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PLEURAL EFFECTS OF INDIUM PHOSPHIDE IN B6C3F1 MICE: NONFIBROUS PARTICULATE INDUCED PLEURAL FIBROSIS

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Abstract

The mechanism(s) by which chronic inhalation of indium phosphide (InP) particles causes pleural fibrosis is not known. Few studies of InP pleural toxicity have been conducted because of the challenges in conducting particulate inhalation exposures, and because the pleural lesions developed slowly over the 2-year inhalation study. The authors investigated whether InP (1 mg/kg) administered by a single oropharyngeal aspiration would cause pleural fibrosis in male B6C3F1 mice. By 28 days after treatment, protein and lactate dehydrogenase (LDH) were significantly increased in bronchoalveolar lavage fluid (BALF), but were unchanged in pleural lavage fluid (PLF). A pronounced pleural effusion characterized by significant increases in cytokines and a 3.7-fold increase in cell number was detected 28 days after InP treatment. Aspiration of soluble InCl_3 caused a similar delayed pleural effusion; however, other soluble metals, insoluble particles, and fibers did not. The effusion caused by InP was accompanied by areas of pleural thickening and inflammation at day 28, and by pleural fibrosis at day 98. Aspiration of InP produced pleural fibrosis that was histologically similar to lesions caused by chronic inhalation exposure, and in a shorter time period. This oropharyngeal aspiration model was used to provide an initial characterization of the progression of pleural lesions caused by InP.

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Keywords

indium phosphide; lung; mice; particulate; pleural fibrosis

Indium phosphide (InP) is an unusual man-made metallic compound used extensively in the microelectronics industry to manufacture semiconductors, injector lasers, solar cells, photodiodes, and light-emitting diodes. Utilization of InP in microelectronics applications has increased because of its superior band gap qualities, high electron mobility, and high breakdown voltage. The increased production and use of InP has increased concern about the safety of semiconductor and photoelectronic industry workers exposed to InP particulates when cutting, grinding, and polishing materials containing this compound. The American Conference of Governmental Industrial Hygienists (ACGIH) recommends a time-weighted average threshold limit value of 0.1 mg/m³ for all indium compounds based upon pulmonary edema [1].

Studies in laboratory animals have shown that InP [2–4], and other “insoluble” indium compounds such as indium arsenide [5–7], copper indium diselenide [8], and indium-tin oxide [9] all cause severe pulmonary inflammation and fibrotic changes after intratracheal instillation of particulates. Workers exposed to indium-tin oxide particulates have been diagnosed with emphysematous changes, pulmonary fibrosis, interstitial pneumonia, and pneumothorax [10–14].

In a 2-year bioassay, inhalation exposure to InP particulate caused alveolar/bronchiolar adenomas and carcinomas in rats and mice [15]. In addition, it was also noted that chronic inhalation exposure to InP particles caused unusual fibrotic lesions throughout the thoracic cavity of mice [15]. Extensive adhesions developed between the lung lobes, as well as to the parietal pleura, the diaphragm, and the pericardial sac in B6C3F1 mice exposed to InP for 2 years. The presence of these adhesions correlated well with histological observations of pleural inflammation, mesothelial cell hyperplasia, and fibrosis in the visceral and parietal pleura of exposed mice. These lesions were unusual because although certain asbestos fibers are known to cause hyperplasia and fibrosis of the pleural mesothelium [16,17], such lesions are rare following inhalation of a nonfibrous particulate like InP.

The mechanism(s) by which InP particles cause pleural fibrosis is unknown. Few mechanistic studies of InP toxicity have been conducted [3,6,15], in part, because of the challenges involved in conducting particulate inhalation exposures, and also because of the length of time required for pleural lesions to develop. The objective of the following studies was to determine if pleural fibrosis would develop more quickly after oropharyngeal aspiration of InP than following inhalation exposure. Mechanistic studies would be considerably more feasible if exposures could be conducted by aspiration and if the time to fibrosis was shortened. Oropharyngeal aspiration is a nonphysiological route of administration that involves treatment with a bolus dose of particles suspended in a liquid vehicle. Administration of particles by oropharyngeal aspiration can result in uneven particle distribution, and the potential for lung overload, thus aspiration is not appropriate for toxicity screening of insoluble particulate compounds. However, oropharyngeal aspiration is often used in mechanistic studies of insoluble particles because it is technically simple to perform and does not require a complex inhalation exposure system.

In this study, we demonstrated that administration of InP to mice by oropharyngeal aspiration resulted in a pronounced pleural effusion followed by fibrosis, and that fibrosis developed much faster after aspiration than after inhalation exposure. Using this model, we characterized the progression of parenchymal and pleural lesions after aspiration of InP. The pleural response

was also evaluated after direct, intrapleural injection of InP in order to eliminate potentially confounding effects resulting from InP injury to the parenchyma. A comparative multichemical approach was also utilized to determine if the pleural effusion was specific to indium toxicity or produced by other non-indium toxicants. Investigation of the mechanism(s) by which a nonfibrous particulate such as InP causes pleural fibrosis may provide new insights into the pathogenesis of fiber-induced pleural disease.

MATERIALS AND METHODS

Chemicals

Electronic grade polycrystalline InP (99.999% purity) was obtained from Johnson-Matthey (Ward Hill, MA.), and was from the same lot used in the previous 2-year study [15]. The InP was premilled to a particle size of 1 to 3 microns and was micronized prior to use. Inductively coupled plasma-atomic emission spectrometry (ICP/AES) analyses indicated purities of 97.1% for indium and 96.9% for phosphorus relative to theoretical values. The mass median aerodynamic diameter of the micronized InP particles was 1.2 microns with a geometric standard deviation of 1.8 [15]. Mice (25 g) received 1 mg/kg of InP, or approximately 5.8×10^6 particles per dose. Indium trichloride (InCl_3), a soluble indium compound, was purchased from Sigma-Aldrich, St. Louis, MO (catalog no. 334065). Because InCl_3 readily dissolved in the saline vehicle, particle number is not relevant. The following chemicals were also utilized: amosite (obtained from CIIT, Research Triangle Park, NC, the kind gift of Dr. David Coffin), vanadium pentoxide (V_2O_5 , Sigma-Aldrich, catalog no. 204854), copper chloride (CuCl_2 , Sigma-Aldrich, catalog no. 451665), iron chloride (FeCl_3 , Sigma-Aldrich, catalog no. 157740), cadmium chloride (CdCl_2 , Fluka, catalog no. 28811), chromium chloride (CrCl_2 , Sigma-Aldrich, catalog no. 244805), nickel chloride (NiCl_2 , Sigma-Aldrich, catalog no. 339350), tin chloride (SnCl_2 , Sigma-Aldrich, catalog no. 204722), lead chloride (PbCl_2 , Sigma-Aldrich, catalog no. 268690), and titanium dioxide (TiO_2 , Cerac). The mass median aerodynamic diameter of the TiO_2 particles was 0.61 microns, with a geometric standard deviation of 0.154. The mean amosite fiber length was 3.25 microns and the mean fiber diameter was 0.29 microns [18].

