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Overload of Lung Clearance Is Associated with Activation of Alveolar Macrophage Tumor Necrosis Factor and Fibronectin Release

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ABSTRACT

This report summarizes recent findings on the relationships among overloaded lung clearance, activation of alveolar macrophages (AM) release of inflammatory mediators and the development of fibrosis using TiO2 as a model nuisance type dust. Briefly rats were intratracheally instilled with 2-100 mg TiO2/kg body weight and AM tumor necrosis factor or fibronectin release determined ex vivo 1, 7, 14 and 28 days after exposure. Lung dust burdens were determined 1 and 28 days after exposure. Histopathology was assessed 28 and 90 days after exposure. Intratracheal instillation of ≥50 mg/kg TiO2 resulted in overloaded lung clearance. TiO2 doses ≥50 mg/kg stimulated transient increases in AM TNF release and a persistent increase in AM fibronectin secretion. Histopathology demonstrated dose-related interstitial inflammation with fibrosis developing only after treatment with ≥50 mg/kg TiO2. Results from these studies suggest activation of AM secretory activity may play a key role in adverse pulmonary responses to high dust burdens of relatively innocuous materials. Studies investigating in vitro responses of AM to dust indicated that direct TiO2:AM interaction does not stimulate release of TNF or fibronectin, however, pre-exposure to γ-interferon can render AM responsive to TiO2 with respect to increased TNF release.

INTRODUCTION

The secretory activity of alveolar macrophages (AM), while important to lung defense, is also thought to play a key role in the pathogenesis of interstitial lung disease. A variety of studies have demonstrated that pneumotoxic dusts such as crystalline silica and asbestos can activate AM to release factors which recruit and activate inflammatory cells, as well as, stimulate fibroblast proliferation and collagen synthesis (Davis, 1986). Two factors whose release is increased after SiO2 or asbestos exposure are tumor necrosis factor (TNF) and fibronectin (Rom et al., 1986; Dubois et al., 1989; Driscoll et.al., 1990a). TNF is a 17 kd peptide which possesses a number of pro-inflammatory activities. Fibronectin, a 440 kd glycoprotein, is a chemotactic and growth factor for mesenchymal cells. Thus, both these macrophage-derived mediators have the potential to play an important role in pulmonary inflammation and tissue repair processes.

Key Words: alveolar macrophage, tumor necrosis factor, fibronectin, fibrosis, titanium dioxide, clearance overload

In contrast to SiO_2 and asbestos, nuisance-type dusts, under most circumstances, are not associated with adverse pulmonary responses. However, exposure to relatively innocuous dusts can result in chronic inflammation and fibrosis when pulmonary dust burdens are reached which overload normal particle clearance mechanisms (Morrow, 1988; Oberdorster, 1988). Chronic inflammation and fibrosis have been observed at excessive lung burdens of titanium dioxide (TiO_2), diesel exhaust and test toner; materials thought to possess a low degree of toxicity (Lee et al, 1986; Wolff et al., 1987; Muhle et al., 1988). At present, there is limited information on similarities and/or differences in mechanisms underlying the pulmonary responses to highly toxic dusts versus relatively innocuous dusts under conditions of clearance overload.

This manuscript summarizes some of our recent findings on activation of alveolar macrophage secretory activity after exposure to excessive lung burdens of TiO_2, a material generally regarded as a nuisance-type dust. Results presented demonstrate that at TiO_2 lung burdens which overload clearance, AM become activated to release TNF and increased levels of fibronectin, responses associated with an infiltration of inflammatory cells and development of fibrosis, respectively. Additionally, results from *in vitro* exposure of AM to TiO_2 or SiO_2 indicate the lung environment plays a key role in AM activation under conditions of overloaded clearance and suggest γ-interferon may represent one factor which is important in rendering AM susceptible to activation by TiO_2.

MATERIALS AND METHODS

Animals

Specific pathogen-free male Fischer 344 rats weighing 180-200g (Charles River Breeding Laboratories, Kingston, NY) were housed in an air conditioned room (25°C, 50% RH) on a 12 hour light/dark cycle. Animals were provided Purina Rodent Chow (5001) and tap water *ad libitum* throughout the study.

