Toxicology and Applied Pharmacology **160,** 250–261 (1999)
Article ID taap.1999.8762, available online at http://www.idealibrary.com on IDEAL®

Inflammatory Effect of Intratracheal Instillation of Ultrafine Particles in the Rabbit: Role of C-fiber and Mast Cells

A. Nemmar,* A. Delaunois,* B. Nemery,§ C. Dessy-Doizé,† J-F. Beckers,‡ J. Sulon,‡ and Pascal Gustin[*,1]

**Department of Pharmacology and Toxicology, †Department of Histology, and ‡Department of Physiology of Reproduction, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium; and §Laboratorium voor Pneumologie, Eenheid voor Longtoxicologie, Katholieke Universiteit Leuven, Herestraat 49 O&N B-3000 Leuven, Belgium*

Received April 27, 1999; accepted July 27, 1999

Inflammatory Effect of Intratracheal Instillation of Ultrafine Particles in the Rabbit: Role of C-fiber and Mast Cells. Nemmar, A., Delaunois, A., Nemery, B., Dessy-Doizé, C., Beckers, J-F., Sulon, J., and Gustin, P. (1999). *Toxicol. Appl. Pharmacol.* **160, 250–261.**

The effects of ultrafine polystyrene carboxylate-modified (fluorospheres) on inflammatory processes are being investigated in rabbit lungs. One milliliter of sterile NaCl (0.9%) containing 4 mg of ultrafine particles (UFP) was intratracheally instilled into anesthetized rabbits. The control animals were only instilled with sterile NaCl (0.9%). Twenty hours after being instilled, the rabbits were killed and their lungs were excised and then tracheally perfused with phosphate-buffered physiological solution (PBS). The lung effluents, collected from small holes made in the pleura, were analyzed for substance P (SP) and histamine content by radioimmunoassay (RIA) methods, after administration of drugs. In addition, in other groups of rabbits, the lung wet/dry (W/D) weight ratio was monitored, as were the cellular and protein contents in bronchoalveolar lavage (BAL). Electron microscopy examination was also performed. In tracheally superfused experiments, UFP induced a significant enhancement of both SP and histamine releases after administration of capsaicin (10^{-4} M), to stimulate C-fiber, and carbachol (10^{-4} M), a cholinergic agonist. A significant increase in histamine release was also recorded in the UFP-instilled group following the administration of both SP (10^{-6} M) plus thiorphan (10^{-5} M) and compound 48/80 (C48/80) (10^{-3} M) to stimulate mast cells. In addition, the BAL fluid analysis of UFP groups showed an influx of neutrophils and an increase in total protein concentration. An increase in the lung WW/DW ratio was also recorded. Both epithelial and endothelial injuries were observed in the lungs of UFP-instilled rabbits. The pretreatment of rabbits *in vivo* with a mixture of either SR 140333 and SR 48368, a tachykinin NK_1 and NK_2 receptor antagonist, or a mixture of terfenadine and cimetidine, a histamine H_1 and H_2 receptor antagonist, prevented UFP- induced neutrophil influx and increased total proteins and lung WW/DW ratio. Therefore, it can be concluded that chemicaly inert, electrically charged UFP induce a pulmonary inflammatory process during which the release of SP and histamine from C-fibers and mast cells was enhanced after various stimuli. These latter mediators can also modulate the inflammatory process. © 1999 Academic Press

Key Words: **ultrafine particles; lung; rabbit; substance P; histamine; neutrophils; mast cells; C-fibers.**

Numerous epidemiological studies have demonstrated an association between peaks in particulate air pollution and increasing mortality, mainly in elderly people with preexisting cardiorespiratory disease (Andersson *et al.*, 1990). Increased morbidity due to reduction in pulmonary function (Pope *et al.*, 1993) and chronic respiratory and cardiovascular disease (Van Der Lende *et al.*, 1981) has also been described in polluted areas. Although such epidemiological associations have been well demonstrated, their biological plausibility and the mechanisms possibly underlying these effects remain unclear. It has been proposed that ultrafine particles (UFP) (aerodiameter below 0.1 mm) are mainly involved in these health effects because they have a larger surface area than the particles of larger size, thus causing greater inflammatory response in lungs, including alveoli and the interstitium, assessed by lung lavage parameters in rat (Oberdörster *et al.*, 1992; Ferrin *et al.*, 1990).

Until now, the studies that have described the effects of UFP on the respiratory tract were performed with UFP of various surface chemistry such as cobalt, nickel, titanium dioxide, and carbon (Faux *et al.*, 1994; Terashima *et al.*, 1997; Zhang *et al.*, 1998). By contrast, the effects of chemically inert but electrically charged UFP on the lungs and airways have never been described so far. Deposition of charged particles in lung airways has only been investigated in hollow-cast models of human airways (Cohen *et al.*, 1998). Some of the effects of UFP described in the literature were related to a free radical generation, which could be responsible for the development of inflammatory process (Li *et al.*, 1997; Zhang *et al.*, 1998). By contrast, the possible mechanisms involved in UFP-induced effects have not been documented, in particular their interactions with C-fibers and mast cells. Substance P (SP) is a tachykinin localized in C- fibers, which belong to the excita-

[1] To whom correspondence should be addressed. Fax: 00-32-4-366-41-76; E-mail: p.gustin@ulg.ac.be.

0041-008X/99 $30.00

tory nonadrenergic, noncholinergic nervous system. These nervous fibers innervate airways in several species and are found beneath the epithelium, around blood vessels (Lundberg *et al.*, 1985; Hislop *et al.*, 1990), and close to mast cells (Dimitriadou *et al.*, 1994). Substance P can be released by various irritants, infectious agents, and inflammatory mediators (Hazbun *et al.*, 1993; Martins *et al.*, 1991; Saria *et al.*, 1988). Substance P activates tachykinin receptors, resulting in an increase in vascular permeability (Delaunois *et al.*, 1993), bronchoconstriction (Advenier *et al.*, 1987), vasodilatation (Delay-Goyet *et al.*, 1992), and submucosal secretion (Rogers *et al.*, 1989). Yet the role of the C-fiber and mast cells and their mediators has been described for other atmospheric pollutants such as ozone (O_3) (Delaunois *et al.*, 1997; Campos *et al.*, 1992).

The aim of the present work was to investigate the effects of chemically inert, charged ultrafine particles on inflammatory processes in rabbit lungs, by assessing various parameters such as analysis of bronchoalveolar lavage (BAL) fluid, wet/dry weight ratio, and morphological integrity of lung tissue. The roles of C-fibers and mast cells in the UFP-induced effects were also considered.

MATERIALS AND METHODS

Particle Description

The particles used for this study were polystyrene carboxylate-modified microspheres (fluorospheres; Molecular Probes, Leiden, The Netherlands). These particles were red-fluorescent and had a diameter of 44 ± 5 nm. The dye excitation and emission wavelenghts are 580 and 605 nm, respectively. One milligram of particles corresponded to 21.4×10^{12} particles.

TABLE 1

Groups of Rabbits Used for the Measurement of the Release of SP and Histamine in Lung Effluents 3 min before and 3 min during Continuous Perfusion with Capsaicin, Carbachol, Exogenous SP plus Thiorphan,[a] and Compound 48/80 Stimulation without (Controls) or with Pretreatment with UFP

	n	UFP	Drugs	Parameters measured in the lung effluents
Group 1a	4	−	Capsaicin (10^{-4} M)	SP + histamine
Group 1b	4	+	Capsaicin (10^{-4} M)	SP + histamine
Group 2a	5	−	Carbachol (10^{-4} M)	SP + histamine
Group 2b	5	+	Carbachol (10^{-4} M)	SP + histamine
Group 3a	4	−	Substance P (10^{-6} M) + thiorphan (10^{-5} M)	Histamine
Group 3b	4	+	Substance P (10^{-6} M) + thiorphan (10^{-5} M)	Histamine
Group 4a	4	−	C 48/80 (10^{-3} M)	Histamine
Group 4b	4	+	C 48/80 (10^{-3} M)	Histamine

[a] SP alone was not used in the present experiments because it has been previously shown that SP alone failed to induce a significant effect on the release of histamine (Nemmar *et al.*, 1999).

TABLE 2

Groups of Rabbits Used for the Cellular Determination and Protein Concentrations in BAL as well as the Assessment of Wet Weight/Dry Weight (WW/DW) Lung Ratios without or with Pretreatment with Antagonists before UFP Administration

	n	UFP	Pretreatment drugs	Measured parameters
Group 1	7	−	—	Cellular counts
				Protein concentration; WW/DW
Group 2	8	+	—	Cellular counts
				Protein concentration; WW/DW
Group 3	4	+	SR140333 (1 mg/kg) + SR48968 (1 mg/kg)	Cellular counts
				Protein concentration; WW/DW
Group 4	4	+	Terfenadine (0.25 mg/kg) + cimetidine (10 mg/kg)	Cellular counts
				Protein concentration; WW/DW

Intratracheal Instillation of Particles

New Zealand white rabbits (males and females) weighing 2.5–3 kg were anesthetized with a single intramuscular injection of ketamine (50 mg/kg) (Imalgène 1000, Rhône Mérieux, Brussels, Belgium) and xylazine (2.6 mg/kg) (Rompun, Bayer, Leverkusen, Germany). The tracheal zone was shaved and desinfected with dermal iso-Betadine (SARGET Merignac, Brussels, Belgium), and 1 ml of sterile NaCl 0.9% containing 4 mg of fluorospheres was instilled in the trachea by means of a 27-gauge needle. The control animals were instilled with 1 ml of sterile NaCl 0.9% alone. The animals were then allowed to recover. This protocol was accepted by the local ethical committee.

