Metalworking Fluid with Mycobacteria and Endotoxin Induces Hypersensitivity Pneumonitis in Mice

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Background: Human cases of hypersensitivity pneumonitis (HP) have been reported among machinists for over 10 yr. Although mycobacteria have been implicated as causal agents, this has not been established in experimental studies and the mechanisms remain unclear. Other constituents of in-use metalworking fluids (MWFs) may also contribute to the development of lung disease. We investigated the potential for *Mycobacterium immunogenum* (MI) in MWFs to induce HP.

Methods: Mice were exposed intranasally for 3 wk to MI (isolated from MWFs), *Saccharopolyspora rectivirgula* (positive control), saline, endotoxin, MWFs spiked with endotoxin and/or MI, used MWFs, and particulate-fortified used MWFs. Responses were assessed 96 h after the last exposure.

Results: Mice exposed to MI in MWFs developed lung pathology consistent with HP along with significantly more monocytes and neutrophils in lung lavage, increased CD4⁺/CD8⁺ T-lymphocyte ratio, and marked pulmonary lymphocytosis on histologic examination when compared with saline-treated control mice. Mice with Grade 2 or higher pathology (0–4 point scale) exhibited significantly elevated macrophage inflammatory protein-1 α and IL-10 and a trend toward higher RANTES 96 h after the final dose. Endotoxin coexposure augmented lung pathology.

Conclusion: MWFs containing mycobacteria induced granulomatous lung lesions, peribronchiolar lymphocytosis, increased cell concentrations in lavage, and up-regulation of several cytokines. These findings are consistent with HP.

Keywords: endotoxin; hypersensitivity pneumonitis; metalworking fluids; mycobacteria; *Saccharopolyspora rectivirgula*

Occupational exposure to metalworking fluid (MWF) aerosols has been linked to cases of hypersensitivity pneumonitis (HP) (1–5). MWFs are oils, oil emulsions, or synthetic mixtures used for machining, forming, treating, or protecting metals. In-use MWF systems are complex mixtures of water, emulsified oils, tramp oil, antifoam agents, rust inhibitors, metals, thermal breakdown products of oils, biocides, microorganisms, and microbial products (6). Epidemiologic studies have shown that inhalation of mists from in-use MWFs can result in acute respiratory symptoms (7), increased frequency of bronchitis (8, 9), and cross-shift declines in lung function (10). Inhalation toxicology studies in guinea pigs and mice have demonstrated the importance of MWF-induced lung inflammation (11, 12).

HP is a group of immunologically mediated lung diseases caused by inhalation of a wide variety of antigenic materials (13, 14). The most common causative agents of HP are inhaled thermophilic microorganisms, fungal spores and fragments, avian dusts and fecal proteins, bacterial proteolytic enzymes and industrial chemicals such as diphenylmethane diisocyanate and trimellitic anhydride (15–20).

Human cases of HP have been reported among machinists for over 10 yr. The largest reported outbreak of work-related respiratory illness in the machining environment in the United States occurred from October 2000 to April 2001 in Ohio. Of the 32 workers, 12 (38%) met a definition for HP (4). Analyses of MWF systems at this plant revealed predominant growth of *Mycobacterium immunogenum* (MI). An earlier assessment described clusters of HP occurring in workers exposed to aerosolized water-based MWFs in eight automotive plants. Mycobacteria were isolated from MWFs in four plants (2). A recent study reported that 95% of mycobacterial isolates recovered from industrial MWFs in plants with cases of HP belong to the fast growing, nonpigmented species MI (21). Our group found that MI was the most important culturable microorganism found in the in-use MWF samples obtained from an engine plant reporting cases of HP (22).

HP is a T-lymphocyte helper 1 (Th1)-type granulomatous lung disease characterized by lung neutrophilia (within 48 h), followed by lymphocytosis (counts above 15%) (23–25). The acute phase of HP is marked by increased neutrophils and CD4⁺ T lymphocytes (26, 27). In contrast, an increased number of CD8⁺ T lymphocytes is observed in subacute/chronic phase (27, 28). Histologically, HP is characterized by a triad of findings: (1) cellular bronchiolitis; (2) interstitial mononuclear cell infiltrates; and (3) scattered, small, nonnecrotizing lymphocytic granulomas and fibrosis in the chronic stage. Between involved areas, there is usually normal lung parenchyma (29).

It has been postulated that other constituents of used MWFs, such as endotoxins produced by circulating bacteria and biofilms (30), may contribute to the lung inflammation and may potentiate immunologic responses and act as an adjuvant or cofactor for the development of HP. Blunted responses in endotoxin-resistant (TLR4-deficient) mice versus sensitive mice and endotoxin dose-dependent responses for production of proinflammatory cytokines, lung neutrophilia, and bronchoconstriction demonstrated that endotoxin in machining plants could be a significant hazard (11, 12).

