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Mycobacterium immunogenum Causes Hypersensitivity Pneumonitis-Like Pathology in Mice

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A surprising number of cases of hypersensitivity pneumonitis have been observed at work sites employing automotive machinists. Because hypersensitivity pneumonitis is not typically associated with exposure to metalworking fluid aerosols, this study examined whether *Mycobacterium immunogenum* (*M. immunogenum*), a rapidly growing mycobacterium isolated from several affected work sites, could induce hypersensitivity pneumonitis in mice. Hypersensitivity pneumonitis-like histologic changes occurred in mice treated with heat-killed and lysed *M. immunogenum*. These lung lesions were characterized by peribronchial and perivascular lymphohistiocytic inflammation and noncaseating granulomas in the parenchyma. The pathologic changes observed in mice instilled with *M. immunogenum*-contaminated used metalworking fluid were indistinguishable from those observed with *M. immunogenum* alone. The role of genetic factors in *M. immunogenum*-induced lung lesions was examined by comparison of the response of eight inbred strains of mice. The observed immunologic changes in the lung were significantly greater in C57Bl/6, 129, and BALB/*c* mice than in the other strains, suggesting that genetic factor(s) contribute to the susceptibility of workers exposed to *M. immunogenum*-contaminated metalworking fluid aerosols. Thus, these studies provide indirect evidence that *M. immunogenum* is an unrecognized class of microorganisms capable of causing hypersensitivity pneumonitis and plays a role in the outbreaks of hypersensitivity pneumonitis in automotive plants.

Hypersensitivity pneumonitis has recently been linked to metalworking fluid aerosol exposure in automotive machinists (Beckett et al., 2005; Bernstein et al., 1995; CDC, 1996; Fox et al., 1999; Kreiss & Cox-Ganser, 1997; Zacharisen et al., 1998). In the cases reported by Bernstein and colleagues, several microbes were isolated from the metalworking fluid and precipitin tests demonstrated that antibodies to a number of microbes were present in the serum of affected workers (Bernstein et al., 1995). However, the causative antigen was not identified. At least eight outbreaks of hypersensitivity pneumonitis have been identified in automotive machining plants in North America (CDC, 1996; Hodgson et al., 2001; Kreiss & Cox-Ganser, 1997; Trout et al., 2003; Zacharisen et al., 1998). Initial findings regarding these hypersensitivity pneumonitis outbreaks were reported in the workshop summary of a joint United Automobile Workers/Chrysler meeting (January 1997) and reviewed by Kreiss and Cox-Ganser (1997) at an American Automobile Manufacturers Association meeting (September 1997).

Hypersensitivity pneumonitis (also known as extrinsic allergic alveolitis) is an interstitial lung disease that is typically associated with a hypersensitive immune response to inhaled antigenic agents. Microbial agents and animal protein are usually the underlying cause of hypersensitivity pneumonitis, although chemical agents such as toluene diisocyanate have been associated with this disease (Charles et al., 1976). The microbes cultured from most "in use" metalworking fluids (Mattsby-Baltzer

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et al., 1989) have not typically been associated with the induction of hypersensitivity pneumonitis. Hypersensitivity pneumonitis has been associated with occupational exposure to microbes such as *Thermoactinomyces* species (Salvaggio, 1991), but these microbes are rarely reported in metalworking fluids.

Strong evidence has been presented suggesting that *Mycobacterium immunogenum* and the closely related *Mycobacterium chelonae*, mycobacteria that are not typically considered to be important human pathogens except in immune-compromised individuals, may play a key role in the production of hypersensitivity pneumonitis in workers exposed to contaminated metalworking fluid aerosols. First, *M. immunogenum* or *M. chelonae* was isolated from the metalworking fluid storage tanks at half of the affected work sites (Kreiss & Cox-Ganser, 1997), and they have since been identified in numerous other samples (Wallace et al., 2002; Selvaraju et al., 2005) are highly associated with the use of triazines as a biocide (Watt, 2003). In addition, serum antibodies to *M. immunogenum* were more prevalent in exposed workers than in nonexposed control subjects (Kreiss & Cox-Ganser, 1997). These findings, however, are not sufficient proof for establishing causality of hypersensitivity pneumonitis by the *M. immunogenum* fraction of metalworking fluid aerosols (Shelton et al., 1999).

