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ANALYSIS OF THE EFFECTS OF INHALED DIESEL EXHAUST ON THE ALVEOLAR INTRAVASCULAR AND INTERSTITIAL CELLULAR COMPONENTS OF RODENT LUNGS

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Abstract

Transmission electron microscopy (TEM) was used to determine the effect of diesel engine exhaust (DEE) on the intravascular and interstitial cellular population of the lungs of exposed rats and guinea pigs. Animals with matched controls were subjected to environments of either 250, 750, 1500 or 6000 $\mu g/m^3$ for either 2 weeks, 6 weeks, 10 weeks or 18 months. These animals were sacrificed immediately following the exposure periods and their lungs perfused with fixative. Following dissection, random stratified biopsies from the lungs of these animals were made. Ultrathin sections from the alveolar lung were prepared and conventionally processed for TEM and randomly photographed to compose a micrograph database. These micrographs were analyzed by point counting using a Zeiss MOP 3 Digital Image Analyzer. The results indicated no significant intravascular cellular response but a significant increase in the mononuclear population in the interstitium.

KEY WORDS: Diesel exhaust, diesel engine exhaust, rats, guinea pigs, intravascular, interstitium, morphometric evaluation

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Introduction

The effectiveness of the diesel engine as a propulsive power plant producing low emission of gaseous pollutants is well established.[21] Concern focuses on the increasing number of vehicles contributing freshly emitted particulate and non-particulate agents into the atmosphere.[14,16,18,23] Studies have revealed potentially toxic pollutants, some of which contain compounds known to be carcinogenic, mutagenic [10,15,26] and immunodepressant.[11]

Previous studies have morphometrically evaluated the parenchymal lung of diesel engine exhaust (DEE) exposed rats and guinea pigs.[4,5,6,8,9] This paper presents results from an extension of these studies meant to determine the cellular make-up of the capillary and interstitial space in DEE exposed rodents using morphometric methods. The questions generated from this last study were: 1)What was the lung's cellular response to the presence of DE? What cells comprised the intravascular population? Would there be a significant difference between control and DE exposed intravascular populations? 2)What was the cellular composition of the interstitium? Would there be an increase in fibroblasts indicating chemotactic response to functioning neutrophils, monocytes, and macrophages? Would there be a significant difference in interstitial cells between the 2 species chronically exposed to DE?

To test these hypotheses, we analyzed and quantitated the cellular components of the alveolar capillary and intersitial compartments of the experimental animals (guinea pigs and rats) that had been acutely and chronically exposed to DE.

Materials and Methods

Animals

Male Hartley guinea pigs and Fisher rats were obtained from Charles River Breeding Laboratory and were housed at the General Motors Biomedical Research Laboratory for an initial quarantine period of two weeks prior to entering the controlled exposure regimens.

Exposure Conditions

Three animals with matched controls were placed in either a clean air chamber or one

receiving freshly emitted diluted diesel engine exhaust to achieve particle concentrations of either 250, 750, 1500 or 6000 µg/m^3. The DE exhaust was emitted by a 1978 Oldsmobile 5.75 L, 4 cycle, indirect injection diesel engine. It was maintained to simulate a 65 km/h (40 mph) cruise situation. Amoco type 2D federal compliance fuel and Amoco 200 30W oil were used. Air flow of the dispersed DE particulate matter measured 2.8 L/min. Chamber temperature was maintained at 22± 2°C with 56± 6% relative humidity. The exposure times were 2 weeks, 6 weeks, 10 weeks, and 18 months. Daily exposure was 20 h, 5.5 days/week (110 h weekly). At the end of the exposure periods, the animals were delivered to Wayne State University for sacrifice, dissection and tissue processing for the ultrastructural studies.

Animal and Tissue Preparation

Guinea pigs and rats were anesthetized with an intraperitoneal injection of sodium pentobarbital. A tracheotomy was done and a plastic cannula (Abbot Butterfly-19 set without needle) was secured in the trachea. After collapsing the lungs by letting air enter the thoracic cavity, the intact lungs were reinflated and fixed *in situ* by instillation of pH 7.4 cacodylate buffered 1% glutaraldehyde of 300 mOms at a pressure of 20 cm H_2O above the hilus. The trachea was tied to insure preservation of the intra-pulmonary fixative volume and the still intact lungs were removed from the chest and placed in fresh fixative. Lung volumes were determined after removal of extrapulmonary structures.

