology techniques to measure mutations in selected genes in the DNA from the tumors. Mutations in portions of the *K-ras* protooncogene and the *p53* tumor suppressor gene were targeted for analysis because patterns of mutations in these genes previously have been associated with exposure to carcinogens in laboratory animals and humans.

### **Results and Implications**

The investigators did not detect any significant increase in the frequency or pattern of *K-ras* or *p53* mutations in rat lung tumors induced by diesel engine exhaust or carbon black particles. This suggests that the tumors developed by a pathway that did not involve mutations in the *K-ras* or *p53* genes. Because these findings were negative, and because only limited information is available on carcinogen-induced gene mutations in rats, we cannot draw any definitive conclusions from this study about the mechanism by which diesel exhaust or carbon black particles cause lung tumors in rats.

# HEI Statement

## Synopsis of Research Report Number 68 Part III

### **No Evidence For Genetic Mutations Found In Lung Tumors From Rats Exposed To Diesel Exhaust or Carbon Black**

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#### BACKGROUND

Diesel engine exhaust contains gases and carbon particles that have many mutagenic or carcinogenic chemicals adsorbed onto them. Some epidemiologic studies suggest that workers exposed occupationally to diesel exhaust have an increased risk of lung cancer. Inhaling high concentrations of diesel exhaust causes lung cancer in rats when the particles accumulate in their lungs. Recent inhalation studies comparing diesel exhaust with carbon black particles (which contain little adsorbed organic material) indicate no differences in the kinds and numbers of lung tumors found in rats. Thus, in rats, lung cancer induced by diesel exhaust appears to be due to the particles themselves, and not to the adsorbed organic compounds.

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# TABLE OF CONTENTS

## Research Report Number 68

### Pulmonary Toxicity of Inhaled Diesel Exhaust and Carbon Black in Chronically Exposed Rats

#### Part III: Examination of Possible Target Genes

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<b>I. STATEMENT Health Effects Institute</b> .....	<b>i</b>
This Statement, prepared by the HEI and approved by the Board of Directors, is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.	
<b>II. INVESTIGATORS' REPORT</b> .....	<b>1</b>
When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The Report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in the selection or management of the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.	
Abstract .....	1
Introduction .....	1
Specific Aims .....	3
Methods .....	3
Experimental Design .....	3
Experimental Procedures .....	3
Results .....	8
Mutational Analysis of <i>K-ras</i> .....	8
Immunohistochemistry of p53 Protein .....	9
Mutational Analysis of <i>p53</i> .....	9
Immunohistochemistry of mdm2 Protein .....	10
Discussion and Conclusions .....	10
General Conclusion .....	10
Mechanisms Underlying <i>p53</i> Dysfunction .....	10
<i>K-ras</i> Gene and Rodent Lung Cancer .....	12
Mechanisms of Nongenotoxic Induction of Cancer .....	12
Current Information Needs .....	12
Acknowledgments .....	12
References .....	12
About the Authors .....	15
Publications Resulting from This Research .....	16
Abbreviations .....	16
<b>III. COMMENTARY Health Review Committee</b> .....	<b>17</b>
The Commentary on the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health.	
Introduction .....	17
Regulatory Background .....	17
Scientific Background .....	18
Carcinogenicity of Diesel Engine Exhaust and Carbon Black Particles .....	18
Oncogenes, Tumor Suppressor Genes, and Cancer .....	19
Technical Evaluation .....	20
Objectives and Study Design .....	20
Results and Interpretation .....	20
Implications for Future Research .....	21
Conclusions .....	22
Acknowledgments .....	22
References .....	22
<b>IV. RELATED HEI PUBLICATIONS</b> .....	<b>25</b>

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### ABSTRACT

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In a previous investigation funded jointly by the Health Effects Institute and the Department of Energy, F344/N rats exposed chronically by inhalation to either diesel exhaust or carbon black developed pulmonary neoplasms that were similar in type and number. Diesel exhaust contains soot particles with adsorbed mutagenic organic compounds, while carbon black is a soot particle analogue virtually free of mutagens. The results of that carcinogenicity study suggested that perhaps it was not the organic carcinogens present in the diesel exhaust that were involved in cell transformation and progression to neoplasia, but rather the effects of a high lung burden of carbonaceous particles that induced neoplasia in the rats exposed to diesel exhaust. However, the presence of organic carcinogens in the diesel exhaust could influence the pathway of cell transformation.

The purpose of the investigation reported here was to determine the frequency and pattern of mutations in the *K-ras* and *p53* genes in lung neoplasms from control rats and rats exposed to diesel exhaust or carbon black. *K-ras* and *p53* were chosen for this study because mutation patterns of these genes in lung neoplasms have been associated with exposures to other carcinogens. Mutation of the *K-ras* gene was not common in neoplasms from control or exposed rats. Mutations were identified in codons 12 or 61 in 3 of 50 neoplasms. Immunoreactive levels of p53 protein, suggesting gene dysfunction, were present in 7 of 13 squamous cell or adenosquamous carcinomas. The frequency of neoplasms displaying elevated p53 protein levels

did not differ between exposure groups. Analysis of single-strand conformational polymorphism and direct sequencing of *p53* did not detect any mutations in these neoplasms. No immunoreactivity or mutation in *p53* was observed in adenocarcinomas. The increased level of p53 protein in the squamous cell and adenosquamous carcinomas was not explained by stabilization by the *mdm2* gene product, because this protein was not overexpressed according to immunohistochemical analysis.

No pattern of mutation or protein immunoreactivity was detected that would suggest a differential mechanism of carcinogenicity between diesel exhaust and carbon black. The low frequency of neoplasms with mutations in the genes selected for this investigation does not support or negate a role for the mutagenic organic constituents of diesel exhaust in the development of neoplasms associated with exposure to it. However, the inactivation of the *p53* pathway may have a role in the induction by both diesel exhaust and carbon black of neoplasms with a squamous cell carcinoma component.

