

Exposure to Cigarette Smoke Impairs Alveolar Macrophage Functions during Halothane and Isoflurane Anesthesia in Rats

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Background: Smoking alters numerous alveolar macrophage functions and is an important risk factor for postoperative pulmonary complications. The authors therefore tested the hypothesis that smoke exposure impairs antimicrobial and proinflammatory responses in alveolar macrophages during halothane and isoflurane anesthesia with mechanical ventilation.

Methods: Thirty control rats and 30 rats exposed to cigarette

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smoke were mechanically ventilated with 1.5 minimum alveolar concentration halothane and isoflurane. Ten smoke-exposed and control animals were assigned to one of three different anesthetic durations (0, 2, and 6 h). The fraction of aggregated cells and cell distribution were determined. Opsonized and unopsonized phagocytosis was measured. Microbicidal activity was determined as the ability to kill *Listeria monocytogenes*. The expression of interleukin (IL)-1 α , IL-1 β , IL-6, macrophage inflammatory protein-2, interferon- γ , and tumor necrosis factor- α was measured by semiquantitative reverse-transcription polymerase chain reaction. Pulmonary lavage concentrations of these cytokines were measured by enzyme-linked immunosorbent assay.

Results: During both halothane and isoflurane anesthesia, the fraction of aggregated macrophages increased, whereas unopsonized and opsonized phagocytosis and microbicidal activity decreased significantly over time in both groups. Responses observed in smoke-exposed rats were almost twice as great as those observed in the control rats. Gene expression and production of all proinflammatory cytokines except IL-6 increased 2–20-fold during anesthesia. The increases in IL-1 β , interferon- γ , and tumor necrosis factor- α in the control rats were 1.5–8 times greater than those in the smoke-exposed rats.

Conclusion: Antimicrobial and proinflammatory responses of alveolar macrophages during anesthesia were markedly suppressed by smoke exposure. Our data suggest that smoke exposure reduces the efficacy of immune defenses during anesthesia. (Key words: Immune response; mechanical ventilation; smoking; volatile anesthetic.)

ALVEOLAR macrophages are the first line of pulmonary defense. Among their antimicrobial responses are chemotaxis, phagocytosis, and microbicidal activity against foreign invaders. Furthermore, alveolar macrophages provide critical proinflammatory functions, including medulating secretion of various proinflammatory cytokines and neutrophil chemoattractants.

Alveolar macrophage functions are significantly altered during anesthesia and surgery.¹⁻³ For example, antimicrobial activities such as phagocytic and microbicidal activities of alveolar macrophages decrease progressively during anesthesia and surgery.^{1,2} Macrophage aggregation and neutrophil influx also increased during

anesthesia and surgery. In both cases, changes became evident 2 h after induction of anesthesia and peaked after 6 h. Isoflurane provokes greater macrophage aggregation and neutrophil influx than propofol.² Inhalation of volatile anesthetics and mechanical ventilation each provoke inflammatory reactions.³ Thus, available data suggest that anesthesia, mechanical ventilation, and surgery independently initiate inflammatory responses in alveolar macrophages.¹⁻³

Smoking is among the most important risk factors for postoperative pulmonary complications, even in the absence of underlying pulmonary disease or abnormal pulmonary function tests.^{4,5} Smoking often reduces phagocytic and microbicidal activities of alveolar macrophages.^{6,7} Furthermore, cigarette smoking is reported to significantly impair gene expression and production of proinflammatory cytokines against inhaled noxious substances.⁸⁻¹⁰ It is therefore likely that both antimicrobial and proinflammatory reactions are further impaired during general anesthesia. Consistent with this theory, Hegab and Matulionis¹¹ examined the morphologic changes of the lungs in smoke-exposed mice during spontaneous inhalation of halothane and reported that inhalation of halothane in smoke-exposed mice markedly increased macrophage aggregation and influx of inflammatory cells to the distal airway. However, from their study, the changes in antimicrobial and proinflammatory functions of alveolar macrophages that result from smoke exposure during anesthesia remain unclear.

We examined how prior chronic exposure to cigarette smoke affects macrophage aggregation, neutrophil influx, and antimicrobial activities, such as phagocytic microbicidal activity in alveolar macrophages during halothane and isoflurane anesthesia with mechanical ventilation, in rats. To evaluate proinflammatory function, we measured gene expression and production of key proinflammatory cytokines in alveolar macrophages, including interleukin (IL)-1 α , IL-1 β , IL-6, macrophage inflammatory protein-2 (MIP-2), interferon- γ (IFN- γ), and tumor necrosis factor- α (TNF- α).

Methods

The protocol of the study was approved by the institutional animal care at the University of Hirosaki. We studied a total of 120 male Wistar rats weighing approximately 200–250 g. All rats were maintained under standard conditions with free access to water and rodent laboratory food. Artificial light was present from 7 A.M. to

7 P.M. each day, and room temperature was maintained between 22°C and 24°C. Sixty rats were exposed to filter-tipped cigarettes. (Hilite; Japan Tobacco, Tokyo, Japan) for 30 min/day over 60 days using a Hamburg II smoking machine (Leybold-Heraeus, Hamburg, Germany) at a rate of 1 puff/min. Puff duration and volume were 2 s and 35 ml, respectively. Cigarette smoke was diluted with air at a ratio of 1:10. The remaining 60 sham-exposed rats served as controls and were applied to the smoking machine as were smoke-exposed rats, but they were not exposed to cigarette smoke.