Lungs remained submerged in glutaraldehyde for 18-24 h prior to selection of tissue samples for microscopy. The TEM specimens (cubes 0.2 cm thick) were cut from tissue slices of the right middle and left lower lobe representing dorsal and ventral parts, doubly stained with uranyl acetate and lead citrate, examined and photographed in an electron microscope operated at 50 kV. Figure 1 illustrates the regions sectioned for evaluation. A typical thin section is shown in Figure 2.

Morphometry

A minimum of 100 photographs were taken from five randomly selected blocks of alveolar lung per animal. Only the 2000x magnification was used for point counting, establishing the basic data bank. The 4000x magnifications in this text were used only to depict more clearly the morphology of the cells counted. Analysis and evaluations for this specific study concentrated on 1142 micrographs representing 23 rats, control and exposed, for 2 weeks, 6 weeks, 10 weeks, and 18 months to 1500 µg and 6000 µg of diesel exhaust and 250 micrographs of guinea pigs, control and exposed to 1500 µg for 18 months.

For determinations of volume densities and absolute cell numbers of the major tissue components, the Weibel[24] coherent multipurpose test system of 168 test points was placed over the micrographs. The distribution of points falling on structures and cell nuclear profiles in measured micrograph areas were counted using a Zeiss MOP 3.

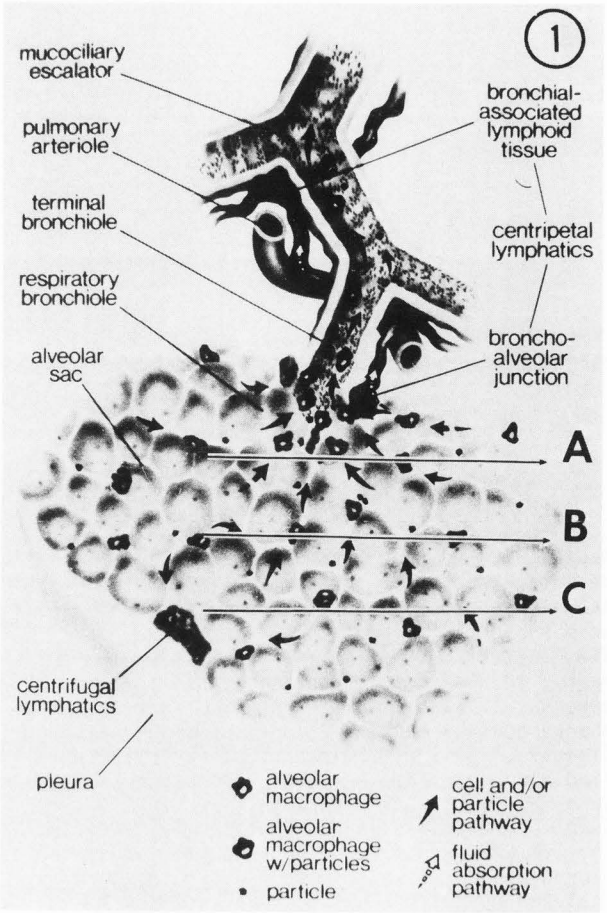


Fig. 1. Schematic Diagram of the Lung. The lines, A, B and C designate the area where random slices of the alveolar lung were taken. (Courtesy of Gareth M. Green, Lung Disease: State of the Art 1976-1977).

Mean caliper diameters for nuclei of all cell types were obtained from previous results with this population of animals.[3,4] These were used for the computation of numerical density. Formulae used for morphometry calculations were:

