Intratracheal Instillation versus Intratracheal Inhalation: Influence of Cytokines on Inflammatory Response

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Our laboratory has developed a method of particle exposure whereby anesthetized rats intratracheally inhale, at a regulated breathing rate and pressure, an aerosolized test material. This method is capable of delivering considerable doses in a short time period and, unlike the commonly used method of intratracheal instillation, does so with an even particle distribution throughout the lung. Early studies comparing the response of male Fischer 344 rats exposed to TiO₂ particles of two differing primary particle sizes showed that at similar particle doses animals exposed by the two methods showed differences in response, as measured by bronchoalveolar lavage (BAL) parameters. Building on this, we sought to study the roles that macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor alpha (TNF-α), two cytokines thought to have proinflammatory roles in the lung, may play in the differences observed. Increases in MIP-2 protein levels in the lavaged cells, but not the supernatant, were observed in those groups where increased polymorphonuclear cells (PMN) in the lung lavage were found, but not in those where no increase in PMN levels was observed. BAL TNF-α levels, measured by enzyme-linked immunosorbent assay, showed no apparent correlation with cellular or biochemical BAL parameters for either particle size or dosing method. Increases in immunocytochemical staining for TNF-α, compared to unexposed controls, were observed in several particle-exposed groups. Thus, it appears that increased BAL MIP-2 protein levels, but not TNF-α, correlate well with the inflammatory response, as measured by PMN numbers in lavaged cells, for both exposure systems. — Environ Health Perspect 105(Suppl 5):1265–1271 (1997)

Key words: intratracheal inhalation, MIP-2, TNF-α, particle, lavage

Introduction

Intratracheal instillation is routinely used for exposure of animals to particles. It has the advantages of accurate administration of test material very rapidly to the lower respiratory tract, bypassing upper respiratory tract defenses and confining the dose to a small volume, which reduces greatly the amount of equipment that must be decontaminated after exposure to highly toxic, carcinogenic, or radioactive materials. However, instillation is a less physiologic method of exposure than inhalation, and particles administered by instillation may not behave like similar particles in an aerosol. After instillation, particles are increasingly deposited in the basal regions of the lung (1), and have a significantly less homogeneous particle distribution than an inhalation exposure (2,3). It has also been suggested that the use of a vehicle for suspending the particles may alter the study results or sensitivity early after administration (4).

However, standard inhalation systems, either nose-only or whole-body chambers, present difficulties when attempting to achieve high lung particle burdens in a short period of time. The aerosol concentrations required for such exposures are difficult to achieve and may not be well tolerated by the animals as well as requiring very large amounts of test material and the maintenance and postexposure cleaning of large exposure chambers. Much of the test material would be expected to deposit in the upper respiratory tract or on the fur, making ingestion of large amounts of material a consideration. Additionally, particles that are respirable in humans may not be so in a rodent inhalation system (5,6), making their study in those models more problematic.

Our laboratory has developed a method of intratracheal inhalation (ITIH) in rats (7) in which the animals are ventilated at a constant rate with an aerosol of test material. This system has the advantages of an intratracheal administration, such as eliminating deposition on skin and fur, bypassing upper respiratory tract defenses, including nasal filters for larger particles, and direct deposition of particles in the lower respiratory tract. However, it is an inhalation system and would be expected to deposit particles in a homogeneous pattern similar to that in other inhalation systems. We have observed this to be the case with our ITIH methodology, as will be detailed in a future report. ITIH is also a closed system that allows for the filtration and removal of potentially hazardous or radioactive particles before exhaust, thus greatly facilitating cleaning and decontamination procedures.

We previously observed differences in bronchoalveolar lavage (BAL) parameters in rats after exposure by ITIH or instillation to two different sizes of titanium dioxide: fine and the more reactive ultrafine TiO₂. Animals exposed by ITIH showed a decreased response in both persistence and severity (Osier et al., unpublished data). As several reports have linked events in the inflammatory process to the production of a number of soluble cytokines or chemokines, we elected to investigate the role that two of these—macrophage inflammatory protein-2 (MIP-2) and tumor necrosis factor alpha (TNF-α)—played in the differences in response to particles that we observed between intratracheal instillation and intratracheal inhalation exposures.