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### INTRODUCTION

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Diesel exhaust is a pulmonary carcinogen in rats exposed to high concentrations for long times (reviewed in Mauderly 1992). Studies with filtered exhaust indicated that the carcinogenicity of diesel exhaust is related to its particle fraction (Heinrich et al. 1986; Brightwell et al. 1989). The organic compounds on diesel particles are mutagenic. As more than 450 organic compounds have been identified in diesel exhaust (Opresko et al. 1984), it is difficult to determine the relative contribution of any single compound to its carcinogenicity. However, polynuclear aromatic hydrocarbons and nitropyrenes, because of their established mutagenicity (Hasegawa et al. 1988; Keane et al. 1991) and carcinogenicity (Moon et al. 1990; Cavalieri et al. 1991; Maeda et al. 1991), have been suggested as the principal carcinogenic constituents of inhaled diesel exhaust.

The size of diesel soot makes it readily respirable. Approximately 20% to 30% of the inhaled particles in dilute exhaust could be deposited in the lungs and airways of

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This Investigators' Report is Part III of Health Effects Institute Research Report Number 68, which also includes a Commentary by the Health Review Committee and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Steven Belinsky, Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P.O. Box 5890, Albuquerque, NM 87185-5890.

Although this document was produced with partial funding by the U.S. Environmental Protection Agency under Assistance Agreement 816285 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and, therefore, may not necessarily reflect the views of the Agency, and no official endorsement should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

of human lung adenocarcinomas (Rodenhuis et al. 1988; Suzuki et al. 1990; Reynolds et al. 1991). The major activating mutation is localized to codon 12 and involves a GC-to-TA transversion. This mutation is also frequently detected in *ras* genes of mouse lung tumors induced by benzo[a]pyrene (You et al. 1989), suggesting that mutagens in cigarette smoke that give rise to bulky hydrophobic adducts could be responsible for activating the *K-ras* gene via this mutation in human adenocarcinomas. Lung tumors induced in the mouse by other chemical carcinogens (e.g., methyl-*N*-nitrosourea, vinyl carbamate, dimethylnitrosamine) also contain an activated *K-ras* gene with a unique mutation profile for each class of compound (Belinsky et al. 1989; You et al. 1989).

Approximately 65% of all human lung tumors contain mutations within the *p53* gene (reviewed in Hollstein et al. 1991). Transversion mutations are more prevalent in lung tumors associated with smoking, and they are distributed in exons 4 through 9. Molecular epidemiological studies have associated mutational profiles within the *p53* tumor suppressor gene with exposures to specific carcinogens. For example, 31% of lung cancers from uranium miners exposed to high levels of radon contained the same AGG-to-ATG transversion at codon 249 (Taylor et al. 1994). Liver tumors associated with exposure to aflatoxin (Hsu et al. 1991) and skin tumors resulting from ultraviolet radiation (Brash et al. 1991) have also been correlated with mutational hot spots in *p53*, suggesting that some carcinogens might produce a specific and recognizable mutational profile in this gene. Thus determining the frequency and pattern of mutation in the *K-ras* and *p53* genes may help clarify whether the mutagenic organic constituents of diesel exhaust have a role in its carcinogenicity. We also evaluated carcinomas for elevated levels of *mdm2* protein, which, when overexpressed, can functionally inactivate *p53* (Oliner et al. 1992; Leach et al. 1993).

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## SPECIFIC AIMS

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The goal of this investigation was to use molecular analyses as tools to determine whether different mechanisms are involved in the pulmonary carcinogenesis induced by diesel exhaust and carbon black in the F344/N rat. The specific aims of this initial study were to determine the following:

1. The frequency and pattern of mutations in the *K-ras* protooncogene;
2. The frequency and pattern of mutations in the *p53* tumor suppressor gene; and
3. The frequency of alterations in the expression of the *mdm2* gene.

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## METHODS

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### EXPERIMENTAL DESIGN

Adenocarcinomas, squamous cell carcinomas, and adenocarcinomas previously induced by diesel exhaust or carbon black (Nikula et al. 1995) were examined for alterations in the *K-ras*, *p53*, and *mdm2* genes. Immunohistochemical and single-strand conformational polymorphism (SSCP)\* analyses were used as methods to screen for gene dysfunction. Specific mutations were identified by direct sequencing of the exon thought to harbor a mutation. A restriction fragment length polymorphism (RFLP) assay was used to determine if *K-ras* mutations were contained in a small percentage of cells rather than the whole neoplasm. Lung neoplasms from control rats were used for comparison with neoplasms induced by diesel exhaust and carbon black.

### EXPERIMENTAL PROCEDURES

#### Exposures and Sampling

Details of exposure conditions and sampling are described elsewhere (Nikula et al. 1995). Briefly, 1,150 male and female F344/N rats (7 to 9 weeks old) were divided into five groups and exposed chronically (16 hours/day, 5 days/week, for 24 months) by inhalation to either diesel exhaust or carbon black at target concentrations of 2.5 or 6.5 mg/m<sup>3</sup>, or to filtered air (control rats). The exposure groups and tumor frequencies from Nikula and associates (1995) are shown in Table 1. Lung tumors were fixed in 4% buffered paraformaldehyde or in 10% neutral buffered formalin. These samples were embedded in paraffin, cut in 5- $\mu$ m sections, stained with hematoxylin and eosin, and examined by light microscopy for histologic diagnosis of the tumor phenotype. Serial sections were also cut for immunohistochemical assays and DNA analysis. The samples amenable to analysis (Table 2) totaled 38 adenocarcinomas, 10 squamous cell carcinomas, and 3 adenocarcinomas. Some of these neoplasms were not included in all analyses because of their small size.