The present study examined whether *M. immunogenum* could produce histological evidence of hypersensitivity pneumonitis in a mouse model. Using an established mouse model of hypersensitivity pneumonitis (Donnelly et al., 1996; Perez Zrellano et al., 1992), mice were treated repeatedly by tracheal instillation with *M. immunogenum* that had been isolated from a metalworking fluid sample taken at an automotive plant in which excess cases of hypersensitivity pneumonitis occurred.

METHODS

Male mice (21–32 g; C57Bl/6, 129/Sv, BALB/*c*, C57BL/10, AKR, DBA/2, C3H/HeJ, and MRL/Mp strains; Jackson Laboratory, Bar Harbor, ME) were fed standard rodent chow (Purina, St. Louis, MO) and water ad libitum and housed in polycarbonate cages with corn-cob bedding. Animal rooms were maintained at 20 to 23°C with a 12-h light/dark cycle.

In the first experiment, C57Bl/6 mice were instilled twice a week for 3 wk with sterile, injectable water ($n = 8$, Abbott Laboratories, North Chicago, IL), *Mycobacterium immunogenum* (180 μg protein/instillation, $n = 12$), used metalworking fluid ($n = 8$), and unused metalworking fluid ($n = 7$). The used semisynthetic metalworking fluid sample was composed of 95% water and 5% metalworking fluid and diluted 1:8 with sterile, pyrogen-free water before treatment. The unused semisynthetic metalworking fluid was the same formulation and diluted to 5% with 95% sterile water and then diluted 1:8 in a manner identical to that used for the used metalworking fluid. The used metalworking fluid sample was collected from a storage tank located at a machining operation where cases of physician-diagnosed cases of hypersensitivity pneumonitis were reported. The used sample had 3.3×10^6 colony-forming units (CFU)/ml bacteria (predominantly *M. immunogenum*), no measurable endotoxin, and no detectable CFU/ml of fungi. *M. immunogenum* isolated from a metalworking fluid sample (discussed later) was grown in Middlebrook 7H10 broth containing AODC supplements for 4 to 6 wk; the bacterial suspension was harvested by centrifugation, washed once and resuspended in sterile, pyrogen-free saline. The bacterial suspension was processed in an all glass tissue grinder to make a finely divided suspension. The *M. immunogenum* instillate contained approximately 5×10^9 CFU/ml and was heat-pasteurized at 60°C for 30 min and lysed by freeze/thawing. To insure that any observed effects were not due to living, and thus potentially infectious, microbes within the used metalworking fluid, four additional mice were instilled with heat-pasteurized used metalworking fluid.

Isolates of *Mycobacterium* were obtained from a contract microbiology laboratory that performed the initial isolation and identification of microbial contaminants in bulk metalworking fluid samples. Six isolates of *M. chelonae* identified according to standard microbiological techniques were recovered from the metalworking fluid samples, and represented the predominant microbial contaminant found in the metalworking fluid bulk samples. The identity of the microbial isolates was confirmed by a second commercial laboratory. The isolates came from separate metalworking fluid samples but were otherwise identical and were assumed to be *M. chelonae* from a common source. More recent studies have identified a rapidly growing mycobacterial species in metalworking fluid that appears to be closely related to *M. chelonae* but is a separate species (Bernstein et al., 1995; Moore et al., 2000; Wilson et al., 2001). This newly recognized species, *M. immunogenum*, differs only slightly from *M. chelonae* with respect to biochemical and growth characteristic (CDC, 1996), and it is likely that the *M. chelonae* isolate used in this study is this new recognized species. Greater than 90% of the mycobacterial isolates from metalworking fluid have been identified as *M. immunogenum* when the appropriate biochemical or molecular (polymerase chain reaction, PCR) assays were performed (Selvaraju et al., 2005; Wallace et al., 2002).

With the exception of recovery mice, animals were sacrificed 4 days after the final instillation. To examine recovery, mice were sacrificed at 1 mo ($n = 1$ for water and $n = 3$ for *M. immunogenum*) and 2 mo ($n = 1$ for water and $n = 3$ for *M. immunogenum*) after the final instillation.