Volume density = Pi/Pt

Numerical density = Nai/$\bar{D}$i

Where Pi = number of points falling on structure

Pt = total number of points counted

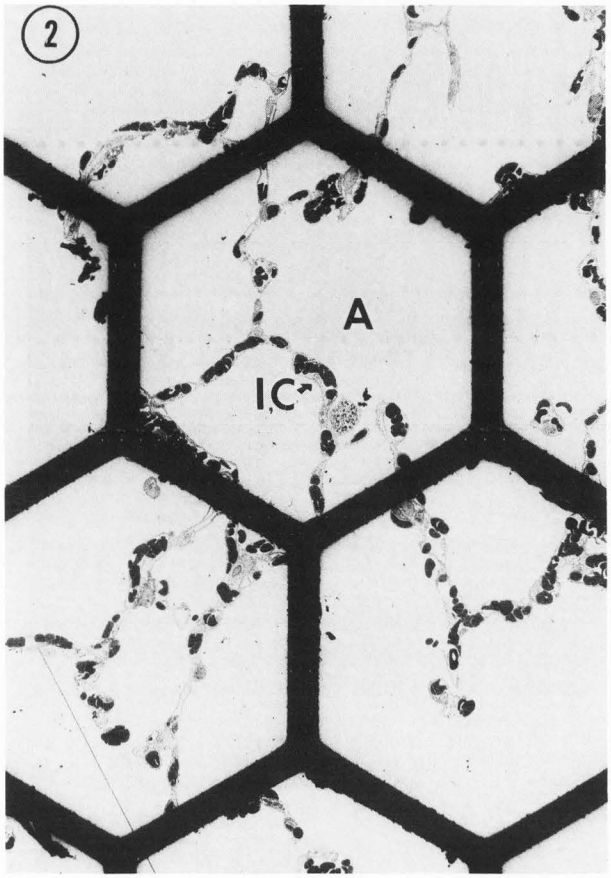
Nai = Cell nuclei per total area of micrograph

$\bar{D}$i = mean caliper nuclear diameter for cell type i

Student's "t" test was used to determine the existence of significant differences between control and exposed groups.[13]

Results

Selected micrographs of the regions studied in this work are shown in Figures 3-7. Each cell type identified in either the capillary intravascular space or the alveolar interstitium is displayed.



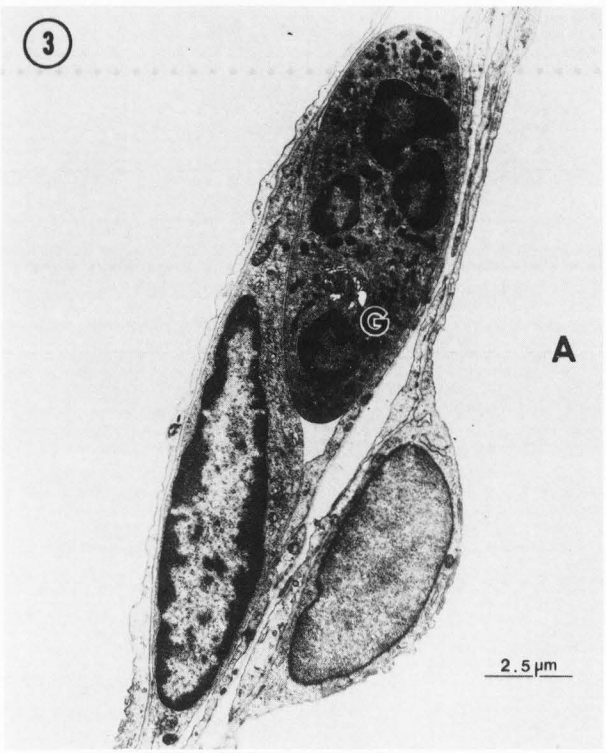


Fig. 2. Low power view of an electron microscope grid bearing a thin section of alveolar lung. Air Spaces (A), and alveolar capillary and interstitium (IC) are shown.
Fig. 3. TEM of Intravascular Area. Granulocyte (G) has multilobulated nuclei which in thin sections may look like separate nuclei; the cytoplasm contains primary granules (large) and specific granules (small and more numerous); cytoplasmic organelles are scarce. Air Space (A).