Protocol

The rats were anesthetized with 50 mg/kg intraperitoneal pentobarbital. A catheter was inserted into the trachea *via* a neck incision. Catheters were also inserted into the femoral artery and vein. Lactated Ringer's solution was infused at a rate of 10 ml \cdot kg⁻¹ \cdot h⁻¹ through the venous catheter. We continuously monitored mean arterial pressure and heart rate from the femoral arterial catheter. The rats were placed on a heating pad (Small Animal Warmer BWT-100; BRC Company, Nagoya, Japan), and rectal temperature was maintained between 37.0°C and 37.5°C.

Thirty smoke-exposed rats and 30 control rats were mechanically ventilated with 1.5 minimum alveolar concentration halothane (1.6%). Ten exposed and control animals were assigned to one of three different anesthetic durations. Ten smoke-exposed and control rats were killed and underwent whole pulmonary lavage immediately after induction of anesthesia (0 h). Ten others in each group were killed 2 and 6 h after onset of anesthesia. A rodent ventilator (model 683; Harvard apparatus, South Natick, MA) set to 10 ml/kg was used in the mechanically ventilated animals. The respiratory rate was controlled to produce an initial arterial carbon dioxide partial pressure between 35 and 45 mmHg. Muscle relaxants were not administered. Arterial blood was sampled for analysis of pH and oxygen and carbon dioxide partial pressures immediately after the start of inhalation of volatile anesthetics (*initial* values) and again at the end of experiment (*final* values) except in the 0-h group. The procedures were similar in the remaining 30 smoke-exposed and 30 control rats except that they were anesthetized with 1.5 minimum alveolar concentration isoflurane (2.1%) rather than halothane.

Whole Pulmonary Lavage and Cell Treatment

Five milliliters of 0.9% saline solution containing 16 mM lidocaine hydrochloride titrated with NaOH to a pH of

7.4 was gently instilled with a syringe, then withdrawn. This procedure was repeated 10 times, so that a total of 50 ml of the saline solution was instilled. Three-milliliter aliquots of lavage fluid were reserved for total cell count, viability, and cell differentiation and aggregation. Total cell number was determined with a hemocytometer. The viability of alveolar cells was evaluated by an ability to exclude 0.2% trypan blue as described previously.^{1,2} Cell differentiation and aggregation were examined by counting 500 cells on a Wright-Giemsa-stained slide. Lavage fluid was divided into three equal volumes for determination of phagocytosis, bactericidal activity, and gene expression of proinflammatory cytokines by reverse-transcription polymerase chain reaction (PCR).

RNA Isolation and cDNA Synthesis

The bronchoalveolar lavage fluid was centrifuged at $200 \times g$ for 10 min. After the supernatant was decanted for measurement of cytokine concentrations, the cell pellets were dissolved in 0.5 ml of guanidinium buffer solution (4 M guanidinium isothiocyanate, 50 mM Tris HCl, 10 mM EDTA, 2% sarcosyl, and 100 mM mercaptoethanol). The following molecular analysis of proinflammatory cytokines was based on our previously reported method.³ RNA was isolated from the guanidinium buffer by the well-established acid guanidinium-phenol-chloroform method.¹² The amount of isolated RNA was measured by a spectrophotometer (Model DU-65; Beckman, Tokyo, Japan). We obtained 1.2–3.2 μg RNA from each sample. By incubation at 40°C for 60 min, cDNA was synthesized from 0.2 μg RNA with 20 μl total reaction mixture that included Tris-HCl buffer (pH 8.3), 1 mM dNTPs, 0.125 μM oligo dT primers, 20 U RNase inhibitor, and 0.25 U avian myeloblastosis virus reverse transcriptase. After 60 min of incubation, the reverse transcriptase was inactivated at 95°C for 5 min.

Semiquantitative Reverse-Transcription PCR

The reverse-transcription PCR mixture (50 μl) contained cDNA synthesized from 0.2 μg RNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl_2 , 0.2 mM dNTP, 0.2 μM 5' and 3' oligonucleotide primers, and 2.5 U Taq polymerase (Takara, Company, Tokyo, Japan).

The reaction mixture was then amplified in a DNA thermocycler (Perkin-Elmer, Irvine, CA). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 56°C (for IL-6 and IFN- γ) or 59°C (for other cytokines) for 1 min, and extension at 72°C for 1 min. The optimal number of PCR cycles for each primer set was deter-

mined in preliminary experiments so that the amplification process was conducted during the exponential phase of amplification.¹³ The number of PCR cycles was as follows; 26 for β -actin, 32 for IL-1 α , 31 for IL-1 β , 35 for IL-6, 28 for IFN- γ , 30 for MIP-2, and 28 for TNF- α . The sequence of cytokine-specific primer pairs, 5' and 3', were the same as in our previous study.³ cDNA for each cytokine and β -actin were then coamplified in single tubes. The β -actin primers were added after several cycles with only cytokine primer so that the final number of PCR cycles was optimal for both the cytokine and β -actin.

The PCR products were quantified by densitometry measurements. The PCR products were separated by electrophoresis on a 1.8% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. PCR products were visualized on a transilluminator (Model FBTIV-816; Fisher Scientific, Pittsburgh, PA) at 312-nm wavelength and photographed with Polaroid 667 film (Japan Polaroid, Tokyo, Japan). The band images were obtained by scanning the photograph with a ScanJet 3P (Hewlett-Packard, Cupertino, CA). The total intensity (average intensity \times total pixels) of each band was measured with Mocha software (Jandel Scientific Software, San Rafael, CA). To evaluate the relative amount of cytokine mRNA in each rat, the cytokine/ β -actin ratio of the intensity of ethidium bromide luminescence for each PCR product was calculated.