The aim of this study was to investigate the potential for mycobacteria in MWFs to induce HP in an established mammalian model and to characterize the cellular and immunologic responses to the exposures. We also sought to determine if endotoxin in MWFs acts as a cofactor or adjuvant for the production of HP from MWF microbial antigens. Some of the results have been previously reported in the form of an abstract (31).

METHODS

Exposure Protocol

We followed the HP induction protocol of Gudmundsson and colleagues (32, 33). The number of mice was determined from power

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calculations based on preliminary data. Female C57Bl/6J mice were dosed with the preparation appropriate to their experimental group (50 μ l) by nasal instillation 3 consecutive d/wk for 3 wk. Mice were necropsied 96 h after the last dose. The purposes for the treatments and the selected doses for the experimental groups are listed in Table 1. Mice exposed to unused MWFs (MWF 0) served as the referent group in Models 1 and 3. The dose of mycobacteria administered was three times what a machinist would receive (per unit lung surface area) when exposed to contaminated MWFs at the permissible exposure limit.

Materials

Nonviable, endotoxin-free preparations of *Saccharopolyspora rectivirgula* (SR) were prepared as previously described (34, 35). The used MWF sample (MWF-U) was obtained from an automotive engine plant machining area where prior HP cases had been identified. Unused MWFs of the same formulation (MWF 0) was used in the experiment in the same concentration (6%) as samples of used MWFs. Used fortified MWF (MWF-UF) was prepared as follows: 1 ml of MWF-U was centrifuged (1 h at $12,000 \times g$), the supernatant was discarded, and 1 ml of MWF-U diluted with water (1:5 dilution) was added to the pelleted solids and vortexed.

MI was isolated from MWF-U and identified by culture-based methods, polymerase chain reaction, and DNA sequencing (22). Endotoxin concentrations were quantified using a kinetic chromogenic *Limulus* amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD) as previously described (36, 37).

Histopathologic Evaluation

Each lung was scored for inflammatory cells infiltrates, lymphoid agglomerates, granulomas, and giant cells and graded for severity on a five-point scale (0–4). The following criteria were used for determination of histologic score (scale 0–4): 0 = no lung abnormality, 1 = presence of

inflammation and granulomas involving < 10% of the lung parenchyma, 2 = lesions involving 10–30% of the lung, 3 = lesions involving 30–50% of the lung, and 4 = lesions involving > 50% of the lung (38).

Cytokine/Chemokine Assays

Cytokine/chemokine assays of bronchoalveolar lavage (BAL) were performed using ELISA (interleukin 6 [IL-6] and IL-10) or using a multiplex, suspension array system (Bio-Rad Laboratories, Hercules, CA) (IL-1 α , IL-4, IL-5, IL-12[p40], tumor necrosis factor α [TNF- α], IL-2, IFN- γ , macrophage inflammatory protein 1 α [MIP-1 α], and RANTES).

Statistical Analyses

Statistical analyses were performed in SAS (version 8; SAS, Inc., Cary, NC) and SPSS (version 10.0; SPSS, Inc., Chicago, IL). Experimental groups were compared with saline-exposed control mice using *t* tests for equal or unequal variances. Secondary analyses reported in the online supplement were performed using Duncan's multiple-range test to simultaneously assess differences between groups of treated mice. Nonparametric one-way procedure was used for analysis of histopathology data; *t* testing for equal or unequal variances was used to compare responses of mice exposed to MI stratified by histology scores < 2 or ≥ 2 . In all analyses, $p < 0.1$ was considered suggestive of an effect, and a *p* value below 0.05 was considered significant. Additional details on the methods are provided in the online supplement.

RESULTS

Experiments were performed in four experimental models to address our primary hypothesis that MI-contaminated MWF can produce hypersensitivity pneumonitis (Table 1). The first model (HP Model) used a positive control to establish that the exposure