Animals

Male B6C3F1 mice (Charles River Laboratories, Raleigh, NC) were 6 to 7 weeks old upon receipt and were acclimated for 7 days prior to treatment. During acclimation, mice were uniquely identified, weighed, and randomized to treatment and control groups. All procedures conformed to federal guidelines for the use of animals in research and all procedures were approved by the National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee. Mice were provided with food (NIH-31) and tap water ad libitum. Animals were housed in a humidity- and temperature-controlled, high-efficiency particulate arresting (HEPA)-filtered, mass air displacement room in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. Animal rooms were maintained with a light-dark cycle of 12 h (light from 0700 to 1900 hours). The B6C3F1 mouse was selected for these studies because pleural lesions were reported in this strain after exposure to InP by inhalation [15].

InP Time Course Study

The progression of lesions in the lung and in the pleura of InP-treated mice was evaluated at various times after oropharyngeal aspiration. B6C3F1 mice ($n = 5$) were administered sterile saline, or 1 mg/kg InP by oropharyngeal aspiration. Bronchoalveolar lavage fluid (BALF) and pleural lavage fluid (PLF) were collected 1, 3, 14, and 28 days after aspiration and evaluated for lactate dehydrogenase (LDH), total protein, cell counts, and cytokines. Tissues were collected for histopathology 7, 14, 28, and 98 days after treatment.

Oropharyngeal Aspiration—Individual body weights of all mice were obtained on study day −1, and these weights were used to calculate a mean body weight used in dose calculation. InP particles were suspended in sterile saline and shaken at high speed overnight to produce a homogeneous suspension. The InP suspension was vortexed thoroughly just prior to treating each animal on day 0. The volume of solution aspirated (0.05 mL) was the same for all mice. Mice (5/group) were anesthetized with isoflurane prior to aspiration of either saline or 1.0 mg/kg InP. Briefly, aspiration was performed by extending the tongue with forceps and depositing the dosing solution in the back of the mouth. The nasal passages were blocked, causing the mouse to inhale through its mouth and the dosing solution was aspirated into the lungs via the gasp reflex. Oropharyngeal aspiration was shown to result in better lung distribution than intratracheal instillation in mice [19]. We routinely use oropharyngeal aspiration when treating mice [20–22] and we have found that aspiration results in fewer dosing errors and complications than when treating by intratracheal instillation.

Pleural and Bronchoalveolar Lavage—Animals were euthanized by intraperitoneal (i.p.) sodium pentobarbital (Nembutal; Abbott Laboratories) overdose followed by exsanguination. Prior to lavaging the pleural cavity, all peritoneal organs except the liver were removed. Two small punctures were made in the diaphragm near the chest wall on the left and right sides. It was difficult to avoid tearing the thin mediastinum during the initial lavage, thus no differentiation was made between left and right sides of the pleural cavity. The pleural cavity was lavaged 3 times with 0.85 mL cold saline.

After completion of the pleural lavage, the lungs were lavaged. The chest cavity was opened to expose the lungs and the tissue surrounding the trachea was trimmed away. A small incision was made in the trachea and a catheter was gently inserted into the trachea. Lungs were lavaged 3 times with 1.0 mL cold saline. The pleural and lung lavage fluid volumes were determined empirically to be optimal for lavaging the lung or pleural compartments of 7- to 8-week-old mice without causing tissue damage by over-inflating.

Samples of BALF and PLF were centrifuged at 2000 rpm ($770 \times g$) for 10 minutes at 4°C. Cell-free fluid from the first lavage was used for LDH and total protein analyses. The remainder was stored at −70°C for cytokine analysis. Total cells were counted electronically (Coulter ZB; Coulter Electronics, Marietta, GA). Cell differentials were determined from manual cell counts of cytopspin preparations stained with Wright Giemsa stain (Fisher, Kalamazoo, MI). Differentials were based upon a total of 300 cells counted per cytopspin.

Histopathology—Separate groups of animals were used for histopathology and lavage analysis in the InP time course study. Mice were euthanized by Nembutal overdose and exsanguination. The chest cavity was opened and the lungs with half the trachea attached were removed and weighed. The airways were perfused with 10% neutral-buffered formalin via the trachea until the lungs were filled to their normal inspiratory volume, and then the trachea was ligated. Tissues were fixed at room temperature in formalin, then embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin, or Masson's trichrome stain.

Cytokine Analyses—BALF or PLF samples (250 μ L) were analyzed for 58 analytes simultaneously using Luminex technology (Rules Based Medicine, Austin, TX). Cytokine levels in day 3 and day 28 BALF from control animals were not significantly different ($P > .05$), therefore these control values were combined and used for comparisons with InP-treated animals. Pilot studies (unpublished data) showed that none of the 58 analytes were changed by InP treatment in PLF prior to day 28. Therefore only day 28 PLF samples (5 treated, 5 control) were collected for cytokine analysis in the InP time course study. The transforming growth factor (TGF)- β 1 enzyme-linked immunosorbent assay (ELISA) kit was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

LDH and Protein Analyses—The acellular PLF and BALF samples were analyzed for total protein and lactate dehydrogenase (LDH) activity. Total protein was measured as an indication of pulmonary vascular leakage caused by InP exposure. Total protein in the PLF and BALF fluid samples was measured using a microplate adaptation of the Sigma-Aldrich Bradford reagent method (Sigma-Aldrich, catalog no. 23208). Absorbance of the protein-dye complex was measured at 592 nm [23]. Because LDH is a cytosolic enzyme, detection of extracellular LDH activity is used as a marker of cell membrane damage. LDH activity was measured in samples using a microplate adaptation of a commercially available liquid LDH Reagent kit (catalog no. L7572; Pointe Scientific, Canton, MI). The rate of reduction of NAD was measured as an increase in absorbance at 340 nm and was directly related to LDH activity.

Indium Analysis—PLF was centrifuged to collect the cells for cytospin slide preparation and calculation of cell differentials. The cell-free PLF fraction was submitted for indium analysis by inductively coupled plasma mass spectrometry (ICP-MS). Because identification of the different indium species was not possible, total indium was measured. All values obtained were below the detection limit of the assay (0.25 ng/mL PLF) (data not shown).

InP and InCl₃ Intrapleural Injection Study

The pleural effects of InP or InCl₃ were evaluated after direct injection into the pleural space in order to eliminate any indirect effects on the pleura resulting from toxicity to the lung parenchyma. B6C3F1 mice ($n = 5$ for each treatment group) received an intrapleural injection of 0.05 mL of sterile saline (controls), or 0.05 mL sterile saline containing 1.0 mg/kg InP or 1.2 mg/kg InCl₃. These doses were chosen so that the InP and InCl₃ solutions contained equimolar concentrations of indium. Unlike InP particles, the InCl₃ particles are readily soluble in saline at pH 7. PLF was collected on days 3 and 28 and evaluated for LDH, total protein, and cell counts as described above. Tissues were scheduled to be collected on day 98 to evaluate pleural histopathology, but were collected early on day 35 because of a lack of changes in PLF parameters at day 28. Tissues were collected and processed for histopathology as described above.

Intrapleural Injection—Mice were anesthetized with isofluorane prior to chemical treatment. The pleural space was expanded by lifting the sternum during injection. A 1-cc syringe, with the needle cover cut in half to limit depth of injection, was used to inject saline, InCl₃, or InP into the pleural cavity after gently puncturing the rib cage.