#### DNA Preparation

DNAs were prepared by the method of Levi and colleagues (1991) from tumor tissue microdissected from 15- $\mu$ m-thick, unstained paraffin sections. Tissue sections were incubated for two hours at 55°C in 400  $\mu$ L of lysis buffer, which consisted of 10 mM tris(hydroxymethyl)amino-

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\* A list of abbreviations appears at the end of the Investigators' Report.

**Table 2.** Number of Carcinomas Analyzed for Gene Dysfunction by Histologic Type

Exposure Atmosphere	Adeno-carcinomas	Squamous Cell Carcinomas	Adeno-squamous Carcinomas
Diesel exhaust	21	6	1
Carbon black	14	3	1
Filtered air	3	1	1

0.001% gelatin, 0.1 mM of each dinucleotide triphosphate, 2.5 units of *Taq* polymerase, and the primer concentration described below for the individual amplifications. All amplifications included negative controls consisting of the amplification reaction mixture, and deionized water or paraffin extract was added in place of template DNA. Following amplification, a fraction of the material from each reaction was checked for proper amplification product molecular weight and the presence of contaminating DNA in 1.5% agarose gels stained with ethidium bromide.

All oligonucleotides described were synthesized using a model 391 synthesizer (Applied Biosystems, Foster City, CA). The oligonucleotides used for *K-ras* PCR amplifications (Table 3) were designed from published rat sequences (Nickell-Brady et al. 1994). To produce DNA template amenable to mutational analysis, pairs of nested or partially nested primers were used in two separate rounds of PCR for

**Table 3.** Amplification and Sequencing Primers for *K-ras* Gene

Primer <sup>a</sup>	Exon	DNA Sequence 5'–3'	Annealing Temperature (°C)
1A	1	TTTTTATTATTAGGCTGCT	52
1B	1	GCATTACCTCTATCGTAGG	52
1C	1	GCCTGCTGAAAATGACTGAGTATA	52
1D	1	CTCTATCGTAGGATCATATTTCATC	52
1E	1	ACTGAATATAAACTTGTGGTAGTTGGACCT	52
1S	1	TTCTGAATTAGCTGT	
2A	2	CTCCTACAGGAACAAGTAG	42
2B	2	CTATAATGGTGAATATCTTC	42
2C	2	AAACAAGTAGTAATTCATGGAGAAA	49
2D	2	CTTCAAATGATTAGTATTATTAT	49
2S	2	GGAGAAACCTGTCTC	

<sup>a</sup> A = 5' outer primer; B = 3' outer primer; C = 5' inner primer; D = 3' inner primer; E = the 5' *Bst*NI primer; S = the sequencing primer.

each target fragment. All were amplified for 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing for 30 seconds at temperatures shown in Table 3, and extension at 72°C for 30 seconds. The first round of amplification of *K-ras* exon 1 used 50 pmol each of primers 1A and 1B. Three percent of the amplification product from the first round was used as the template DNA for the second round of amplification, using nested primers, in which primers 1C and 1D (50 pmol each) were cycled as above. First and second rounds of amplification of *K-ras* exon 2 were as described for exon 1 using the primers and annealing temperatures shown in Table 3.

**Table 4.** Amplification and Sequencing Primers for *p53* Gene

Primer <sup>a</sup>	Exon	DNA Sequence 5'–3'	Annealing Temperature (°C)
4A	4	GGTTCCTCTTTGGCCCATCC	54
4C	4	CATCCACAGCCCACCACAG	54
4B	4	AGCAACTCTTCAGGCCCACT	54
5A	5	GCCCCACTTTGACCCTTGATCC	60
5B	5	AGGAGCCAGGCCTAAGAGCAAG	60
5C	5	TGATCCTTAGTTGGCTTGTCC	55
5D	5	GAGCAAGAATAAGTCAGAGGC	55
5S	5	CCTTTGATTCTTTCTCC	
6/7A	6/7	TGGGGTTAGAAGCTGGTTG	52
6/7B	6/7	GGCTCATGGAACAAAAACAGGC	52
6D	6	TGGATAGTGGTATAGTCGGA	52
7C	7	ACAGCGTGGTGGTACCGTAT	52
6/7S	6/7	TCCAGGGTCTCCCGG	
8A	8	AGGTAGGGCCTGGTTTACAG	54
8B	8	AGAGCAAGGGGTGACTTTGG	54
8C	8	TGGTTTACAGTCAGGATGGAGC	53
8D	8	ACTTTGGGGTGAAGCTGAAGG	53
8S	8	ACCTTCCTTTGTCTCTG	
9A	9	AGCAGGCAGGACAAAAGAAGG	50
9B	9	TAATCCAATAATAACCTTGG	50
9C	9	CAGCTTCACCCCAAGTCAC	50

<sup>a</sup> A = 5' outer primer; B = 3' outer primer; C = 5' inner primer; D = 5' inner primer; S = the sequencing primer.

two sets of nondenaturing conditions as follows: 6% acrylamide, 5% glycerol, and  $1 \times$  Tris–borate–ethylenediamine–tetraacetic acid (EDTA) buffer, run at room temperature for 16 to 18 hours at 3 to 5 W; 6% acrylamide, 10% glycerol, and  $0.5 \times$  Tris–borate–EDTA buffer, run at 4°C for 8 to 16 hours at 15 to 30 W.

