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Effects of Welding Fumes of Differing Composition and Solubility on Free Radical Production and Acute Lung Injury and Inflammation in Rats

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The goals of this study were to examine acute lung damage and inflammation, as well as free radical production, caused by welding fumes of different chemical compositions and solubilities. The fumes were from a gas metal arc welding using a mild-steel (GMA-MS) or stainless-steel electrode (GMA-SS) and a manual metal arc welding using a stainless-steel electrode (MMA-SS), which was further separated into soluble and insoluble fractions. The MMA-SS was the only fume to contain soluble chromium. Free radical production was observed only in suspensions of MMA-SS fume under various conditions. Male Sprague-Dawley rats were intratracheally instilled with either a welding fume suspension at 2 mg/rat or a saline vehicle, and various parameters of inflammation and damage were measured at 3 h and days 1, 3, and 6. Only the MMA-SS treatment caused a continued increase in lung weight until day 6 and elevated lipid peroxidation at day 3. All of the fumes caused increases in macrophages and neutrophils obtained by lavage, but the increased cellularity was extended through day 6 following the MMA-SS treatment only. Only the MMA-SS treatment led to an increased recovery of eosinophils and damage to the alveolar–capillary barrier. While all of the fumes produced increases in cytotoxicity, the MMA-SS treatment caused the maximal response at day 3. These findings indicate that different welding fumes caused varied responses in the lungs of rats, correlated to their metal composition and ability to produce free radicals. Additionally, both the soluble and insoluble fractions of the MMA-SS fume were required to produce most effects, indicating that the responses are not dependent exclusively on the soluble metals.

Key Words: **welding fumes; free radicals; lung inflammation; lung injury; electron spin resonance.**

Arc welding is the process of joining two pieces of metal that have been rendered liquid by heat as electricity passes from one electrical conductor to another. The high temperatures of the welding process (~4000°C) heat the base metal pieces to be joined, as well as a consumable metal electrode that is continuously fed into the weld during the process (Howden, 1988). Fumes are formed at the tip of the electrode where the electric arc occurs by the evaporation of the metals and fluxes coating the electrode, if used, with the majority of the fumes coming from the electrode wire. These metal vapors are oxidized on contact with air and form small particles composed of a complex mixture of metal oxides. These resulting metal complexes, and thus the respiratory exposure of welders, vary according to the materials and welding process used. Stainless-steel (SS) and mild-steel (MS), two of the most common types of electrode wire used, have different elemental constituents. SS fumes contain two metals, Cr and Ni, which alone or in combination have been shown to be cytotoxic to pulmonary cells and associated with lung disease (Camner and Johansson, 1992; Lees, 1991). MS fumes contain neither Cr nor Ni.

There are an estimated 800,000 full-time welders employed worldwide (Sundin, 1988) and approximately 410,040 workers employed as welders, cutters, solderers, and brazers in the United States (Bureau of Labor, 1999). Many more are estimated to perform intermittent welding as part of their employment. Epidemiological studies have indicated that large numbers of welders experience some type of respiratory illness. Respiratory effects observed include acute and chronic bronchitis, airway irritation, chemical pneumonitis, occupational asthma, and a possible increase in lung cancer (Sferlazza and Beckett, 1991).

Some studies have been undertaken to evaluate the toxicity of welding fumes using both *in vitro* and *in vivo* models. Stern and Pigott (1983) and Pasanen *et al*. (1986) demonstrated that SS fumes from manual metal arc welding were much more cytotoxic to rat macrophages than fumes from a variety of other welding processes. Antonini *et al*. (1996, 1997) demonstrated that welding fumes generated from SS materials were more cytotoxic to rat macrophages while inducing a greater release of reactive oxygen species (ROS) from them. The SS fumes were also more pneumotoxic *in vivo* and were cleared from the lungs at a slower rate than fumes collected from MS welding. In addition, the SS fume treatment caused increased

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levels of tumor necrosis factor-(TNF)-α and interleukin-(IL)-1β in the lungs of exposed rats. The authors noted in these studies that the lung response to MS fumes was transient and reversible, similar to the pulmonary response to iron oxide, a mineral particle characterized as a nuisance dust with little pneumotoxic potential. The effects that they observed may have been due to differences in the metal composition and solubility of the SS and MS welding fumes, the persistence of the SS fumes, and presence of inflammatory cytokines following SS fume treatment.