TABLE 1. MODELS USED IN THIS STUDY AND THEIR PURPOSE AND DOSING REGIMEN

Group	Exposure	Purpose	Dose [†]
HP Model		To test the induction of HP in this protocol	
Saline	Sterile pyrogen-free saline	Negative control	50 μ l sterile pyrogen-free saline (0.9% NaCl)
SR	<i>Saccharopolyspora rectivirgula</i>	Positive control for HP	200 μ g/50 μ l (2.2×10^5 CFU)
Model 1		To test the influence of endotoxin	
ENDO	Endotoxin	Positive control for nonspecific airway inflammation during exposure	250 EU/50 μ l
MWF 0	Virgin MWF	Control	In-use concentration 6% further diluted 1:5 w/ water
MWF 0+ENDO	Virgin MWF spiked with endotoxin	Control	The above plus endotoxin at 250 EU/50 μ l
Model 2		To test the response after MI exposure	
MI	<i>Mycobacterium immunogenum</i>	Characterize response and test for development of HP	MI isolated from in-use MWF: 500 μ g/50 μ l (1.2×10^6 cfu)
MWF 0+MI	Virgin MWF spiked with MI	Test for development of more severe HP	In-use concentration 6% further diluted 1:5 w/water, spiked with MI 500 μ g/50 μ l
MWF 0+MI+ENDO	Virgin MWF spiked with endotoxin and MI	Test for development of more severe HP	The above plus endotoxin at 250 EU/50 μ l
Model 3		To test the response of MWF contamination	
MWF 0	Virgin MWF	Control	In-use concentration 6% further diluted 1:5 w/water
MWF-U*	In-use MWF as received	Test for development of HP	In-use concentration 6% further diluted 1:5 w/water
MWF-UF	In-use MWF fortified	Test for development of HP	Sixfold concentration of suspended particles by centrifugation (1 h at $12,000 \times g$) dosed as 1:5 dilution

Definition of abbreviations: ENDO = endotoxin alone; HP = hypersensitivity pneumonitis; MI = *Mycobacterium immunogenum*; MWF = metalworking fluid; MWF 0 = unused MWF; MWF-U = used MWF; MWF-UF = used fortified MWF; SR = *Saccharopolyspora rectivirgula*.

* MWF-U was obtained from an automotive engine plant machining area where prior cases of HP had been identified.

[†] Listed dose was administered by nasal instillation three sequential days per week for 3 wk.

Figure 1. Micrographs of lung sections stained with Masson's trichrome. Control group: mice exposed to saline alone with no sign of hypersensitivity pneumonitis (HP) (A, B); expression of HP with fibrosis in mice exposed to *Saccharopolyspora rectivirgula* (positive control for HP) (C, D). The areas indicated by the box in the low-magnification figures are shown at higher magnification in the micrographs on the right.

protocol could produce HP in these mice (Figure 1). The next model (Model 1) evaluated responses to MWF and endotoxin without mycobacterial contamination. Model 2 investigated HP induction in MI-exposed mice and with MWF spiked with MI. Model 3 tested a used MWF sample that was associated with human cases of HP among machinists and was shown to contain MI (22). We used two statistical approaches to evaluate and present the data in Models 1, 2, and 3. In Figures 2A–2C, data are presented with asterisks indicating statistically significant differences compared with saline-exposed control mice based on pairwise *t* tests. Secondary analyses are presented in Tables E2, E3, and E4 of the online supplement using Duncan's multiple range test and grouped data.

All the C57Bl/6J mice exposed in the four models exhibited normal weight gain and were not significantly different from sentinel mice or control mice treated with saline. No changes were observed in body condition, but moderate changes in animal behavior (including sluggishness) were observed in animals exposed to SR or experimental groups with virgin and used MWF. There were significant changes observed in lung cytology, cytokine production, and histology.

HP Model

In the model of induction of HP (Table 2), we found that SR-treated mice (positive control) had a significantly higher concentration of total cells, macrophages, neutrophils, and lymphocytes in the BAL ($p = 0.002$, $p = 0.003$, $p = 0.006$, and $p = 0.004$, respectively) than saline-treated mice (negative control).

Mice treated with SR had the highest cellular response of all groups tested. The mean concentrations of most of the listed cytokines and chemokines were higher in the SR-treated mice than in saline-treated control mice (Table 2). IL-1 α and IL-12(p40) were significantly higher, and RANTES approached significance. All BAL fluid (BALF) samples (HP Model and Models 1, 2, and 3) had very low concentrations of IL-2 and IFN- γ , with most values near or below the lower detection limit (IL-2: group means 3.16–8.17 pg/ml, overall range 0.14–16.4 pg/ml; IFN- γ : group means 1.26–3.62 pg/ml, overall range 0.55–6.90 pg/ml).

The Th2 cytokines IL-4 and IL-5 were no different and the inflammatory cytokine TNF- α also was no different from negative control mice (data not shown). Phenotypic analyses of T lymphocytes in the BALF showed a significantly higher CD4⁺/CD8⁺ ratio in SR-treated mice as compared with control mice (4.7 versus 1.6) (Table 2). This was due to a doubling of CD4⁺ T lymphocytes and a nearly twofold reduction in CD8⁺ cells. Bradford assay of BAL supernatant for total protein did not reveal a difference between these two groups of animals.