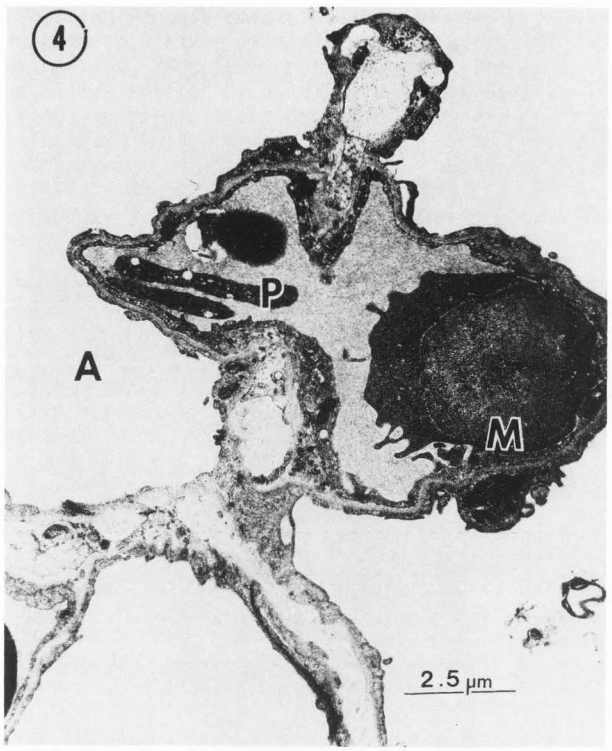
The results of the identification and quantitation of the cellular population in the alveolar capillaries are summarized in Table 1. There was no significant difference in either cell type or number between control and exposed animals. There also was no observable difference in response between species.[17]

Computed volume densities of all components of the interstitium and capillaries are summarized in Table 2. No significant differences existed between control and exposed animals for any intravascular constituent. Significant increases in volume density of the tissue component of the ten week and eighteen month exposed animals can be noted. Likewise, there is a significant increase in the cellular contents of the interstitium of these two groups. With respect to the pulmonary alveolar macrophages, increases can be seen for all exposures, reaching significant differences in the 6 week and 10 week exposure groups.[13] Numerical densities for interstitial cells in the rats and guinea pigs are shown in Table 3. Significant increases in total number of cells are seen in the 10 week exposed rats and 18 month exposed rats and guinea pigs. Increases in both cell types can be observed for virtually all exposures reaching significant differences for mononuclear cells in both rats and guinea pigs at 18 month exposures. Guinea pigs demonstrated more mononuclear cells than the rat in both control animals and exposed animals.

Discussion

The effects of diesel exhaust upon the lungs have been extensively studied with major focus on functional impairment. Williams and Chook[27] isolated and quantitated the specific emissions found in diesel exhaust. Of the fractions, Benzo (a) pyrene, a polynuclear aromatic hydrocarbon (PAH) is known to be both a carcinogenic and mutagenic agent.[3,11,12,19,23]

Andersen[1] noted that NO and NO_2 increase airway resistance causing structural changes in the lung. In the same study, the effect of the pollutant, CO, when inhaled, caused the formation of carboxyhemoglobin, thus reducing O_2 transport of the blood. Barfknecht et al[2] showed that inert particulate material may act as a cytotoxic



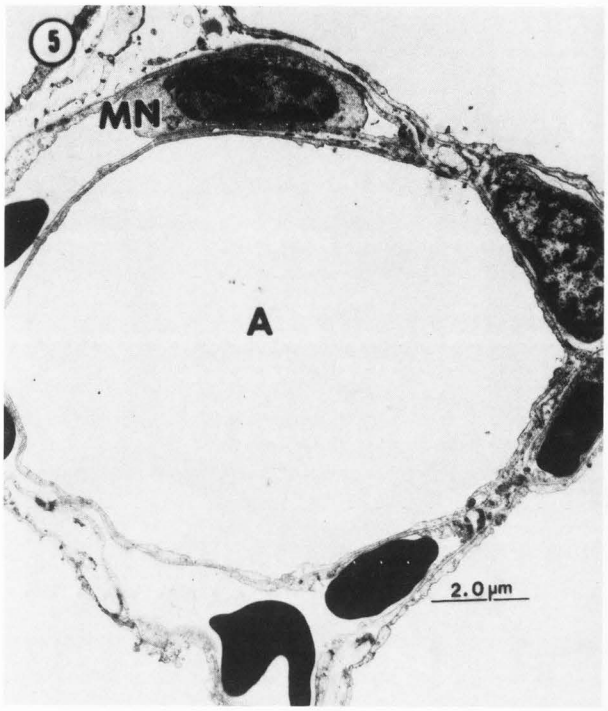
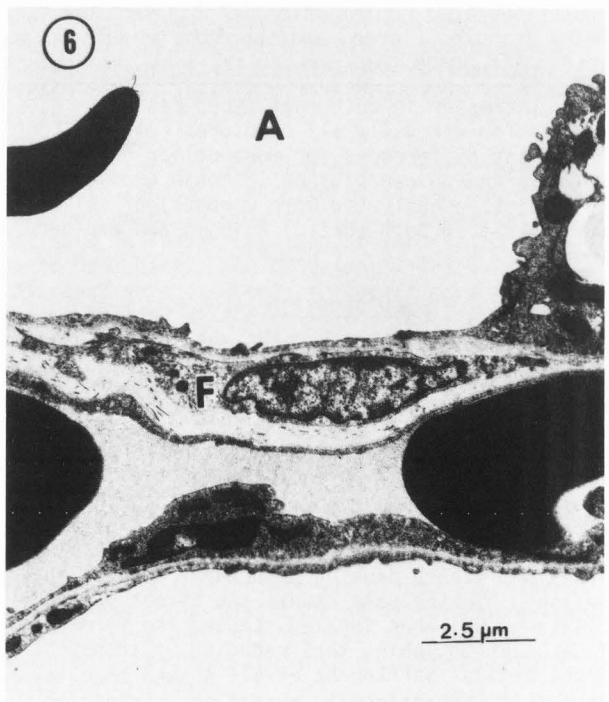