Prior to instillation, mice were rapidly anesthetized using isofluorane (Abbott Laboratories, King of Prussia, PA). Once the desired anesthetized state was reached, mice were placed on a restraining stand angled at 45–55°. A modified laryngoscope was maneuvered into the mouth and a modified hypodermic needle was slowly guided through the epiglottis to the carina. The needle was withdrawn 3 to 4 mm from the carina and the instillate rapidly dispensed as a bolus.

Mice were euthanized with pentobarbital (260 mg/kg). The lungs were fixed with 2% gluteraldehyde, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). A blinded, reproducible assessment and grading of all

lung sections was performed with severity of each lesion type graded on a score from 0 (no change) to 5 (severe change).

In the interstrain susceptibility study, mice were instilled twice/week for 5 wk with *Mycobacterium immunogenum* (180 μg protein/instillation, $n = 6$/strain) and the lungs were analyzed at 4 days after the last instillation as described earlier.

Data were analyzed with a one-factor analysis of variance (ANOVA) (independent factor of treatment or strain in the two studies) followed by a Student–Newman–Keuls test for comparison of multiple groups to each other. Values of $p < .05$ were considered statistically significant.

RESULTS

Comparison of *M. immunogenum* and *M. immunogenum*-Contaminated Metalworking Fluid

Tracheal instillation of *M. immunogenum* resulted in lesions consistent with hypersensitivity pneumonitis in the lungs of mice. Histologic assessment resulted in two categories of inflammatory changes that were used in enumerating pathologic changes: (1) an immune-mediated granulomatous pneumonitis, characterized by organized, epithelioid lymphocyte granulomas, presence of lymphocytes and plasma cells in peripheral granulomas, and increased bronchus-associated lymphoid tissue (BALT); and (2) a cellular interstitial pneumonitis/toxic pneumonitis consisting of diffuse or focal cellular infiltration in the alveolar parenchyma, characterized by disorganized, scirrhous histiocytic inflammation with diffuse interstitial fibroplasia and type II pneumocyte hyperplasia.

As shown in Table 1 and Figure 1, in the majority of lung sections, the *M. immunogenum*-induced lesions consisted of the immune-mediated granulomatous inflammatory pattern with inflammatory cell infiltrates in the lung parenchyma composed primarily of lymphocytes, plasma cells, macrophages, and scattered neutrophils and eosinophils. These infiltrates were disseminated through the alveolar interstitium, sometimes occurring in the alveolar air spaces, and were concentrated around the conducting airways and blood vessels. In the perivascular and peribronchial locations, the cells formed dense cuffs without infiltration into the walls of the vessels or airways. In the lung sections with the greatest severity of disease, large aggregates of inflammatory cells were present around the airways and vessels and occasionally in the peripheral lung parenchyma. This more severe disease was also characterized by extensive peribronchial and perivascular lymphohistiocytic inflammation and discreet, noncaseating granulomas in the lung parenchyma. The granulomas were small and loosely organized, and composed of moderately differentiated macrophages admixed with lymphocytes, scattered neutrophils, and rarely erythrocytes (Figure 1). Additionally, induction of bronchial-associated lymphoid tissue (BALT) and hyperplasia of lung-associated lymph nodes (LALN) were present in sections of lung from most mice. These findings recapitulate the lung lesions typically associated with hypersensitivity pneumonitis in humans and animal models of the disease (Reyes et al., 1982; Schuyler et al., 1991).

TABLE 1
Immune-mediated response of mice to treatment

Treatment	Number of mice	Number affected[a]	Severity score[b] (mean ± SD)
Water	6	0	0
M. immunogenum	6	6	2.3 ± 0.5[c]
Used, contaminated metalworking fluid	8	8	2.8 ± 0.7[b]
Unused, clean metalworking fluid	7	1	0.4 ± 1.1

[a]Number of animals with immune-mediated pneumonitis—lymphocyte infiltration, granulomas, and induced BALT with LALN hyperplasia.

[b]Severity grading scale for immune-mediated response: 0 = no change; 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe.

[c]Statistically different ($p < .05$) from water-treated and unused metalworking fluid-treated groups.