Cytokine Enzyme-linked Immunosorbent Assay in Whole Pulmonary Lavage Fluid

Concentrations of IL-1 β , IL-6, MIP-2, IFN- γ , and TNF- α in pulmonary lavage fluid were evaluated in duplicate by an investigator blinded to anesthetic type and sample time using commercially available enzyme-linked immunosorbent assay kits (TFB Co., Tokyo, Japan). The minimum detection levels were 5, 3, 1, 7, and 0.7 $\mu\text{g}/\text{ml}$, respectively. The concentration of cytokines under minimum detection levels were considered as zero. We did not measure IL-1 α because its function is mainly intracellular and may be released by cellular damage. In our laboratory, the intraassay and interassay coefficient of variation of each cytokine measurement was less than 5% and 8%, respectively. The absorbance of each well was determined at 450 nm with a microplate reader. Background absorbency of blank wells was subtracted from the standard and unknowns before determination of sample concentration.

Phagocytosis and Microbicidal Activity

Phagocytosis and microbicidal activity were evaluated with a slightly modified version of our previously described technique.² Alveolar macrophages were separated from bronchoalveolar lavage fluid by centrifugation at $200 \times g$ for 10 min. After the supernatant was decanted, alveolar macrophages were resuspended at a concentration of 0.25×10^6 cells/ml in a balanced saline solution containing NaCl (125 mM), KCl (6 mM), dextrose (10 mM), CaCl_2 (0.3 mM), and MgCl_2 (1.0 mM), titrated with NaOH to a pH of 7.4.

Resuspended alveolar macrophages were incubated as suspensions at 37°C in 20-ml sterile centrifuge tubes on a shaking platform (60 cycles/min). Unopsonized and opsonized (1.0 μm diameter) particles were added to the separate centrifuge tubes, each containing a sample of the cell suspension; the particle-to-cell ratio was 15:1. The tubes were incubated for 15 min, and the phagocytosis was then stopped by addition of 2 ml ice-cold balanced saline solution. The cell suspension was placed on a glass slide, fixed, and stained. We recorded the fraction that ingested at least one particle and the number of fluorescent particles per positive phagocytic alveolar macrophage.

Bactericidal ability of the alveolar macrophages was determined by their ability to kill *Listeria monocytogenes* using a modification of a previously described method.² *Listeria* were stored at a concentration of 1×10^9 colony-forming unit/ml in the RPMI-1640 medium (Gibco BRL, Life Tech, Inc., Rockville, MD) and stored at -80°C until use. Alveolar macrophages were separated as in the phagocytosis assay at 2-h intervals and at the end of surgery. We resuspended each set of alveolar cells at a concentration of 0.25×10^6 cells/ml in RPMI-1640 and plated them in 24-well dishes. After removal of nonadherent cells by washing with RPMI-1640, the adherent cells (> 98% macrophages) were finally resuspended in 0.5 ml RPMI containing 10% normal human serum.

The bacteria were resuspended in the same medium at a concentration of 2×10^6 colony-forming unit/ml. Resuspended aliquots of *Listeria* (0.5 ml) were mixed and incubated for 30 min and 120 min in 5% CO_2 /air. Total reaction volume was 1.0 ml. The pellets of alveolar macrophages were lysed by adding 10 ml of sterilized distilled water and vortexing for 30 s to release bacteria. The viable fraction of *Listeria* bacteria was determined by plating serial 10-fold dilutions on agar plates. The number of colonies of *Listeria* was counted after 48 h on one of the plates. The rate at which alveolar macro-

phages killed *Listeria* was calculated by dividing the fraction of the initial inoculum of *Listeria* killed by the fraction of the initial inoculum surviving in the control (cell-free) tubes.

Statistical Analysis

Time-dependent data in each smoke-exposed and control group were analyzed using one-way analysis of variance and Dunnett tests for comparison with control values. Differences between smoke-exposed and control groups at each time point were evaluated using two-tailed, unpaired *t* tests. Intragroup differences in hemodynamic and respiratory parameters were evaluated by two-tailed, paired *t* test. Data are expressed as mean \pm SDs; $P < 0.05$ was considered statistically significant.

Results

Demographic Data and Cell Recovery by Whole-lung Lavage

There were no significant time-dependent (0, 2, and 6 h of anesthesia), intragroup (initial vs. final) and intergroup (smoke-exposed vs. control) changes in hemodynamic or respiratory parameters during halothane and isoflurane anesthesia (tables 1 and 2). Although all rats were ventilated with an inhaled oxygen fraction of 0.21, arterial oxygen partial pressure exceeded 80 mmHg in all rats.

Concentration and total number of alveolar cells in the lavage fluid was fivefold greater in smoke-exposed than in control rats at all evaluation points during halothane anesthesia ($P < 0.001$). In both smoke-exposed and control rats, macrophage aggregation and neutrophil influx increased over the course of 6 h of halothane anesthesia ($P < 0.01$). Six hours after induction of anesthesia, macrophage aggregation in the smoke-exposed rats was almost twice that in the control rats ($P < 0.05$; table 3). These cellular responses were similar during isoflurane anesthesia (table 4).