Twenty hours after intratracheal instillation of fluorospheres, the lungs of each animal were excised and submitted to either tracheally superfused experiments or BAL analysis or lung edema determination or electron microscopy studies.

Tracheally superfused lungs and determination of SP and histamine effluent content. Twenty hours after intratracheal instillation of particles, the rabbits were anesthetized with a single intramuscular injection of ketamine (50 mg/kg) and xylazine (2.6 mg/kg). The trachea was isolated and cannulated. Pancuronium (0.2 mg/kg) was administered via a marginal ear vein to prevent reflex movements of the chest as a result of anoxia during the midsternal incision. The animals were ventilated with a small animal respirator (Palmer, Analis, Namur, Belgium) and 2000 U/kg of heparin were injected into the right ventricle before exsanguination. The lungs were removed from the chest, and numerous small holes were made in the pleural surface with a 25-gauge needle to facilitate the exit of the superfusate, as described by Martins *et al.* (1991) and recently adapted to our model (Nemmar *et al.*, 1999). The lungs were superfused via the trachea at a flow rate of 40 ml/min with a phosphate-buffered physiological solution (PBS), pH 7.4, containing the following (in mM): 137 NaCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 2.68 KCl, 0.6 $NaHCO_3$, 0.13 NaH_2PO_4, and 0.87 Na_2HPO_4. This perfusion buffer was kept at 37°C and passed through a bubble trap before entering the lungs via the tracheal cannula. The lung effluents were collected in polypropylene tubes containing pure acetic acid to yield a final concentration of 5% by volume, and then kept on ice.

Each perfusate collected was purified and concentrated using Sep Pak C_{18} cartridges (Waters, Milford, MA). These cartridges had been conditioned with 20 ml of methanol and washed with 20 ml of distilled water. The samples were then administered to the cartridges and washed with 3 ml of distilled water. The peptide was eluted with 8 ml of aqueous solution containing 70% aceto-

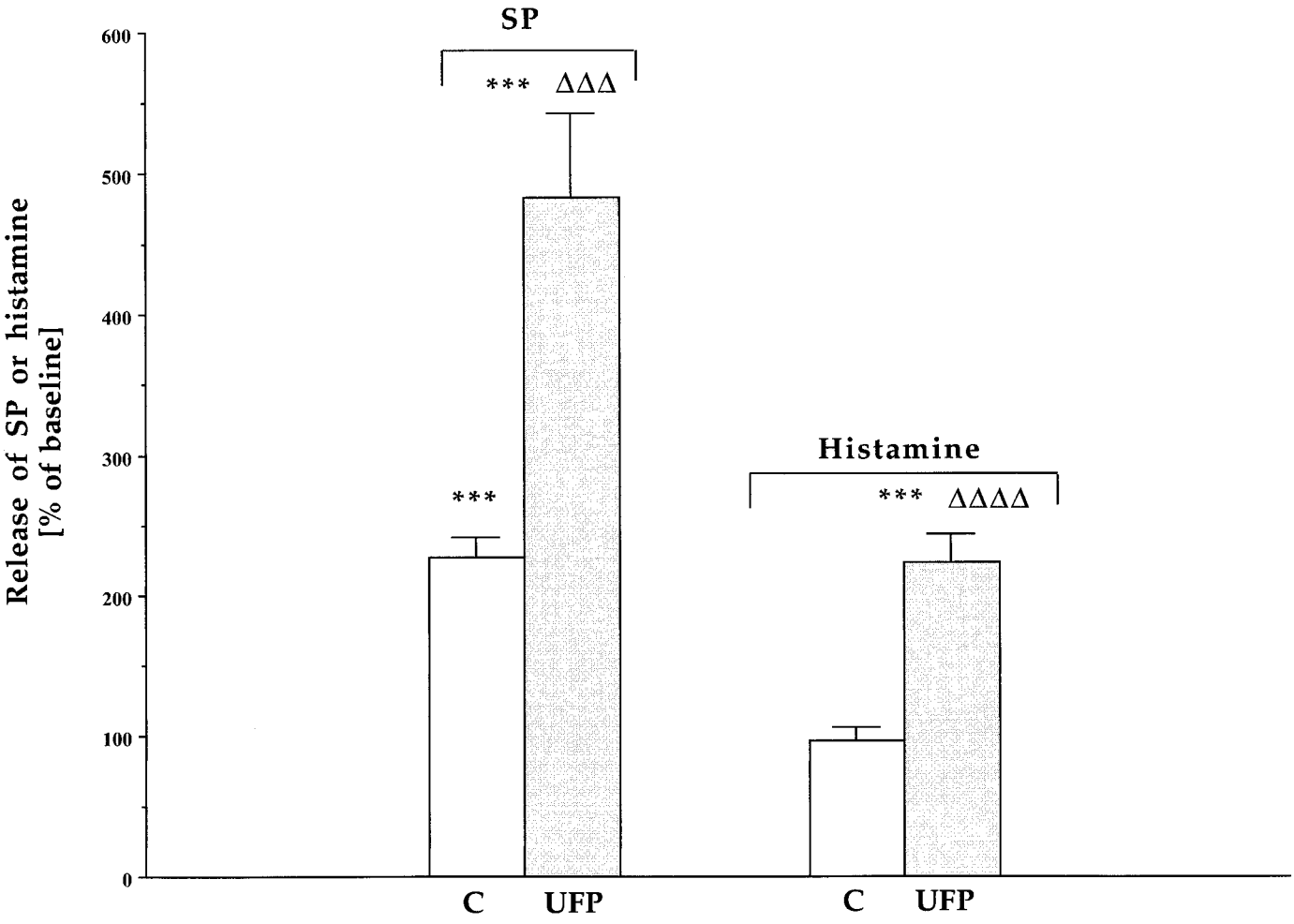

FIG. 1. Capsaicin (10^{-4} M)-induced release of SP and histamine in lung effluents, expressed as % of baseline value, recorded in control (C) rabbits ($n = 4$) and in rabbits instilled with ultrafine particles (UFP) ($n = 4$). Baseline values, considered as 100%, were not indicated in the figure. *Value significantly different from the baseline value (100%) in the same group (***$p < 0.005$). $^{\Delta}$Value significantly different from the corresponding value measured in the control group ($^{\Delta\Delta\Delta}p < 0.005$; $^{\Delta\Delta\Delta\Delta}p < 0.001$).

nitrile and 0.1% trifluoracetic acid (TFA). The eluates were dried under vacuum and kept at −20°C until the assays were performed. Using this protocol, the recovery of added SP in lung effluents was 75 ± 4.5%.

Substance P-Like Immunoreactivity (SP-LI) was determined by radioimmunoassay (RIA), according to a method described by Nemmar *et al.* (1998). The lower limit of detection was 7.4 PM. The assay had 0.1% cross-reactivity with Neurokinin A (NKA), 3% with Neurokinin B (NKB), and no cross-reactivity was recorded with either SP 1–7 or SP 1–9.

The histamine content of lung effluents was determined by means of a commercially available radioimmunoassay kit (Immunotech, Marseille, France). The lower limit of detection of this assay was 0.2 nM and its specificity was 100% against several endogenous analogues.

After an equilibration period of 15 to 20 min during which the lungs were tracheally perfused with PBS, the lung effluents were collected in two fractions corresponding to two consecutive perfusion periods of 3 min. During the first period, PBS was perfused, while, during the second period, capsaicin or carbachol or SP plus thiorphan or C 48/80 was included in the perfusate. All drug concentrations given in the text are final concentrations in perfusates. Several groups of rabbits were used for the tracheally superfused lung studies (Table 1).

BAL procedure. In rabbits undergoing BAL, a tube was connected to the tracheal cannula. The lungs were washed with 3 × 10 ml saline (9 g/liter). The recovered fluid aliquots were pooled (the mean recovered 15.3 ± 3.15 ml, i.e., 51% of the instilled liquid). No difference in the amount of recovered fluid was observed between the different groups.

The counting of the cells was performed in a Thoma hemocytometer after staining with 1% gentian violet, and the designation of macrophages versus neutrophiles was accomplished on a slide preparation after staining with hemacolor. The liquid was centrifuged (800*g* at 4°C for 15 min), and the supernatant was then stored at −20°C until further analysis. Total protein concentrations were measured by the method of Lowry *et al.* (1956).

Assessment of edema. In order to assess lung edema, small pieces of lungs were weighed immediately after lung excision (wet weight) and after drying in an oven at 65°C for 48 h (dry weight). The wet weight/dry weight ratios (WW/DW) were then determined in control lungs and in lungs pretreated with fluorospheres.