Indirect evidence of a possible role of free radical production by the SS fumes in lung toxicity was found by Antonini *et al*. (1998). They demonstrated that freshly generated SS fumes induced greater lung injury and inflammation than aged fumes, indicating a possible presence of reactive components on the fume surface. In addition, an *in vitro* study by Antonini *et al*. (1999) found differences in the metal compositions and solubilities of fumes collected during gas metal arc (GMA) welding or flux-covered manual metal arc (MMA) welding with both MS and SS electrodes. The MMA-SS fume was found to be much more water-soluble than either the GMA-SS or GMA-MS fumes, and the soluble fraction of the MMA-SS fumes was comprised mainly of Cr. The small soluble fraction of the GMA-MS sample contained Mn with little Fe, whereas a more complex mixture of Mn, Ni, Fe, Cr, and Cu was found in the GMA-SS fume. When rat alveolar macrophages were treated *in vitro* with the soluble fractions of the fumes, the MMA-SS sample was shown to be most cytotoxic and have the greatest impact on their function, reducing their ability to produce ROS when stimulated.

The goals of the present study were to examine the acute effects of three different welding fumes (GMA-MS, GMA-SS, and MMA-SS) of differing compositions and solubilities on lung inflammation and injury in rats. Furthermore, the effects of the water-soluble and insoluble components of the MMA-SS fume were also examined in an attempt to identify the constituents of the fume responsible for the toxicity. These effects were correlated with the free radical production potential of each fume.

MATERIALS AND METHODS

Welding fume collection. The collection of the welding fumes used in this study was previously described by Antonini *et al*. (1999). The fumes were obtained from Lincoln Electric courtesy of Kenneth Brown. Briefly, the fumes were generated in a cubical open-front fume chamber (volume = 1 m^3) by a skilled welder using a manual or automatic technique appropriate for the electrode. The fumes were collected on 0.2-μm Nuclepore filters (Nuclepore Co., Pleasanton, CA) and changed after every 2 min of collection. The fume samples were generated in three different ways: gas metal arc welding (with argon and CO_2 shielding gasses) using a mild-steel electrode (GMA-MS); gas metal arc welding using a stainless-steel electrode (GMA-SS); and manual metal arc welding using a flux-covered stainless-steel electrode (MMA-SS).

Preparation of welding fume samples. The three-fume particle samples (GMA-MS, GMA-SS, MMA-SS) were suspended in sterile phosphate-buffered saline (PBS), pH 7.4, and sonicated for 1 min. The MMA-SS fume was further divided into soluble and insoluble components. The MMA-SS suspension (MMA-SS-Tot; 6.67 mg/ml) was incubated overnight at 37°C with shaking and then centrifuged at 12,000 $\times$ *g* for 30 min. The supernatant (MMA-SS-Sol) was recovered and filtered with 0.22-μm filters (Millipore Corp., Bedford, MA). The pellet (MMA-SS-Insol) was resuspended with the original volume of PBS. Because GMA-MS and GMA-SS were found to be mostly water-insoluble in a previous study (Antonini *et al*., 1999), they were not further divided into soluble and insoluble components for animal treatment in this current study.

Sample characterization. The characterization of the fumes used in this study was previously reported by Antonini *et al*. (1999). Briefly, the amounts of seven different metals commonly found in welding fumes (Cr, Cu, Fe, Mn, Ni, Ti, and V) were measured using inductively coupled argon plasma atomic emission spectroscopy (NIOSH, 1994). The particle sizes of all three fumes were found to be of respirable size, ranging from 0.92- to 1.38-μm count mean diameters, as reported in Antonini *et al*. (1999).

Laboratory animals. All studies were performed on adult male pathogen-free Sprague-Dawley rats (Hla: [SD] CVF; 200–300g; Hilltop Laboratory Animals, Scottdale, PA). They were given a conventional laboratory diet and water *ad libitum* and housed in an AAALAC-approved animal facility with restricted access and HEPA-filtered air, monitored free of pathogens, and allowed to acclimate for at least one week before treatment.