Gene Expression of Proinflammatory Cytokines

During halothane anesthesia, relative expression of MIP-2, IFN- γ , and TNF- α in alveolar cells increased significantly in control and smoke-exposed rats over the course of 6 h of anesthesia ($P < 0.001$). The increases in IFN- γ and TNF- α were greater in control than smoke-exposed rats ($P < 0.005$). There were no differences in expression of MIP-2 between the groups. Relative expression of IL-1 α increased in both smoke-exposed and

SMOKE EXPOSURE AND ALVEOLAR MACROPHAGES

Table 1. Cardiorespiratory Responses and Body Temperature during Halothane Anesthesia

		Control			Smoke-exposed		
		0 h	2 h	6 h	0 h	2 h	6 h
MAP (mmHg)	Initial	81 ± 7	81 ± 10	83 ± 9	86 ± 9	88 ± 9	82 ± 6
	Final	—	85 ± 10	82 ± 6	—	86 ± 10	80 ± 7
HR (beats/min)	Initial	402 ± 13	404 ± 25	403 ± 16	405 ± 12	411 ± 21	404 ± 12
	Final	—	404 ± 18	404 ± 12	—	404 ± 10	410 ± 17
pH	Initial	7.41 ± 0.04	7.43 ± 0.04	7.40 ± 0.04	7.39 ± 0.02	7.42 ± 0.04	7.39 ± 0.03
	Final	—	7.41 ± 0.03	7.39 ± 0.03	—	7.42 ± 0.04	7.40 ± 0.04
P _{CO₂} (mmHg)	Initial	38 ± 2	37 ± 2	38 ± 2	38 ± 1	37 ± 2	38 ± 1
	Final	—	38 ± 1	38 ± 2	—	37 ± 2	38 ± 1
P _{O₂} (mmHg)	Initial	95 ± 8	98 ± 8	96 ± 10	97 ± 8	97 ± 9	94 ± 6
	Final	—	100 ± 9	97 ± 7	—	97 ± 6	96 ± 8

Initial values identify the beginning of the study; final values are those obtained at the end of the experiments. Results are presented as mean ± SD. There were no significant time-dependent, intragroup, or intergroup differences.

HR = heart rate; MAP = mean arterial pressure.

control rats at 6 h after anesthesia, but there was no difference between smoke-exposed and control rats. Relative expression of IL-1 β in the smoke-exposed and control rats increased significantly at 2 and 6 h of anesthesia, respectively ($P < 0.01$), and the increase was significantly greater in control than smoke-exposed rats at 6 h of anesthesia ($P < 0.05$). Expression of IL-6 was minimal at all evaluation points in both groups (fig. 1).

Expression of genes for proinflammatory cytokines during isoflurane anesthesia was nearly the same as halothane anesthesia. However, expression of IFN- γ did not increase significantly until 6 h of anesthesia. Furthermore, expression of IL-1 α and IL-1 β failed to increase over time in the smoke-exposed rats (fig. 2).

During halothane anesthesia, the concentration of IL-1 β did not change over time in either group. The concentration of MIP-2 increased significantly after 2 and

6 h of anesthesia in control and smoke-exposed rats ($P < 0.001$); however, there were no significant differences between groups. Concentrations of IFN- γ and TNF- α increased after 6 h of anesthesia in the control rats ($P < 0.01$), and increases were significantly greater in the control than in the smoke-exposed rats ($P < 0.05$). During the entire course of anesthesia, IL-6 was undetectable in the lavage fluid (fig. 3). Cytokine concentrations during isoflurane anesthesia were similar to those during halothane anesthesia (fig. 4).

Phagocytic and Bactericidal Activities

After 2 and 6 h of halothane anesthesia, phagocytosis of both unopsonized and opsonized particles decreased significantly in the smoke-exposed and control rats, respectively ($P < 0.001$). At these time points, the decreases in both unopsonized and opsonized phagocytosis

Table 2. Cardiorespiratory Responses and Body Temperature during Isoflurane Anesthesia

		Control			Smoke-exposed		
		0 h	2 h	6 h	0 h	2 h	6 h
MAP (mmHg)	Initial	86 ± 10	83 ± 9	81 ± 8	86 ± 10	85 ± 6	85 ± 5
	Final	—	85 ± 10	79 ± 8	—	87 ± 8	83 ± 8
HR (beats/min)	Initial	403 ± 18	401 ± 21	398 ± 13	406 ± 14	408 ± 19	406 ± 21
	Final	—	402 ± 12	398 ± 12	—	404 ± 16	401 ± 16
pH	Initial	7.41 ± 0.03	7.42 ± 0.02	7.41 ± 0.03	7.40 ± 0.04	7.43 ± 0.03	7.40 ± 0.03
	Final	—	7.41 ± 0.03	7.42 ± 0.02	—	7.42 ± 0.02	7.39 ± 0.02
P _{CO₂} (mmHg)	Initial	38 ± 1	37 ± 2	37 ± 2	38 ± 1	37 ± 1	38 ± 1
	Final	—	37 ± 2	38 ± 1	—	37 ± 2	38 ± 1
P _{O₂} (mmHg)	Initial	98 ± 6	98 ± 7	96 ± 9	97 ± 8	100 ± 9	95 ± 7
	Final	—	97 ± 9	97 ± 8	—	100 ± 8	96 ± 6

Initial values identify the beginning of the study; final values are those obtained at the end of the experiments. Results are presented as mean ± SD. There were no significant time-dependent, intragroup, or intergroup differences.

HR = heart rate; MAP = mean arterial pressure.