There were no pathologic changes and no evidence of HP or inflammation in the lungs of the control mice treated with saline (Figures 1A and 1B). Histopathology evaluation of the lungs from mice treated with SR demonstrated the development of lesions consistent with HP, including significant peribronchiolar lymphocytosis with giant cells, interstitial inflammation and fibrosis, and nonnecrotizing granulomas (Figures 1C and 1D). At the SR dose used in this model, 50% of the animals exhibited Grade 2 or higher lung pathology, which was consistent with HP (Figure 3). Four of the six saline-treated mice were scored at Grade 0, and two were scored Grade 1. None had Grade 2 or higher lung pathology. The same examinations were performed blindly in sentinel mice, and data revealed completely normal lung histology (all Grade 0).

Model 1. The ability of virgin MWF, with or without added endotoxin, to induce HP was investigated in experiments with virgin MWF (MWF 0), a low dose (250 EU) of endotoxin alone (ENDO), and virgin MWF spiked with low-dose endotoxin (MWF 0+ENDO). These data are presented in Figures 2A–2C. Results from Duncan test are presented in Table E3. MWF 0 and MWF 0+ENDO had significantly higher numbers of macrophages in BALF (39.0 and $38.8 \times 10^3/\text{ml}$, respectively) compared with saline-treated mice ($18.0 \times 10^3/\text{ml}$). The innate endotoxin content of the MWF 0 was 10 EU in 50 μl . The proportion of lymphocytes in the lavage did not differ significantly among groups in this model. Endotoxin-treated mice were no different from saline control mice for any of the outcome measures, with the exception of IL-1 α (Figures 2A–2C and Table E2). The fact that total cell and neutrophil concentrations in BALF from

Figure 2. Results of bronchoalveolar lavage fluid analyses. Values are expressed as mean and SE. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as compared with mice exposed to saline (t test for equal and unequal variances). (A) Differential cells counts. (B) Cytokines. (C) Cytokines and chemokines.

TABLE 2. COMPARISON OF OUTCOMES FROM HYPERSENSITIVITY PNEUMONITIS MODEL EXPOSURES (SALINE AND SACCAROPOLYSPORA RECTIVIRGULA GROUPS)

Parameter	Negative Control (n = 10)	Positive Control (n = 9)	p Value
Lavage cells ($\times 10^3$ /ml)			
Total cells	19.07 $\pm$ 4.87	128.71 $\pm$ 31.67 [†]	0.002
Macrophages	18.08 $\pm$ 4.60	100.29 $\pm$ 24.51	0.003
Neutrophils	0.22 $\pm$ 0.08	15.87 $\pm$ 5.30	0.006
Lymphocytes	0.90 $\pm$ 0.35	11.08 $\pm$ 3.22	0.004
Cytokines, pg/ml			
IL-10	76.1 $\pm$ 5.6	94.1 $\pm$ 15.0	0.257
IL-12 (p40)	57.9 $\pm$ 15.0	126.5 $\pm$ 27.8	0.045
IL-6	8.4 $\pm$ 1.2	8.4 $\pm$ 1.0	0.998
IL-1 α	1.8 $\pm$ 0.6	5.7 $\pm$ 1.4	0.024
Chemokines, pg/ml			
MIP-1 α	5.6 $\pm$ 2.2	9.5 $\pm$ 2.0	0.204
RANTES	5.5 $\pm$ 1.8	11.3 $\pm$ 2.5	0.079
T lymphocytes			
% CD4 ⁺	23.6 $\pm$ 1.8	41.6 $\pm$ 6.2	0.017
% CD8 ⁺	15.7 $\pm$ 1.6	9.7 $\pm$ 1.0	0.009
CD4 ⁺ /CD8 ⁺ ratio	1.6 $\pm$ 0.2	4.7 $\pm$ 0.9	0.006
Total protein, μ g/ml	71.3 $\pm$ 6.8	78.2 $\pm$ 9.7	0.567
Histology score*	0.33 $\pm$ 0.21	2.00 $\pm$ 0.68	0.055

Definition of abbreviations: IL = interleukin; MIP-1 α = macrophage inflammatory protein 1 α .

* n = 6 in each group.

[†] One animal from the positive control group was excluded from the database due to its identification as an outlier (total cells of this sample was 1623×10^3 /ml bronchoalveolar lavage fluid [BALF]; macrophages, neutrophils, and lymphocytes/ml BALF were as follows: 840×10^3 , 37×10^3 , and 747×10^3 , respectively).

endotoxin-treated mice did not differ from negative control mice demonstrated that mild endotoxin-induced, neutrophil-mediated inflammation apparent 4 h postexposure (data not shown) had resolved by the time of necropsy, 96 h after the last exposure (39). Concentrations of IL-10 and RANTES were significantly higher in groups exposed to virgin MWF and virgin MWF spiked with endotoxin. MWF 0 had also significantly higher values of IL-6 (Figures 2B and 2C and Table E2). MIP-1 α was also

increased in this group of animals (Figure 2C). There was no difference between groups in IL-12 (p40) concentrations or in IL-2, IL-4, IL-5, and IFN- γ (not shown). No difference in CD4⁺ or CD8⁺ cell ratio was observed between groups in Model 1 (Figure 4 and Table E2). Total protein was higher in BAL supernatant from mice exposed to MWF 0 (Table E2).