Similar hypersensitivity pneumonitis-like histological changes were also observed in mice repeatedly instilled with used metalworking fluid that was contaminated with *M. immunogenum*. As shown in Table 1 and Figure 1, there were no significant differences in the type and severity of pathology between the groups of mice instilled with *M. immunogenum* only or with *M. immunogenum*-contaminated metalworking fluid. Treatment with either type of *M. immunogenum*-containing solution produced an immune-based pathology. Because the same types of immunologic lung changes were observed in mice instilled with pasteurized used metalworking fluid (data not shown), we could conclude that infection did not play a role in the production of hypersensitivity pneumonitis-like changes in the lungs of mice instilled with used metalworking fluids.

Repeated instillation of clean, unused metalworking fluid diluted in sterile, pyrogen-free water caused pulmonary lesions that were characterized by a toxic pneumonitis that was significantly different than the immunologic lung lesions seen in the lungs of animals repeatedly instilled with *M. immunogenum*-contaminated metalworking fluid (Table 2 and Figure 1). The pulmonary lesions observed in mice treated with unused metalworking fluid were characterized by cellular interstitial pneumonitis and a more severe toxic pneumonitis. The pattern of inflammation did not contain an apparent involvement of the lymphoid immune components of the lung, with BALT and LALN hyperplasia rarely observed in mice dosed with the unused metalworking fluid. Rather, the pattern of inflammation was diffuse and consisted of poorly organized macrophages forming broad clusters and scirrhous sheets of loose inflammation. Lymphocytes and epithelioid differentiation of macrophages were rarely seen. The inflammation more readily effaced and distorted the lung tissue and was accompanied by an early interstitial fibrous