Fig. 4. TEM of Intravascular Area. Monocyte (M) demonstrates variable features according to the state of activity. Platelets (P) are anucleate bodies that have a disc shape. The finely granular cytoplasm contains a variety of organelles, large dense granules and membrane-bound granules. Air Space (A).20

Fig. 5. TEM. Intravascular Area. Mononuclear cells (MN) (lymphocyte) possess a small rounded nucleus with moderate condensed chromatin and sparse cytoplasm containing few organelles. Air Space (A).



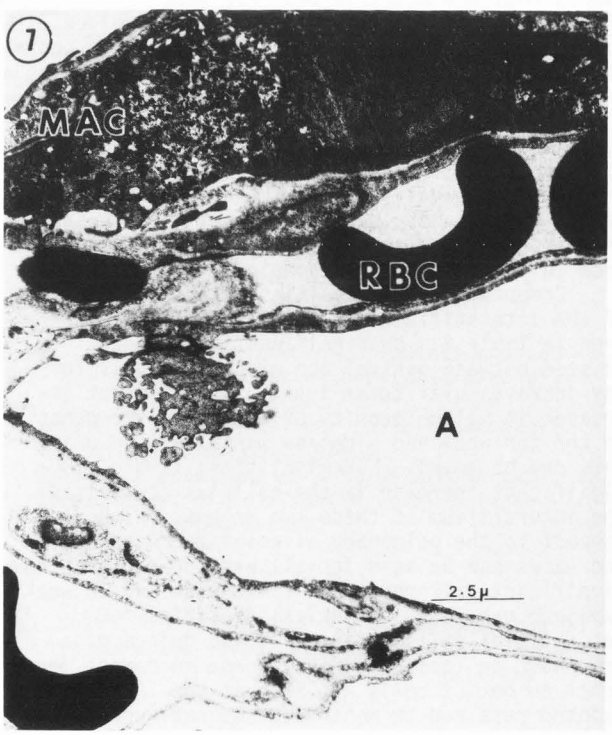


TABLE 1

WHITE BLOOD CELL POPULATIONS WITHIN CAPILLARIES OF RODENTS:

CHARACTERIZATION BASED ON COUNTS OF ACTUAL CELL NUMBERS

CONDITIONS	TOTAL NO. WBC	GRANULOCYTES NO.	GRANULOCYTES %	MONONUCLEAR CELLS NO.	MONONUCLEAR CELLS %
RATS:					
2 wk C	61	29	47.5	32	52.5
2 wk E[a]	71	23	32.4	48	67.6
6 wk C	28	7	25.0	21	75.0
6 wk E[a]	28	6	21.4	22	78.6
10 wk C	28	4	14.3	24	85.7
10 wk E[b]	26	4	15.4	22	84.6
18 mo C	13	5	38.5	8	61.5
18 mo E[b]	16	7	43.7	9	56.2
GUINEA PIGS:					
18 mo C	15	2	13.3	13	86.7
18 mo E[b]	20	8	40.0	12	60.0

C = Control E = exposed DE = Diesel Exhaust wk = week mo = month m = meter a = exposed to 6000 μg DE/m^3 b = exposed to 1500 μg DE/m^3

Mononuclear cells include monocyte, macrophage, lymphocyte. No basophils or eosinophils were seen.

agent to human fibroblasts inducing a greater rate of mutation.