Multichemical Comparison Study

To determine if the delayed pleural effusion is unique to indium compounds, male B6C3F1 mice were treated with a number of chemicals by oropharyngeal aspiration, and BALF and PLF cell numbers were evaluated at days 3 and 28. Control mice received sterile saline. The following chemicals were evaluated: amosite, V₂O₅, TiO₂, CuCl₂, FeCl₃, CdCl₂, CrCl₂, NiCl₂, SnCl₂, and PbCl₂. Amosite is an insoluble fiber, V₂O₅ is a soluble particle, TiO₂ is a relatively inert, insoluble particle, and all the metal chlorides are freely soluble in saline. The doses of all metal chlorides were approximately 2 mM (molar equivalent to InP dosage). Amosite, V₂O₅, and TiO₂ were administered at 1 mg/kg.

Statistics

Two-way analysis of variance (ANOVA) analysis was performed on the BALF and PLF end points. A Tukey's honestly significant difference (HSD) test was used to determine which treatment-day combinations differed. Dunnett's test was also used to compare means. A P value $< .05$ was considered statistically significant.

RESULTS

Time-Course Study

Total Protein in BALF and PLF—Early responses to InP in the lung and pleura were evaluated in BALF and PLF. Total protein was increased 4-fold in BALF on day 14 and remained significantly ($P < .05$) elevated at day 28 (Figure 1). Although there was a trend of increasing total protein in PLF of InP-treated mice, these changes were not significantly different from controls. The P value for the day 28 InP total protein concentration in PLF was .058 in comparison to the control group, and thus has borderline significance.

LDH Activity in BALF and PLF—LDH activity in the BALF was increased significantly ($P < .05$) on day 3, reached peak levels on day 14, and remained significantly ($P < .05$) elevated at 28 days after treatment (Figure 2). In contrast, there were no significant changes in LDH activity in the PLF of InP-treated mice at any time point.

BALF Cells—An immediate response of the lung to InP aspiration was a significant ($P < .05$) influx of polymorphonuclear neutrophils (PMNs) and a significant ($P < .05$) decrease in the number of alveolar macrophages on day 1 (Table 1). The numbers of PMNs in the BALF remained elevated through day 28. The numbers of macrophages remained decreased through day 14 and then increased significantly ($P < .05$) to about 2-fold control numbers at day 28. A delayed but significant ($P < .05$) influx of lymphocytes into the BALF was observed on day 14, with decreased numbers present at day 28. The total BALF cell numbers in InP-treated mice were similar to controls at early time points, but were significantly increased ($P < .05$) at day 28 (Table 1, Figure 3). This change represented about a 3-fold increase in the total number of BALF cells in InP-treated mice relative to controls.

PLF Cells—In the pleura of InP-treated mice, the total cell numbers were not significantly ($P < .05$) increased until day 28 (Table 2, Figure 3). Although the total PLF cell numbers were increased by 3.7-fold at day 28, the percentages of macrophages, eosinophils, lymphocytes, and mast cells remained relatively constant throughout the study, indicating that there were similar increases in all PLF cell types (Table 2). There were no PMNs present in the PLF of either control or treated animals. It should be noted that the normal basal levels of cell numbers and types differ between the PLF and BALF, with typically 10-fold more cells in the PLF. The PLF often contains free-floating mesothelial cells in certain species [24,25]; however, in this study fluorescence-activated cell-sorting (FACS) analysis demonstrated that all cells in the PLF from treated and control mice were 100% CD45⁺ (mesothelial cells are CD45[−] [data not shown]).

Indium Analysis—Cell-free samples of PLF were analyzed for total indium by ICP-MS. All values obtained were below the detection limit of the assay (0.25 ng/mL PLF) (data not shown). InP particles were not observed within PLF cells; however, indium could have been present in a soluble form. Additional studies are in progress; however, the amount of indium reaching the pleural cavity is unknown at this time.

Histopathology—The lungs of saline-treated mice appeared normal at all time points (Figure 4A). The lungs of InP-treated mice at 7 days post exposure were characterized by increased intra-alveolar eosinophilic proteinaceous exudate containing small numbers of admixed InP particles and a mild pleocellular inflammatory infiltrate consisting of increased numbers of alveolar macrophages and neutrophils (Figure 4B). By 14 days after exposure, lungs had mild multifocal areas of inflammatory cell infiltrates forming septal thickening with associated mild alveolar epithelial cell hypertrophy and proliferation (Figure 4C). Pleocellular inflammation was present in perivascular regions (Figure 4C). At 28 days post exposure, the InP-treated

lungs had multifocal areas of moderate to severe inflammation with associated alveolar epithelial cell proliferation (Figure 4D). Inflammation was characterized by an increased component of lymphocytes as well as macrophages and neutrophils. A prominent angiocentric pattern of inflammation was present in addition to centriacinar inflammation. InP-exposed lungs at the 98-day time point (Figure 4E) were characterized by multifocal regions of severe chronic inflammation with associated proliferative pneumonia. Proliferative changes consisted of alveolar septal thickening with inflammatory cells associated with alveolar epithelial cell hypertrophy and proliferation. Perivascular inflammation was prominent with extensive lymphoid proliferation (Figure 4F).

At all time points, the visceral pleura of saline-treated mice had a normal appearance characterized by a single layer of mesothelium with no evidence of fibrosis or thickening (Figure 5A and C). The visceral pleura of InP-treated mice appeared similar to controls at time points prior to day 28; however, multifocal areas of thickened pleural membrane with inflammatory cells and overlying mesothelial cell hypertrophy and hyperplasia were present at day 28 (not shown). By day 98, the visceral pleura of InP-treated mice was characterized by extensive areas of fibrotic thickening (Figure 5B). Intense Masson trichrome staining is evident in these fibrotic areas (Figure 5D).

Cytokine Analyses—In these initial studies, a large Luminex array (58 analytes) of cytokines, chemokines, and growth factors was evaluated in order to obtain as much information as possible about InP-induced changes in the lung and pleural cavity. Only analytes that demonstrated greater than a 2-fold difference over controls are shown. It is recognized that fold-increase is not necessarily indicative of the importance of a mediator in driving the disease process; however, because almost all analytes were changed to some extent, the 2-fold cut-off was selected arbitrarily as a method to simplify the data.

BALF Cytokines—Cytokine analysis was performed on acellular BALF collected 1, 3, 14, and 28 days after InP or saline aspiration (Table 3). Complex changes were demonstrated in many chemokines and cytokines, and generally reflected the ongoing inflammation in the lungs of InP-treated mice. Most analytes increased in concentration with time after treatment, with the highest concentrations present in BALF samples collected on day 28. A number of chemokines were significantly increased in the BALF and could be correlated with the increased numbers of macrophages and monocytes (monocyte chemoattractant protein [MCP]-1, MCP-3, MCP-5, macrophage colony-stimulating factor [M-CSF], vascular endothelial growth factor [VEGF]), lymphocytes (lymphotactin, macrophage inflammatory protein [MIP]-1 α , MIP-1 β), and neutrophils (RANTES [regulated upon activation, normal T-cell expressed, and secreted; CCL5], granulocyte chemoattractant protein [GCP]-2, KC/GRO- α , stem cell factor [SCF]) recovered from the BALF at various times after treatment. A number of cytokines involved with tissue repair, matrix metalloproteinase-9 (MMP-9), tissue factor, fibrinogen, fibroblast growth factor-basic (FGF-basic), FGF-9, and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) were also significantly elevated in the BALF.