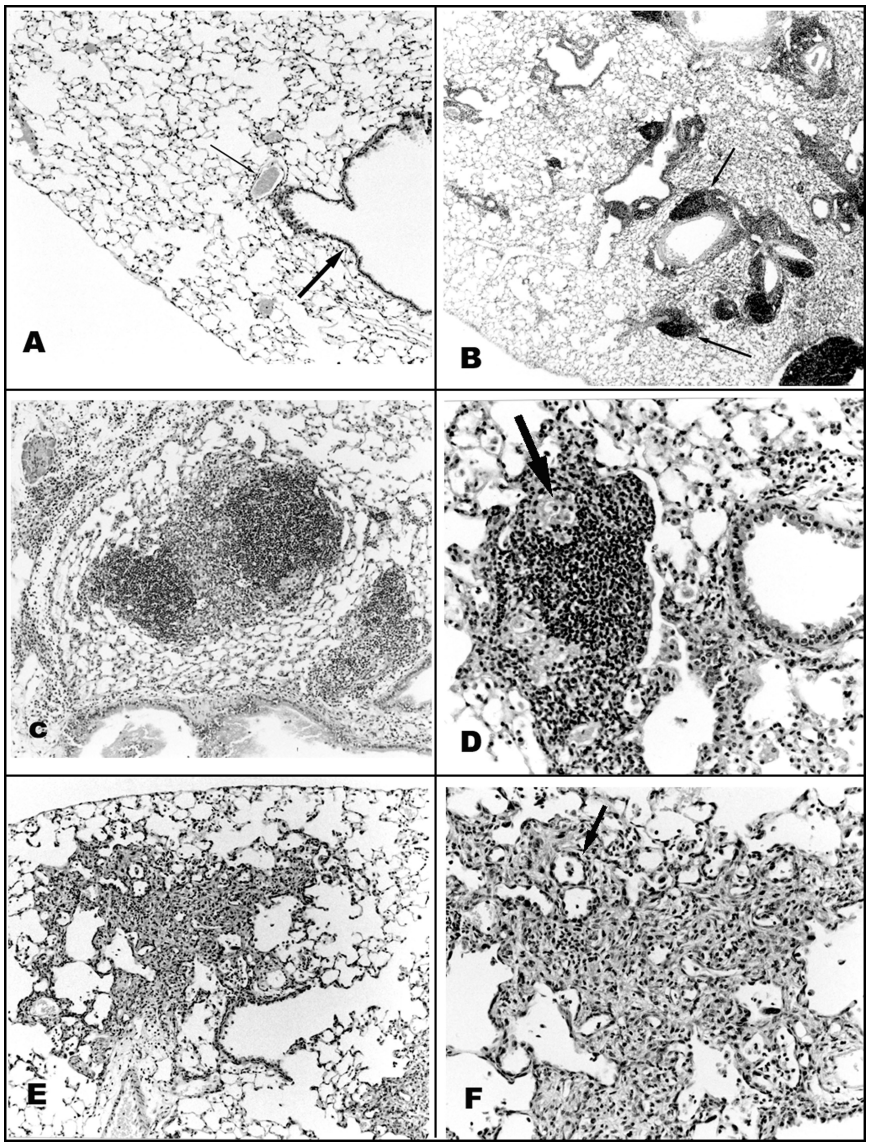

FIG. 1. Histopathology of lung alterations in representative mice exposed to instillates of water (A), or *Mycobacterium immunogenum* lysate (B, C), metalworking fluid naturally contaminated with *M. immunogenum* (D), or uncontaminated metalworking fluid (E, F). (A) Normal lung in control mouse instilled with sterile water (vehicle). Vascular and airway profiles are present. H&E, 40× (B) Multifocal lymphocytic and histiocytic perivascular and peribronchiolar pattern of lung inflammation, following instillation with *M. immunogenum* lysate. H&E, 20×. (C) Focal lymphohistiocytic inflammation organized into a discreet granuloma within the lung parenchyma, following instillation with *M. immunogenum* lysate. H&E, 100×. (D) Discreet, mixed lymphocytic and histiocytic granuloma within the lung parenchyma, following instillation with *M. immunogenum*-contaminated metalworking fluid. H&E, 200×. (E) Focally extensive interstitial fibrosis with scattered lymphohistiocytic inflammation following instillation with uncontaminated metalworking fluid. H&E, 40×. (F), Higher magnification of (E), with arrow indicating type II pneumocyte hyperplasia lining the alveoli. H&E, 200×.

tissue reaction. Moreover, there was loss of alveolar fine architecture and replacement by dilated alveolar spaces lined by proliferative type II pneumocytes. Overall, this pattern is consistent with a toxic injury response in the lung (Haschek & Witschi, 1991) and suggested that, at the dose administered, the metalworking fluid was inherently toxic to the mouse lung.

The majority of control animals had no pulmonary changes (4/6), and the changes seen in two control mice were minimal and nonspecific. The latter likely represented minor secondary pulmonary inflammation related to the instillation procedure.

The induction of hypersensitivity pneumonitis-like changes by *M. immunogenum* was reversible. In mice allowed to recover

TABLE 2
Toxic pneumonitis response to treatment

Treatment	Number of mice	Number affected[a]	Scores[b] (mean ± SD)
Water	6	0	0
M. immunogenum	6	0	0
Used, contaminated machining fluid	8	0	0
Unused, clean machining fluid	7	5	3.3 ± 2.3[c]

[a]Number of animals with toxic pneumonitis—scirrhous histiocytic inflammation with fibroplasia and type II pneumocyte hyperplasia.

[b]Severity grading scale for toxic pneumonitis response: 0 = no change; 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe.

[c]Statistically different ($p < .05$) from water-treated, *M. immunogenum*, and used metalworking fluid-treated groups.

for 1 mo after the final instillation of *M. immunogenum*, there were only a few granulomatous-like lesions. At 2 mo after exposure, the lungs appeared normal, thus suggesting that continual presence of antigenic material is necessary for maintaining hypersensitivity pneumonitis-like changes in the *M. immunogenum*-treated animals. This result is similar to that observed in human patients who recover from hypersensitivity pneumonitis if removed from the harmful environment early enough in the course of disease.

Interstrain Differences in Response to *M. immunogenum*

Significant interstrain differences in response were observed in inbred mice repeatedly instilled with *M. immunogenum* (Table 3). Although DBA/2, C3H/HeJ, and MRL/Mp mice were resistant to the immunologic changes induced by *M. immunogenum*, C57Bl/6, 129/Sv, and BALB/*c* mice were classified as sensitive strains. The same involvement of lymphoid immune components of the lung seen in the study comparing *M. immunogenum* and *M. immunogenum*-contaminated metalworking fluid was observed in the three sensitive inbred strains.