Dust Exposures for Bronchoalveolar Lavage Studies

The general experimental design and intratracheal instillation procedure have been described previously (Driscoll et al., 1990a). Briefly, animals were intratracheally instilled with sterile saline (control group) or saline suspensions of TiO_2 (Anatase; Fisher Scientific, Fair Lawn, NJ). TiO_2 was heated to 200°C for two hours for sterilization prior to use. The particle size (Feret's diameters) determined microscopically after 15 minutes of sonication was 2.1 ± 1.5 μm and surface area determined by nitrogen adsorption was 8.8 m^2/g. Instillations were performed at a dosing volume of 1.0 ml/kg body weight with TiO_2 doses of 5, 10, 50, and 100 mg/kg body weight. Intratracheal instillations were performed on lightly anesthetized animals (Na pentabarbital, intraperitoneal injection; 3 mg/kg) using a pediatric laryngoscope and a #20 ball tipped dosing needle attached to a 1 ml syringe placed 1 cm into the trachea.

Bronchoalveolar Lavage and Cell Culture

At 1, 7, 14, and 28 days post-instillation 5 or 6 animals/treatment group were sacrificed by intraperitoneal injection of Na pentobarbital (50 mg/kg) and exsanguination via the abdominal aorta. Bronchoalveolar lavage (BAL) and AM culture were performed as described previously (Driscoll et al., 1990a). Briefly, the trachea was cannulated and the lung infused 6 times with Ca/Mg-free phosphate buffered saline solution (pH=7.2; PBS) at a volume of 8 ml/wash. The bronchoalveolar lavage fluid (BALF) was centrifuged at 300 x g for 10 minutes, and the cell pellet resuspended in RPMI 1640 (Gibco, Grand Island, NY) containing 25 mM HEPES buffer. BALF cell number and viability were determined by hemocytometer counting and trypan blue exclusion, respectively, and cell differentials were performed on cytocentrifuge preparations, fixed in methanol and stained with Diff Quik™ (American Scientific). The BALF cell suspensions

were adjusted to a concentration of 1 X 10^6 viable AM/ml and one ml seeded into 35mm plastic tissue culture dishes. AM were allowed to adhere for 60 minutes in a humidified incubator (37°C and 5 % CO_2) after which adherent monolayers were rinsed vigorously 3 times with RPMI 1640 media. The rinsed monolayers were incubated (37°C and 5% CO_2) for 24 hr in 1 ml RPMI 1640 containing 2 mg/ml fraction V bovine serum albumin (BSA; Sigma). The AM conditioned media was filtered (0.45 µm pore size) to remove nonadherent cells and frozen at -70°C until analysed for the presence of TNF or fibronectin. TNF bioactivity was determined using an L929 cell lysis assay and immunoreactive fibronectin was determined by ELISA as described previously (Driscoll et al., 1990a, b).

Histopathology

On days 28 and 90 (at 100 mg/kg, day 60) after instillation, five animals/treatment group (at 100 mg/kg, 3 animals/treatment) were sacrificed by ether inhalation and exsanguination via the abdominal aorta. The lungs were infused to 25cm H_2O with 10% buffered neutral formalin. Paraffin embedded sections from both lungs were stained with hematoxylin and eosin (H&E) or Masson's trichrome for light microscopic examinations.

In Vitro Exposure of AM

Methods used for in vitro exposure of F344 rats alveolar macrophages are described in detail elsewhere (Driscoll et al., 1990b and c). Briefly, AM were obtained by bronchoalveolar lavage of untreated F344 rats, suspended in RPMI 1640 media and 5 x 10^5 cells/well seeded for experiments examining TNF release and 2 x 10^6 AM/well seeded for experiments examining fibronectin release. Heat sterilized (200°C x 2 hr) SiO2 or TiO2 was suspended at concentrations of 10, 30, 100, or 300 µg/ml in RPMI 1640 containing 2 mg/ml BSA and 1 ml of the dust suspension added to cultures of adherent AM for 48 hr. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2. AM conditioned media was analysed for several consituents including LDH, TNF and fibronectin. The experiment was repeated 3 times, using cells pooled from at least 5 animals. Additional experiments were conducted to examine the effects of 24 hr pretreatment with 500 units/ml rat γ-interferon on TNF release upon a subsequent 24 hr exposure to 100 µg/ml SiO2 or, 100 and 1000 µg/ml TiO2.

Pulmonary Dust Retention

Groups of 15 rats were intratracheally instilled as described above with TiO2 at 2, 5, 10, 50, and 100 mg/kg body weight. Five animals/group were sacrificed at 1, 7, and 28 days after treatment, the lungs removed enbloc, trimmed, weighed and lyophylized. Titanium levels in lung tissues were determined as described previously (Driscoll et al., 1990b).