PLF Cytokines—Cytokine analyses of PLF from early time points (up to 14 days after InP aspiration) did not show significant changes in any of the 58 analytes relative to saline controls (data not shown), and for this reason only day 28 PLF cytokine data are shown (Table 4). The Luminex array demonstrated significantly increased protein levels of a number of cytokines in the PLF at day 28. The largest increases were observed in the concentration ratios of fibrinogen, MMP-9, myeloperoxidase (MPO), and tissue inhibitor of metalloproteinase (TIMP)-1. A 2-fold reduction in interleukin-1 α (IL-1 α) concentration was observed in PLF of InP-treated animals.

Intraleural Injection Study

Protein in PLF—Intraleural injection of InP caused only mild and transient changes in PLF toxicity endpoints (Table 5). Injected InP caused a statistically significant increase ($P < .05$) in PLF protein at day 28; however, because the saline control value at this time point was unusually low, the biological significance of this change is questionable. In contrast, intraleural injection of InCl₃ caused a significant ($P < .05$) 3-fold increase in PLF protein levels at day 3 that resolved by day 28.

LDH Activity in PLF—LDH activity was significantly ($P < .05$) increased at 3 days after intraleural injection of InP, but returned to control levels by day 28 (Table 5). Intraleural injection of the soluble InCl₃ caused a transient increase in LDH activity that was significant ($P < .05$) only at day 3.

PLF Cells—Intraleural injection of InP did not cause significant changes in PLF total cell numbers (Table 5). However, intraleural injection of InCl₃ caused about a 3-fold increase in cell numbers at day 3, which resolved by day 28.

Histopathology—Because there were few changes in the PLF of InP-treated mice at day 28, the mice originally designated for histopathology at day 98 were sacrificed early at day 35 to ensure that the InP particles were injected into the pleural space and not the lung. InP particles were macroscopically visible in the pleural cavities as dark particles adhering to the parietal pleura of the thoracic wall and the mediastinum at day 35 (not shown). However, microscopic examination did not reveal histopathological changes in visceral pleura, or on the surface of the diaphragm in sections examined.

Multichemical Comparison Study

BALF Cells—InCl₃ and NiCl₂ aspiration significantly increased ($P < .05$) BALF cell numbers on days 3 and 28. InP demonstrated a similar trend to that observed in the time course study; however, the increase on day 28 was not significant, although neutrophil influx was demonstrated. Several of the soluble metal chlorides (CuCl₂, FeCl₃), amosite, and V₂O₅ caused transient increases in BALF cell numbers that were significant ($P < .05$) only at day 3 (Figure 6). CdCl₂ caused a delayed increase in BALF cell numbers that was significant ($P < .05$) at day 28. CdCl₂ also caused the death of 2 of 5 mice and the BALF of survivors was contaminated with blood. Aspiration of TiO₂, CrCl₂, PbCl₂, and SnCl₂ had no effect on BALF cell numbers at either time point.

PLF Cells—Only InP, InCl₃, and CdCl₂ caused pleural effusions after oropharyngeal aspiration (Figure 7). InCl₃ caused a pleural effusion at day 3 as well as at day 28. InP and CdCl₂ caused a pleural effusion only on day 28. Two of the 5 CdCl₂-treated mice died during the experiment leaving only 3 mice for the final day 28 time point. All other toxicants had no effects on the pleural cavity.

DISCUSSION

In this study, we characterized the progression of parenchymal and pleural lesions in mice after a single oropharyngeal aspiration of InP particles. We found that when mice were exposed to InP particles by aspiration, pleural fibrosis developed in approximately 14 weeks. Previously, when exposed daily to InP by inhalation, there were no pleural effects at 13 weeks and pleural fibrosis was reported only after inhalation exposure for 2 years [15]. Although oropharyngeal aspiration is a nonphysiological route of administration, the more rapid development of pleural fibrosis after a single InP aspiration greatly facilitates the ability to conduct mechanistic studies.

In this initial report, we used this aspiration model to characterize the progression of InP effects in the parenchyma and in the pleura.

Aspiration of InP caused an early inflammatory response in the lung parenchyma similar to that caused by many insoluble particulates. Insoluble particles are typically phagocytized by alveolar macrophages (AMs), resulting in AM activation and the release chemokines that attract PMNs [26]. Increased levels of chemokines and an influx of PMNs were noted in BALF on the day following InP aspiration. The number and concentrations of chemokines and cytokines in the BALF continued to increase with time after InP aspiration.

Decreased numbers of AMs were noted in the lungs of InP-treated mice, suggesting that InP particles were cytotoxic for AMs. The phagocytized InP particles may be solubilized by lysosomal enzymes to a reactive indium species that cause AM death, and the release of reactive indium into the parenchyma. Lysed AMs and injured parenchymal cells may be the source of the increased LDH activity observed in the BALF of treated mice. LDH is a cytoplasmic enzyme normally not found in BALF. By day 14, an increase in total protein was detected in BALF, indicating an increase in alveolar membrane permeability. Injury to the type I epithelial cells that line the alveoli can result in loss of tight junctions and the transudation of serum proteins into the alveoli [26]. All of these indices of lung inflammation continued to increase for up to 28 days after InP aspiration, suggesting that InP was not rapidly cleared from the lung and that parenchymal injury was ongoing. The half-life of InP in the lung during repeated inhalation exposure was reported to be about 150 days [15].

In contrast to the immediate changes measured in the BALF of treated mice, there were no significant changes in PLF toxicity parameters until day 28, at which time the number of cells in the pleural space increased dramatically by 3.7-fold. This delayed pleural effusion following InP treatment was noteworthy because exudative pleural effusions have been reported to precede pleural fibrosis [27–31]. Although inflammation in the lung parenchyma is commonly observed in response to insoluble particulates, the delayed pleural effusion and subsequent pleural fibrosis have only been reported for insoluble fibers. It is possible that pleural effects may have been present in other studies of nonfibrous particulates, but they were overlooked or missed due to their delayed appearance.

The delayed, exudative pleural effusion following InP aspiration was also characterized by significant increases in cytokines and chemokines in the PLF. Although many different cytokines were increased in the PLF of InP-treated mice, the greatest increases were observed in cytokines associated with tissue remodeling and repair. The concentrations of fibrinogen were increased 24-fold in the PLF of InP-treated animals. Fibrinogen plays an important role during the initial process of wound healing by stimulating the formation of a fibrin matrix over the injured area. Continuous injury to the pleura by InP and prolonged release of fibrinogen may result in an abnormal wound healing process and pleural fibrosis. Abnormal fibrin turnover has been reported to be involved in pleural fibrosis [31,32]. Other up-regulated PLF cytokines were MMP-9 (20-fold increase) and TIMP-1 (13-fold increase), both of which are involved in maintenance of the extracellular matrix. MMP-9 is a metalloproteinase that breaks down specific protein components of the extracellular matrix. The activity of MMP-9 is controlled by specific tissue inhibitors (TIMPs), including TIMP-1 [33]. Disruption of the balance between metalloproteinases and their TIMPs has been implicated in pleural effusions and pleural thickening [34]. Surprisingly, transforming growth factor-1 β (TGF-1 β) was not elevated in the PLF. TGF-1 β is a profibrotic growth factor central to the predominant pathway leading to fibrosis [28,35]. TGF-1 β may have been elevated at time points later than day 28, but unfortunately PLF was not available at these time points. TGF-1 β was not assayed in the BALF and is an area of future investigation.