Histopathology scores shown in Table 3 demonstrate that, for Model 1, all mean scores were less than 1, and as a group they were not significantly different from saline control mice. More significant histologic changes were observed in a few mice in the MWF 0-exposed groups, with 18% (2 of 11) and 14% (1 of 7) Grade 2 responses in those exposed to MWF 0 without and with endotoxin, respectively.

Model 2. Our principal hypothesis was that MI exposure in MWF can induce HP. To test this, we exposed mice to MI alone, virgin MWF spiked with MI (MWF 0+MI), and virgin MWF spiked with MI and ENDO (MWF 0+MI+ENDO). Significant increases were observed in a host of measured outcomes as compared with control mice. Mice exposed to MWF 0+MI or MWF 0+MI+ENDO had elevated macrophages, neutrophils, and lymphocytes in the lavage compared with saline-exposed mice (Figure 2A and Table E3). The concentration of IL-10 was significantly higher in MWF 0+MI+ENDO and MWF 0+MI groups of mice compared with saline-treated mice. These experimental groups did not demonstrate significantly different concentrations of IL-12 (p40), IL-1 α , MIP-1 α , (Figures 2B and 2C), IL-2, IL-4, IL-5, or IFN- γ (not shown) from negative control mice. The MI-exposed animals had significantly higher levels of IL-6 and IL-1 α compared with saline group. Both chemokines studied (MIP-1 α and RANTES) and total protein were significantly elevated in MWF 0+MI+ENDO group in secondary analyses where all groups in this model were considered together (Table E3). The CD4⁺/CD8⁺ ratio was significantly higher in the MWF 0+MI group compared with control group and all other groups in the model.

Histologic evaluation of the lungs showed that 55% (11 of 20) of the mice exposed to the MWF 0+MI and 100% (7 of 7) of those exposed to MWF 0+MI+ENDO had focal peribronchiolar lymphocytosis with granulomas scoring Grade 2 or higher HP (Figure 3). Groups of mice exposed to MWF 0+MI+ENDO

Figure 3. Percentage of experimental groups yielding lung histopathology grade 2 or higher (gray bars) or Grade 3 or higher (black bars). Mean histology grade of negative control mice (saline) was significantly different from the groups MWF0+MI ($p < 0.05$), MI and MWF-UF ($p < 0.01$), and MWF0+MI+ENDO ($p < 0.001$). A t test was used for comparison.

Figure 4. CD4⁺/CD8⁺ T-lymphocyte ratio (mean and SE) in the bronchoalveolar lavage fluid of exposed and control mice. Mean of negative control mice was significantly different from the groups MWF 0+MI (* $p < 0.05$), SR, MWF-U and MWF-UF (** $p < 0.01$), and MI (** $p < 0.001$). A t test was used for comparison.

TABLE 3. HISTOLOGY SCORES FOR THE STUDY GROUPS

Group	Histology Score (mean $\pm$ SE)	n	χ^2 p Value*
Saline	0.33 $\pm$ 0.21	10	Referent
Model 1			
ENDO	0 $\pm$ 0	8	
MWF 0	0.73 $\pm$ 0.24	11	
MWF 0+ENDO	0.72 $\pm$ 0.29	7	0.171
Model 2			0.003
MI	1.60 $\pm$ 0.24	8	
MWF 0+MI	1.55 $\pm$ 0.26	20	
MWF 0+MI+ENDO	2.86 $\pm$ 0.26	7	
Model 3			0.028
MWF-U	0.67 $\pm$ 0.42	6	
MWF-UF	1.90 $\pm$ 0.31	10	

Definition of abbreviations: ENDO = endotoxin alone; MI = *Mycobacterium immunogenum*; MWF 0 = unused metalworking fluids; MWF-U = used metalworking fluids; MWF-UF = used fortified metalworking fluids.

* Groups within the models are compared with saline-treated mice using a nonparametric one-way analysis of variance.

demonstrated a higher mean histology score than those exposed to MI without ENDO (2.86 vs. 1.60 and 1.55 in MI and MWF 0+MI groups, respectively; Table 3). This pathology was clear evidence that MI in MWF induced HP in these mice.

Model 3. To test the hypothesis that used MWF without spiked MI can cause HP, we compared responses of mice to used MWF found to contain MI and associated with human cases of HP (MWF-U) and the same MWF fortified to enhance the quantity of suspended solids (MWF-UF). We reasoned that fortifying the amount of particulate matter and mycobacteria in the MWF would simulate a more highly contaminated MWF. As a comparison, the data for the virgin MWF were included in the statistical analyses using Duncan's multiple range test (Table E4) to control for the effects of the MWF without MI. The MWF 0 contained 10 EU/50 μ l of innate endotoxin, and MWF-U contained 7 EU/50 μ l.