Intratracheal treatment. The rats were intratracheally (i.t) instilled with 2 mg of welding fume in 0.3-ml PBS, or the equivalent volume of the soluble fraction of MMA-SS, or the saline vehicle. This dose of fume was selected based on a dose–response study reported by Antonini *et al*. (1996). A lower dose of fume (0.2 mg/100 g body weight) did not produce inflammatory responses, while a higher dose (5.0 mg/100 g body weight) produced drastic pulmonary inflammation and injury 1 day post-treatment. Also in that study, the responses to a positive control, silica, and a negative control, iron oxide, were reported to help gauge the responses observed after welding fume treatment. Prior to instillation, the animals were anesthetized with Brevital (sodium methohexital, 3 mg/kg), and the instillations were preformed as described in Taylor *et al*. (2000).

Bronchoalveolar lavage (BAL). To harvest the pulmonary cells for morphologic and functional analysis and to obtain acellular fluid for damage indicator analysis, the rats were euthanized at the respective time points with an overdose of sodium pentobarbital and then exsanguinated by severing the left renal artery. BAL was performed at 3 h and 1, 3, and 6 days after i.t. welding fume or vehicle treatment by washing out the lungs with aliquots of calcium- and magnesium-free PBS. The left lung was clamped off and removed prior to BAL. The first BAL volume was administered as 1 ml per 100 g body weight, and this volume was instilled into the lungs for 30 s with light massaging, withdrawn, and again instilled into the lungs for another 30 s. Once withdrawn, this aliquot (designated the first BAL fraction) was kept separate from the rest of the BAL fluid. BAL was continued with similar aliquots and pooled until 40 ml of BAL fluid, containing BAL cells, were recovered. The recovered fluid was then centrifuged (500 $\times$ *g*, 10 min, 4° C), the supernatant decanted, and the cells resuspended. The first BAL fraction was centrifuged separately, and the supernatant was assayed for lactate dehydrogenase activity and albumin content as described below. The cells from the first BAL fraction were then pooled with the rest of the cells recovered from that animal, and the total BAL cells were counted using a Coulter Counter equipped with a Channelizer (model Z_b, Coulter Electronics, Hialeah, FL). Cell differentials were performed visually following cytospin preparations of microscope slides (Shandon Cytospin II, Shandon Inc., Pittsburgh, PA) and Wright-Geimsa staining (Hema-Tec 2000, Bayer Corp., Elkhart, IN).

Analysis of albumin. The integrity of the alveolar–capillary barrier was evaluated by measuring the amount of albumin, a protein from the blood, in the first BAL fraction. BAL fluid albumin was determined according to a Sigma Diagnostics method utilizing the reaction of albumin with bromcresol green. The reaction product was then measured with a spectrophotometer at 628 nm and quantified against known concentrations of bovine serum albumin.

Analysis of lactate dehydrogenase (LDH) activity. LDH activity in the first BAL fraction was used as an indicator of cellular integrity. LDH leaks from cells as a result of damage or when membrane integrity is lost at cell death. LDH activity was determined by the oxidation of lactate coupled to the reduction of NAD^+ at 340 nm over time. Measurements were performed with a Cobas Mira analyzer (Roche Diagnostics Systems, Montclair, NJ).

Free radical measurements. Electron spin resonance (ESR) with spin trapping was used to examine free radical generation. Spin trapping involves the addition reaction of a short-lived radical with a paramagnetic compound (i.e., spin trap) to form a relatively long-lived free radical product, termed the spin adduct, which can be studied with conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radical trapped, and the pattern of hyperfine splittings of the spin adduct is generally characteristic of the original, short-lived, trapped radical. To determine the presence of hydroxyl radicals, 5, 5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin trap in these studies. Measurements were made with a Bruker ESP 300E spectrometer and a flat cell assembly (Bruker Instruments Inc., Billeria, MA). *Spex 300* software (Clarksville, MD) was used for data collection and analysis. The Fenton reaction ($Fe_2SO_4 + H_2O_2$) was used to generate hydroxyl radicals as a positive control for one system, while Cr(VI), NADPH, and glutathione reductase (GSSG-R) were used in another one. Final concentrations for the reactants are listed in the figure legends.