Electron microscopy studies. Lung tissue sampled from animals treated with UFP ($n = 4$) and without treatment with UFP ($n = 3$) devoted to electron microscopy investigation were fixed in 4% Millonig's phosphate-buffered glutaraldehyde, pH 7, and postfixed in 2% aqueous osmium tetroxide. Four

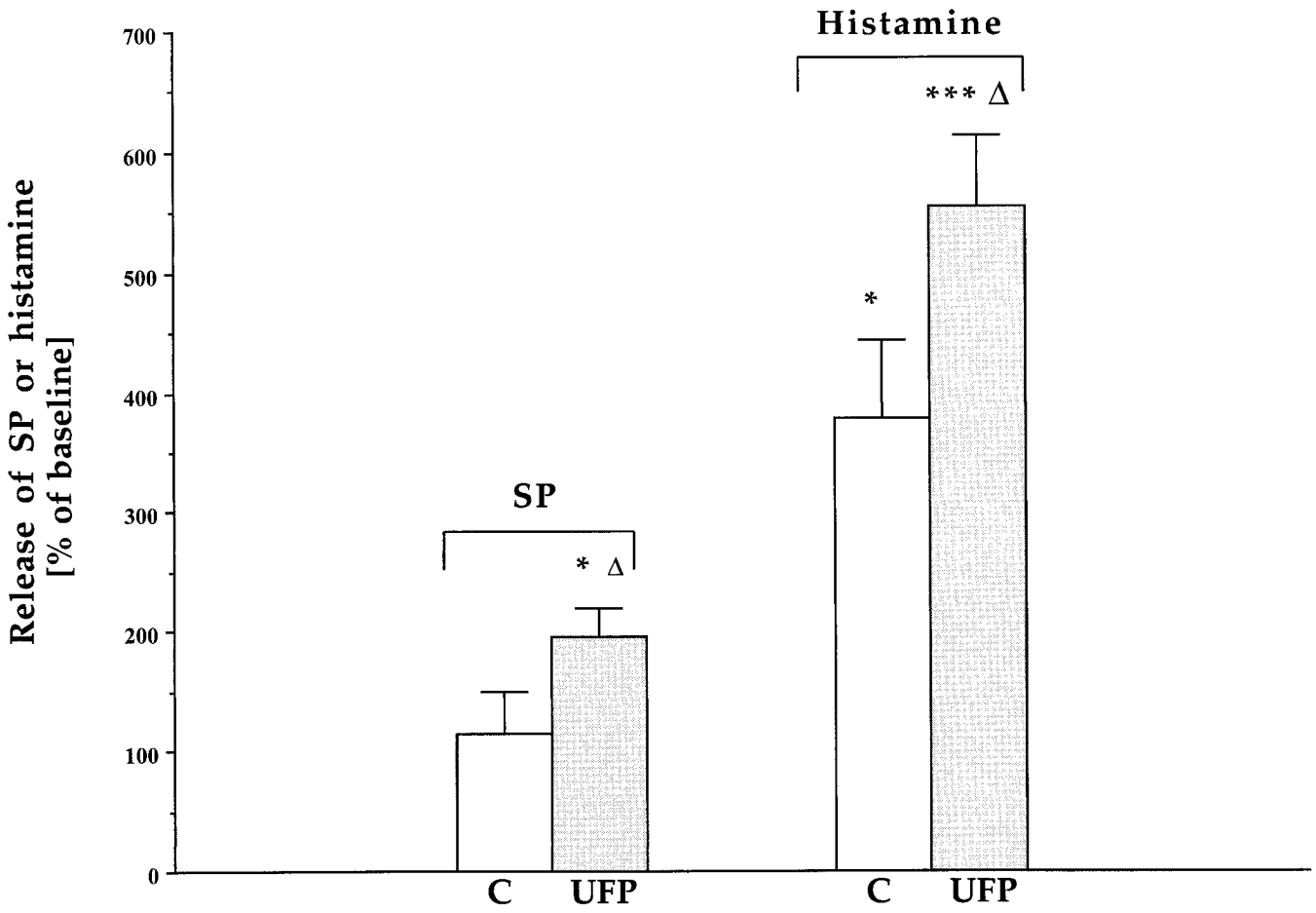

FIG. 2. Carbachol (10^{-4} M)-induced release of SP and histamine in lung effluents, expressed as % of baseline value, recorded in control (C) rabbits (n = 5) and in rabbits instilled with ultrafine particles (UFP) ($n = 5$). Baseline values, considered as 100%, were not indicated in the figure. *Value significantly different from the baseline value (100%) in the same group ($*p < 0.05$; $***p < 0.005$). $^{\Delta}$Value significantly different from the corresponding value measured in the control group ($^{\Delta}p < 0.05$)

tissue blocks per animal were embedded in Epon. Thin sections were contrasted with uranyl acetate followed by lead citrate. Sections were examined with a Zeiss 910 electron microscope, to assess injuries at the level of the blood–alveolar barrier.

Investigation of the Role of Neurokinins and Histamine Receptors

In order to assess the role of neurokinins and histamine on cellular response, airway mucosa permeability, and edema production, the following experiments were performed. Ten minutes before instillation of fluorospheres, as described above, two groups of rabbits were pretreated by iv administration of a mixture of SR140333 (1 mg/kg) and SR48968 (1 mg/kg) ($n = 4$), which are NK_1 and NK_2 receptor antagonists, respectively, or a mixture of terfenadine (0.25 mg/kg) and cimetidine (10 mg/kg) ($n = 4$), which are H_1 and H_2 receptor antagonists, respectively. Ten hours after the first injection, this pretreatment was renewed. The rabbits were euthanized 10 h after the second injection. BAL fluid analyses and WW/DW ratios were then performed. The different groups of animals used for this part of the protocol are summarized in Table 2.

Drugs

Carbamylcholine chloride and SP (Sigma Chemical Co., St. Louis, MO) were dissolved in PBS. Capsaicin and thiorphan (both from Sigma Chemical Co.) were dissolved in ethanol (0.3 ml) and then diluted in perfusion liquid. SR 140333, SR 48968 (Sanofi Recherche, Monpellier, France), terfenadine (Sigma Chemical Co.), and cimetidine (SmithKline Beecham Pharma, Genval, Belgium) were dissolved in 25% ethanol. The solvents used to dissolve the drugs had no effect on the parameters measured in this study.

Statistics and Calculations

Data are expressed as the means ± SEM. A paired or unpaired Student's *t* test was used for the comparison of two means. Where necessary, the probability (p) values for significance were corrected by Bonferroni's method to allow multiple comparisons to be made (Wallenstein *et al.*, 1980). Differences were considered significant when $p < 0.05$.

For each lung, the SP and histamine values recorded during the prestimulation period were considered as the baseline and expressed as 100%; the values of SP and histamine recorded during stimulation periods were then expressed as percentages of their corresponding baseline values.

RESULTS

Tracheally Superfused Rabbit Lung Studies

The mean baseline concentrations of SP and histamine recorded in effluents collected from rabbit lung controls were 4.6