TABLE 3
Interstrain differences in response to *M. immunogenum* instillation

Strain	Mean score[a]	SEM[b]
C57Bl/6	1.93	0.28
129/Sv	1.78	0.34
BALB/*c*	1.55	0.17
C57BL/10	0.95	0.21
AKR	0.73	0.10
DBA/2	0.49	0.11
C3H/HeJ	0.58	0.09
MRL/Mp	0.60	0.00

[a]Mean severity score for each strain for immune-mediated pneumonitis, which included lymphocyte infiltration, granulomas, and induced BALT with LALN hyperplasia. Severity grading scale for immune-mediated response: 0 = no change; 1 = minimal; 2 = slight; 3 = moderate; 4 = marked; 5 = severe.

[b]Standard error of the mean.

DISCUSSION

Metalworking fluid aerosols are an occupational hazard for nearly 1 million workers in the United States (NIOSH, 1998). Despite the strong evidence that metal working fluid aerosols produce adverse respiratory effects (NIOSH, 1998; Woskie et al., 1996; Zacharisen et al., 1998), identification of the metalworking fluid components responsible for these adverse effects has been difficult. Metalworking fluids used during machining processes contain many potentially toxic or irritating agents. Some of these components are intentionally added to the metalworking fluid for lubrication, cooling, antimicrobial (e.g., formaldehyde-releasing agents), or antifoaming purposes. Inadvertent growth of microbes in the metalworking fluids can also present a health hazard. Identification of the factor(s) contributing to the production of adverse respiratory effects is further complicated by the different types of metalworking fluids in use (straight, soluble, semisynthetic, and synthetic), which contain many different potentially toxic agents.

A number of cases of physician-diagnosed hypersensitivity pneumonitis have occurred in automotive workers exposed to metalworking fluid aerosols. The causative agent has not been identified and could be any of a number of components of in-use metalworking fluids. The potentially responsible agents include the individual chemical components of metalworking fluids, additives (including biocides), and contaminants. Because the chemical components varied among the affected work sites (Fox et al., 1999; Kreiss & Cox-Ganser, 1997), a great deal of interest has focused on the potential ability of microbial contaminants to cause hypersensitivity pneumonitis. Microbial agents such as those found in pigeon dropping extract (Wilson et al., 1982) and heated swimming pools (Rose et al., 1998) are implicated in hypersensitivity pneumonitis.

Of the numerous microbes present in in-use metalworking fluids, investigators have suggested that *M. immunogenum* may play a significant role in the recent hypersensitivity pneumonitis outbreaks (Fox et al., 1999; Kreiss & Cox-Ganser, 1997). First, *M. immunogenum* was cultured from several of the metalworking fluid samples collected from the affected work sites (Wallace et al., 2002). The presence of this rapidly growing mycobacterium in metalworking fluid could have resulted from the overuse of biocides to eliminate other microbes, such as gram negative bacteria, commonly associated with metalworking fluid storage facilities (Kreiss & Cox-Ganser, 1997; Mattsby-Baltzer et al., 1989; Watt, 2003). Alternatively, these reports of *M. immunogenum* in the microbial sampling of metalworking fluids may simply reflect the fact that mycobacteria have not been looked for in the routine assays of microbial-contaminated

metalworking fluids. Thus, it is not clear whether the recent presence of mycobacteria in metalworking fluids is actually novel or had never before been examined.

Atypical mycobacteria species have the potential to cause adverse effects but usually only in immunocompromised individuals (Swetter et al., 1993). *Mycobacterium immunogenum* is a common environmental soil contaminant. It is relatively slow-growing like other mycobacteria, and resistant to drying, and thus survives a long time in the environment. While simple heat treatment (e.g., milk pasteurization) can kill *M. immunogenum*, it can be hard to eradicate, as demonstrated by the difficulty in removing it from PVC water supply pipes and bronchoscopes using standard sterilization techniques (Fraser et al., 1992; Kiely et al., 1995). Thus, under the right conditions, *M. immunogenum* appeared to become the dominant microorganism in the metalworking fluids at the affected work sites and could have reached biologically significant levels in the metalworking fluids and the airborne particles generated by the machining processes. The presence of whole or fragmented *M. immunogenum* in respirable metalworking fluid aerosols has not been reported for the affected work sites.