Measurement of lipid peroxidation (LPO) markers. The lipid peroxidation products malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) were measured using the BIOXYTECH® LPO-586™ Colorimetric Assay for Lipid Peroxidation Markers (Oxis International, Inc., Portland, OR). Briefly, the left lungs from treated or control animals were removed, weighed, and homogenized with a PowerGen 700 (Fisher Scientific, Pittsburgh, PA) for 30 s. The homogenate was centrifuged at $1,500 \times g$ for 10 min, and the supernatant was decanted and frozen at −80°C for subsequent analysis. The LPO-586 assay is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole with MDA and 4-hydroxyalkenals at 45°C. One molecule of either MDA or 4-HNE reacts with two molecules of the chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm.

Cytokine analyses. Levels of the cytokines TNF-α, IL-1β, IL-6, and IL-10 were assayed in the first fraction of BAL fluid 3 h and 1, 3, and 6 days after i.t. welding fume or saline treatment. Cytokine protein concentrations were determined with enzyme-linked immunosorbent assay (ELISA) kits from Biosource International (Camarillo, CA). The results of this colorimetric assay were obtained with a Spectramax 250 plate spectrophotometer using *Softmax Pro 2.6* software (Molecular Devices Corp., Sunnyvale, CA).

Statistics. All data are presented as means ± standard error of measurement (SEM). Comparisons between means were made using a two-way ANOVA followed by Tukey's protected *t* post-hoc test (GB-STAT, Dynamic Microsystems Inc, Silver Spring, MD). Statistical significance was established when $p < 0.05$.

RESULTS

Metal analysis of the fumes revealed that different types of substrates produced fumes with differing metal compositions, as reported by Antonini *et al*. (1999) (Table 1). The GMA-MS fume was comprised mainly of Fe and Mn, while the SS fumes had Fe and Mn along with Cr and Ni. The GMA-MS and GMA-SS fumes were found to be mostly water-insoluble, while the MMA-SS fume was partly water-soluble. The soluble fraction of the MMA-SS fume contained mostly Cr.

ESR was used to assess the ability of the fumes to produce free radicals in cell-free systems. Only MMA-SS fume reacted with H_2O_2 in the presence of DMPO to produce a characteristic DMPO-$^\bullet$OH spin adduct signal showing the generation of hydroxyl radicals (Fig. 1A). The spectrum produced consists of a 1:2:2:1 quartet with the splitting of $a_H = a_N = 14.9$ G. Based on these splitting constants, the 1:2:2:1 quartet was assigned to a DMPO-$^\bullet$OH adduct. When the MMA-SS was separated into its Sol and Insol fractions, it was found that both fractions produced a DMPO-$^\bullet$OH signal, with Insol producing a greater signal than Sol, although the MMA-SS-Tot gave the greatest signal (Fig. 1B). To measure the presence of reactive Cr(VI), NADPH and GSSG-R were added to the samples with DMPO. While Cr(VI) cannot be measured directly with ESR, its reduction to Cr(V) and the resulting $^\bullet$OH production can be monitored (Shi and Dalal, 1989). Only the MMA-SS sample produced a Cr(V) signal with an accompanying DMPO-$^\bullet$OH signal, with most of the activity coming from the Sol fraction (Fig. 1C).

TABLE 1
Concentrations of Various Metals in the Welding Fume Samples (μg/mg total weight)

Sample	Fe	Mn	Cr	Ni
GMA-MS	111.0	19.0	0.1	0.0
GMA-SS	87.6	38.2	30.6	8.0
MMA-SS	33.4	13.6	23.2	2.1
MMA-SS-Sol	0.2	5.1	38.2	0.3

Note: Soluble metals were measured in the soluble fraction of the MMA-SS fume (MMA-SS-Sol), whereas the GMA-MS and GMA-SS fumes were found to be largely water-insoluble. The data presented were modified from Antonini *et al*. (1999).