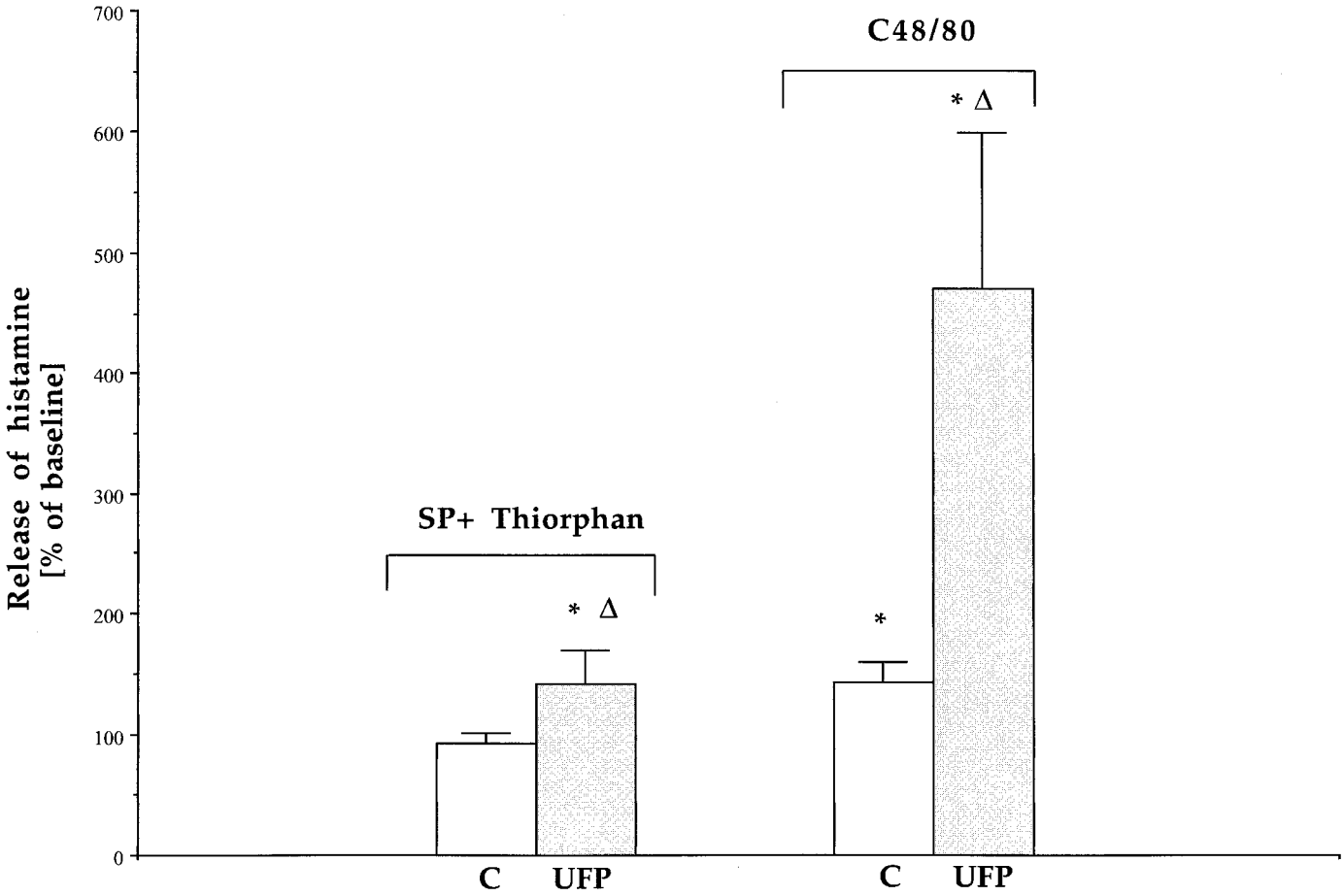

FIG. 3. Exogenous SP (10^{-6} M) plus thiorphan(10^{-5} M) and compound 48/80 (10^{-3} M) (C48/80)-induced release of histamine in lung effluents, expressed as % of baseline value, recorded in control (C) rabbits ($n = 4$) and in rabbits instilled with ultrafine particles (UFP) ($n = 4$). Baseline values, considered as 100%, were not indicated in the figure. *Value significantly different from the baseline value (100%) in the same group ($^{*}p < 0.05$). $^{\Delta}$Value significantly different from the corresponding value measured in the control group ($^{\Delta}p < 0.05$). SP alone was not used in the present experiments because it has been previously shown that SP alone failed to induce a significant effect on the release of histamine (Nemmar *et al.*, 1999).

pM and 2.7 nM, respectively, and the corresponding values observed in rabbit lungs pretreated with ultrafine particles were similar, being 4.0 pM and 3.8 nM respectively.

Effect of Ultrafine Particles on Capsaicin-Induced SP and Histamine Release

Figure 1 illustrates the effects of capsaicin (10^{-4} M), to stimulate C-fiber, on the release of SP and histamine recorded in rabbit lung effluents 20 h after intratracheal instillation of ultrafine particles. In control rabbits, capsaicin induced a significant release of SP but not of histamine. The values recorded were 227 ± 14% ($p < 0.005$) and 96 ± 9% (NS) of baseline, respectively. In rabbits instilled with 4 mg of ultrafine particles, the capsaicin-induced releases of SP (483 ± 60%) ($p < 0.005$) and histamine (223 ± 20%) ($p < 0.001$) were significantly enhanced compared to control rabbits.

Effect of Ultrafine Particles on Carbachol-Induced SP and Histamine Release

In control rabbit lungs, carbachol (10^{-4} M), a cholinergic agonist, failed to induce a significant release of SP (115 ± 35%), while a significant release of histamine was observed (378 ± 66%) ($p < 0.05$). In rabbits instilled with ultrafine particles, carbachol, like capsaicin, caused an increase of the release of both SP (195 ± 24%) and histamine (556 ± 58%) (Fig. 2), which was significantly more pronounced compared to control ($p < 0.05$) for both effects.

Effect of Ultrafine Particles on Exogenous SP plus Thiorphan and on Compound 48/80 (C48/80)-Induced Histamine Release

Figure 3 shows the effect of SP (10^{-6} M) plus thiorphan (10^{-5} M) as well as the effect of C48/80 (10^{-3} M) to stimulate mast cells on the release of histamine in lung perfusates.

TABLE 3

Protective Effect of SR 14033/SR48968 and Terfenadine/Cimetidine on UFP, Induced Changes in the Number of Cells after Staining with 1% Gentian Violet and Type of Cells after Staining with Hemacolor in BAL Collected 20 h after Intratracheal Instillation of UFP in Rabbits

			Percentage of different cell types	
	n	Total number of cells ($\times 10^6$/ml)	Macrophage (%)	Neutrophil (%)
Controls	7	19.2 ± 3.0	100	0
UFP without pretreatment[a]	8	35.0 ± 3.9**	48.3 ± 10.0****	51.6 ± 10.0**
UFP + SR 14033 + SR48968[b]	4	20.0 ± 2.0$^{\Delta}$	91.5 ± 3.0$^{\Delta\Delta\Delta\Delta}$	8.5 ± 3.0$^{\Delta\Delta\Delta\Delta}$
UFP + cimetidine + terfenadine[c]	4	17.0 ± 2.0$^{\Delta}$	95.7 ± 3.0$^{\Delta\Delta\Delta\Delta}$	4.2 ± 2.5$^{\Delta\Delta\Delta\Delta}$

[a] The dose of UFP used was 4 mg, dissolved in 1 ml of NaCl 0.9%.
[b] The doses of SR 140333 and SR 48968 used were 1 mg/kg iv for each one.
[c] The doses of terfenadine and cimetidine used were 0.25 and 10 mg/kg iv, respectively.
** $p < 0.01$ and **** $p < 0.001$, value significantly different from corresponding value in control group.
$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta\Delta\Delta}$ $p < 0.001$, value significantly different from value recorded in UFP group.

In control rabbits, SP plus thiorphan did not induce any effect on histamine release (92 ± 9% of baseline). In rabbits instilled with 4 mg of ultrafine particles, SP plus thiorphan induced a low but significant release of histamine compared to control group. The value recorded was 141 ± 28% of baseline value ($p < 0.05$).

In control rabbit lungs, compound 48/80 (10^{-3} M) induced a significant release of histamine (143 ± 17%) ($p < 0.05$). Instillation of rabbits with ultrafine particles enhanced significantly the C48/80-induced histamine release compared to control group (471 ± 128%) ($p < 0.05$), (Fig. 3).

Bronchoalveolar Lavage Fluid Studies

Pharmacological modulation of the effects of UFP on BAL fluid cell composition. Table 3 shows the effects of UFP on the number and type of BAL fluid cells. Intratracheal instillation of UFP induced a significant increase in the total number of cells (35 ± 3.9 × 10^6 cells/ml) compared to the control group (19.2 ± 3x10^6 cells/ml) ($p < 0.01$). The ultrafine particles also produced an influx of neutrophils in BAL compared to the control group, which accounted for 51.6 ± 10% ($p < 0.001$), while the percentage of macrophages was 48.3 ± 10% ($p < 0.001$). The BAL cells in controls consisted in 100% macrophages (Table 3).

Pretreatment of rabbits with a mixture of SR140333 and SR48968 strongly inhibited the increase in total cell number (20 ± 2x10^6 cells/ml) ($p < 0.05$) and the influx of neutrophils (8.5 ± 3% of total cells) ($p < 0.001$) observed in the BAL collected from the instilled group (Table 3).

The increase in the total cell number and the influx of neutrophils recorded in the instilled group were also completely blocked after pretreatment of rabbits with a mixture of terfenadine and cimetidine. The values recorded were 17 ± 2 × 10^6 cells/ml ($p < 0.05$) and 4.2 ± 2.5% of total cells ($p < 0.001$), respectively (Table 3).

Pharmacological modulation of the effect of UFP on total protein BAL content. The intratracheal instillation of UFP induced a significant increase in total protein concentrations in BAL fluid (1.68 ± 0.05 mg/ml) compared to control values (0.66 ± 0.07 mg/ml) ($p < 0.001$) (Fig. 4).

Pretreatment of rabbits with a mixture of SR140333 and SR48968 partially prevented the increase in total protein BAL content recorded in the instilled rabbits (1.23 ± 0.08 mg/ml) ($p < 0.05$) (Fig. 4).

Terfenadine plus cimetidine also partially inhibited this effect. The value recorded in this group (1.24 ± 0.05 mg/ml) was significantly different from the value recorded in the instilled group ($p < 0.05$) (Fig. 4).

Pharmacological modulation of the effect of UFP on WW/DW ratio. Figure 5 illustrates the effect of intratracheal instillation of UFP on the WW/DW ratio. UFP induced a significant increase in this parameter. The values recorded were 10.36 ± 0.32 in UFP instilled rabbits and 5.22 ± 0.16 in the control group ($p < 0.001$).

Pretreatment of rabbits with a mixture of SR140333 and SR48968 completely prevented the UFP-induced increase in WW/DW ratio. The value recorded was 5.14 ± 0.05, which was significantly different from the value recorded in instilled group ($p < 0.001$).