Spector[22], in his morphometric studies, theorized that the irritant material phagocytized by the macrophages could illicit cellular (fibroblastic) responses. Several studies conducted by our group involved the effect of acute and chronic exposure of DE inhalation on the lungs of experimental animals.[4,5,6,8,9] The major finding was that neither pathological nor neoplastic changes were evident, although signs of inflammation appeared in lungs under higher (> 750 μg DE/m^3) DE exposure regimens. Secondary findings on broncholavaged cells included morphological changes such as increased cell size, bizarre nuclei in some monocytes and surface changes on macrophages. Phagocytic ability of macrophages for fluorescent microspheres declined as exposure to DE increased both in dosage and time. Additional work demonstrated that granulocytes are recruited into alveolar spaces. A third study dealt with further morphometric analysis of the alveolar lungs of guinea pigs chronically exposed to DE for 6 months. Gross examination and light microscopy revealed lungs extensively discolored with suggestions of fibrous tissue formation. Quantitative morphometry done on alveolar lung sections from guinea pigs exposed to 750 μg DE/m^3 or greater concentration revealed that alveolar septal tissue, specifically the interstitial region, was thicker than age matched control tissue.

Identification and quantitation of the cellular contents of the interstitium then became the objective of this present study. Results clearly indicate a significant increase in macrophages and mononuclear cells, the guinea pig demonstrating a slightly higher number than the rat. Both, therefore, exhibit normal chemotactic response. Though not reaching significant levels, both species appear to have an increase of fibroblasts from the 6th week of DE

Fig. 6. TEM. Interstitium Area. Fibroblasts (F) are cells whose nucleus is moderately condensed and have a small quantity of cytoplasm with characteristic fine tapering of the cytoplasmic extensions; organelles present become more prominent while active in synthesis of collagen. Air Space (A).[7,25]

Fig. 7. TEM of the Interstitium Area. Macrophage (MAC), the active form of the monocyte, is known for its large size (up to 50 μm in diameter), its far reaching cytoplasmic tentacles, an irregular nucleus with heterochromatin typically clumped around the nuclear envelope. The cytoplasm can demonstrate a few to many organelles (quiescent state/phagocytic state). Red Blood Cell (RBC) has high electron density and rare cytoplasmic organelles. The profile shape depends on the position when cut. Air Space (A).

TABLE 2

VOLUME DENSITIES OF SELECTED COMPONENTS IN RAT ALVEOLAR LUNG

(UNITS = cm^3/cm^3 TOTAL PARENCHYMAL LUNG VOLUME)

	CONDITIONS	TOTAL CAPILLARY SPACE	CONTENTS CAPILLARY SPACE		
			PLASMA	RED BLOOD CELLS	OTHER CELLS
RATS:	2 wk C	0.075 ± 0.016	0.029 ± 0.003	0.034 ± 0.024	0.011 ± 0.012
	2 wk E[a]	0.058 ± 0.015	0.021 ± 0.006	0.032 ± 0.009	0.005 ± 0.002
	6 wk C	0.079 ± 0.009	0.037 ± 0.002	0.039 ± 0.009	0.003 ± 0.001
	6 wk E[a]	0.060 ± 0.022	0.024 ± 0.008	0.033 ± 0.015	0.003 ± 0.001
	10 wk C	0.050 ± 0.009	0.020 ± 0.003	0.024 ± 0.005	0.005 ± 0.002
	10 wk E[a]	0.077 ± 0.053	0.030 ± 0.016	0.043 ± 0.035	0.004 ± 0.002
	18 mo C	0.148 ± 0.042	0.053 ± 0.020	0.090 ± 0.024	0.005 ± 0.002
	18 mo E[b]	0.105 ± 0.034	0.032 ± 0.019	o.069 ± 0.019	0.004 ± 0.000

C = control E = exposed DE = Diesel Exhaust a = exposed to 6000 µg DE/m^3 b = exposed to 1500 µg DE/m^3