Multifocal areas of visceral pleural inflammation were associated with the increased cytokine levels in PLF at day 28. Pleural fibrosis developed after the effusion and was detected by 98 days after InP aspiration. Chronic injury to the pleura and/or an abnormal repair response to InP-induced damage may contribute to the progression of pleural thickening to pleural fibrosis. Additional studies are planned to investigate more thoroughly changes in PLF mediators that occur as the parenchymal inflammation resolves and pleural thickening progresses to fibrosis. These studies may help to identify the primary mediators involved in the development of pleural fibrosis.

Asbestos fibers are thought to directly induce pleural effects after translocation from the alveoli [36]; however, it is not known if InP particles are translocated to the pleura after oropharyngeal aspiration. The distinct black InP particles could not be detected microscopically in the pleural membrane, the free pleural cells or in the PLF after oropharyngeal aspiration. It is possible that indium may have been present in a soluble form that could not be detected microscopically. In addition, indium may have been present in PLF, but at levels below the limit of quantification of the ICP-MS method (0.25 ng/mL). Interestingly, InP particles injected directly into the pleural cavity did not cause a pleural effusion or pleural pathology. The InP particles were visible in the pleural cavity for up to 35 days after injection with no toxic effects. These results suggest that pleural effusion and fibrosis are not caused by InP particles that have been translocated to the pleural cavity.

Although InP particles had no effect when injected into the pleural cavity, an immediate pleural effusion was observed after intrapleural injection of InCl₃, a soluble form of indium. These results suggest that a soluble ionic form of indium may be responsible for the toxicity observed in the parenchyma as well as in the pleura. InP particles have been shown to be insoluble in saline and synthetic lung fluid [2,37] and therefore are unlikely to be soluble in lung lining fluid or pleural fluid of mice. However, alveolar macrophages (AMs) are present in large numbers in the lung and are highly efficient in digesting particulate matter [38]. Dissolution of InP particles in the lysosomes of activated AMs followed by release of a reactive form of InP by exocytosis, or after AM necrosis, may be the mechanism for damage to lung parenchymal cells. In vitro studies are being conducted to evaluate this potential mechanism.

The lack of pleural effects after intrapleural injection of InP particles suggests that pleural macrophages may be less able to phagocytize and/or dissolve InP particles than AMs. Alveolar macrophages have been reported to exert stronger phagocytic and antibactericidal activities than pleural macrophages [39]. The potential differences in the responses of alveolar and pleural macrophages to InP particles are being evaluated. The pleural effects of InP may be due to a reactive form of InP formed in the parenchyma and cleared to the pleura by inflammatory cells, or via the lymph. Although we were unable to detect indium in the pleura, we cannot exclude the possibility that indium was present but at levels below the method detection limit.

Alternatively, the pleural effects of InP may be caused by inflammatory mediators passively transported from the injured parenchyma. Because of its close proximity to the lung, the pleura is prone to react to inflammatory events occurring in the lung parenchyma [40,41]. InP caused a significant inflammatory response in the lung with the increased release of a large number of cytokines and chemokines. Many of the same cytokines that were elevated in the BALF were also increased in the PLF, suggesting that these mediators could have been passively transferred to the pleural space resulting in the pleural lesions.

Compounds with different chemical and physical attributes were administered to mice by aspiration at similar doses, and the effects on BALF and PLF cell numbers were evaluated. Of the compounds tested, only InP, InCl₃, and to a lesser extent CdCl₂ caused a pleural effusion

in mice. The pleural effusion caused by InCl_3 was present at day 3 and day 28, whereas the pleural effusions caused by InP and CdCl_2 did not appear until day 28. The early effect of InCl_3 is likely due to its high solubility. Although CdCl_2 is also a soluble metal salt, it had no effect on pleural cell numbers at day 3, and caused a small but statistically significant increase on day 28. The biological significance of the pleural effects of CdCl_2 is questionable because of its significant acute pulmonary toxicity. Two of 5 CdCl_2 -treated mice died shortly after treatment, and the BALF and PLF cell counts from the 3 surviving CdCl_2 -treated mice were contaminated with blood. Also, CdCl_2 caused only about a 1.4-fold increase in pleural cells at day 28, whereas InCl_3 and InP caused 5.4- and 3.3-fold (compared to 3.7-fold demonstrated in the time course study) increases in cell numbers, respectively. Interestingly, other soluble compounds such as CuCl_2 and NiCl_2 caused large increases in cell numbers in the lung; yet neither chemical caused a pleural effusion. V_2O_5 , a soluble metal oxide, and amosite, an insoluble asbestos fiber, had only transient effects on BALF cell numbers and no effect on PLF cell numbers. The amosite used in this study was previously shown to cause mesothelioma in rats [18] and was therefore tested as a positive control in this study. The lack of amosite-induced changes in PLF cell numbers is most likely a result of the dose given or the relatively short duration of the study. TiO_2 , a relatively inert, poorly soluble particle, had no effects on lung or pleural cell numbers. Although the ability of chemicals to affect the BALF cell numbers is influenced by chemical (solubility, reactivity) and physical (particle, fiber) attributes, the ability to cause a pleural effusion appears to be more complex. These limited data suggest that pleural effusions observed after oropharyngeal aspiration and 28-day duration may be unique to InP and other indium compounds.

In summary, oropharyngeal aspiration of InP particles caused an initial inflammatory response in the lung parenchyma followed by a delayed exudative pleural effusion and the development of pleural fibrosis. These pleural effects are normally associated with pulmonary exposure to fibrous particulates. Pleural lesions developed much faster after a single oropharyngeal aspiration of InP than after inhalation exposure. Out of 12 chemicals administered by oropharyngeal aspiration model, only InP , InCl_3 and possibly CdCl_2 caused significant pleural effusions in mice, suggesting that indium compounds may act through a unique mechanism. Additional studies using this aspiration model are in progress to further characterize the mechanism of InP -induced pleural fibrosis. Studies with InP are being conducted to more clearly identify the potential role of pulmonary inflammation in the development of the pleural effusion, and to better characterize the gap between day 28 when the pleural effusion first forms and day 98 when pleural fibrosis is first evident. This model is also being used to evaluate the ability of other indium compounds to cause pleural fibrosis. In vitro studies are in progress to evaluate InP particle dissolution by alveolar macrophages, and to identify the reactive component(s).