The enhancement of WW/DW ratio was also strongly inhibited after pretreatment of rabbits with a mixture of terfenadine and cimetidine. The value recorded was 4.98 ± 0.11, the latter being significantly different from the value recorded in instilled group ($p < 0.001$).

Electron Microscopy Studies

Control lung tissue appeared intact. Capillary endothelium was separated from the alveolar epithelium by a continuous basement membrane, and the integrity of both epithelium and endothelium structures was maintained (Fig. 6A).

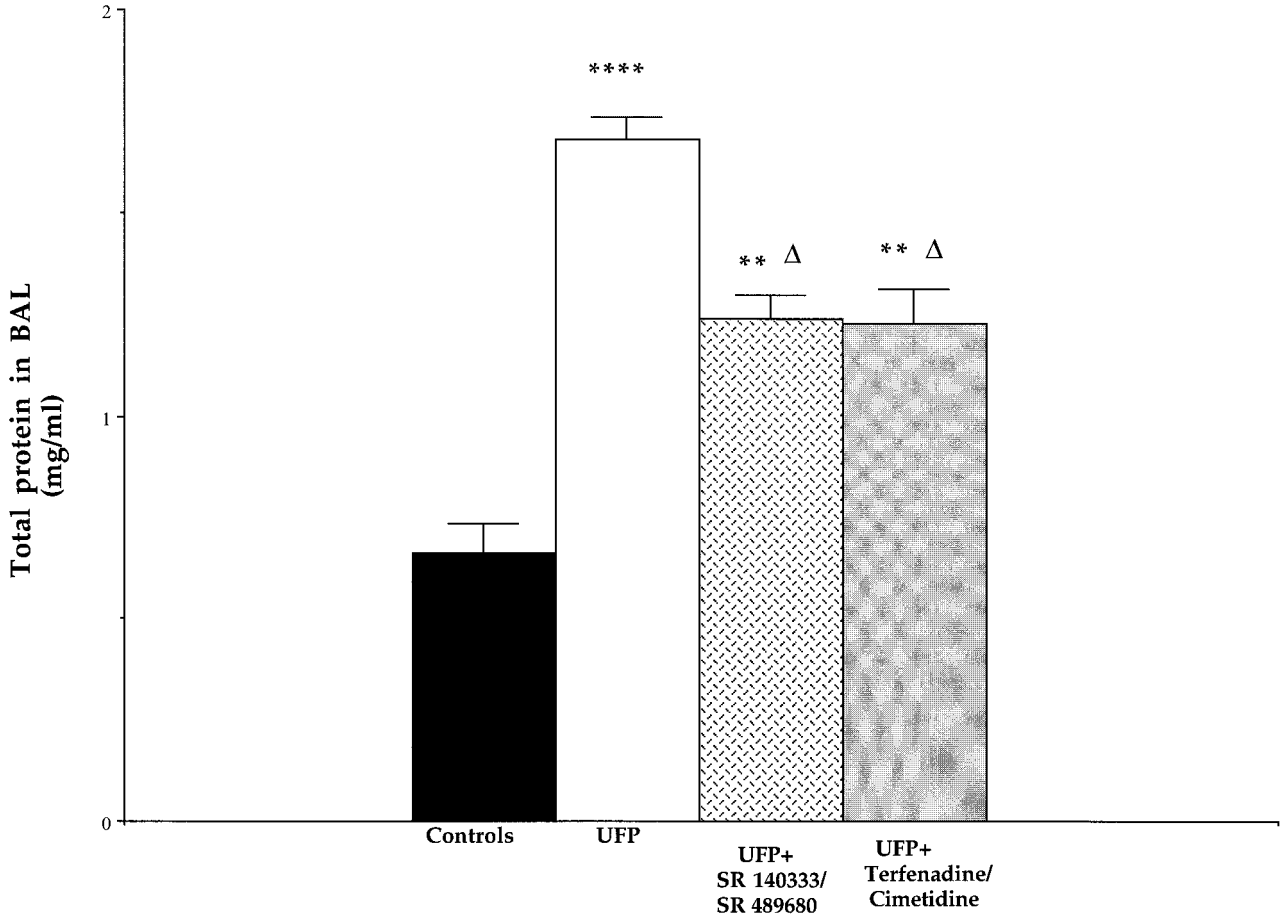

FIG. 4. Values of total protein concentrations in bronchoalveolar lavage (BAL) fluid, expressed as mg/ml, recorded in control rabbits ($n = 7$) and in rabbits instilled with ultrafine particles (UFP) without ($n = 8$) or with pretreatment with a mixture of SR 140333 (1 mg/kg) and SR 48968 (1 mg/kg) ($n = 4$) or a mixture of terfenadine (0.25 mg/kg) and cimetidine (10 mg/kg) ($n = 4$). *Value significantly different from the control value (**$p < 0.01$; ****$p < 0.001$). $^{\Delta}$Value significantly different from the corresponding value measured in the UFP group ($^{\Delta}p < 0.05$).

The examination of specimens collected 20 h after intratracheal instillation of UFP revealed the presence of severe damage. The alveolar epithelium presented abnormal swelling and numerous vacuoles of various sizes. In places, the basement membrane showed disorganization and swelling (Fig. 6B). The endothelial damages were most pronounced. The endothelium showed cell edema with capillary dilation in several areas and a wide-spread cytoplasmic disorganization (Fig. 6C).

DISCUSSION

The present study demonstrates that the intratracheal instillation of chemically inert, but negatively charged UFP produced pulmonary edema and neutrophil inflammation. These changes are accompanied, and presumably mediated by, stimulation of C-fibers and mast cells as shown by the inhibitory effect observed after pretreatment of rabbits with both mixture of tachykinin NK_1 and NK_2 receptor antagonist SR 140333 and SR 48968 and a mixture of tachykinin H_1 and H_2 receptor antagonist terfenadine and cimetidine. Furthermore, in tracheally perfused experiments, the intratracheal instillation of UFP induced a significant enhancement of SP and histamine releases after administration of capsaicin to stimulate C-fiber, carbachol, a cholinergic agonist, SP plus thiorphan, and C 48/80 to stimulate mast cells.

Several epidemiological studies have shown that peaks of air pollution, in particular due to particulate matter with an aerodiameter of ≤ 10 μm (PM_{10}), are associated with increased morbidity and mortality (Schwartz *et al.*, 1994a; Pope *et al.*, 1995). A decline in pulmonary function (Pope and Kranner, 1993) as well as increased incidence of chronic respiratory and cardiovascular diseases (Van Der Lende *et al.*, 1981) and hospital admissions (Schwartz *et al.*, 1994b) were frequently observed and correlated to air pollution with PM_{10}. Although the mechanisms underlying respiratory morbidity due to PM_{10} remain unclear, it is thought that ultrafine particles with aerodiameter ≤ 0.1 μm are of greatest concern to health effects (Peters *et al.*, 1997), since they have a larger surface area than