Statistical Analysis

All data are expressed as actual values except dust retention data which are presented as a percentage of the day 1 group mean lung burdens. Differences between treatments were evaluated using a one-way ANOVA, followed by pairwise comparisons using the Newman-Keuls test (Zar, 1984). Statistical significance was considered at $p<0.05$.

RESULTS

The lung burdens determined 24 hr after instillation of 2, 5, 10, 50 or 100 mg/kg TiO2 were 0.4 ± 0.1, 1.1 ± 0.2, 2.0 ± 0.3, 8.8 ± 0.5, 15.2 ± 0.5 mg TiO2/g lung weight, respectively. Dust retention 28 days after instillation as a

percentage of the day 1 lung burdens are shown in Figure 1. Instillation of 50 and 100 mg/kg TiO2 resulted in increased dust retention relative to that observed for instilled doses of 2, 5, or 10 mg/kg.

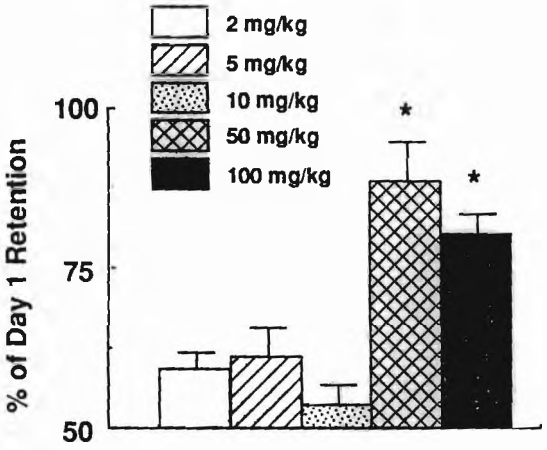

Figure 1. Retention of TiO2 28 days after instillation. Results are presented as a percentage of lung burdens determined 24 hr post exposure, X±SE; N=5. (*) denotes a significant difference from 2, 5, and 10 mg/kg dose groups; $p<0.05$.

Principal histological changes 28 days after TiO2 treatment consisted primarily of particle laden macrophages and interstitial inflammation with responses 90 days after exposure being of similar or decreased severity relative to those at day 28. Masson's trichrome stained sections were evaluated for increased prominence of collagen, which was interpreted to reflect fibrosis. At 28 days no fibrosis was observed for TiO2 exposed rats, however, at the later times fibrosis was present at TiO2 doses ≥50 mg/kg.

The effects of dust exposure on AM release of TNF or fibronectin are described in detail elsewhere (Driscoll et al., 1990a and b) and summarized with histopathology (collagen staining) and lung burden data in Table 1. TiO2 at doses of 50 or 100 mg/kg stimulated transient increases in AM TNF release, with lower dose levels eliciting no significant TNF response. Instillation of 50 or 100 mg/kg TiO2 stimulated AM to release increased levels of fibronectin, in contrast, exposure to 10 mg/kg elicited only a transient (day 7) increase in fibronectin release and treatment with 5 mg/kg did not stimulate a fibronectin response.

The effects of in vitro SiO2 or TiO2 exposure on release of LDH, TNF and fibronectin are summarized for doses of 30 and 300 µg/ml in Table 2. SiO2, but not TiO2, resulted in increased LDH as well as TNF release. In contrast neither dust had a significant effect on fibronectin secretion.

The effects of γ-interferon pretreatment on dust-induced AM TNF release are shown in Figure 2. Treatment with γ-interferon alone elicited small but significant increases in AM TNF release. Pretreatment with γ-interferon followed by SiO2 resulted in an increase in AM TNF release which was additive (equal to SiO2 alone plus γ-interferon alone). In contrast, combined treatment with 1000 µg/ml TiO2 and γ-interferon resulted in a stimulation of TNF release which was synergistic. Treatment of γ-interferon primed cells with 100 µg/ml TiO2 did not stimulate TNF release over that elicited by γ-interferon alone.

TABLE 1.

DISCUSSION

A number of studies have demonstrated that lung burdens of low solubility dusts which overload normal lung clearance mechanisms are associated with development of chronic pulmonary inflammation, fibrosis and in some instances lung tumors (Morrow, 1988; Oberdorster, 1988). Our laboratory has been investigating mechanisms underlying the pathogenesis of dust-induced interstitial lung disease. In the present report we summarize our recent observations on activation of AM secretory activity after exposure of rats to high and low lung burdens of TiO2, a material generally considered to be a nuisance-type dust. The results discussed demonstrate an association between overload of lung clearance, stimulation of AM

TABLE 1.