Exposure to the used MWF associated with cases of HP among machinists also produced granulomatous lesions in the mice with 33% (2 of 6 mice) of the MWF-U-exposed mice exhibiting Grade 2 pathology (mean score = 0.67). Exposure to MWF-UF produced a higher degree of pathology with a mean score of 1.90, and 70% (7 of 10) of the mice exhibited Grade 2 or higher pathology (Table 3 and Figure 3). Granulomatous inflammation caused by used MWF-U and MWF-UF was characterized by accumulation of inflammatory cells, principally large macrophages and giant cells usually surrounded by a rim of lymphocytosis (Figure 5). Total protein was elevated in all groups in this model compared with negative control mice (Table E4). Mice treated with MWF-UF had significantly elevated macrophages, neutrophils, and lymphocytes in the lavage over saline-exposed control mice (Figure 2A). IL-6 levels were significantly higher in MWF-U-exposed mice compared with the saline group, and IL-10 was significantly elevated in mice exposed to MWF-UF (Figure 2B). There were no significant differences found in IL-1 α , MIP-1 α , RANTES, IL-2, IL-4, IL-5, or IFN- γ concentrations in comparison with saline-exposed mice. The CD4⁺/CD8⁺ ratio (Figure 4) was increased approximately threefold in the groups exposed to MWF compared with control mice. In mice exposed to MWF-UF, this ratio was increased nearly fourfold over control mice and was associated with an increase in the percentage of CD4⁺ cells and a decrease in CD8⁺. The mean histology score for the MWF-UF group was 1.90 (Table 3), and 70% of these mice had Grade 2 or 3 pathology (Figure 3).

Evaluation of Responders

As with humans exposed to agents that cause HP, only some of the exposed animals developed lung lesions consistent with HP (40). To further consider the responses of the mice exposed to MI in MWF (Groups MWF 0+MI, MWF 0+MI+ENDO, MWF-U, and MWF-UF), we divided the mice into those with Grade 2 or higher lung pathology and those with Grade 0 or 1 histology scores. Grade 2 and higher lesions were found in 63% of mice (27 of 43) with exposure to MI in MWF. Comparison of these strata is shown in Table 4. Animals with Grade 2 or greater pathology had nearly double the concentration of total cells and macrophages in the lavage. They also demonstrated significantly increased IL-10 and MIP-1 α over mice with Grade 0 or 1 histology scores. There was no difference between these groups in T-lymphocyte markers.

In total, these data support our primary hypothesis that MWF containing MI can induce HP. Of mice exposed to MWF-U with an enhanced amount of particulate matter, 40% had Grade 2 and 30% had Grade 3 pathology.

DISCUSSION

Machining fluids provide a suitable medium for the growth of microorganisms. Often the predominant organisms are gram-negative bacteria, such as *Pseudomonas* (6). These organisms give rise to endotoxin, which can achieve relatively high concentrations and pose a risk of inflammatory lung disease (11, 12, 30, 41). When machining fluids are repeatedly treated with certain biocides, resistant organisms survive, whereas others are eliminated. This leads to selection of niche organisms such as mycobacteria. Outbreaks of lung disease characterized as HP have been observed among workers exposed to MWF over the past decade (1–5). Many of these outbreaks were linked to MWF containing mycobacteria. To date, 102 of 107 (95%) MWF isolates (from 10 industrial sites within United States and Canada) were found to contain MI. All of these industrial sites were colonized with the same genotypes or MI clones, a genotype that is relatively infrequent among clinical isolates of the species (42). Mycobacteria of the species *immunogenum*, *chelonae*, and *avium* are approximately 0.5 by 1.5 μ m rod-shaped, acid-fast, slow-growing bacteria comprised of a trilaminar rough, waxy cell wall. This cell wall contains resistant lipids and polysaccharides that allow the organism to survive extreme physical and chemical environments.

We sought to determine if MI-contaminated MWF could induce granulomatous lung disease in mice and if endotoxin in MWF acts as an adjuvant for the production of HP. To do this, we adapted an established murine model of HP using *Saccharopolyspora rectivirgula*, a thermophilic bacterium that contaminates "moldy" hay and causes farmers' lung disease. HP is an immunologic disease regarded as a Th1 response and in humans is characterized by the development of lymphocytic granulomas and fibrosis and the release of IFN- γ , IL-2, and IL-12 in the early stages (23). Conversely, Th2 responses are exemplified by IgE-mediated allergic responses in which cells commonly up-regulate IL-4 and IL-5. Inflammatory responses from exposure to endotoxin produce IL-6 (39, 43).