MIP-2 is a basic,heat-stable, single-chain protein with an approximate molecular weight of 6 kD. It is chemotactic for neutrophils but does not induce an oxidative burst (8). It is mitogenic for epithelial cells...
but not for fibroblasts (9). mRNA for MIP-2 has been demonstrated in macrophages, neutrophils, fibroblasts, and epithelial cells (10–11). MIP-2 expression has been reported after exposure to dusts (11), lipopolysaccharide (LPS) (10), and other inflammatory stimuli (12). As a result of these studies and others, MIP-2 is believed to play an important role in regulating inflammatory responses within the lung.

TNF-α is a 17-kD (by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]) protein with a wide variety of biological activities. mRNA for TNF-α has been reported in macrophages, neutrophils, fibroblasts, and epithelial cells (10–11). TNF-α activity or mRNA expression has been reported in response to various stimuli, including dusts (13), LPS (10), and other inflammatory stimuli (14). Antibodies to TNF-α ameliorate the fibrotic response seen after silica exposure (15) as well as decreasing tissue injury during endotoxic shock (16). TNF-α also promotes neutrophil adhesion (17) and induction of other cytokines such as MIP-1, MIP-2, interleukin-1β, interleukin-6, and interleukin-8 (11,16).

The present study investigates the roles that TNF-α and MIP-2 may play in the observed differences in BAL parameters, specifically neutrophil influx. It was our hypothesis that alterations in protein concentrations of these cytokines would correspond to changes in BAL neutrophil numbers.

**Methods**

**Animals**

Specific pathogen-free male Fischer 344 rats weighing 175 to 225 g were group housed in wire-bottom cages. Animals were fed Purina Laboratory Chow and water ad libitum.

**Selection of Particles**

In this study, two types of titanium dioxide were used. The first had a mean primary particle size of 0.25 μm and will be referred to as fine TiO₂ (Fisher Scientific, Springfield, NJ). The second had a mean primary particle size of approximately 0.021 μm and will be referred to as ultrafine TiO₂ (Degussa A.G., Frankfurt am Main, Germany). Both particle types were of the anatase crystalline form.

**Intratracheal Inhalation Exposures**

Intratracheal inhalation exposures were performed as previously reported by our laboratory (7). Briefly, animals were anesthetized with halothane and intubated intratracheally with a modified 14-gauge cannula. The open end of the cannula was attached to a port on the inhalation apparatus such that an airtight seal was formed. The animals were ventilated at a frequency of 30 breaths/min (1.5-sec inhalation, 0.5-sec exhalation) at a maximum inhalation pressure of 15 cm H₂O with an aerosol of either water (sham-exposed) or test material. A schematic of the apparatus is shown in Figure 1.

TiO₂ aerosols were generated from a sonicated aqueous suspension using a Lovelace nebulizer, and dried through a drying tube. Aerosols had mass median aerodynamic diameter ranges from 1.0 to 1.2 and Fg values of 1.6 to 2.2. Target concentration was 125 mg/m³. Concentration was monitored with a light-based particle sensor connected to a chart recorder. Additional material was added as needed to the nebulizer cup through a custom-installed inlet, allowing either water or particle suspension to be added as required. Halothane (0.75% final concentration) was used to maintain anesthesia during exposure. Animals were placed on a heating pad to prevent a fall in body temperature during exposure. Exposure duration was 2 hr. After exposure the tracheal catheter was removed, and the animals were allowed to recover on a heating pad, usually 5 to 20 min, before being returned to their cages. Animals (three per group) were sacrificed on days 0, 1, 3, and 7 postexposure.

**Intratracheal Instillation Exposures**

Animals were anesthetized with halothane and a small catheter, attached to a 1-ml syringe, was inserted into the trachea via a modified otoscope. An amount of particles equivalent to that present in the day 0 ITIH group (500 μg fine TiO₂, 750 μg ultrafine TiO₂) was instilled suspended in a volume of 0.2 ml saline. TiO₂ in the lungs of the ITIH groups was determined by chemical analysis, as described below. This led to lung burdens on day 1 postexposure that were slightly on day 1 postexposure that were significantly, lower than those of animals receiving particles by ITIH. Particle suspensions were placed in an ultrasonic bath for 10 min before instillation to ensure an even suspension. Animals were allowed to recover, then returned to their cages. Groups of animals (three per group) were sacrificed on days 0, 1, 3, and 7 postexposure.