In separate experiments, each welding fume (GMA-MS, GMA-SS, and MMA-SS), different fractions of MMA-SS (MMA-SS-Tot, MMA-SS-Sol, and MMA-SS-Insol), or saline was given to rats intratracheally on day 0. Three h and 1, 3, and 6 days after i.t. treatment, the rats were euthanized. The left lungs were removed, weighed, and assayed for lipid peroxidation, and the right lung lobes were subjected to BAL. The cells recovered by BAL were counted and identified. Table 2 displays the cell numbers from rats treated with the three different fumes at different time points. Differences in the cellular response to the welding fume treatments were observed. The GMA-SS fume caused an increase in cellularity of the BAL on day 1, attributable to a large influx of neutrophils at that time point. The MMA-SS fume also caused a significant increase in neutrophils at day 1, although it was not as great as the relatively insoluble GMA-SS fume. By day 3, the increases in total cells seen after treatment with all three fumes were mainly due to significant increases in alveolar macrophages recovered by BAL. The MMA-SS fume treatment also led to the only increase in eosinophil recovery at day 3, indicating a differential immune response to that fume. By day 6, only the rats treated with the MMA-SS fume had increased BAL cellularity, with increased macrophage recovery along with increased eosinophils. The MMA-SS fume was separated into its soluble