HP animal models have demonstrated that increases in IL-12 can result in an amplification of the severity of HP (32), whereas the presence of IL-10 modulates the disease (33). IL-10 production by dendritic cells in the lung and lymph nodes helps promote T-cell tolerance (54). Lung cells from mice that developed severe granulomatous pneumonia after treatment with *Mycobacterium tuberculosis* produced high levels of IL-10 as an antiinflammatory response (55). In our studies of MWF-induced HP, we did not observe significant differences in IL-12

Figure 5. Micrographs of lung sections (stained with Masson's trichrome) from mice exposed to used MWFs (A, B, C) and lung sections (hematoxylin and eosin staining) from mice exposed to used fortified MWFs (D, E, F). The areas indicated by the box in the low-magnification figures are shown at higher magnification in the micrographs. Granulomas and fibrosis (see blue staining) representing chronic forms of HP and giant cells (arrow) developed in the lungs exposed to used MWF samples (MWF-U and MWF-UF).

(p40), IL-2, or IFN- γ concentration in BAL collected at necropsy. In addition, levels of IL-6 associated with murine exposures to contaminated MWF were not different to a biologically important degree. In previous studies we demonstrated that acute inflammatory responses mediated by the production of IL-6 resolved by 24 h after exposure (39). In the current study, necropsy of the animals was performed 96 h after the last exposure, by which time many of the proinflammatory and Th1 cytokines and chemokines in the BALF had likely returned to baseline levels. Future studies of cytokine responses in this model of HP will require addition of further experimental groups for collection of BALF in closer association with MWF or MI exposures.

It is postulated that endotoxin exposure can shift the Th1/Th2 balance toward Th1 responses. Inhaled endotoxin is recognized as a potent inducer of airway inflammation and is a leading suspect for acute respiratory effects related to MWF. We hypothesized that low-level endotoxin contamination of MWF contributes to lung inflammation, potentiates immunologic responses, influences Th1/Th2 balance, and may act as an adjuvant or cofactor for the development of HP. On the fourth day after the last dose, when animals were killed, we did not observe any residual

marked inflammation in the lungs from mice treated by endotoxin alone. It has been suggested that the Th1/Th2 shift depends on the level of endotoxin. A significant reduction of endotoxin burden may induce a shift to Th2 responses. However, extremely high endotoxin loads may bring about a second switch, away from Th1 and again toward Th2 responses (44, 45). We found that coexposure to endotoxin at 250 EU per mouse and MWF 0 with MI as compared with MWF 0+MI without endotoxin led to higher BALF concentrations of IL-10, IL-1 α , MIP-1 α , and RANTES. MIP-1 α has been reported as being released by alveolar macrophages from patients with acute HP (56), but it does not seem to be a requirement for the expression of pulmonary inflammation in experimental models of HP (57). In this study, MIP-1 α was elevated in several groups of mice (e.g., MWF 0, MWF 0+ENDO, MI, MWF 0+MI+ENDO) but with a wide distribution of values within animal groups.

Evidence that MWF may be harmful to the airways through changes in pH or the addition of biocides, which can irritate the respiratory tract (46), was found in our experiment. Total lavage cells and neutrophil concentration were higher in mice that were exposed to virgin MWF (pH = 9) than in control mice (Figure 2A and Table E2). Prior inhalation studies of MWF

TABLE 4. COMPARISON OF RESPONSES OF MICE WITH A HISTOLOGY SCORE OF 2 OR GREATER WITH THOSE WITH SCORES OF 0 OR 1 FOR GROUPS OF MICE EXPOSED TO *MYCOBACTERIUM IMMUNOGENUM*

Parameter	Histology Score < 2 (mean)	Histology Score $\geq$ 2 (mean)	Comparison by Histology Score (p Value)
Lavage cells/ml			
Total cells	47,300	82,810	0.019
Macrophages	39,130	66,700	0.028
Neutrophils	5,995	11,890	0.121
Lymphocytes	3,442	5,065	0.208
Cytokines, pg/ml			
IL-10	96.9	142.9	0.003
IL-12 (p40)	71.4	107.4	0.163
IL-6	10.0	8.6	0.434
IL-1 α	3.4	3.9	0.370
Chemokines, pg/ml			
MIP-1 α	7.8	13.7	0.010
RANTES	8.8	10.3	0.407
T lymphocytes			
% CD4 ⁺	36.3	39.2	0.504
% CD8 ⁺	9.6	10.6	0.592
CD4 ⁺ /CD8 ⁺	5.7	4.8	0.482
Total protein, μ g/ml	93.8	127.9	0.061

For definition of abbreviations, see Table 2.