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References

1. ACGIH. Threshold Limit Values for Chemical Substances and Physical Agents, and Biological Exposure Indices. Cincinnati, OH: American Conference of Governmental Industrial Hygienists; 2007. p. 35
2. Kabe I, et al. In vitro solubility and in vivo toxicity of indium phosphide. *J Occup Health* 1996;38:6–12.
3. Oda K. Toxicity of a low level of indium phosphide (InP) in rats after intratracheal instillation. *Ind Health* 1997;35(1):61–68. [PubMed: 9009503]

4. Uemura T, et al. Effects of intratracheally administered indium phosphide on male Fischer 344 rats. *J Occup Health* 1997;39:205–210.
5. Omura M, et al. Testicular toxicity of gallium arsenide, indium arsenide, and arsenic oxide in rats by repetitive intratracheal instillation. *Fundam Appl Toxicol* 1996;32:72–78. [PubMed: 8812231]
6. Tanaka A, et al. Chronic toxicity of indium arsenide and indium phosphide to the lungs of hamsters. *Fukuoka Igaku Zasshi* 1996;87:108–115. [PubMed: 8690334]
7. Tanaka A, et al. Comparative study of the toxic effects of gallium arsenide, indium arsenide and arsenic trioxide following intratracheal instillations to the lung of Syrian golden hamsters. *Fukuoka Igaku Zasshi* 2000;91:21–33. [PubMed: 10714013]
8. Morgan DL, et al. Acute pulmonary toxicity of copper gallium diselenide, copper indium diselenide, and cadmium telluride intratracheally instilled into rats. *Environ Res* 1995;71:16–24. [PubMed: 8757234]
9. Tanaka A, et al. Pulmonary toxicity of indium-tin oxide and indium phosphide after intratracheal instillations into the lung of hamsters. *J Occup Health* 2002;44:99–102.
10. Chonan T, Taguchi O, Omae K. Interstitial pulmonary disorders in indium-processing workers. *Eur Respir J* 2007;29:317–324. [PubMed: 17050566]
11. Homma T, et al. Interstitial pneumonia developed in a worker dealing with particles containing indium-tin oxide. *J Occup Health* 2003;45:137–139. [PubMed: 14646287]
12. Homma S, et al. Pulmonary fibrosis in an individual occupationally exposed to inhaled indium-tin oxide. *Eur Respir J* 2005;25:200–204. [PubMed: 15640342]
13. Hamaguchi T, et al. Exposure to hardly soluble indium compounds in ITO production and recycling plants is a new risk for interstitial lung damage. *Occup Environ Med* 2008;65:51–55. [PubMed: 17626138]
14. Taguchi O, Chonan T. Three cases of indium lung. *Nihon Kokyuki Gakkai Zasshi* 2006;44:532–536. [PubMed: 16886812]
15. NTP. National Toxicology Program Technical Report Series. 2001. Toxicology and carcinogenesis studies of indium phosphide (CAS no. 22398–90-7) in F344/N rats and B6C3F1 mice (inhalation studies); p. 7-340.
16. McNulty JC. Malignant pleural mesothelioma in an asbestos worker. *Med J Aust* 1962;49:953–954. [PubMed: 13932248]
17. Wagner JC, Sleggs CA, Marchand P. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. *Br J Ind Med* 1960;17:260–271. [PubMed: 13782506]
18. Hesterberg TW, et al. Studies on the inhalation toxicology of two fibreglasses and amosite asbestos in the syrian golden hamster. Part I. Results of a subchronic study and dose selection for a chronic study. *Inhal Toxicol* 1999;11:747–784. [PubMed: 10477658]
19. Foster WM, et al. Methodology for the measurement of mucociliary function in the mouse by scintigraphy. *J Appl Physiol* 2001;90:1111–1117. [PubMed: 11181627]
20. Card JW, et al. Gender differences in murine airway responsiveness and lipopolysaccharide-induced inflammation. *J Immunol* 2006;177:621–630. [PubMed: 16785560]
21. Card JW, et al. Cyclooxygenase-2 deficiency exacerbates bleomycin-induced lung dysfunction but not fibrosis. *Am J Respir Cell Mol Biol* 2007;37:300–308. [PubMed: 17496151]
22. Kirby PK, et al. Pleural effects of indium phosphide in B6C3F1 mice: nonfibrous particulate induced pleural fibrosis. *Exp Lung Res*. 2009 in press.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
24. Noppen M. Normal volume and cellular contents of pleural fluid. *Curr Opin Pulm Med* 2001;7:180–182. [PubMed: 11470970]
25. Noppen M. Normal volume and cellular contents of pleural fluid. *Paediatr Respir Rev* 2004;5(Suppl A):S201–S203. [PubMed: 14980271]
26. Henderson, RF. Bronchoalveolar lavage: a tool for assessing the health status of the lung. In: McClellam, RO.; Henderson, RF., editors. *Concepts in Inhalation Toxicology*. New York: Hemisphere; 1989. p. 415-444.

27. Stephens M, et al. Asbestos induced diffuse pleural fibrosis: pathology and mineralogy. *Thorax* 1987;42:583–588. [PubMed: 3660310]
28. Huggins JT, Sahn SA. Causes and management of pleural fibrosis. *Respirology* 2004;9:441–447. [PubMed: 15612954]
29. Jantz MA, Antony VB. Pleural fibrosis. *Clin Chest Med* 2006;27:181–191. [PubMed: 16716812]
30. Mutsaers SE, et al. Growth factors in pleural fibrosis. *Curr Opin Pulm Med* 2006;12:251–258. [PubMed: 16825876]
31. Mutsaers SE, et al. Pathogenesis of pleural fibrosis. *Respirology* 2004;9:428–440. [PubMed: 15612953]
32. Jantz MA, Antony VB. Pathophysiology of the pleura. *Respiration* 2008;75:121–133. [PubMed: 18332619]
33. Gomez DE, et al. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur J Cell Biol* 1997;74:111–122. [PubMed: 9352216]
34. Iglesias D, et al. Metalloproteinases and tissue inhibitors of metalloproteinases in exudative pleural effusions. *Eur Respir J* 2005;25:104–109. [PubMed: 15640330]
35. Decolonne N, et al. TGF-beta1 induces progressive pleural scarring and subpleural fibrosis. *J Immunol* 2007;179:6043–6051. [PubMed: 17947678]
36. Davis JM, Jones AD. Comparisons of the pathogenicity of long and short fibres of chrysotile asbestos in rats. *Br J Exp Pathol* 1988;69:717–737. [PubMed: 2848570]
37. Dittmar TB, et al. Surface concentrations of indium, phosphorus, and oxygen in indium phosphide single crystals after exposure to Gamble solution. *Anal Chem* 1992;64:2929–2933.
38. Galle P, Berry JP, Galle C. Role of alveolar macrophages in precipitation of mineral elements inhaled as soluble aerosols. *Environ Health Perspect* 1992;97:145–147. [PubMed: 1396450]
39. Gjomarkaj M, et al. Phenotypic and functional characterization of normal rat pleural macrophages in comparison with autologous peritoneal and alveolar macrophages. *Am J Respir Cell Mol Biol* 1999;20:135–142. [PubMed: 9870927]
40. Antony VB. Immunological mechanisms in pleural disease. *Eur Respir J* 2003;21:539–544. [PubMed: 12662014]
41. Quintana HK, et al. Identification with MRI of the pleura as a major site of the acute inflammatory effects induced by ovalbumin and endotoxin challenge in the airways of the rat. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L651–L657. [PubMed: 16728527]

FIGURE 1.

Total protein in lung and pleural lavage fluid. Bronchoalveolar lavage (BALF) and pleural lavage fluids (PLF) were collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg), and were analyzed for total protein. Values represent means from InP-treated mice expressed as percent of saline-treated controls, $n = 4-5$. ^aSignificantly different from saline-treated controls ($P < .05$).

FIGURE 2.

LDH activity in lung and pleural lavage fluid. Bronchoalveolar lavage (BALF) and pleural lavage fluids (PLF) were collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg), and were analyzed for lactate dehydrogenase (LDH) activity. Values represent means from InP-treated mice expressed as percent of saline-treated controls, $n = 4-5$. ^aSignificantly different from saline-treated controls ($P < .05$).