Table 3. Cell Recovery from Bronchoalveolar Lavage Fluid during Halothane Anesthesia

	Control			Smoke-exposed		
	0 h	2 h	6 h	0 h	2 h	6 h
Recovery rate (%)	84 ± 4	83 ± 3	85 ± 3	84 ± 3	84 ± 3	84 ± 3
Cell concentration (×10 ⁵ /cm ³)	1.6 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	5.4 ± 1.3†	5.3 ± 1.1†	6.0 ± 1.5†
Total cell (×10 ⁶)	6.5 ± 1.4	6.3 ± 1.1	6.2 ± 1.4	22.9 ± 6.1†	22.3 ± 4.9†	25.3 ± 6.8†
Macrophage (%)	96 ± 2	96 ± 2	90 ± 2*	96 ± 2	96 ± 2	89 ± 2*
Lymphocyte (%)	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 2	3 ± 1
Neutrophil (%)	2 ± 1	2 ± 1	8 ± 2*	2 ± 1	2 ± 2	9 ± 3*
Aggregation (%)	1.2 ± 1.2	2.0 ± 1.5	4.1 ± 1.7*	2.4 ± 1.7	4.1 ± 2.2	9.3 ± 3.6*†
Viability (%)	97 ± 2	97 ± 2	97 ± 2	98 ± 1	97 ± 2	97 ± 2

Results are presented as mean ± SD.

* Significant differences from control values.

† Significant differences between smoke-exposed and control rats.

sis were significantly greater in smoke-exposed than in control rats ($P < 0.005$). Microbicidal activity at both 30- and 120-min incubations decreased significantly after 2 and 6 h of anesthesia in both groups ($P < 0.001$). At 6 h of anesthesia, microbicidal activity in the smoke-exposed rats had decreased twice as much as in the control rats ($P < 0.001$; fig. 5).

Changes in phagocytic and microbicidal activities during isoflurane anesthesia were almost similar to those observed during halothane anesthesia. However, there was no significant difference of opsonized phagocytosis between smoke-exposed and control rats after 2 h of isoflurane anesthesia (fig. 6).

Discussion

Gene Expression and Production of Proinflammatory Cytokines

We found that gene expression and production of proinflammatory cytokines increased over time in both

smoke-exposed and control rats. These results are consistent with those of our previous study.³ Gene expression and production of proinflammatory cytokines increase after inhalation of noxious substances such as aerosolized lipopolysaccharide or bacteria,¹⁴⁻¹⁸ and even 100% oxygen.¹⁹ Furthermore, mechanical ventilation induces expression of the gene for TNF- α .²⁰ Our results with these previous studies suggest that inhalation of volatile anesthetics during mechanical ventilation induces an inflammatory reaction in the distal airway.

Among the proinflammatory cytokines, IL-1 and TNF- α are the most important mediators secreted from alveolar macrophages.²¹ It is interesting that both expression and concentration of TNF- α increased, whereas the production of IL-1 β did not. Although we cannot explain the mechanism, expression of TNF- α is generally much faster than that of IL-1. For example, administration of lipopolysaccharide causes a marked increase in gene expression for TNF- α within 30 min. Furthermore, even the production TNF is faster than that of IL-1.^{17,18} TNF- α

Table 4. Cell Recovery from Bronchoalveolar Lavage Fluid during Isoflurane Anesthesia

	Control			Smoke-exposed		
	0 h	2 h	6 h	0 h	2 h	6 h
Recovery rate (%)	84 ± 3	85 ± 2	85 ± 3	84 ± 3	85 ± 3	84 ± 3
Cell concentration (×10 ⁵ /cm ³)	1.5 ± 0.3	1.6 ± 0.3	1.5 ± 0.4	5.9 ± 1.5†	5.9 ± 0.9†	6.5 ± 1.2†
Total cell (×10 ⁶)	6.4 ± 1.2	6.9 ± 1.3	6.3 ± 1.6	24.9 ± 6.3†	25.1 ± 4.3†	27.3 ± 5.2†
Macrophage (%)	97 ± 2	96 ± 1	90 ± 2*	96 ± 3	95 ± 2	89 ± 2*
Lymphocyte (%)	1 ± 1	2 ± 1	2 ± 1	2 ± 2	3 ± 2	3 ± 1
Neutrophil (%)	2 ± 1	2 ± 1	8 ± 2*	2 ± 1	3 ± 1	8 ± 3*
Aggregation (%)	1.2 ± 0.9	1.6 ± 1.4	3.1 ± 1.7*	2.2 ± 1.5	2.8 ± 1.2	7.3 ± 3.9†
Viability (%)	97 ± 2	98 ± 1	97 ± 2	97 ± 2	97 ± 2	98 ± 1

Results are presented as mean ± SD.

* Significant differences from control values.

† Significant differences between smoke-exposed and control rats.

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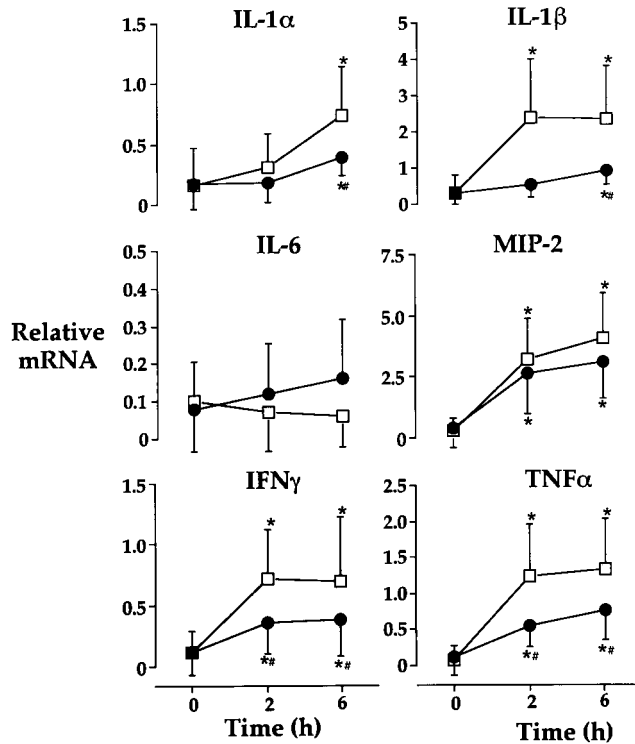