Groups MWF 0+MI, MWF 0+MI+ENDO, MWF-U, and MWF-UF. The comparison was performed by *t* test for equal or unequal variances.

aerosols in guinea pigs (47) showed that pH and osmolarity had significant effects on the response to unused semisynthetic machining fluids. Adjustment of the alkalinity and hypotonic fluid to pH 7 and 300 mOsm alone or in combination significantly reduced lung injury and inflammation (47).

Phenotype characteristics of lymphocytes from BALF from previous HP studies are mixed. Examination of BAL lymphocyte subsets in patients with symptomatic HP reveals activated T lymphocytes, often with a predominance of CD8⁺ cells having suppressor/cytotoxic functions. CD4⁺/CD8⁺ ratios are usually less than 1. However, a number of investigators have shown that these ratios vary widely in patients with HP, with some having normal or increased numbers of CD4⁺ cells. Reasons for this variability are unclear, but data suggest that the forms of HP (acute vs. chronic) and the timing of last antigen exposure in relation to BAL may affect cellular phenotypes. Laflamme and colleagues (48) found that CD4⁺/CD8⁺ ratios in HP patients ranged from 0.1 to 10.6 (mean $\pm$ SD, 2.0 $\pm$ 2.8) compared with the ratios for the normal control group that ranged from 0.3 to 5.1 (1.7 $\pm$ 1.2). A CD4⁺-predominant BAL lymphocytosis has been described in patients with the fibrotic stage of HP (14). This is in line with our study where for MI-exposed animals, Masson's trichrome staining revealed the presence of fibrosis in the lungs with marked peribronchiolar lymphocytosis with increased CD4⁺ cells and reduced CD8⁺ cells. In another human study of HP, lung fibrosis was accompanied by a higher CD4⁺/CD8⁺ ratio in BALF as compared with a nonfibrosis group (2.59 $\pm$ 2.63 vs. 0.31 $\pm$ 0.13; *p* < 0.03). In addition, results from this study suggested that long-term exposure induced a relative increase of CD4⁺ cells as compared with an increase of CD8⁺ cells at the early stage (49).

We used a similar animal model of HP induction as Gudmundsson and colleagues (32, 33), and our results with SR for phenotype characterization of BALF lymphocytes are consistent with their results. Our study validates the previous finding that BALF lymphocytes of mice exposed to SR were more commonly CD4⁺ than CD8⁺. Activated CD4⁺ lymphocytes accumulations have been reported in the lung and other involved extrapulmonary organs in sarcoidosis (50). HP is usually characterized

by lymphocytosis of predominantly CD8⁺ T-suppressor cells. This is in contrast to sarcoidosis, in which BALF usually demonstrates CD4⁺ T-helper cell lymphocytosis. For these reasons, the CD4/CD8 ratio has been often used in the differential diagnosis of sarcoidosis and HP (51). However, BAL findings likely vary depending on the timing of the last antigen exposure and the stage of the disease. Soon after acute exposure, neutrophils predominate. Later, as the disease progresses to the chronic form, the ratio of CD4⁺ to CD8⁺ T cells increases (52, 53).

This study had several limitations. First, collection of BALF 96 h after dosing complicated the interpretation of cytokine data *vis-à-vis* other published studies where early-onset induction of cytokines was assessed. Second, dosing by nasal instillation used in this murine model of HP, although convenient, has several potential shortcomings. Inhalation of mycobacterial bioaerosols in MWF environments is likely to produce a more proximal and focal distribution than generated by nasal instillation. Further, this dosing protocol exposes the mice to components of the MWF that could volatilize in the workplace, and animals in the model were exposed to a bolus dose on each day, whereas human exposure typically occurs over a work shift as a number of task-based peak periods of exposure overlying a lower exposure level. These deficiencies are mitigated by the fact that this model was adopted to determine if *M. immunogenum* alone or in MWF can induce HP and not to determine safe limits of exposure.

Definitive diagnosis of HP requires histologic evidence of lymphocyte accumulation in the pulmonary interstitium with the formation of granulomas. Scoring of lungs for evidence of these changes demonstrated low, but nonzero, mean scores for MWF with or without endotoxin. When MI was added to the MWF, the mean histology score was markedly higher (1.55 vs. 0.73). Addition of endotoxin further increased the mean histology score to a level in excess of the positive control (2.86 vs. 2.00). This shows that mice exposed to MI in MWF develop lesions consistent with HP and that these are worsened by the addition of small amounts of endotoxin. Experiments with the MWF-UF sample collected from a machining facility with cases of HP also induced histopathologic changes consistent with HP and histologic scores comparable to the positive control.

This study demonstrates that MWF spiked with MI and MWF-U naturally contaminated with mycobacteria induce granulomatous lung lesions, peribronchiolar lymphocytic infiltrates, and increased cell concentrations in lung lavage. Thus, control of mycobacteria and endotoxin contamination of in-use MWF is likely of importance for the prevention of HP among machinists.

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