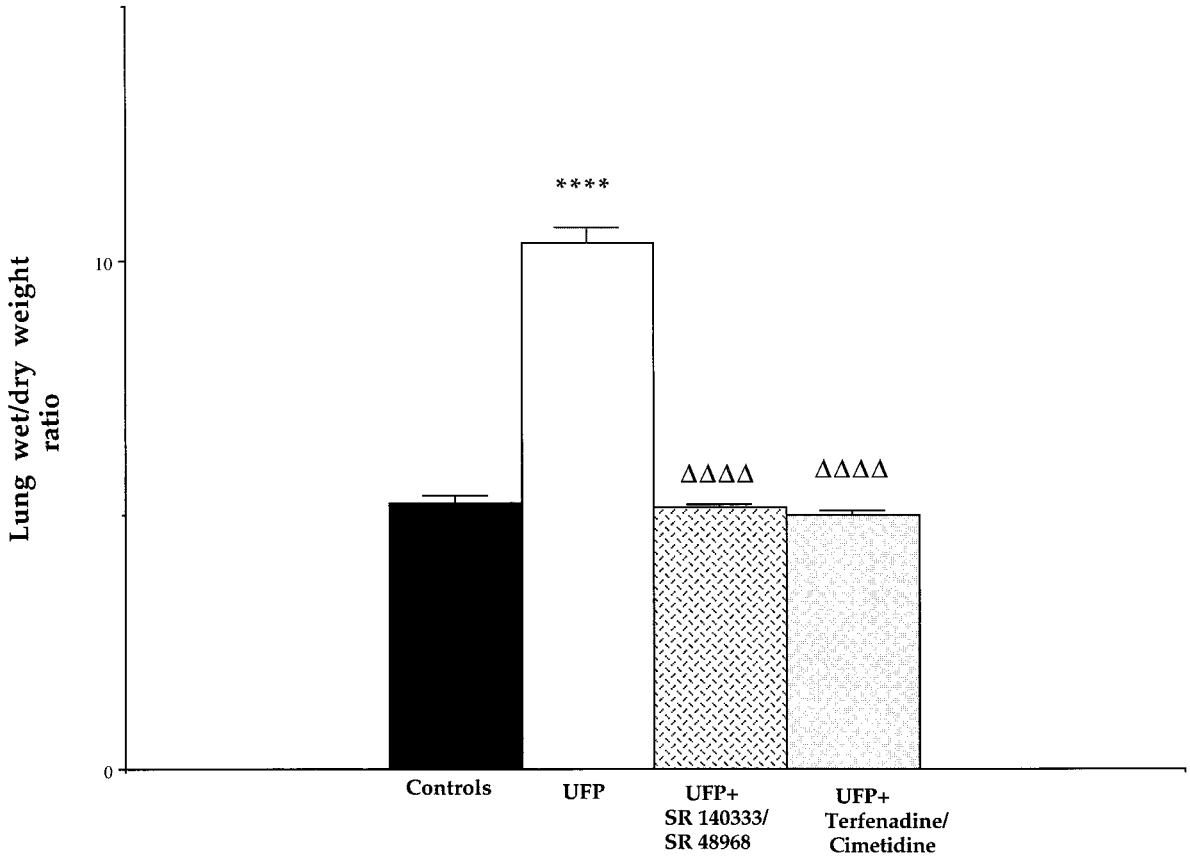

FIG. 5. Values of lung wet/dry weight ratio in control rabbits ($n = 6$) and in rabbits instilled with ultrafine particles (UFP) (4 mg) without ($n = 7$) or with pretreatment with a mixture of SR 140333 (1 mg/kg) and SR 48968 (1 mg/kg) ($n = 4$) or a mixture of terfenadine (0.25 mg/kg) and cimetidine (10 mg/kg) ($n = 4$). *Value significantly different from the control value (****$p < 0.001$). $^{\Delta}$Value significantly different from the corresponding value measured in the UFP group ($^{\Delta\Delta\Delta\Delta}p < 0.001$).

the particles of larger size; thus, they are able to be more toxic than the larger size particles (Pope al., 1995). In addition to the diameter of particles, the surface chemistry seems to play an important role in their effects. In fact, many particles, such as quartz (Vallyathan *et al.*, 1988), asbestos (Dalal *et al.*, 1989), cobalt, nickel, and titanium dioxide (TiO_2) (Zhang *et al.*, 1998), that cause lung damage, appear to exert a free radical activity at their surface. Furthermore, the role of iron in producing hydroxyl radicals was also suggested to explain particle toxicity (Donaldson *et al.*, 1995; Zhang *et al.*, 1998).

Since the studies regarding the UFP have often been performed with particles of various surface chemistry, the present study was designed to explore the effects of chemically inert, but charged UFP on lungs, as well as their pharmacological modulation. The choice of the charged particles was based on the fact that the most ambient particles are charged (Cohen *et al.*, 1998). Moreover, it has been demonstrated in hollow-cast models of human airways that particle charge causes an increase in respiratory tract deposition (Cohen *et al.*, 1998).

In order to assess whether charged UFP can increase the response of C-fibers and mast cells to various substances, the release of SP and histamine in a tracheally superfused model has been performed. This experimental model has previously been developed in rabbit in order to study the interactions between C-fibers, cholinergic fibers, and mast cells (Nemmar *et al.*, 1999). Briefly, it has been demonstrated that SP released upon the action of capsaicin can activate cholinergic fibers. Endogenous acetylcholine can, in turn, activate C-fibers and mast cells with a subsequent release of SP and histamine. In the present study, no histamine release following capsaicin stimulation was recorded. While this discrepancy between our present and previous data cannot be readily explained, it can be suggested that the surgical stress imposed on the animals of the present study could interfere with mediators release. The results obtained in the present work demonstrated that, in lungs from rabbits treated with UFP, capsaicin-induced SP and histamine release in lung effluents were significantly enhanced compared to controls (Fig. 1). Moreover, in rabbits instilled with UFP, carbachol, which has been show to directly activate mast cells and C-fibers, also caused a significant increase in the release of both SP and histamine (Fig. 2). In turn, exogenous SP plus thiorphan failed to modify the histamine release in control lungs, while a low but significant increase was observed in UFP- pretreated rabbits (Fig. 3). Thiorphan was

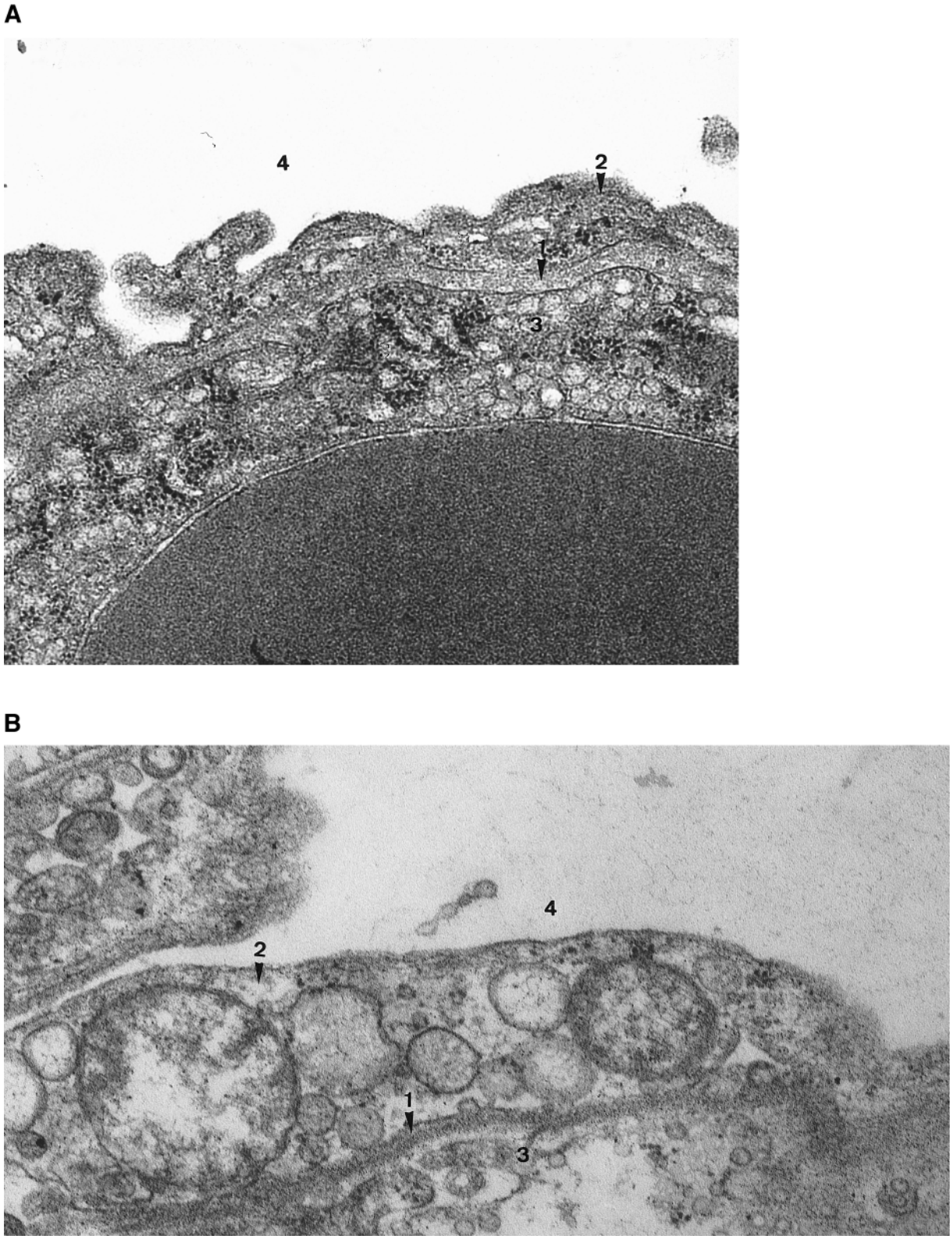

FIG. 6. Photomicrograph of lung tissue collected from control rabbits (A) and from rabbits treated with UFP (B and C). Scale, 1 cm = 0.2 μm. 1, basal lamina; 2, alveolar epithelium; 3, capillary endothelium; 4, alveolus, 5, capillary.