**Tissue Titanium Dioxide Determination**

Analysis was performed by atomic emission spectroscopy using techniques previously described in our laboratory (18). Samples of lung tissue, aliquots of lavage supernatant, and lavage cell pellets were dried overnight, ashed, and then fused in platinum.
crucibles with a 2:1 (w/w) mixture of sodium carbonate and sodium borate. The melt was dissolved in 7% (v/v) sulfuric acid; titanium content was determined by plasma atomic emission spectroscopy, reading emission at the 334.91-nm emission line. Titanium dioxide concentration was calculated from the titanium content.

**Bronchoalveolar Lavage**

Bronchoalveolar lavage (BAL) was performed as previously described in our laboratory (19). Briefly, animals were anesthetized with sodium pentobarbital and sacrificed by exsanguination via the carotid artery. The trachea was cannulated and the trachea, heart, and lungs removed en bloc. The heart and fascia were removed, and the lungs lavaged 10 times with 5 ml each of sterile saline at room temperature. The first two washes were pooled, as were the final eight. The washes were centrifuged at 400 x g for 10 min at 4°C in a swinging-bucket centrifuge. The supernatant from the first two washes was frozen in aliquots and saved for later analysis, and the cells from all washes were pooled and resuspended in saline to 15 ml final volume.

**Measurement of Cytokine Levels**

Aliquots of BAL supernatant and lysed cell pellet were analyzed for MIP-2 and TNF-α levels by enzyme-linked immunosorbent assay (ELISA) using commercially available, rat-specific kits (BioSource, Camarillo, CA). Because of low TNF-α levels in most samples, the samples were concentrated by lyophilization prior to TNF-α analysis.

**Immunohistochemical Staining or TNF-α**

Exposed animals were sacrificed, and the lungs perfused with sterile, room-temperature saline through the pulmonary artery. The lungs were inflated with fix and then fixed in 10% neutral-buffered formalin and embedded in paraffin. Five micron-thick sections were cut onto Superfrost slides (VWR Scientific, San Francisco, CA) and dried in a 60°C oven overnight.

Slides were rehydrated through xylene and alcohols to phosphate-buffered saline (PBS), then blocked with normal serum for 30 min at room temperature. Slides were washed in PBS, then incubated with anti-TNF-α antibody at a 1:50 dilution for 90 min at room temperature. The slides were again washed in PBS and secondary antibody from the Vectastain staining kit (Vector Labs, Burlingame, CA) was added for 60 min at room temperature. Slides were washed and 10 µg/ml fluorescein avidin B (Vector Labs) added for 30 min in the dark at room temperature. Slides were washed in distilled water in the dark, then overlapped with Vectashield mounting medium (Vector Labs) and stored in the dark at 4°C until examined. Examination was performed in a blinded fashion, and a qualitative rating scale was used to rank the samples.

**Statistical Analysis**

Data were analyzed with the SigmaStat statistics package (Jandel). An analysis of variance was performed, and Tukey's test was used to determine significance of individual groups. Comparisons were made between unexposed, sham-exposed, and particle-exposed groups as well as between similarly exposed groups at different time points. A group was considered significant if the p value was less than 0.05.

**Results**

**Lung TiO2 Burdens**

The TiO2 lung burdens of the ITIH-exposed animals immediately after exposure were 490 ± 119 µg for the fine TiO2 and 765 ± 81 µg for the ultrafine TiO2. These levels were used to determine the instilled doses. Lung TiO2 content on day 1 for the fine TiO2 particles was 393 ± 44 µg and 498 ± 59 µg for the instilled and inhaled groups, respectively. These levels were not significantly different from each other (p = 0.07). The burdens for the ultrafine TiO2 groups were 559 ± 35 µg for the instilled group and 690 ± 133 µg for the inhaled group, which also were not significantly different from each other (p = 0.175).