### DNA Sequencing

The PCR products were directly sequenced using the dideoxy chain termination method with Sequenase DNA polymerase (US Biochemical Corp., Cleveland, OH). Sequencing primers (Tables 3 and 4), end-labeled with [ $\gamma$ - $^{32}$ P]deoxyadenosine triphosphate by T4 polynucleotide kinase (US Biochemical Corp.), were annealed to 300 to 600 ng of heat-denatured, amplified DNA and extended for 1 to 2.5 minutes depending on the fragment length. The products of this reaction were separated on an 8% acrylamide denaturing gel and visualized by exposure to Kodak XAR film at  $-80^{\circ}\text{C}$  with an intensifying screen.

### K-ras Exon 1 Restriction Fragment Length Polymorphism Analysis

All samples were analyzed for K-ras codon 12 mutations using a mutant allele enrichment method (Kahn et al. 1991). This method relies on the use of a 5' exon 1 mismatch primer (1E) that replaces the G residue at the first position of codon 11 with a C, thereby creating a *Bst*NI restriction site (CCTGG) that overlaps codon 12 when the wild-type allele is amplified. Treatment of the sample with the *Bst*NI restriction enzyme (New England Biolabs, Beverly, MA) then digests amplimers of the wild-type allele into fragments of 89 and 27 base pairs (bp), leaving intact mutants at either of the first two positions in codon 12. Subsequent rounds of amplification with the same primers will selectively amplify the mutant allele. A second PCR round and enzyme digestion increases the sensitivity with which a mutant allele is detected to one in  $10^4$  copies of the wild-type allele.

An initial PCR round of 30 cycles was performed using primers 1A and 1B as described above. Three percent of the product from this round was used as the template for a second round of amplification using 50 pmol each of primers 1E and 1B. The cycling pattern consisted of  $94^{\circ}\text{C}$  for 30 seconds,  $53^{\circ}\text{C}$  for 30 seconds, and  $72^{\circ}\text{C}$  for 30 seconds for 30 cycles. These PCR products were digested for three hours with the restriction endonuclease *Bst*NI. Six percent of the digested sample was used as the template for a final PCR amplification of 30 cycles. Following digestion by *Bst*NI, mutant and wild-type K-ras exon 1 restriction fragments were resolved by electrophoresis of the DNA fragments through an 8% nondenaturing polyacrylamide gel. Mutant

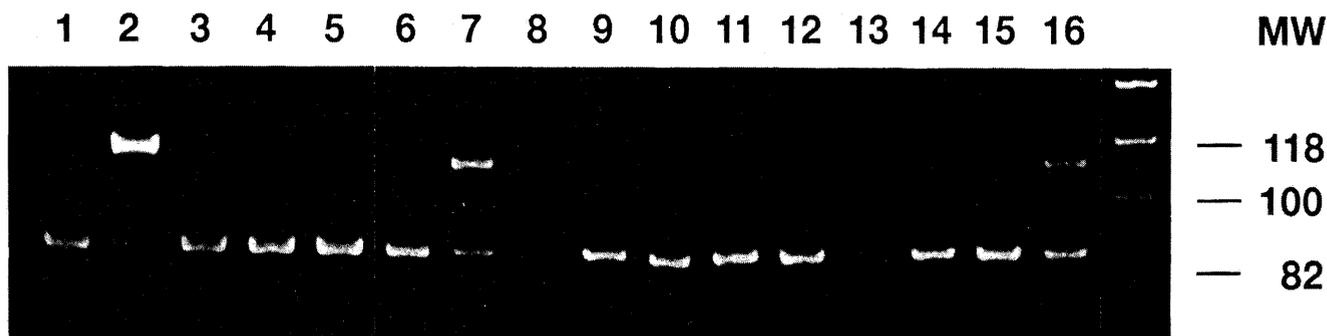
and wild-type fragments were also examined following the second PCR amplification and restriction enzyme digestion.

### Immunohistochemical Analyses of p53 and mdm2 Gene Products

Fixed lung tumor specimens embedded in paraffin blocks were sectioned, and 5- $\mu\text{m}$ -thick sections were mounted on slides for immunohistochemical analysis. Prior to immunostaining, the sections were deparaffinized, rehydrated through a graded series of alcohols, rinsed in distilled water and Automation Buffer (Biomedica, Foster City, CA), and subjected to antigen retrieval as described by the manufacturer (BioGenex, San Ramon, CA). Then the slides were incubated in 0.15% trypsin at  $37^{\circ}\text{C}$  for 30 minutes, and in 0.1% hydrogen peroxide in phosphate-buffered saline at  $25^{\circ}\text{C}$  for 30 minutes, and blocked in 2% goat serum with 0.2% bovine serum albumin at  $25^{\circ}\text{C}$  for 20 minutes. Rat p53 was immunostained overnight at  $4^{\circ}\text{C}$  with the anti-p53 antibody CM1 (SigNet, Dedham, MA, 1:10 diluted stock) diluted 1:50 (final dilution 1:500) in Automation Buffer containing 1% bovine serum albumin. Serum controls were also incubated overnight in a 1:500 dilution of rabbit serum. Bound p53 antibody was detected by a biotinylated secondary antibody and an avidin biotin peroxidase system (Vector Laboratories, Burlingame, CA). The chromagen was 3,3'-diaminobenzidine (Vector Laboratories) with a hematoxylin counterstain (Harleco, Gibbstown, NJ).

The conditions described above for the detection of a rat p53 protein by the CM1 antibody were defined using a rat 2 cell line (provided by R. Frisque, Pennsylvania State University, State College, PA) that had been immortalized by SV40 (Picksley et al. 1994) as a positive control. Rat 2 cells were inoculated subcutaneously into athymic nude mice, and the resulting sarcoma was removed and fixed in neutral buffered formalin. The tissue was then embedded in paraffin, and 5- $\mu\text{m}$  tissue sections were prepared for immunohistochemistry. The distribution of p53 immunoreactivity was evaluated by light microscopy. In each assay, the positive control slides showed p53 nuclear immunoreactivity, while nuclear immunoreactivity was absent in the serum control slides. If p53 immunoreactivity was detected in a neoplasm, the immunohistochemistry was repeated on a serial section to confirm the positive finding.