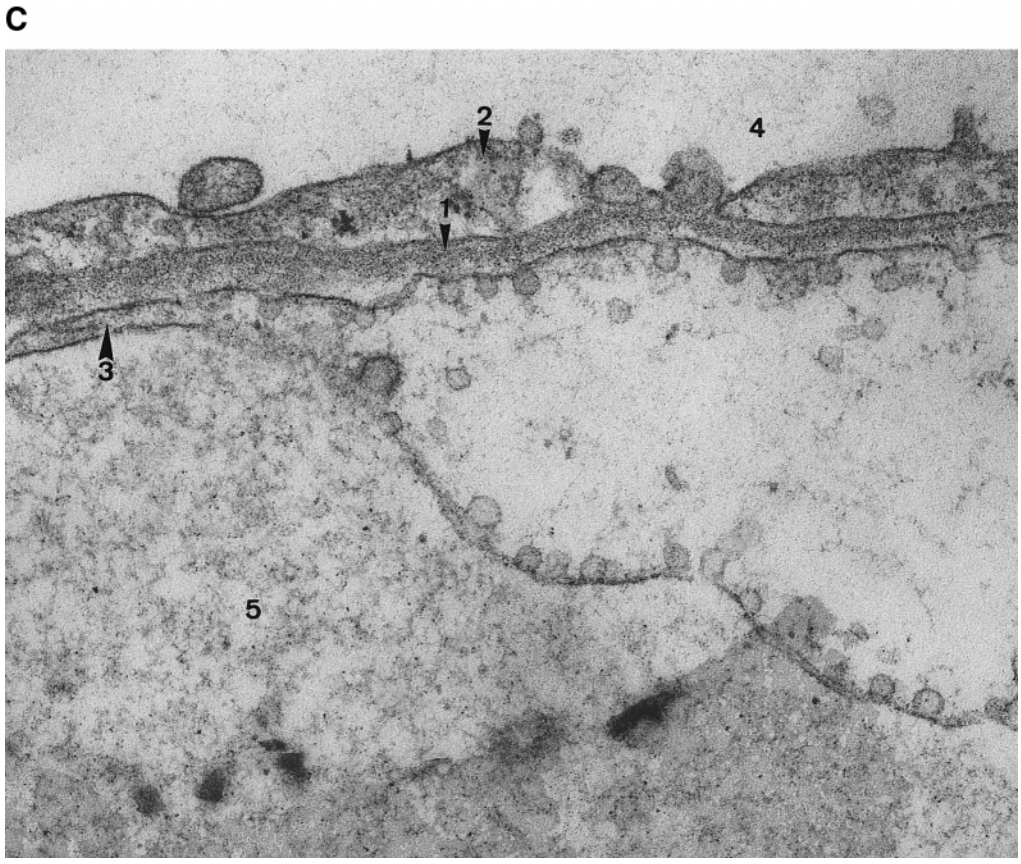

FIG. 6—*Continued*

added to avoid SP degradation by lung tissue (Nemmar *et al.*, 1999). Again, the absence of effect of SP in control lungs is not readily explained but could be related to surgical stress present in this study. In addition, the release of histamine due to C48/80 significantly increased in lung effluents from rabbits pretreated with UFP compared to control lungs (Fig. 3). All these results suggest that UFP could increase the sensitivity or the response of C-fibers and mast cells to various stimuli since the release of both SP and histamine was enhanced in UFP-exposed rabbits, after administration of various agonists known to act on C-fibers and mast cells. Such enhancement of the release of inflammatory mediators has never been described so far with UFP or even with larger-size particles. Airway hyperresponsiveness to various agonists (SP, histamine, and methacholine) after exposure to other atmospheric pollutants such as O_3 has been well described in several animal species, and even in man (Holtzman *et al.*, 1979; Gordon *et al.*, 1984; Campos *et al.*, 1992), and has been partly attributed to airway epithelium damage, leading to enhanced vagal reflex (Holtzman *et al.*, 1983) and C-fiber activation (Campos *et al.*, 1992). The epithelial injury recorded in the present study in the UFP-treated lungs argues in favor of a similar mechanism in rabbits. Other mechanisms could also be involved. Like O_3, which can act as a chemotactic and an activating factor for mast cells (Murlas and Roum, 1985; Shields and Gold, 1987), UFP could directly activate these cells. However, C-fibers and mast cells could also be indirectly activated by mediators released from inflammation (Li *et al.*, 1997).

Our data show that intratracheal instillation of 4 mg of UFP in rabbits can cause a neutrophil influx and an increase in total protein concentrations in BAL fluid (Fig. 4). A similar effect has been reported in rats 6 h after intratracheal instillation of ultrafine carbon black particles (Li *et al.*, 1997). In addition, an increase in lung WW/DW ratio was also recorded (Fig. 5), indicating pulmonary edema formation. The increase in lung weight ratio was described in rat after intratracheal instillation with quartz but not with carbon black or diesel particles (Murphy *et al.*, 1998). Moreover, in the present work, photomicrographs from lungs collected 20 h after intratracheal instillation of UFP showed both epithelial and endothelial alveolar damage (Figs. 6B and 6C). The latter can be related to an increase in the permeability of the alveolar–blood barrier as indicated by the increase of both WW/DW ratio and protein concentrations in BAL. Thus, intratracheal instillation of inert charged UFP can induce an inflammatory process, characterized by a neutrophil influx in the lung tissue with an increase in both epithelial and endothelial permeability leading to pulmonary edema.

While the baseline values of SP and histamine concentra-

tions recorded in tracheally superfused rabbit lungs were not modified by UFP pretreatment, it can be hypothetized that activation of C-fibers and mast cells as observed *in vitro* can also occur *in vivo* and can be related to the neutrophil influx, the increase in protein concentration in BAL fluid, and the increase in lung WW/DW ratio. To assess this hypothesis, two groups of rabbits were pretreated with a mixture of SR 140333 and SR 48968, NK_1 and NK_2 receptor antagonists, respectively, or a mixture of terfenadine and cimetidine, H_1 and H_2 receptor antagonists, respectively. In both cases, the strong inhibition of the increase in total cell number and of the neutrophil influx in BAL fluid (Table 3) are in favor of a determinant role of neuropeptides, probably SP and histamine, in these UFP-induced effects. The role of SP in the release of inflammatory mediators from various cell types and in the stimulation of inflammatory cellular activities, including lymphocyte proliferation, mast cells degranulation, and modulation of activity of PMN, has been previously described in human and rat (Fewtrell *et al.*, 1982; Perianin *et al.*, 1989; DeRose *et al.*, 1994). Moreover, it was also reported that histamine can both directly activate equine neutrophils by acting on H_1 receptors located on these cells (Benbarek, 1998) and stimulate human neutrophils adhesion by acting on H_1 and H_2 receptors located on endothelial cells (Schaefer *et al.*, 1998). Pulmonary edema recorded in our model could be due to activation of neutrophils by SP and histamine, with a subsequent release of reactive oxygen species leading to damage in endothelium as indicated above (Perianum *et al.*, 1989; Benbarek, 1998; Schaefer *et al.*, 1998). Moreover, in rabbits, SP and histamine are also able to directly induce pulmonary edema through the alteration of microvascular permeability (Delaunois *et al.*, 1993). The fact that the inhibition of the UFP-induced increase in total proteins of BAL fluid by tachykinin and histamine receptor antagonists (Fig. 4) was only partial, while a total blockade of the increase in the lung WW/DW ratio was recorded (Fig. 5), could be explained by a possible increase in protein epithelial secretion or release, contributing to the total increase in protein content of BAL fluid. Indeed, Murphy *et al.* (1998) described an increased secretion of pulmonary surfactant in rats after intratracheal instillation of crystalline quartz particles. Histamine and neurokinins could not influence this latter effect or partially mediated it. Moreover, the possible limited accessibility of the antagonists, injected intravenously, at the epithelial level could explain this partial blockade

Based on our data, the following sequence of events can be suggested: intratracheal administration of charged UFP results in an inflammatory process in the lung tissue, characterized by neutrophil influx and an increase in both epithelial and endothelial permeability confirmed by morphological studies. An enhanced sensitivity of C-fibers to various stimuli was recorded, leading to a possible increase of the release of SP and histamine *in vivo*, which are both determinant for the neutrophils influx and pulmonary edema formation. Further investigation should be performed to study the role of the particles charge, size, and time dose response.

ACKNOWLEDGMENTS

The authors thank Dr Edmonds-Alt (Sanofi Recherche, Montpellier, France) for his kind provision of SR 140333 and SR 48968. This work was supported by the Special Research Funds of the University of Liège.

REFERENCES

Advenier, C., Naline, E., Drapeau, G., and Regoli, D. (1987). Relative potencies of neurokinins in guinea pig trachea and human bronchus. *Eur.J. Pharmacol.* **139,** 133–137.

Andersson, P. J., Wilson, J. D., and Hiller, F. C. (1990). Respiratory tract deposition of ultrafine particles in subjects with obstructive and restrictive lung disease. *Chest* **97,** 1115–1120.

Benbarek, H. (1998). Studies on the stimulation of equine neutrophils using an ex-vivo model. PhD thesis, University of Liège.

Campos, M. G., Segura, P., Vargas, M. H., Vanda, B., Ponce-Monter, H., Sellman, M., and Mantano, L. M. (1992). O_3 induced airway hyperresponsiveness to non cholinergic system and other stimuli. *J. Appl. Physiol.* **73,** 354–361.

Cohen, B. S., Xiong, J. Q., Faug, C. P., and Li, W. (1998). Deposition of charged particles on lung airways. *Health Phys.* **74,** 554–560.

Dalal, N.S., Suryan, M. M., Vallyathan, V., Green, F. H. Y., Jafari, B., and Wheeler, R. (1989). Detection of reactive free radicals in fresh coal mine dust and their implications for pulmonary injury. *Ann. Occup. Hyg.* **33,** 79–84.

Delaunois, A., Gustin, P., and Ansay, M. (1993). Effects of capsaicin on the endothelial permeability in isolated and perfused rabbit lungs. *Fundam. Clin. Pharmacol.* **7,** 81–91.