**PMN Numbers in BAL Pellet**

Total numbers of polymorphonuclear leukocytes (PMN) in the lavage cell pellet are shown in Figure 2. No changes were seen following sham exposure. Exposure to fine TiO2 resulted in an increased number of PMNs only after instillation, and then only at the day 1 time point. No significant changes in PMN number were seen after intratracheal inhalation of fine TiO2. When ultrafine TiO2 was given by intratracheal inhalation, an increase in PMN number was seen on day 1, which decreased to control levels by day 3 postexposure. Following instillation of "ultrafine" TiO2, a significant increase in PMN number was seen on days 1, 3, and 7 postexposure. This increase was also significantly elevated when compared to the ultrafine group, which was exposed by intratracheal inhalation.

**Macrophage Inflammatory Protein Levels**

MIP-2 protein levels in the lavage supernatant, measured by ELISA, are shown in Figure 3A. Decreases in immunoreactive MIP-2 were seen on days 0, 1, and 3 for those animals exposed to ultrafine TiO2 by intratracheal inhalation, with a decrease observed for those animals exposed to ultrafine TiO2 by instillation...
only at the day 1 time point. No other significant changes were observed in the lavage supernatant.

Figure 3B shows MIP-2 levels in the lysed lavage cell pellet. An increase in MIP-2 concentration was observed in animals exposed to fine TiO$_2$ by instillation, but this difference was not significant. No other changes in pellet MIP-2 levels were observed in animals exposed to fine TiO$_2$. Animals exposed to ultrafine TiO$_2$ by instillation showed significant increases in pellet MIP-2 protein levels on days 1, 3, and 7 postexposure. After intratracheal inhalation of ultrafine TiO$_2$, increased pellet MIP-2 levels were seen only at day 1 postexposure.

**Tumor Necrosis Factor Alpha Levels**

TNF-α levels in the lavage supernatant and cell pellet are shown in Figure 4A, B. No significant changes were observed in either fraction at any time point, particle type, or exposure method. Recovery in samples spiked with TNF-α and following concentration by lyophilization was greater than 95%.

As shown in Figure 5A, animals intratracheally instilled with saline showed very low amounts of TNF-α at day 1 when lung sections were examined immunocytochemically. Similarly, at day 1, animals sham exposed by ITIH showed little TNF-α immunoreactivity (Figure 5B). However, animals receiving ultrafine TiO$_2$ by instillation (Figure 5C) or ITIH (Figure 5D) showed increased TNF-α activity. Smaller increases were seen in fine TiO$_2$-exposed animals.

The results of our TNF-α immunocytochemical studies are summarized qualitatively in Table 1. No response was seen in sham-instilled animals at any time point observed. Sham-exposed ITIH animals showed an increase in TNF-α staining on day 0, which decreased to undetectable by day 3. Animals exposed to fine TiO$_2$ showed little staining at any time point or exposure method examined, with staining confined predominantly to particle-containing macrophages. Animals instilled with ultrafine TiO$_2$ showed increased staining on day 1, which rapidly decreased by day 3. By contrast, animals exposed to ultrafine TiO$_2$ by ITIH showed significant staining at all time points examined, with slightly less staining on day 1 than at other time points.

**Discussion**

Using the two different methods of intratracheal inhalation and intratracheal instillation, we administered TiO$_2$ to rats. The ITIH animals had slightly higher dust levels in their lungs on day 1 postexposure, although the lung burdens achieved using ITIH and instillation were not significantly different. In spite of the slightly greater lung burden in the ITIH animals, the ITIH-exposed animals in general showed a lesser response than the instillation-exposed animals for each particle type.