Immunohistochemistry for detection of mdm2 was performed on all 13 squamous cell carcinomas or adenocarcinomas, and on 20 adenocarcinomas (10 induced by carbon black and 10 by diesel exhaust). Serial sections of the tumors used for the mdm2 analyses were prepared to the protein block step as for the p53 assays. The



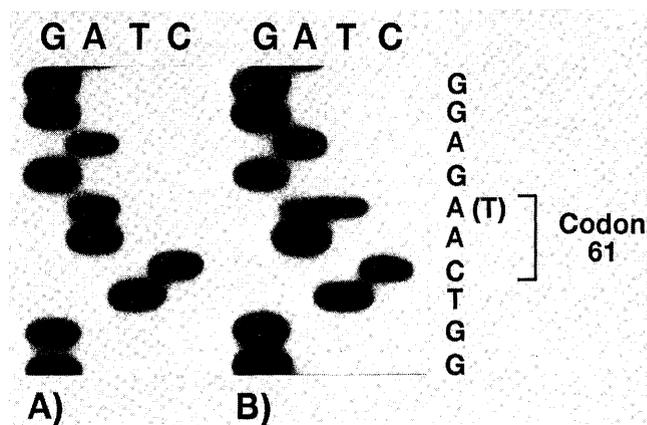
**Figure 3.** *Bst*NI-digested products from a first-round PCR amplification with the mismatch primer. Lanes 2 and 7 contain the uncleaved 116-bp fragment that is produced by amplification of the mutant sequence from an adenocarcinoma and squamous cell carcinoma, respectively; lane 16 is a sample from a mouse lung tumor cell line that is heterozygous for a GGT-to-GAT transition in codon 12. The 89-bp cleavage product of the wild-type amplicon can be seen in all lanes except 8 and 13, which are water-blank controls. The approximate ratio of wild-type allele to mutant in the sample can be determined by comparing the intensities of the 116-bp and 89-bp fragments present on the gel. MW = molecular weight.

#### IMMUNOHISTOCHEMISTRY OF p53 PROTEIN

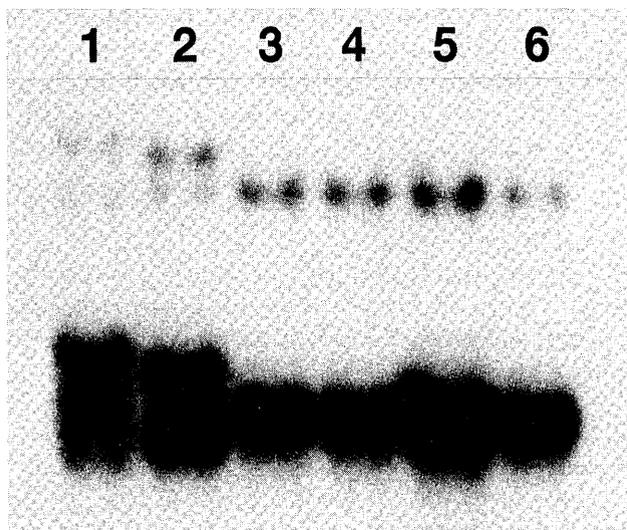
None of the 37 adenocarcinomas was immunoreactive with the p53 antibody. Three of six squamous cell carcinomas in rats exposed to diesel exhaust and one of three squamous cell carcinomas in rats exposed to carbon black exhibited nuclear p53 immunostaining. Nuclear immunoreactivity was not apparent in the squamous cell carcinoma from a control rat subjected to sham exposure. All three adenosquamous carcinomas had nuclear p53 immunoreactivity. The immunoreactive nuclei in the adenosquamous carcinomas were found predominantly in the squamous portion of the carcinoma, but faint immunoreactivity was present in a few nuclei in the adenocarcinomatous portion of the neoplasms from the rats exposed to diesel exhaust and carbon black. The immunostained nuclei in the squamous cell carcinomas and adenosquamous carcinomas were generally distributed throughout the neoplasm in the basilar layers of the neoplastic cords and in the poorly differentiated portions of the neoplasms. Little to no nuclear reactivity was observed in the more differentiated, polyhedral, keratinizing cells toward the centers of neoplastic cords. Therefore, in those neoplasms with positive immunoreactivity, the proportion of the neoplastic nuclei that was immunostained varied inversely with the degree of differentiation and keratinization. Examples of immunoreactivity are depicted in Figure 5, and the estimated ranges of positively stained nuclei for each neoplasm are indicated in Table 5.

#### MUTATIONAL ANALYSIS OF p53

Despite the immunoreactivity observed in seven squamous cell carcinomas or adenosquamous carcinomas, no mutations were detected in *p53* exons 4 through 9 by SSCP analyses. Of the 46 neoplasms analyzed by SSCP, only one mutation was detected in a squamous cell carcinoma induced by diesel exhaust (Figure 6). This mutation was determined to be silent in exon 8 at the third position of codon 272 (GTT → GTC, data not shown). Conditions of SSCP were validated by detection of a point mutation in rat *p53* exon 5 generated by a mismatch primer, and by the detection of known point mutations in exons 5, 8, and 9 of



**Figure 4.** The sequence of a region surrounding K-ras codon 61. (A) Wild-type sequence. (B) An A-to-T transversion found in an adenocarcinoma induced by diesel exhaust.