**p = 0.05 Significant at 95% level of confidence
***p = 0.02 Significant at 98% level of confidence

exposure through the 18th month. The number of fibroblasts in the exposed animals almost doubled those of the matched controls. These cells displayed no observable structural changes in either the nuclear or the cytoplasmic areas.[25] The question arises regarding the stimulus that orchestrates the chemotactic response. Two possible explanations are available. Macrophages are known to secrete a chemotactic factor which induces fibroblasts into the interstitial area. The macrophages containing phagocytized irritants lie in close proximity to fibroblasts. They release lysosomal substances illiciting both an increase in the fibroblasts together with apparent accelerated fibroblastic activity. This may result in the increased synthesis of collagen visualized within the interstitium.[22]

Another possibility considers chemotactic factors liberated by epithelial type 1 cells which illicit a response from both the macrophage and the fibroblast. Each then responds independently of the other. In an earlier study[3] it was noted that epithelial type 1 cells demonstrate the presence of membrane-bound DE particulates within the cytoplasm. It has been theorized[22] that type 1 cells engage in particular secretory activity together with phagocytosis and digestion. These cells are of histiocytic-macrophage derivation and as such have demonstrated the ability to phagocytize DE particulate matter. Morphometric techniques alone are not sufficient to resolve this question.

Conclusions

Our findings from this study lead us to suggest the following conclusions: 1) Exposure to 6000 µg DE/m^3 for 2 weeks was insufficient to illicit any cellular response. 2) Both species demonstrated adaptive multi-cellular response to DE. 3) Increased numbers of fibroblasts are found in the interstitium, possibly due to chemotactic stimuli initiated by alveolar macrophages or epithelial type 1 cells, and 4) Further work needs to be done to evaluate the activity of the fibroblasts within the interstitium.

Acknowledgement

The authors express their sincere gratitude for the expert technical assistance of Steve Barmatoski.

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TABLE 2 (continued)

VOLUME DENSITIES OF SELECTED COMPONENTS IN RAT ALVEOLAR LUNG

(UNITS = cm^3/cm^3 TOTAL PARENCHYMAL LUNG VOLUMES)

TISSUE EXCLUDING CAPILLARY CONTENTS	ALVEOLAR MACROPHAGES	INTERSTITIAL SPACE	CONTENTS OF INTERSTITIAL SPACE	
			CELLULAR	NON-CELLULAR
0.098 ± 0.014	0.012 ± 0.014	0.044 ± 0.009	0.018 ± 0.012	0.026 ± 0.002
0.125 ± 0.025	0.018 ± 0.011	0.049 ± 0.003	0.026 ± 0.002	0.023 ± 0.005
0.127 ± 0.011	0.006 ± 0.003	0.056 ± 0.003	0.025 ± 0.005	0.031 ± 0.002
0.129 ± 0.020	0.016 ± 0.005***	0.050 ± 0.003	0.024 ± 0.002	0.026 ± 0.005
0.114 ± 0.010	0.005 ± 0.004	0.044 ± 0.004	0.022 ± 0.005	0.022 ± 0.003
0.166 ± 0.020***	0.029 ± 0.009***	0.060 ± 0.004***	0.035 ± 0.003***	0.025 ± 0.004
0.105 ± 0.013	0.006 ± 0.004	0.046 ± 0.003	0.016 ± 0.004	0.030 ± 0.002
0.131 ± 0.004***	0.030 ± 0.011***	0.047 ± 0.003	0.022 ± 0.001**	0.025 ± 0.005

C = control E = exposed to DE = Diesel Exhaust a = exposed to 6000 µg DE/m^3 b = exposed to 1500 µg DE/m^3

**p = 0.05 Significant at 95% level of confidence
***p = 0.02 Significant at 98% level of confidence

TABLE 3

COMPARISON OF EFFECTS OF DE EXPOSURE ON THE ABSOLUTE NUMBER OF INTERSTITIAL CELLS (IC) IN ALVEOLAR LUNGS

(MEAN ± SD)