Our results demonstrate increases in MIP-2 protein levels within the BAL pellet in those groups where increased PMN influx was observed. As MIP-2 is chemotactic for neutrophils (8), this finding is not surprising. However, PMNs express mRNA for MIP-2 (10). This raises the
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Figure 5. TNF-α immunocytochemistry. (A) Sham-exposed instilled rat. (B) Sham-exposed instilled rat. (C) Rat instilled with ultrafine TiO₂. (D) Rat exposed to ultrafine TiO₂ by THH. All figures are x 170, and at day 1 postexposure time point.
Table 1. TNF-α protein levels, as measured by immunocytochemistry.

| Group                  | Day 0 | Day 1 | Day 3 | Day 7 |
|------------------------|-------|-------|-------|-------|
| Instilled saline       | −     | −     | ND    | ND    |
| ITH H<sub>2</sub>O     | ++ (+) | −     | ND    | ND    |
| Instilled fine TiO<sub>2</sub> | − (+) | −     | ND    | ND    |
| ITH fine TiO<sub>2</sub> | −     | +     | ND    | ND    |
| Instilled ultrafine TiO<sub>2</sub> | − (+) | −     | ND    | ND    |
| ITH ultrafine TiO<sub>2</sub> | +++ (+) | +     | ND    | ND    |

ND, not done. Samples were rated from − to ++++, with +++ being the highest rating.

question of whether the PMNs were a major source of the increased MIP-2 levels we observed or merely a result of the increased MIP-2 levels seen. Further studies using immunocytochemistry should help us address this issue.

Previous studies have suggested an important role for TNF-α in the inflammatory process, with alterations in TNF-α mRNA or activity seen as early as 0.7 h after inhalation of LPS, a potent inducer of inflammation (20), as well as during dust-induced inflammation (15–17). Therefore, a surprising result of these studies was a lack of changes in TNF-α protein levels, measured by ELISA, in either the lavage supernatant or cell pellet. However, the more sensitive method of immunocytochemistry clearly shows increased TNF-α levels in several lung sections. It is interesting that increases in TNF-α staining did not appear to correlate with inflammatory cell influx, as less significant changes were seen in animals instilled with particles (more inflammation) than in those exposed by ITH (less inflammation). However, macrophages that had phagocytized particles often showed staining for TNF-α, even in those animals where few other responses were seen. Increased staining was also evident in the sham-exposed ITH group of animals, which suggests that ventilation of the animals is sufficient to provoke TNF-α synthesis. Useful future studies might include a) immunocytochemical staining on samples of BAL cell pellet to investigate the discrepancy between BAL ELISA analysis and TNF-α staining on whole lung sections, and b) further investigation of the decreased TNF-α staining in ultrafine TiO<sub>2</sub>-instilled animals compared to the ITH-exposed animals, in which a lessened BAL response was seen.

The PMN response, which was parallelled by increases in MIP-2 in the BAL cell pellet, appears to indicate that, for both sizes of TiO<sub>2</sub> particles, an instilled dose will elicit a more persistent and/or severer response than a similar amount of particles administered by intratracheal inhalation. Several reasons could account for these differences. A number of investigators (1–4) have demonstrated differences in particle distribution or homogeneity of exposure between instillation and inhalation methods. The heterogeneous distribution of particles after an instilled dose would be expected to create areas of high and low concentration within the lung. These focal areas of high particle burden could be expected to react more strongly than in a situation in which the same dose was more evenly distributed throughout the lung.

Differences in the dose rate between instillation and intratracheal inhalation may also play a role in the differences in PMN response observed. Ferin et al. (21) reported significant differences in interstitialization of particles administered by intratracheal instillation or inhalation, and claimed that the difference in dose delivery rates was a decisive factor. However, Henderson et al. (22) found no differences in BAL parameters among animals exposed by inhalation to carbon black at different exposure rates resulting in similar particle burdens. Determination of the respective roles dose rate, dose, and particle distribution that play on the effects seen in this system will require further studies.

Our studies have shown that increases in MIP-2 levels in the BAL cell pellet, but not in the supernatant, appear to correlate with BAL PMN response. TNF-α levels, in either the BAL pellet or supernatant, did not show any significant changes at any exposure method or time point, nor did changes in TNF-α immunocytochemical staining appear to correlate with inflammatory cell influx. As with other BAL parameters, pellet MIP-2 levels seem to be greater in persistence and value after an instilled dose than after an intratracheal inhalation of a similar dose of the same particles. Further study of these differences may give us information about the role of particle distribution and/or dose rate in particle effects, and the role these mediators may play in the process.

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