Delaunois, A., Segura, P., Dessy-Doizé, C., Ansay, M., Mantano, L. M., Vargas, M. H., and Gustin, P. (1997). Ozone-induced stimulation of pulmonary sympathetic fibers: A protective mechanism against edema. *Toxicol. Appl. Pharmacol.* **147,** 71–82.

Delay-Goyet, P., Satoh, H., and Lundberg, J. M. (1992). Relative involvement of substance P and calcitonin gene-related peptide mechanisms in antidromic vasodilatation in rat skin. *Acta Physiol. Scand.* **146,** 537–538.

DeRose, V., Robbins, R. A., Snider, R. M., Spurzem, J. R., Thiele, G. M., Rennard, S. I., and Rubinstein, I. (1994). Substance P increases neutrophil adhesion to bronchial epithelial cells. *J. Immunol.* **152,** 1339–1346.

Dimitriadou, V., Rouleau, A., Dam Trung Tuong, M., Newlands, G. F. J., Miller, H. R. P., Luffan, G., Schwartz, J.-C., and Garbarg, M. (1994). Functional relationship between mast cells and C-sensitive nerve fibers evidenced by histamine H_3 modulation in rat lung and spleen. *Clin. Sci.* **87,** 151–163.

Donaldson, K., Gilmour, P. S., Mac Nee, W., and Oberdörster, G. (1995). Free radical activity associated with ultrafine titanium dioxide particles and PM10 material. *Am. J. Respir. Crit. Care Med.* **151,** A64.

Faux, S.P., Howden, P. J., and Levy, L. S. (1994). Iron-dependent formation of 8-hydroxydeoxyguanosine in isolated DNA and mutagenicity in Salmonella typhimurium TA 102 induced by crocidolite. *Carcinogenesis* **15,** 1749–1751.

Ferrin, J., Oberdörster, G., Penney, D. P., Soderholm, S. C., and Piper, H. C. (1990). Increased pulmonary toxicity of ultrafine particles? Particle clearance, translocation, morphology. *J. Aerosol. Sci.* **21,** 381–384.

Fewtrell, C. M. S., Foreman, J. C., Jordan, C. C., Oehme, P., Renner, H., and

Stewart, J. M. (1982). The effects of substance P on histamine and 5-hydroxytryptamine release in rat. *J. Physiol. (Lond.)* **330,** 393–411.

Gordon, T., Venugopalan, C. S., Amdur, M. O., and Drazen, J. M. (1984). Ozone-induced airway hyperreactivity in the guinea pig. *J. Appl. Physiol.* **57,** 1034–1038.

Hazbun, M. E., Hamilton, R., Holian, A., and Eschenbacher, W. L. (1993). Ozone-induced increases in substance P and 8-epi-prostaglandin F2α in the airways of human subjects. *Am. J. Respir. Cell Mol. Biol.* **9,** 568–572.

Hislop, A. A., Warton, J., and Allen, K. M. (1990). Immunohistochemical localization of peptide-containing nerves in human airways: Age-related changes. *Am. Rev. Respir. Dis.* **141,** 535–539.

Holtzman, M. J., Cunningham, J. H., Sheller, J. R., Irsigler, G. B., Nadel, J. A., and Boushey, H. A. (1979). Effect of ozone on bronchial reactivity in atopic and nonatopic subjects. *Am. Rev. Respir. Dis.* **120,** 1059–1067.

Holtzman, M. J., Fabbri, L. M., O'Byrne, P. M., Gold, B. D., Aizawa, H., Walters, E. H., Alpert, S. E., and Nadel, J. A. (1983). Importance of airway inflammation for hyperresponsiveness induced by ozone. *Am. Rev. Respir. Dis.* **127,** 686–690.

Li, X. Y., Gilmour, P. S., Donaldson, K., and Mac Nee, W. (1997). In vivo and in vitro proinflammatory effects of particulate air pollution (PM10). *Environ. Health Perspect.* **10,** 1279–1283.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Kandall, R. J. (1956). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Lundberg, J. M., Hokfelt, T., and Martling, C. R. (1985). Substance P immunoreactive sensory nerves in the lower respiratory tract of various mammals including man. *Cell Tissue* **235,** 251–261.

Martins, M. A., Shore, S. A., and Drazen, J. M. (1991). Release of tachykinins by histamine, methacholine, PAF, LTD4 and substance P from guinea pig lungs. *Am. J. Physiol. (Lond.)* **261,** L449–L455.

Murlas, C. G., and Roum, J. H. (1985). Sequence of pathology in the airway mucosa of guinea pig during ozone-induced bronchial hyperreactivity. *Am. Rev. Respir. Dis.* **131,** 314–320.

Murphy, S. A., BéruBé, K. A., Podey, F. D., and Richards, R. J. (1998). The response of lung epithelium to well characterized fine particles. *Life Sci.* **62,** 1789–1799.

Nemmar, A., Delaunois, A., Beckers, J-F., Sulon, J., Bloden, S., and Gustin, P. (1999). Modulatory effect of imetit, a histamine H3 receptor agonist, on C-fibers, cholinergic fibers and mast cells in rabbit lungs in vitro. *Eur. J. Pharmacol.* **371,** 23–30.

Nemmar A., Gustin, P., Delaunois, A., Beckers, J. F., and Sulon, J. (1998). Radioimmunoassay of substance P in lung perfusate. *J. Pharmacol. Toxicol. Methods* **39,** 109–114.

Oberdörster, G., Ferrin, J. R., Gelein, R., Soderholm, S. C., and Finkelstein, J. (1992). Role of the alveolar macrophage in lung injury: Studies with ultrafine particles. *Environ. Health Perspect.* **97,** 193–199.

Perianin, A., Synderman, R., and Malfory, B. (1989). Substance P primes human neutrophil activation: A mechanism for a neurogenical regulation of inflammation. *Biochem. Biophys. Res. Commun.* **161,** 520–524.

Peters, A.,Wichmann, H. E., Tuch, T., Heinrich, J., and Heider, J. (1997). Respiratory effects are associated with the number of ultrafine particles. *Am. J. Respir. Crit. Care. Med.* **155,** 1376–1383.

Pope, C. A., and Kranner, R. E. (1993). Acute effects of PM10 pollution on pulmonary function of smokers with mild to moderate chronic obstructive pulmonary disease. *Am. J. Respir. Dis.* **147,** 1336–1340.

Pope, C. A., Thun, J. T., Namboodiri, M. M., Dockery, D. W., Evans, J. S., Speizer, F. E., and Heath, C. W. (1995). Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *Am. J. Respir. Crit. Care Med.* **151,** 669–674.

Rogers, D. F., Aursudkij, B., and Barnes, P. J. (1989). Effects of tachykinins on mucus secretion in human bronchi in vitro. *Eur. J. Pharmacol.* **174,** 283–286.

Saria, A., Martling, C. R., Yan, Z., Theodorsson-Norheim, E., Gamse, R., and Lundberg, J. M. (1988). Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylpiperazinium and vagal stimulation. *Am. Rev. Respir. Dis.* **137,** 1330–1335.

Schaefer, U., Schneider, D., Rixen, D., and Neugebauer, E. (1998). Neutrophil adhesion to histamine stimulated cultured endothelial cells is primarily mediated via activation of phospholipase C and nitric oxide synthetase isoenzymes. *Inflamm. Res.* **47,** 256–264.

Schwartz, J. (1994a). Air pollution and daily mortality: A review and meta-analysis. *Environ. Res.* **64,** 36–52.

Schwartz, J. (1994b). Air pollution and hospital admissions for the eldery in Detroit and Michigan. *Am. J. Respir. Crit. Care Med.* **150,** 648–655.

Shields, R. L., and Gold, W. M. (1987). Effect of inhaled ozone on lung histamine in conscious guinea pigs. *Environ. Res.* **42,** 435–445.

Terashima, T., Wiggs, B., English, D., Hogg, J. C., and van Eeden, S. F. (1997). Phagocytocis of small carbon particles (PM10) by alveolar macrophages stimulates the release of polymorphonuclear leucocytes from bone marrow. *Am. J. Respir. Crit. Care Med.* **155,** 1441–1447.

Vallyathan, V., Shi, X., Dallal, N. S., Irr, W., and Castranova, V. (1988). Generation of free radicals from freshly factured silica dust: potential role in acute silica-induced lung injury. *Am. Rev. Respir. Dis.* **138,** 1213–1219.

Van Der Lende, R., Kok, T. J., Reing, R. P., Quanjer, P. H., Schouten, J. P., and Orie, N. G. (1981). Decrease in VC and FEV1 with time: Indications for effects of smoking and air pollution. *Bull. Eur. Physiopathol. Respir.* **17,** 775–792.

Wallenstein, S., Zucker, C. L., and Fleiss, J. L. (1980). Some statistical tests useful in circulation research. *Circ. Res.* **47,** 1–9.

Zhang, Q., Kusaka, Y., and Sato, K. (1998). Differences in the extent of inflammation caused by intratracheal exposure to three ultrafine metals: Role of free radicals. *J. Toxicol. Environ. Health* **53,** 423–438.