**Figure 6.** Single-strand conformational polymorphism analysis of *p53* exon 8. Lane 1 reveals one sample from an adenocarcinoma induced by diesel exhaust with a shift in electrophoretic mobility of the single-strand amplicon, indicating altered sequence as compared with wild type (lane 3). Lane 2 is a rat exon 8 amplicon that contains a known point mutation. Lanes 4, 5, and 6 are amplicons from adenocarcinomas arising in control rats or rats exposed to carbon black.

nosquamous carcinomas, both SSCP analysis and direct sequencing failed to detect any mutations in these samples. Given the extent and distribution of immunoreactivity in these tissues, and the sensitivity of SSCP analysis (Suzuki et al. 1990) and direct sequencing, it is unlikely that mutant alleles present would not have been detected.

The p53 protein immunoreactivity in these neoplasms may be due to mutations outside exons 4 through 9, although this is unlikely because such mutations have rarely been associated with protein stabilization and occur in less than 5% of human neoplasms with p53 mutations (Hollstein et al. 1991; Bodner et al. 1992). Alternatively, p53 immunoreactivity may be due either to stabilization by other gene products or to the disruption of protein degradation. Stabilization of p53 protein by another protein may correlate with functional inactivation, analogous to its inactivation and extended half-life when bound by the SV40 large tumor antigen. Precedent for this finding exists in several other reports in which detection of p53 by immunohistochemistry was enhanced in the absence of mutations within the conserved region (Lehman et al. 1991;

**Table 5.** Immunoreactivity of p53 Protein and *p53* Gene Mutations in Squamous Cell Carcinomas and Adenosquamous Carcinomas

Sample	Exposure Atmosphere	Neoplastic Phenotype <sup>a</sup>	Percentage of Immunoreactive Nuclei <sup>b</sup>	Mutation Detected
H454	Diesel exhaust	SCC	> 50	None
L355	Diesel exhaust	SCC	10–25	None
H415	Diesel exhaust	SCC	26–50	None
M861	Diesel exhaust	SCC	0	Not analyzed <sup>c</sup>
I456	Diesel exhaust	SCC	0	None
T826	Diesel exhaust	SCC	0 <sup>d</sup>	None
T935	Diesel exhaust	AdSC	< 10 <sup>e</sup>	Not analyzed <sup>c</sup>
T759	Carbon black	AdSC	26–50 <sup>f</sup>	GTT → GTC silent
G744	Carbon black	SCC	26–50	None
T738	Carbon black	SCC	0	None
G757	Carbon black	SCC	0	None
H516	Filtered air	AdSC	< 10 <sup>e</sup>	Not analyzed <sup>c</sup>
E018	Filtered air	SCC	0	Not analyzed <sup>c</sup>

<sup>a</sup> SCC = squamous cell carcinoma; AdSC = adenosquamous carcinoma.

<sup>b</sup> Estimated percentage of neoplastic nuclei that were p53-immunoreactive. Estimates were based on two positively immunostained slides per neoplasm.

<sup>c</sup> Tissue was not available for this analysis.

<sup>d</sup> Immunostained sections taken from the neoplasm were recapitulated in a nude mouse because of the small size of the original neoplasm.

<sup>e</sup> Most of this neoplasm was adenocarcinomatous, and a smaller portion was squamous cell carcinomatous. The p53-immunoreactive nuclei were restricted to the squamous cells; 10% to 50% of the nuclei in the squamous portion were immunoreactive.

<sup>f</sup> Most of this neoplasm was phenotypically a squamous cell carcinoma and a small portion was adenocarcinomatous. The p53-immunoreactive nuclei were primarily in the squamous portion. The percentage of immunoreactivity is estimated from the total population of neoplastic nuclei.

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## INTRODUCTION

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The environment contains a wide variety of pollutants generated by human activities, particularly in heavily industrialized and urbanized settings. A significant portion of these pollutants are emissions derived from the burning of gasoline, diesel fuel, and lubricants in internal combustion engines. Since 1983, the Health Effects Institute has conducted a multidisciplinary scientific research program to understand better the health consequences of motor vehicle emissions and their individual constituents. The impact of most of these pollutants on human health is unknown because their toxicologic effects are not completely understood. The study described in this report was funded to address this need. In 1991, at the time funding for this study was considered, people were concerned about the human health effects of diesel engine exhaust because (1) diesel exhaust contains thousands of chemicals, some of which are known mutagens and carcinogens (International Agency for Research on Cancer 1989), and (2) several laboratories had established that benign and malignant lung tumors appeared in rats after they had inhaled diesel engine exhaust over most of their life spans (reviewed by Mauderly 1992, and Busby and Newberne 1995). Most of the tumors that developed appeared only after long-term exposure to sufficiently high concentrations of exhaust that the lungs accumulated a heavy burden of particles. It was unclear whether the particles themselves or the adsorbed chemicals were responsible for tumor development (Vostal 1986; McClellan 1994). Also, some epidemiologic studies indicated that workers exposed to diesel exhaust for extended periods had a higher incidence of lung cancer than their relatively nonexposed counterparts (reviewed by Mauderly 1992, and Cohen and Higgins 1995). The International Agency for Research on Cancer (1989) classified diesel engine exhaust as a potential human carcinogen.

To resolve the issue about the role of particles in diesel exhaust-induced lung cancer, in 1988 HEI and the U.S. Department of Energy jointly funded Dr. Joe L. Mauderly and collaborators at the Inhalation Toxicology Research Institute (ITRI)\* in Albuquerque, NM perform a long-term carcinogenesis study in which rats were exposed by inhalation to diesel engine exhaust or carbon black particles. The main goal of the Mauderly study was to compare the

carcinogenic effect of equivalent doses of diesel engine exhaust particles (which contained carcinogenic organic compounds) and carbon black particles (which were essentially devoid of organic compounds). This research was expected to provide information on the relative contributions of carbon particles and the particle-associated organic compounds in causing lung tumors in rats.