Fig. 1. Changes in gene expression of proinflammatory cytokines in smoke-exposed (circles) and control (squares) rats anesthetized with halothane. Data are expressed as the ratio of cytokine mRNA to β -actin mRNA. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

may be more sensitive to noxious stimuli to the lungs, as indicated by the fact that mechanical ventilation or inhalation of 100% oxygen increases gene expression of TNF- α .¹⁹

Interleukin-1 β accounts for > 90% of the IL-1 mRNA. Although expression of the gene for IL-1 α increased significantly, we did not measure IL-1 α in the lavage fluid. It is likely that IL-1 α concentrations failed to increase because this cytokine functions primarily intracellularly and is only released in substantial concentrations by cellular damage.²¹

We found that both expression and concentration of MIP-2 increased simultaneously in smoke-exposed and control rats. MIP-2, like IL-8 in humans, is one of the most potent chemoattractants for neutrophils to the distal airway. Neutrophil influx was similar in our smoke-exposed and control rats. This result is consistent with a study showing that the concentrations of IL-8 in bronchoalveolar lavage fluid is similar in smoking and non-smoking patients.⁸ Thus, our results suggest that changes of neutrophil chemoattractance in distal airway

by inhalation of volatile anesthetics under mechanical ventilation were not modulated by prior exposure to cigarette smoke.

Although our methods possibly were insufficiently sensitive, it is notable that we failed to observe either expression or production of IL-6 in alveolar macrophages and lavage fluid. Why this gene is so poorly expressed during anesthesia and surgery remains unknown. McCrea *et al.*⁸ reported that IL-6 concentrations in bronchoalveolar lavage fluid were greater in smokers than nonsmokers. IL-6 is a pleiotropic cytokine released by alveolar macrophages. Like IL-1 β and TNF- α , IL-6 is involved in acute-phase protein production and is an endogenous inflammatory mediator. On the other hand, IL-6 limits inflammation by inhibiting release of TNF- α by macrophages.¹⁶ Even direct instillation of IL-6 abrogates subsequent lipopolysaccharide-induced lung injury, suggesting that IL-6 may exert antiinflammatory effects in the lungs.²²

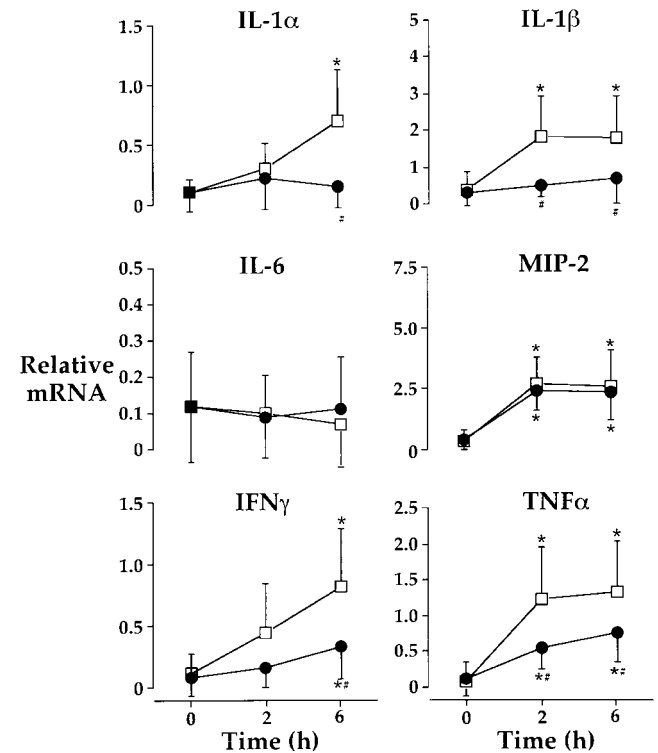


Fig. 2. Changes in gene expression of proinflammatory cytokines in smoke-exposed (circles) and control (squares) rats anesthetized with isoflurane. Data are expressed as the ratio of cytokine mRNA to β -actin mRNA. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

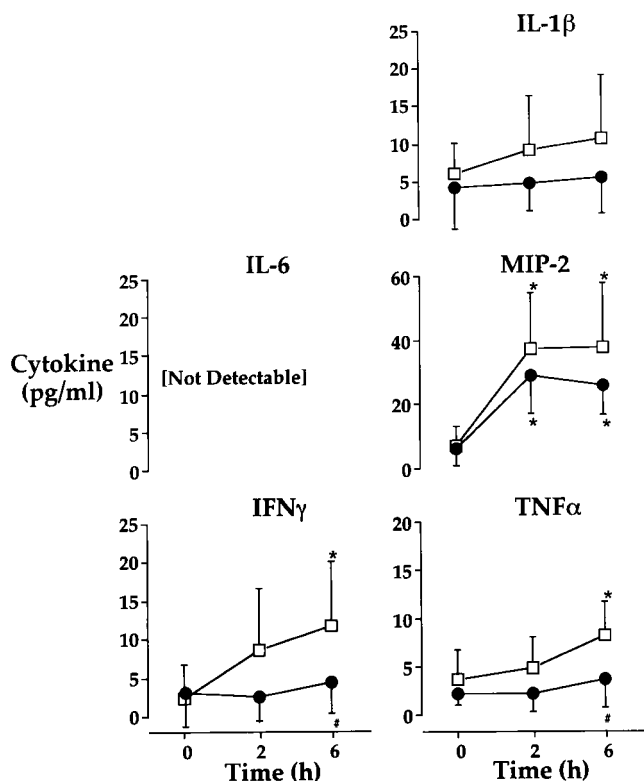


Fig. 3. Changes in absolute pulmonary lavage concentration of proinflammatory cytokines in smoke-exposed (circles) and control (squares) rats receiving halothane under mechanical ventilation. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