CONDITIONS	ABSOLUTE NUMBER OF CELLS				
	TOTAL IC ($X\ 10^{-6}$)	FIBROBLASTS NUMBER ($X\ 10^{-6}$)	% of IC CELLS	MONONUCLEAR CELLS NUMBER ($X\ 10^{-6}$)	% of IC CELLS
RATS (N = 3 for each group)					
2 wk C	468.4 ± 27.8	421.6 ± 25.0	90	46.8 ± 2.8	10
2 wk E	517.7 ± 166.2	425.5 ± 136.6	82	92.1 ± 29.6	18
6 wk C	480.2 ± 113.4	365.9 ± 101.7	76	114.3 ± 27.0	24
6 wk E	561.4 ± 75.0	458.1 ± 61.2	82	103.3 ± 13.8	18
10 wk C	277.2 ± 134.6	204.3 ± 99.2	74	72.9 ± 35.2	26
10 wk E	533.8 ± 55.9**	420.1 ± 44.0***	79	113.7 ± 11.9	21
18 mo C	433.8 ± 192.8	319.7 ± 142.1	74	114.1 ± 50.7	26
18 mo E	731.3 ± 133.7***	514.9 ± 94.1	70	216.5 ± 39.6***	30
GUINEA PIGS (N = 3 for each group)					
18 mo C	1083.0 ± 171	468.9 ± 74.0	43	614.1 ± 97.0	57
18 mo E	1618.0 ± 254.0***	637.5 ± 100.1	39	980.5 ± 153.9***	61

Abbreviations specific as in Table 2

**p = 0.06, marginally significant
***p = 0.05 significant at the 95% level of confidence

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Discussion With Reviewers

Reviewer I: Do these data suggest that inflammatory cells (neutrophils or eosinophils) are recruited from pulmonary capillaries?
Authors: Our results show an increase in these cells in the interstitial space. Morphometric methods are not capable of establishing the origin of these cells. These cells are capable of diapedesis and it is reasonable to speculate that this is their mode of entry.

Reviewer I: What factors may be contributing to the influx of interstitial fibroblasts observed after diesel exposure? Are diesel particles phagocytized by interstitial fibroblasts or macrophages?
Authors: Opsonins synthesized and released by the macrophages may be contributing to the influx of interstitial fibroblasts after diesel exposure. Diesel particles were not observed in the cytoplasm of the interstitial fibroblasts but were observed to be stored within phagosomes of the alveolar macrophages.

Reviewer I: Are diesel particles translocated from air space to capillary endothelium? Were any particles observed phagocytized by granulocytic cells in the vasculature?
Authors: Again, morphometric analysis cannot determine the translocation of diesel particles from one part of the lung to another. Previous studies (6) did publish that the eosinophil had phagocytized diesel exhaust particles.

Reviewer II: Could you comment on studies that have used the exhaust of gasoline engines as a control for the effect of diesel exhaust on the lungs? I am not completely convinced that diesel exhaust is worse than gasoline exhaust or for that matter just dirty air.
Authors: To our knowledge, there have been no published morphometric studies on the effect of gasoline engine emissions on the lung. This indeed would be an interesting study. It would still be necessary to have room air as a control. Our study did not use the gasoline engine as a control because we intended to only address the impact of the diesel particulate. This was decided based on prior information that the adsorbed organic phase on the diesel particle had potentially carcinogenic effects.

Reviewer II: A great potential for bias with transmission electron microscopy exists because of the inherent problems of random sampling that result from the necessity to select areas that are free of preparation artifacts. Could you comment about this in your study and in general.
Authors: We have attempted to remove as much bias from our sampling technique as possible in that each area of a given grid was photographed without regard to specific content as long as it contained a recognizable tissue structure. Areas containing only air spaces were noted as the grid was mapped, but were not photographed. These blank areas were included in the data base. Preparation artifacts were not a significant problem since whole lungs were prepared by intratrachial infusion of glutaraldehyde thus preventing alveolar collapse and preserving the normal anatomical relationships of lung parenchyma and stroma. Sections used in this study were of excellent quality and, for the most part, free of sectioning and stain artifact.

In general, we are aware that pure random sampling alone can introduce bias and give erroneous results especially in non-homogeneous tissues. Therefore our approach was to use tissue samples from different planes, as shown in our Figure 1, thus obtaining a more representative sample. This stratified random sampling technique has been shown to result in smaller standard errors. (See Aherne WA, Dunnill MS (1982) Morphometry, Edward Arnold Ltd., pp. 19-32)