Tumor tissues from Dr. Mauderly's study provided an opportunity to increase understanding about the mechanisms by which diesel engine exhaust and carbon black cause lung tumors in rats. Therefore, in February 1991, Dr. Steven A. Belinsky, also from ITRI, submitted the application, "Identification of Target Genes Involved in Carbon Black and Diesel-Induced Lung Cancer." Dr. Belinsky proposed to measure mutations in certain genes (the *K-ras* protooncogene and the *p53* tumor suppressor gene) in the rat lung tumor tissues. These genes had been found to be involved in other studies of lung cancer in humans and laboratory animals. Any differences noted between the types and locations of mutations (the mutational spectra) induced by the two kinds of particles might clarify whether the organic compounds in diesel emissions had any role in the induction of lung tumors.

The HEI Research Committee approved Dr. Belinsky's study, which began in January 1993 and ended in February 1994. Total expenditures were \$125,323. Dr. Belinsky's final report was received in December 1994 and, following discussion by the Health Review Committee, was accepted for publication in April 1995. During the review of the Investigators' Report, the Review Committee and the investigators had an opportunity to exchange comments and to clarify issues in the Investigators' Report and in the Review Committee's Commentary. This Commentary is intended to place the Investigators' Report in perspective as an aid to the sponsors of HEI and to the public.

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## REGULATORY BACKGROUND

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Diesel engines are employed in light-duty applications such as passenger cars and light trucks, and in heavy-duty usage in larger trucks, buses, locomotives, agricultural and construction equipment, and ships. Interest in diesel engines has been renewed in the United States because of several advantages over gasoline or spark-ignition engines, including increased fuel efficiency, decreased emissions of carbon monoxide and hydrocarbons, and 10% to 25% less emission of carbon dioxide, which has implications for reducing global warming (DeLuchi 1992; Hammerle et al. 1994; Sawyer and Johnson 1995).

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\* A list of abbreviations appears at the end of the Investigators' Report for your reference.

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under these exposure conditions, and suggest that the adsorbed organic compounds do not play a role in tumor development in this species. This implies that the diesel exhaust-induced lung tumors are not primarily due to direct genotoxic activity of the particle-associated mutagens, but result from nongenotoxic processes such as oxidative DNA damage, cell proliferation, and inflammation caused by particle-induced lung overload (Lechner and Mauderly 1994; McClellan 1994; Oberdörster 1994; Nauss et al. 1995).

### ONCOGENES, TUMOR SUPPRESSOR GENES, AND CANCER

Carcinogenesis in humans and laboratory animals is a multistage process involving several mutational and non-mutational events (reviewed by Harris 1991). Mutations in transforming genes such as protooncogenes and tumor suppressor genes, which control cell differentiation and proliferation, play an important role in this process.

Protooncogenes are normal genes that control cell proliferation, but when they are abnormally activated by specific kinds of mutations at specific sites, they are termed "oncogenes" because they can disrupt the normal growth and differentiation pathways of the cell and lead to unregulated cell growth and malignancy (Barbacid 1987; Harris 1991). Tumor suppressor genes are another set of normal genes that control cell proliferation; instead of being abnormally activated like protooncogenes, they are abnormally inactivated by a variety of mutations or other chromosomal abnormalities and losses, any of which can produce unregulated cell growth (Harris 1991; Hollstein et al. 1991). Molecular analysis of tumor tissue to assess the location and kinds of mutations in these genes may show a unique pattern or mutational spectrum for each type of tumor, as well as for the causative agent or agents that induced the tumor (Harris 1991; Lambert 1992). This knowledge is valuable for understanding the basic molecular and cellular mechanisms that underlie tumor induction, and may be useful for estimating cancer risks in individuals exposed to carcinogens in the environment. Extensive literature is available on the genetic alterations in protooncogenes and tumor suppressor genes in tumors from humans and laboratory animals (Harris 1991; Hollstein et al. 1991; Reynolds and Anderson 1991; Lechner and Mauderly 1994). For the purpose of this commentary, the following remarks pertain only to the frequency of mutations in the *K-ras* protooncogene and the *p53* tumor suppressor gene in rat and human lung tumors.

*K-ras*, along with the related *H-ras* and *N-ras*, is a *ras* protooncogene that contains the genetic code for the amino

acid sequence of plasma membrane proteins, which help modulate chemical signals to the cell that control cell proliferation (Barbacid 1987). *K-ras* contains four exons, which are four separate nucleotide sequences within the gene, each of which specifies the amino acid sequence for one of four different sections of the protein. The majority of known mutations that activate the *K-ras* protooncogene occur at localized sites in codons 12 and 13 in exon 1, and at codon 61 in exon 2. Scientists have found *K-ras* mutations in 20% to 60% of human lung adenocarcinomas, but have observed much lower frequencies of *H-ras* and *N-ras* mutations (Rodenhuis et al. 1988; Suzuki et al. 1990; Reynolds et al. 1991; Vainio et al. 1993). Relatively little information has been obtained about *K-ras* mutations in chemically or physically induced lung tumors in rats. *K-ras* mutations were found in 14 of 19 lung tumors in rats exposed chronically to tetranitromethane (Stowers et al. 1987), but in only two of 12 lung tumors induced by inhalation of beryllium metal aerosol (Nickell-Brady et al. 1994). *K-ras* mutations were also detected in 33 of 71 (46%) of the adenomas and malignant tumors induced in the lung by a single inhalation of an aerosol containing the radioisotope plutonium oxide ( $^{239}\text{PuO}_2$ ) (Stegelmeier et al. 1991).