Phagocytic and Microbicidal Activities

Phagocytosis of opsonized and unopsonized particles and microbicidal activity decreased over time during both halothane and isoflurane anesthesia. Antimicrobial activities are generally upregulated by proinflammatory cytokines and colony-stimulating factors,^{23,24} all of which increased during the 6-h anesthesia period. Nonetheless, these activities decreased over time in both groups. Therefore, these reductions presumably resulted in part from the direct anesthetic-induced inhibition of phagocytic and chemotactic activity. In fact, our previous study demonstrated that intraoperative decreases in these activity were more pronounced during isoflurane than in propofol anesthesia.² Similar reductions in oxidative activity of alveolar macrophages have also been reported by exposure of volatile anesthetics.^{25,26} Volatile anesthetics markedly suppress the production of adenosine triphosphate and protein in alveolar macrophages by exposure to volatile anesthetics.^{27,28}

Alveolar macrophages have important immunologic

functions as antimicrobial and proinflammatory cells. The progressive neutrophil influx and macrophage aggregation observed in this study suggest inflammatory responses. Given this situation, alveolar macrophages may function more as proinflammatory cells, with antimicrobial functions such as phagocytic and bactericidal activities being suppressed. This conclusion is consistent with previous reports. It is well established that antimicrobial functions of alveolar macrophages decrease over time, as do functions of other immune cells. In contrast, expression and concentrations of proinflammatory cytokines increase during anesthesia.²⁹

A notable finding in this study is our observation that phagocytic and microbicidal activities decreased almost twice as much in smoke-exposed as in control rats. There are numerous potential explanations for this observation: (1) gene expression and production of proinflammatory cytokines in smoke exposed rats did not increase as much as in control rats; (2) volatile anesthetics and smoking both reduce in metabolic activity by

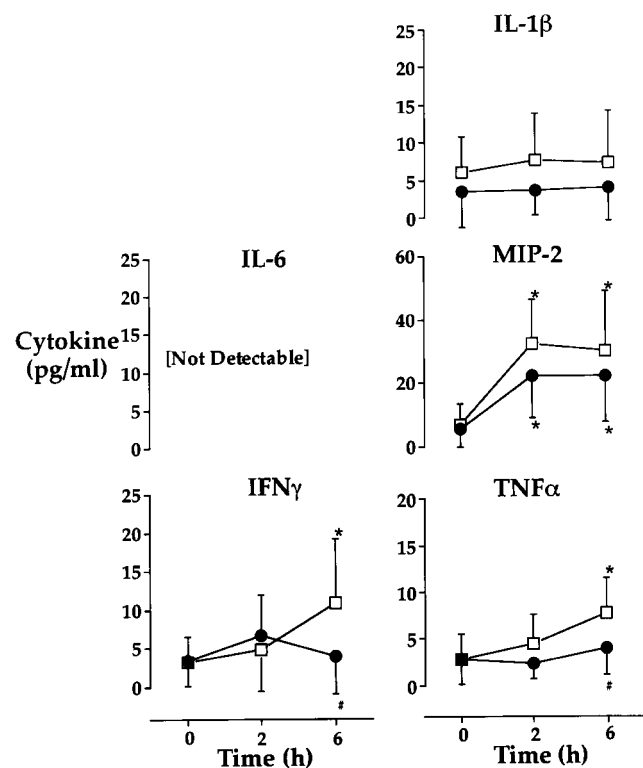


Fig. 4. Changes in absolute pulmonary lavage concentration of proinflammatory cytokines in smoke-exposed (circles) and control (squares) rats receiving isoflurane concentration under mechanical ventilation. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

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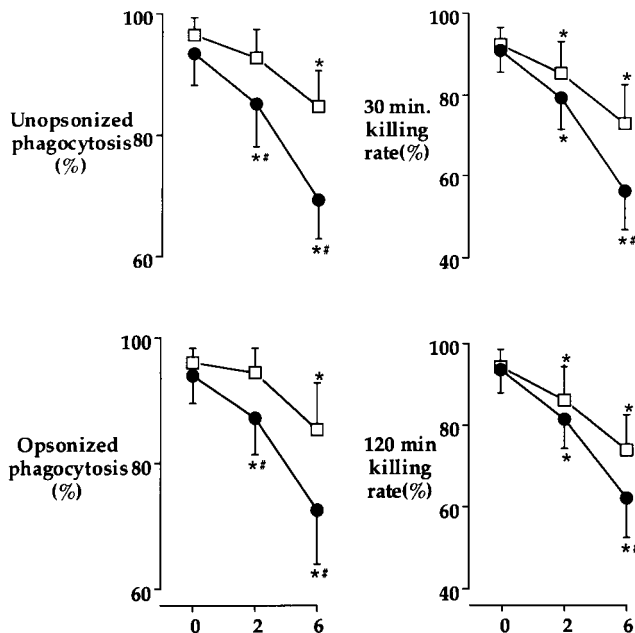