Epidemiology studies have attempted to use the presence of serum precipitins in automotive workers as a means of identifying microbial causes of the hypersensitivity pneumonitis outbreaks (Kreiss & Cox-Ganser, 1997). This type of identification is problematic, however, due to the inherent difficulty in obtaining and testing extracts of all potential antigens and the observation that cell-mediated immune mechanisms are the predominant basis for granulomatous diseases (Rose, 1994). Therefore, to examine the potential role of *M. immunogenum* in the recent hypersensitivity pneumonitis outbreaks in automotive plants, we studied the pathologic changes that occur in mice instilled with *M. immunogenum*. Although nasal instillation of mice with *M. immunogenum* did not have an adverse effect on the lung (data not shown), intratracheal instillation clearly produced hypersensitivity pneumonitis-like lesions in the murine lung. As observed in human hypersensitivity pneumonitis (Rose et al., 1998), noncaseating granulomas and other immune-based changes were observed in mice instilled with *M. immunogenum.*

A particularly important finding in our study was that the type of granulomatous lesion observed in mice instilled with *M. immunogenum* alone was also observed in mice instilled with a used metalworking fluid that was contaminated with *M. immunogenum* at the work site. This similar hypersensitivity pneumonitis-like pathology occurred despite the fact that the amount of *M. immunogenum* was possibly three magnitudes greater in the *M. immunogenum*-only instillations (approximately 10^9 CFU/ml) than in the *M. immunogenum*-contaminated metalworking fluid instillations (10^6 CFU/ml). The actual difference was probably less than three magnitudes because the use of CFUs to assess the presence of microbes in metalworking fluid samples does not account for the presence of dead microbes (Thorne et al., 1996a). These dead microbes may be whole or fragmented but they may still retain biologic, especially antigenic, activity. Our finding that the pasteurized used metalworking fluid also produced immune-type lesions including granulomas is consistent with the hypothesis that innate antigenic properties of *M. immunogenum*, and not infection by the bacteria, are responsible for the observed effects. The identification of the antigen(s)/protein(s) responsible for the induction of immune-based granulomas could have potential utility in identification, prevention, and treatment of hypersensitivity pneumonitis in metalworking fluid workers. Importantly, there is considerable confusion in the identification of nontuberculin mycobacteria. The species of *Mycobacterium fortuitum, immunogenum*, and *chelonae* are closely related and thus difficult to discern except by rigorous biochemical means or comparison of DNA sequences.

The lesion type produced by instillation of clean, unused metalworking fluid was different from the immune-based response to an equal dose of the used metalworking fluid. The pathologic changes of the former were not characterized by involvement of the lymphoid immune components of the lung. A diffuse pattern of inflammation was observed that consisted of damage and regeneration of the alveolar respiratory epithelium, clusters of poorly organized macrophages, and an early fibrous tissue reaction without lymphocyte recruitment or epithelioid differentiation of macrophages into granulomas. Thus, unused metalworking fluid caused a toxic pneumonitis-type reaction in the lung when the delivered dose was given by intratracheal instillation. These changes, however, were not overtly apparent in mice that received *M. immunogenum*-contaminated, used metalworking fluid; rather, in the *M. immunogenum*-exposed mice the immune-mediated inflammatory reaction predominated. This suggests that, other than the possibility that the used metalworking fluid had lost some of its toxic physical–chemical properties, in the presence of an antigen driven response, the higher order immunologic mechanisms override fundamental inflammatory responses to tissue injury and drive the reaction toward lymphocyte-mediated humoral (not evaluated in this model) and cell-mediated hypersensitivity mechanisms (Kunkel et al., 1993).

A key issue to the understanding of the contribution of *M. immunogenum* to the induction of hypersensitivity pneumonitis is whether the observed changes in the murine lung occur at realistic exposure concentrations. This was not addressed in this preliminary study which used the instillation route of exposure. As demonstrated previously in dose-response studies with inhaled metalworking fluid aerosols (Gordon & Galdanes, 1999; Schaper & Detwiler, 1991; Thorne et al., 1996b), the adverse response to the instilled used and unused metalworking fluids was dose dependent (data not shown). Additional studies with realistic concentrations of aerosolized *M. immunogenum* and *M. immunogenum*-contaminated metalworking fluids are necessary to assess the true contribution of *M. immunogenum* to hypersensitivity pneumonitis in automotive workers exposed to metalworking fluid aerosols.