Pulmonary Responses to Intratracheally Instilled TiO_2 Relative to a Saline Instilled Control Group: Macrophage TNF and Fibronectin Release, Dust Retention, and Development of Pulmonary Fibrosis[a].

TiO2	AM TNF Release[b]				AM Fibronectin Release[b]			Dust Retention	Fibrosis	
Dose	day 1	day 7	day 14	day 28	day 7	day 14	day 28	day 28	day 28	day 90[c]
100 mg/kg	+	-	+	-	+	+	+	increased	-	+
50 mg/kg	-	+	+	-	+	+	+	increased	-	+
10 mg/kg	-	-	-	-	+	-	-	normal	-	-
5 mg/kg	-	-	-	-	-	-	-	normal	-	-

[a] changes in dust retention are relative to TiO2 retentions observed for dose groups ≤10 mg/kg

[b] (+) = statistically significant increase above control; (-) not statistically different from control

[c] day 60 for 100 mg/kg groups

TABLE 2.

Response of Rat Alveolar Macrophages to <u>In Vitro</u> Treatment with SiO2 and TiO2 (N=3; X±SE)

Treatment	Lactate Dehydrogenase (units/10^6cells/24 hr)	Tumor Necrosis Factor (units/10^6cells/24 hr)	Fibronectin (ng/10^6cells/24 hr)
Control	1.9 ± 1.4	<2	67.1 ± 7.7
SiO2:			
30 µg/ml	7.3 ± 0.3 *	12.2 ± 2.2 *	68.8 ± 3.9
300 µg/ml	26.0 ± 0.3 *	52.2 ± 5.3 *	68.2 ± 4.6
TiO2:			
30 µg/ml	0.8 ± 0.2	<2	75.6 ± 13.1
300 µg/ml	1.3 ± 0.2	<2	56.9 ± 9.6

* significantly different from control; $p<0.05$

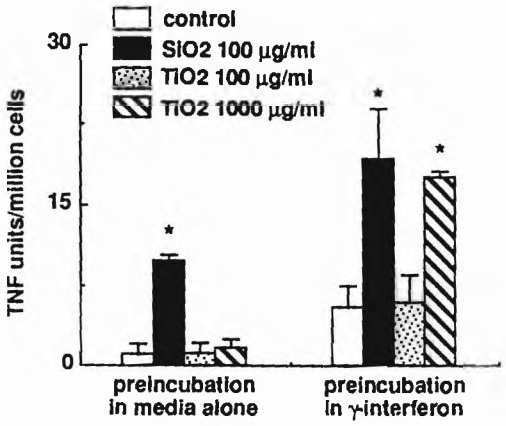

Figure 2. Effect of a 24 hr pretreatment with 500 u/ml rat γ-interferon on TiO2 and SiO2-induced AM TNF release. Data are presented as the X±SD, N=3. (*) denotes statistically significant difference from appropriate non-dust exposed control; $p<0.05$.

to release TNF and fibronectin, and the eventual development of fibrosis. The in vitro data presented indicate that the pulmonary environment plays a key role in activation of AM after dust exposure and suggests γ-interferon represents one factor with the potential to render AM susceptible to activation by TiO2.

To further elucidate the mechanisms underlying the activation of inflammatory processes in the lung after mineral dust exposure, we have focused our attention on the AM. This cell, because of its ability to release mediators which can modulate the activities of inflammatory and immunocompetent cells, has the potential to play a key role in the initiation and perpetuation of inflammatory responses within the lung. TNF represents an AM-derived cytokine with pro-inflammatory activities. This cytokine can stimulate expression of adhesion molecules for inflammatory cells on capillary endothelium (Bevilacqua et al., 1989); activate release of neutrophil and monocyte chemotactic peptides by a variety of cells including macrophages, fibroblasts and endothelial cells (Larsen et al., 1989; Strieter et al., 1989); and stimulate phagocytic cells to release reactive oxygen species and lysosomal enzymes (Klebanoff et al., 1986; Tsujimoto et al., 1986). Thus, this cytokine has the potential to play a key role in the recruitment and activation of inflammatory cells to the lung after inhalation of noxious materials. More recently, the key role of TNF in lung fibrosis was demonstrated by Piguet et al. (1989, 1990) who reported that treatment of SiO2 or bleomycin exposed mice with anti-TNF antibodies markedly attenuated collagen deposition. In the present report we summarize results suggesting a positive association between intratracheally instilled doses of TiO2 which overload lung clearance and activation of AM TNF release. Interestingly, statistical evaluation on an individual animal basis of the relationship between dust-induced increases in ex vivo AM TNF release and numbers of neutrophils in bronchoalveolar lavage fluid reveal a significant positive correlation (Driscoll et al, 1990a). This association strongly suggests a role for TNF in the recruitment of inflammatory cells under conditions of excessive dust exposure. Given the known pro-inflammatory activities associated with this cytokine it is likely that TNF, at least in part, plays a role in the inflammation and fibrosis we observed at excessive lung burdens of TiO2.