FIGURE 3.

Total cells in lung and pleural lavage fluid. Bronchoalveolar lavage (BALF) and pleural lavage fluids (PLF) were collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg), and were analyzed for total cell numbers. Values represent means from InP-treated mice expressed as percent of saline-treated controls, $n = 4-5$. ^aSignificantly different from saline-treated controls ($P < .05$).

FIGURE 4.

Progression of pleural lesions after InP (1 mg/kg) aspiration. (A) Day 28 normal appearing lungs of saline-treated control animal (10×). (B) Day 7 InP. Intra-alveolar accumulation of proteinaceous exudate with increased alveolar macrophages some of which contain InP particles (*small arrow*). Pleocellular inflammatory infiltrate in peribronchiolar interstitial region (*large arrow*) (40×). (C) Day 14 InP. Mild multifocal areas of inflammatory cell infiltrates forming septal thickening (*small arrows*) with associated mild alveolar epithelial cell hypertrophy and proliferation (*large arrows*) (20×). (D) Day 28 InP. The multifocal areas of inflammation have progressed to moderate and severe with associated alveolar epithelial cell proliferation (*small arrow*). Pleocellular inflammation is also present (*large arrow*) (20×).

(E) Day 28 InP. Multifocal areas of pleura are thickened with inflammatory cells and overlying mesothelial cell hypertrophy and hyperplasia (*small arrows*) (20×). (F) Day 98 InP. Perivascular inflammation (*small arrows*) is prominent with extensive lymphoid proliferation (*large arrow*). Multifocal regions of inflammation have progressed to severe with associated proliferative pneumonia (*) (10×).

FIGURE 5.

Pleural thickening at day 98 after InP (1 mg/kg) aspiration. (A) Normal pleura of saline-treated animal (large arrow) (H&E stain, 10 $\times$). (B) Visceral pleura of InP-treated animals are characterized by extensive areas of fibrotic thickening (*large arrow*) (H&E, 10 $\times$). (C) Masson's trichrome staining demonstrated no blue fibrotic tissue in the control pleural membrane (10 $\times$). (D) Masson's trichrome staining demonstrates extensive blue fibrotic tissue in pleural membrane of InP-treated mice (*large arrow*) (10 $\times$).

FIGURE 6.

Effects of chemicals on bronchoalveolar lavage fluid cell numbers. Bronchoalveolar lavage fluid (BALF) was collected 3 and 28 days after oropharyngeal aspiration of chemical (1 mg/kg) or saline (control) and was analyzed for total cell numbers. Values represent means from chemical-treated mice expressed as percent of saline-treated controls, $n = 4-5$. ^aSignificantly different from saline-treated controls ($P < .05$).

FIGURE 7.

Effects of chemicals on pleural lavage fluid cell numbers. Pleural lavage fluid (PLF) was collected 3 and 28 days after oropharyngeal aspiration of chemical (1 mg/kg) or saline (control) and was analyzed for total cell numbers. Values represent means from chemical-treated mice expressed as percent of saline-treated controls, $n = 4-5$. ^aSignificantly different from saline-treated controls ($P < .05$).

TABLE 1

Bronchoalveolar Lavage Cell Differentials

Cell type	Treatment	Days after treatment			
		1	3	14	28
Macrophage	Saline	4.12 ± 0.61(95%)	3.37 ± 0.51(99.4%)	4.14 ± 0.84(99.6%)	3.21 ± 0.32(98.4%)
	InP	1.86 ± 0.24 ^a (59%)	1.28 ± 0.27 ^a (35.3%)	1.86 ± 0.35(39.5%)	6.10 ± 1.40(57.2%)
PMN	Saline	0.20 ± 0.08(4.5%)	0.01 ± 0.01(0.4%)	0.02 ± 0.03(0.4%)	0.04 ± 0.02(1.4%)
	InP	1.28 ± 0.36 ^a (40%)	2.30 ± 0.37 ^a (63.5%)	1.31 ± 0.14(27.8%)	3.80 ± 0.80 ^a (35.8%)
Lymphocyte	Saline	0.02 ± 0.01(0.5%)	0.01 ± 0.01(0.2%)	0.00 ± 0.00(0.0%)	0.01 ± 0.01(0.2%)
	InP	0.02 ± 0.01(1%)	0.04 ± 0.10(1.2%)	1.54 ± 0.16 ^a (32.7%)	0.74 ± 0.16 ^a (7%)
Total cells	Saline	4.34 ± 0.52	3.39 ± 0.51	4.16 ± 0.83	3.26 ± 0.38
	InP	3.16 ± 0.35	3.62 ± 0.37	4.71 ± 0.94	10.64 ± 1.87 ^a

Note. Bronchoalveolar lavage fluids were collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg) and were analyzed for total cell numbers and cell type. Values represent means ($\times 10^5$) $\pm$ SEM, $n = 4-5$. Percentage of total cell number is in parentheses.

^aSignificantly different from controls ($P < .05$).

TABLE 2

Pleural Lavage Cell Differentials

Cell type	Treatment	Days after treatment			
		1	3	14	28
Macrophage	Saline	0.95 ± 0.15(25%)	0.59 ± 0.22(14%)	0.56 ± 0.03(14%)	0.50 ± 0.07(12%)
	InP	0.84 ± 0.17(25%)	0.66 ± 0.07(16%)	1.02 ± 0.22(18%)	1.88 ± 0.40 ^a (12%)
Eosinophil	Saline	0.42 ± 0.07(11%)	0.56 ± 0.06(14%)	0.31 ± 0.03(8%)	0.50 ± 0.07(10%)
	InP	0.55 ± 0.05(16%)	0.63 ± 0.06(16%)	0.54 ± 0.14(10%)	1.97 ± 0.43 ^a (13%)
Lymphocyte	Saline	2.30 ± 0.42(61%)	2.86 ± 0.19(71%)	2.91 ± 0.13(76%)	3.10 ± 0.25(77%)
	InP	1.91 ± 0.29(57%)	2.64 ± 0.30(66%)	3.93 ± 0.39(71%)	11.49 ± 2.43 ^a (74%)
Mast cell	Saline	0.08 ± 0.02(2%)	0.04 ± 0.01(1%)	0.07 ± 0.02(2%)	0.02 ± 0.01(1%)
	InP	0.05 ± 0.02(1%)	0.07 ± 0.02(2%)	0.06 ± 0.01(1%)	0.12 ± 0.03 ^a (1%)
Total cells	Saline	3.75 ± 0.56	4.05 ± 0.20	3.85 ± 0.14	4.12 ± 0.34
	InP	3.35 ± 0.49	4.00 ± 0.30	5.55 ± 0.54	15.40 ± 3.25 ^a

Note. Pleural lavage fluids were collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg) and were analyzed for total cell numbers and cell type. Values represent means ($\times 10^6$) $\pm$ SEM, $n = 4-5$. Percentage of total cell number is in parentheses.

^aSignificantly different from controls ($P < .05$).