Our experiments also demonstrate that interstrain differences in response occur in mice instilled repeatedly with *M. immunogenum*. Because inbred mice strains are genetically homogenous within a strain and less so among strains, this strain dependence in response suggests that genetic factors contribute to the immunologic response to *M. immunogenum*. The contribution of multiple genes to granuloma induction in the mammalian lung has been demonstrated for other immunotoxicants (Rossi et al., 1987; Donnelly et al., 1996; Wilson et al., 1982). In these experimental studies, genes within the H-2 locus (the major histocompatibility complex equivalent in mice) were linked to the induction of granulomas in the mouse lung. C3H/He mice with an H-2^k haplotype responded strongly to the interstitial lung disease (hypersensitivity pneumonitis) produced by pigeon dropping extract (Wilson et al., 1982), whereas C57Bl/10 mice with an H-2^b haplotype had only a mild inflammatory response after repeated challenge with the pigeon dropping extract. Although these results suggested that the H-2 haplotype controls the response to the pigeon dropping extract, C3H.SW mice with an H-2^b haplotype (identical to that of low responder C57BL/10 mice) were also high responders, implying that the induction of granulomas was multigenetic in nature. Similarly, Rossi and colleagues (1987) observed that the development of interstitial lung disease in inbred mouse strains is multifactorial with a variety of immune and nonimmune genes contributing to the susceptibility. Thus, the role of host genetic factors in *M. immunogenum*-induced lung granulomas is also likely to be polygenic in nature. Moreover, such potential genetic susceptibility factors are consistent with the observation that only a fraction of exposed metalworking fluid workers develop hypersensitivity pneumonitis. Although exposure dose may also play a significant role in the development of hypersensitivity pneumonitis in individual workers, more experimental evidence is necessary to determine the relative contribution of dose and genetic susceptibility as well as other susceptibility factors such as health and smoking status.

In conclusion, our study has demonstrated that *M. immunogenum* can produce lung lesions in a test animal and that the immune-based changes in mice are similar to those observed in patients with hypersensitivity pneumonitis (Rose, 1994). Although this finding provides strong evidence that *M. immunogenum* may be responsible for the recent outbreaks of hypersensitivity pneumonitis among automotive workers, other components of in-use metalworking fluids may play significant roles. Based on our results with unused metalworking fluid and the observation that hypersensitivity pneumonitis has occurred in workers exposed to soluble, semisynthetic, and synthetic metalworking fluid aerosols (Fox et al., 1999; Kreiss & Cox-Ganser, 1997), it is unlikely that chemical components of metalworking fluids are solely responsible for hypersensitivity pneumonitis. These chemical components and other contaminants may, however, have a supporting role in the development of hypersensitivity pneumonitis. In addition to the contention that antigen-driven immune responses supersede innate inflammatory responses, it has also been shown that the nature of the innate inflammatory response to general tissue injury can influence the nature of an incipient inflammatory response (Fearon & Locksley, 1996). Additionally, contaminating metals and microbial agents are ubiquitous in metalworking fluids. Certain metals such as nickel and beryllium are known sensitizers (Huang et al., 1992). Moreover, bacterial endotoxin can be an adjuvant in the sensitization process (Rylander, 1994), and mycobacteria species themselves are used as adjuvants (e.g., complete Freund's adjuvant). Thus, it is possible that significant interactions between *M. immunogenum* and other metalworking fluid contaminants are involved in the development of hypersensitivity pneumonitis. Finally, it is apparent that the development of hypersensitivity pneumonitis in automotive workers exposed to metalworking fluid aerosols is complicated. Not all exposed workers become sensitized, and not all sensitized individuals develop hypersensitivity pneumonitis. Thus, host factors such as genetic susceptibility may contribute to hypersensitivity pneumonitis among automotive workers, as it does in many other forms of hypersensitivity pneumonitis (Flaherty et al., 1980). A similar role for a gene–environment interaction exists for the development of adverse immune responses in other occupational diseases such as chronic beryllium disease (Maier & Newman, 1998).

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