In addition to TNF, AM can release fibronectin, a mediator with the potential to influence development of pulmonary fibrosis. Fibronectin is chemotactic for fibroblasts (Postlethwaite et al., 1981); can mediate interactions between a variety of cell types and the extracellular matrix (Mosher, 1984; Macarak and Howard, 1983); and, acting in concert with other growth factors can stimulate fibroblasts to proliferate (Bitterman et al., 1983; Bitterman et al., 1986). Thus, fibronectin is thought to play a critical role in tissue repair; functioning to recruit cells to sites of tissue injury and inflammation, facilitating cell attachment to extracellular matrix proteins and promoting local cell proliferation. Excessive TiO2 lung burdens stimulated release of fibronectin, a response which persisted through the 28-day post-instillation period. In contrast, dust burdens which did not overload lung clearance elicited only transient or no increase in fibronectin release.

Importantly, persistent increases in fibronectin release were associated with eventual increases in collagen deposition (i.e., fibrosis). In this respect, previous studies have demonstrated that individuals with silicosis, asbestosis or coal worker pneumoconiosis have AM populations releasing increased levels of fibronectin (Rom et al., 1986) as well as increased fibronectin deposition at sites of pneumoconiotic lesions (Wagner et al., 1982). The prospective nature of our observations on increased fibronectin release and collagen deposition further support an important and early role for AM-derive fibronectin in the development of pulmonary fibrosis and suggest similarities in mechanisms of pulmonary fibrosis exist between highly toxic dusts and excessive lung burdens of relatively innocuous materials.

The mechanisms by which TiO2 stimulates AM to release TNF and fibronectin are unknown, however, results from our in vitro experiments indicate the pulmonary environment plays a key role. In this respect, the response seen after dust instillation could involve cells and or secondary mediators not present under in vitro exposure conditions. One cytokine, released by activated lymphocytes, which can "prime" macrophages to become more responsive to subsequent stimulation is γ-interferon (Adams and Hamilton, 1984). Since we previously observed that high doses of TiO2 result in persistent increases in BALF lymphocyte numbers (Driscoll et al., 1990a), and thus, potentially increased lymphocyte secretory products, we investigated the effects of in vitro γ-interferon pretreatment on responsiveness of AM to TiO2. These results indicated γ-interferon can prime AM to release TNF upon in vitro exposure to high doses (1000 μg/ml) of TiO2. It is noteworthy that the priming response was only observed for the high dose of TiO2, suggesting that this effect may be unique to AM which become engorged with particulate materials such as occurs in vivo under conditions of overloaded clearance. Interestingly, a similar response was not apparent for SiO2 exposure which suggests differences may exist in the mechanism of SiO2 and TiO2-induced TNF release. These findings demonstrate that secondary factors can increase responsiveness of AM to dust exposure and suggest γ-interferon has the potential, if released upon in vivo dust exposure, to influence AM responsiveness to TiO2.

In conclusion, the findings summarized in this report suggest that similar to highly toxic dusts (e.g., SiO2 and asbestos), the development of interstitial lung disease in animals exposed to excessive lung burdens of nuisance-type dusts may, at least in part, result from activation of AM to release pro-inflammatory factors. The correlation, observed between ex vivo AM TNF release after instillation of TiO2 and neutrophil numbers in BAL fluid clearly suggests a role for this cytokine in dust-induced recruitment and activation of inflammatory cells. Further, the association of persistent AM fibronectin release with the development of fibrosis provides additional support for this mediator in the pathogenesis of interstitial lung disease. Lastly, the in vitro studies indicate the pulmonary environment plays an important role in the activation of AM TNF and fibronectin release after exposure to excessive lung burdens of TiO2 and, suggests that factors such as γ-interferon could be important in modulating AM responsiveness to dusts in vivo.

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