TABLE 3

Cytokine Ratios in Bronchoalveolar Lavage Fluid of InP-Treated Mice

Analyte	Days after treatment			
	1	3	14	28
Apo A1	1.2	1.7	3.6 ^a	4.9 ^a
CD40	1.3	1.9	17.2 ^a	36.3 ^a
Factor VII	2.0 ^a	3.4 ^a	2.9 ^a	3.1 ^a
FGF-9	1.2	0.9	1.0	2.4 ^a
FGF-basic	1.9	2.8 ^a	2.9 ^a	4.2 ^a
Fibrinogen	1.1	1.4	9.8	44.3 ^a
GCP-2	4.3 ^a	6.6 ^a	5.7 ^a	6.5 ^a
GM-CSF	5.9 ^a	7.0 ^a	10.0 ^a	8.0 ^a
GST- α	1.2	0.7	0.0 ^a	0.0 ^a
Haptoglobin	1.2	1.3	2.0 ^a	2.2 ^a
IgA	1.0	1.6	14.5	74.2 ^a
IL-10	1.1	0.9	1.1	2.1 ^a
IL-11	1.3	1.1	1.5	3.1 ^a
IL-18	3.2 ^a	6.2 ^a	2.8 ^a	3.1 ^a
IL-1 α	2.0	1.9	2.1	5.2 ^a
IL-6	8.4 ^a	2.7	4.3	18.8 ^a
IP-10	1.5	1.7	9.4	50.1 ^a
KC/GRO α	(0.5) ^c	(0.5) ^c	(0.4) ^c	(1.0) ^c
LIF	1.2	1.5	1.4	3.0 ^a
Lymphotoxin	1.1	1.0	1.8	4.4 ^a
MCP-1	2.3	8.8	71.3	453.2 ^a
MCP-3	1.7	5.7	65.5	352.6 ^a
MCP-5	1.2	1.3	13.9 ^a	114.2 ^a
M-CSF	3.5	8.1 ^a	12.2 ^a	13.4 ^a
MDC	1.5	4.0	5.9 ^a	10.8 ^a
MIP-1 α	1.8	2.7	2.5	8.9 ^a
MIP-1 β	1.7	2.3	3.9	21.5 ^a
MIP-1 γ	9.9	49.3 ^a	24.8	97.8 ^a
MIP-2	5.4	4.3	4.4	14.2 ^a
MMP-9	16.0	31.5	51.5	545.9 ^a
OSM	1.2	1.1	1.7	3.7 ^a
RANTES	1.6	1.6	3.6	10.4 ^a
SCF	1.1	0.9	1.3	2.6 ^a

Analyte	Days after treatment			
	1	3	14	28
TIMP-1	5.5	6.3	23.3	57.9 ^a
Tissue Factor	2.3	4.3 ^a	4.2 ^a	5.8 ^a
TNF- α	1.3	1.2	1.5	3.3 ^a
TPO	2.2 ^a	3.1 ^a	3.2 ^a	4.0 ^a
VCAM-1	1.9	3.2	10.1 ^a	17.6 ^a
VEGF	2.0	4.1 ^a	13.1 ^a	7.9 ^a
vWF	1.5	1.8	3.3 ^a	4.3 ^a

Note. Bronchoalveolar lavage fluid (BALF) was collected at various times after oropharyngeal aspiration of saline (control) or InP (1 mg/kg) and was analyzed for cytokines.

^a Significantly different from mean control value at respective time point.

^b Values represent the ratio of cytokine levels in InP-treated: controls (mean $\pm$ SEM, $n = 4-5$).

^c KC/GRO α was not present in control BALF so mean values for treated mice are shown.

TABLE 4

Day 28 Cytokine Ratios in Pleural Lavage Fluid

Analyte	Units	Control	InP	Ratio ^c
CD40	pg/mL	4.220 ± 0.492 ^b	8.656 ± 1.548 ^a	2.0
Clusterin	μg/mL	0.345 ± 0.054	1.953 ± 0.593 ^a	5.7
Cystatin-C	ng/mL	21.280 ± 2.670	50.520 ± 12.743	2.4
Fibrinogen	μg/mL	4.722 ± 1.252	111.944 ± 45.135 ^a	23.7
GCP-2	ng/mL	0.005 ± 0.0004	0.017 ± 0.008	3.0
Haptoglobin	μg/mL	0.542 ± 0.024	1.959 ± 0.572 ^a	3.6
IgA	μg/mL	0.405 ± 0.028	1.370 ± 0.381 ^a	3.4
IL-6	pg/mL	3.142 ± 0.237	9.940 ± 3.405	3.2
MCP-1	pg/mL	6.682 ± 0.291	17.046 ± 7.574	2.6
MCP-3	pg/mL	12.580 ± 0.463	25.540 ± 10.964	2.0
M-CSF	ng/mL	0.040 ± 0.004	0.080 ± 0.012 ^a	2.0
MIP-1 γ	ng/mL	0.136 ± 0.013	0.674 ± 0.187 ^a	4.9
MMP-9	ng/mL	0.230 ± 0.012	4.707 ± 1.832 ^a	20.4
MPO	ng/mL	0.128 ± 0.038	5.224 ± 3.171	40.9
Osteopontin	ng/mL	0.934 ± 0.196	4.568 ± 1.373 ^a	4.9
SAP	μg/mL	0.084 ± 0.004	0.233 ± 0.045 ^a	2.8
TIMP-1	ng/mL	0.121 ± 0.008	1.583 ± 0.482 ^a	13.1
VCAM-1	ng/mL	3.610 ± 0.280	9.522 ± 1.604 ^a	2.6
vWF	ng/mL	0.443 ± 0.028	1.394 ± 0.369 ^a	3.2
IL-1 α	pg/mL	16.120 ± 0.398	8.672 ± 1.346 ^a	0.5

Note. Changes in cytokine levels in pleural lavage fluid were measured 28 days after oropharyngeal aspiration of saline (control) or InP (1 mg/kg).

^a Significantly different from controls ($P < .05$).

^b Values represent means ± SEM, $n = 4-5$.

^c Values expressed as ratio of InP-treated:control. Only cytokines with an InP:saline ratio of ≥ 2.0 and a P value $< .05$ are listed.

TABLE 5

Pleural Lavage Fluid indices of Toxicity After Intrapleural Injections

Treatment	End point	Days after intrapleural injection	
		3	28
Saline	Total protein (mg/mL)	0.31 ± 0.05	0.17 ± 0.02
	LDH (units)	45.90 ± 3.37 ^a	42.18 ± 9.61
	Cell number (×10 ⁶)	5.68 ± 0.88	3.73 ± 0.27
InP	Total protein (mg/mL)	0.29 ± 0.04	0.33 ± 0.06 ^a
	LDH (units)	70.74 ± 7.08 ^a	52.03 ± 7.66
	Cell number (×10 ⁶)	5.21 ± 1.01	5.79 ± 1.18
InCl ₃	Total protein (mg/mL)	1.16 ± 0.17 ^a	0.21 ± 0.05
	LDH (units)	105.66 ± 10.30 ^a	30.01 ± 6.90
	Cell number (×10 ⁶)	16.51 ± 3.95 ^a	3.32 ± 1.22

Note. Pleural lavage fluid was collected 3 and 28 days after intrapleural injection of saline (control), indium trichloride (InCl₃), or indium phosphide (InP) and was evaluated for changes in total protein, lactate dehydrogenase (LDH) activity, and total cell numbers.

^aSignificantly different from controls ($P < .05$).

^bValues represent means ± SEM, $n = 4-5$.