*p53* is a tumor suppressor gene that codes for a nuclear phosphoprotein involved in the control of cell proliferation (Harris 1991; Hollstein et al. 1991). Mutations and other genetic alterations in *p53* are the most common genetic change detected in human tumors, with a wide variety of mutational spectra described from different types of tumors; thus, mutations in *p53* genes occur over a much wider range of sites than mutations in *K-ras* genes. Nearly all of the known base substitution mutations are found within exons 5 through 8, a region that includes codons 110 through 307. However, because mutation analyses have been principally confined to this part of the gene, the possibility exists that undetected mutations may occur in other parts of the gene (e.g., in exons 1 through 4 and exon 9) as well. Consistent mutation frequencies of 35% to 67% have been reported for *p53* genes in every type of human lung tumor examined (Kishimoto et al. 1992; Suzuki et al. 1992; Taylor et al. 1994). As with studies of *K-ras* gene activation, data for *p53* gene mutations in rat lung tumors are extremely limited. No *p53* mutations were reported in 12 lung tumors from rats exposed to beryllium aerosol (Nickell-Brady et al. 1994).

In addition to the direct analysis of mutations in *p53* genes, the existence of mutations may be inferred by the presence of increased amounts of the mutant p53 protein in tumor cell nuclei, as measured by immunohistochemical techniques (Bodner et al. 1992; Nickell-Brady et al. 1994).

exposed to carbon black particles. No *K-ras* or *p53* mutations were found in the five tumors from rats exposed to filtered air.

Many studies have observed differences in transforming gene mutations in smaller numbers of tumors than were analyzed in this study. Nonetheless, it is possible that some significant differences in mutations between the diesel exhaust- and the carbon black-induced lung tumors might have been observed if even more tumors had been examined. No statistical analysis was performed by the investigator. However, the numbers of animals, the incidence and kinds of tumors used, and the numbers and types of mutations found in this study could be used to calculate how many animals and tumors would be needed in the future to establish a statistically significant effect at the level of mutations observed by these investigators.

In addition to analyzing *p53* mutations as a direct measurement of *p53* inactivation, the investigators also indirectly examined *p53* function by immunochemical techniques that detected *p53* or *mdm2* proteins. The presence of *p53* protein, or the stabilization of this protein by overexpressed *mdm2* protein, is associated with *p53* inactivation. None of the 37 adenocarcinomas had *p53* protein, as measured by immunoreactivity of the *p53* gene with *anti-p53* antibody, but 7 of the 12 tumors that contained some squamous cells (squamous cell carcinomas and adenosquamous carcinomas) did react. This finding appears inconsistent with the observed lack of *p53* mutations; however, the investigators correctly noted that this immunoreactivity was not evidence for *p53* mutations. Immunochemical techniques are not quantitative and observed differences in levels of protein expression may not be precise. Furthermore, no *p53* mutations were found by the single-strand conformation polymorphism technique, which is a more sensitive indicator of mutations. Also, the investigator has discussed reasonable alternatives to explain this antibody reactivity. Finally, there was no indication that *mdm2* protein was overexpressed in any of the 23 adenocarcinomas or 13 squamous cell carcinomas and adenosquamous carcinomas examined to indicate *p53* inactivation.

At the time this study was funded, little information was available on the occurrence of protooncogene and tumor suppressor gene mutations in different species exposed to different carcinogens. Therefore, the experimental approach and design of this study reflected the state of knowledge at that time. Cancer develops via multiple pathways, and it is now known that those pathways can vary from species to species in any given organ site. For example, although inactivation of *p53* occurs in 35% to 67% of human lung tumors, it is rarely observed in mouse, rat, or hamster lung tumors (Kishimoto et al. 1992; Suzuki et al.

1992; Lechner and Mauderly 1994; Taylor et al. 1994). The possibility remains that the mechanisms underlying the development of lung tumors in rats may differ from those in humans.

When this study commenced, it was considered likely that if the organic compounds adsorbed to the carbonaceous core of diesel exhaust particles contributed to the induction of lung tumors, then mutations in protooncogenes or tumor suppressor genes, shown to be mutated by chemical carcinogens in other studies, would be detected. These mutations were expected to result from a genotoxic mechanism mediated by the formation of DNA adducts with the activated metabolites of some of the known mutagenic polynuclear aromatic hydrocarbons (PAHs) and nitro-PAHs found in diesel exhaust. Because carbon black particles lack significant amounts of these mutagens, tumors induced by exposure to this material were expected to develop by a different, nongenotoxic mechanism.

Evidence in support of a nongenotoxic, particle-induced mechanism for tumor development was provided in an extensive companion study that measured lung DNA adducts in rats (Randerath et al. 1995) from the same prolonged inhalation experiment that Dr. Mauderly carried out to measure tumor formation, and from which Dr. Belinsky analyzed gene mutations. Using analytical techniques that would have detected adducts from the PAHs and nitro-PAHs present in diesel engine exhaust, no evidence was found for the formation of any adduct that was not already present in lung DNA from animals exposed to filtered air. Gallagher and associates (1994) also determined DNA adducts in lungs from a separate carcinogenesis study with rats exposed by inhalation for a prolonged time to diesel exhaust and carbon black particles (Heinrich et al. 1995). These investigators also did not find any new adducts not already present in control animals, although one adduct, thought to originate from an unidentified nitro-PAH, was consistently found at increased levels only in diesel exhaust-exposed animals.

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## IMPLICATIONS FOR FUTURE RESEARCH

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The current state of knowledge about the role of protooncogene activation and tumor suppressor gene inactivation does not yet permit scientists to address the question of how inhaled particles induce lung tumors in the rat. It is possible that analyzing mutations in genes derived from lung tumors focuses attention on a portion of the carcinogenic process that is too late to be useful in elucidating specific mechanisms of action. If this possibility is true, we might expect to see the same frequency and pattern of mutations in

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