Fig. 5. Changes in phagocytosis (left) and microbicidal activity (right) in smoke-exposed (circles) and control (squares) rats that received halothane under mechanical ventilation. Phagocytosis was evaluated by the fraction of alveolar macrophages ingesting both opsonized and unopsonized particles. Microbicidal activity was evaluated by the percentage of *Listeria monocytogenes* killed by alveolar macrophage at 30 and 120 min incubation. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

inhibiting the respiratory burst required for phagocytic and microbicidal activity^{27,30}; and (3) macrophage aggregation is significantly and markedly augmented in smoke-exposed rats. At the very least, aggregation decreases cell membrane surface area available for phagocytosis, thus reducing the ability of alveolar macrophages to ingest foreign particles.² Phagocytosis and bactericidal activity of alveolar macrophages is a key element of pulmonary defense. Smoking markedly reduced both functions, suggesting that smokers have limited ability to resist pulmonary insults.

Macrophage Aggregation and Neutrophil Influx

Neutrophil influx was observed after 6 h of anesthesia in smoke-exposed and control rats, and the increases were similar in each group. Two studies suggest that smoke exposure may enhance chemotactic activity for both monocytes and neutrophils.^{31,32} This observation is consistent with our observation that expression and production of MIP-2 was similar in the two groups.

Aggregation of alveolar macrophages increased in both

groups, but the increase was much greater in smoke-exposed than control rats. Hegab and Matulionis¹¹ reported that inhalation of halothane under spontaneous ventilation for 24 h causes massive macrophage aggregation in smoke-exposed mice, whereas this response was absent in unexposed mice. Adhesion molecules are a critical component of macrophage and neutrophil accumulation. Schaberg *et al.*^{33,34} reported that alveolar macrophages and pulmonary vascular epithelial cells from smokers augmented expression of the leukocyte adhesion molecules CD11a/CD18, CD11b/CD18, CD11c/CD18, and CD54 (intercellular adhesion molecule-1). The accumulated and activated neutrophils up-regulate local adhesion molecules.³⁵ Klut *et al.*³¹ reported that neutrophils within pulmonary microvessels are activated by smoke-exposed rabbits. Taken together, these results suggest that increases in adhesion molecules in smoke-exposed rats promotes accumulation of inflammatory cells to the distal airway and macrophage aggregation.

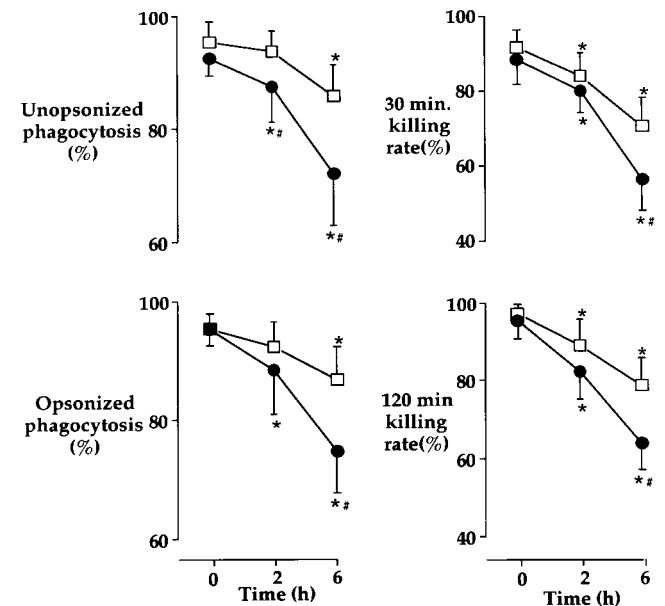


Fig. 6. Changes in phagocytosis (left) and microbicidal activity (right) in smoke-exposed (circles) and control (squares) rats that received isoflurane under mechanical ventilation. Phagocytosis was evaluated by the fraction of alveolar macrophages ingesting both opsonized and unopsonized particles. Microbicidal activity was evaluated by the percentage of *Listeria monocytogenes* killed by alveolar macrophage at 30 and 120 min incubation. *Significant differences from control values; #significant differences between smoke-exposed and control rats. Data are expressed as mean \pm SD.

Limitation and Summary

We did not separate the macrophages from other cells to avoid any artificial influence of the sorting process. For example, even adherence to plastic test tubes for separation of alveolar macrophage activates gene expression of cytokines.³⁶ Macrophages are the most likely source in initial responses.²⁰ However, IFN- γ is a potent stimulator to alveolar macrophages, but this cytokine is mainly secreted by lymphocytes. Although IL-8 is expressed and secreted from alveolar macrophages to recruit neutrophils, migrated and activated neutrophils produce lysosomal enzymes and oxygen free radicals, which facilitate IL-8 secretion in the lungs significantly.^{37,38} Thus, we cannot draw a definite conclusion regarding cell source for the observed increase in gene transcription.

Caution must be paid when extrapolating these findings to humans undergoing general anesthesia and surgery. In fact, we observed less neutrophil influx and macrophage aggregation than in our previous human study.² The most important factor accounting for the difference is contribution of surgical stress. Surgical stress augments leukocyte adhesion molecules and activates neutrophil function.²⁹ Our current results indicate that even a 6-week exposure to cigarette smoke impairs antimicrobial and proinflammatory functions of alveolar macrophages. Although our results may have some clinical significance, the clinical importance of these finding remains to be determined.

We conclude that inhalation of volatile anesthetics during mechanical ventilation modulates alveolar macrophage function. Furthermore, antimicrobial and proinflammatory responses of alveolar macrophages, on both the cellular and histologic levels, are markedly suppressed by smoke exposure.

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