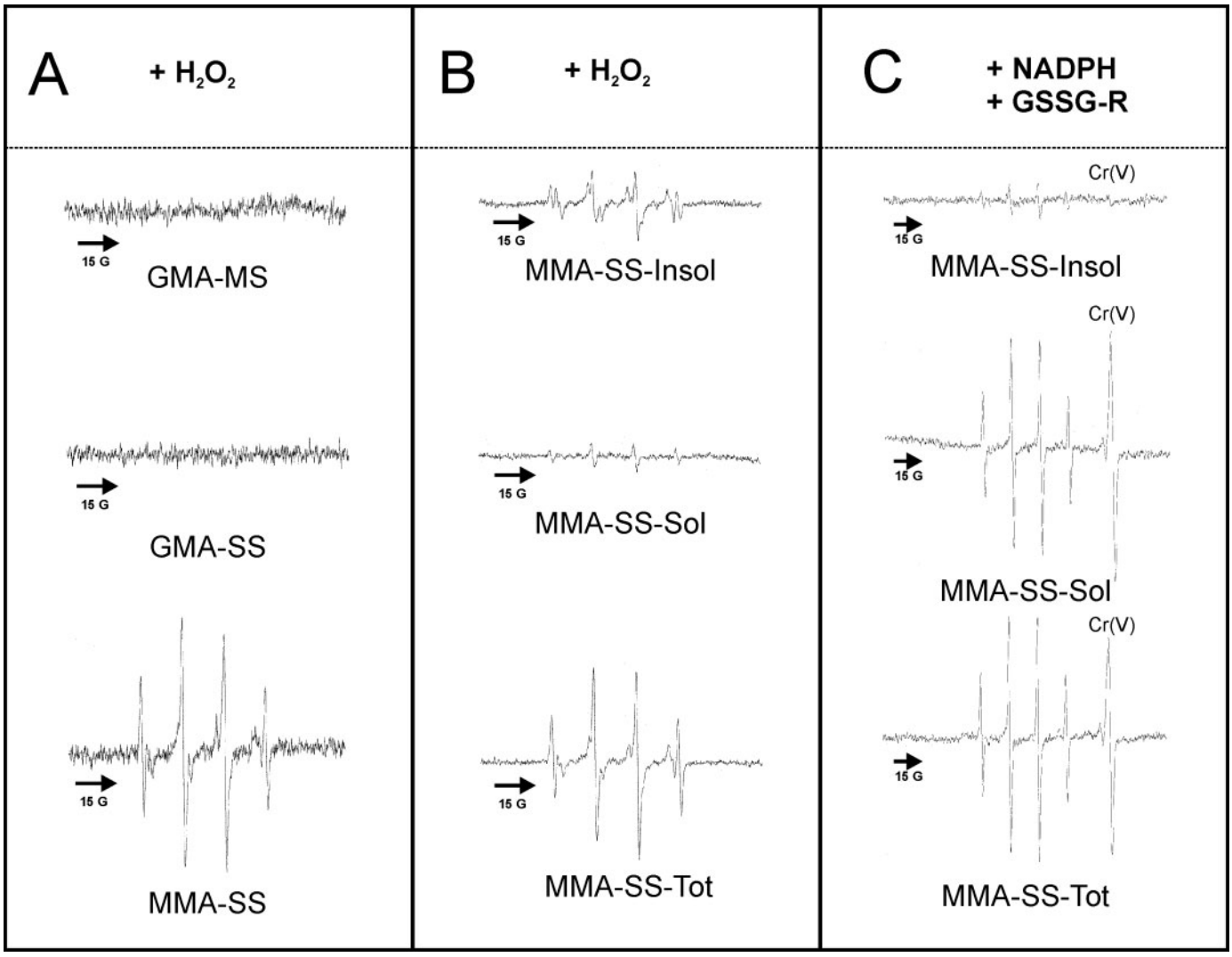

FIG. 1. ESR spectra of welding fumes (1.0 mg/ml) with DMPO (100 mM). (A) GMA-MS, GMA-SS, or MMA-SS with H_2O_2 (10 mM). (B) MMA-SS-Tot, MMA-SS-Sol, or MMA-SS-Insol with H_2O_2 (10 mM). (C) MMA-SS-Tot, MMA-SS-Sol, or MMA-SS-Insol with NADPH (5 mM) and GSSG-R (0.5 mg/ml) with Cr(V) peaks indicated. Arrows represent 15 Gauss and direction of scan.

and insoluble fractions and given to groups of rats along with the total MMA-SS fume and saline for comparison. The number of BAL cells recovered at the various time points are shown in Table 3. No significant differences in cell numbers were observed until day 1, where an increase in cellularity of the BAL fluid from rats treated with MMA-SS-Insol was attributable to a significant increase in neutrophils. By day 3, where the MMA-SS-Tot treatment caused an increase in all cell types, MMA-SS-Insol treatment caused an increase in neutrophils while MMA-SS-Sol treatment caused an increase in eosinophils. By day 6, all three treatments caused an increase in total cells, mainly attributable to the increase in macrophages, whereas increased neutrophils still resulted from MMA-SS-Insol treatment and increased eosinophils followed MMA-SS-Tot treatment.

Several parameters were measured as indicators of pulmonary damage following intratracheal fume treatment. Increases in left lung weight followed both SS fume treatments, but, while the weights of the lungs treated with GMA-SS peaked at day 1, the weights of the lungs from animals treated with MMA-SS continued to increase through day 6, weighing significantly more than the lungs from any other treatment group (Fig. 2A). Figure 2B indicates that this increase in MMA-SS-Tot lung weight is caused by treatment with either the soluble or insoluble fraction until day 6, when the left lung weight of the animals treated with MMA-SS-Tot was greater than the lung weight of those treated with either fraction.

Several markers of pulmonary injury were measured in the first BAL fraction to evaluate the differential toxicity of welding fumes. Albumin, normally found in the blood, can be measured in the BAL fluid, where its increase signifies damage to the delicate alveolar–capillary barrier. First BAL fraction

TABLE 2
Bronchoalveolar Lavage Cell Profiles: GMA-MS, GMA-SS, MMA-SS

	Cell number (10^6)			
Treatment	Total cells	Macrophages	Neutrophils	Eosinophils
Naive	6.52 ± 0.81	6.48 ± 0.82	0.04 ± 0.02	0.00 ± 0.00
3 h				
Saline	5.44 ± 0.51	5.36 ± 0.52	0.09 ± 0.03	0.01 ± 0.00
GMA-MS	4.37 ± 0.52	4.10 ± 0.53	0.23 ± 0.04	0.04 ± 0.02
GMA-SS	4.00 ± 0.38	3.87 ± 0.37	0.13 ± 0.04	0.01 ± 0.00
MMA-SS	4.27 ± 0.36	4.01 ± 0.37	0.22 ± 0.07	0.04 ± 0.01
Day 1				
Saline	6.19 ± 0.52	5.59 ± 0.54	0.58 ± 0.17	0.02 ± 0.01
GMA-MS	10.40 ± 0.69	5.67 ± 0.28	4.71 ± 0.57	0.03 ± 0.02
GMA-SS	17.36 ± 1.96[a]	4.59 ± 0.48	12.71 ± 1.65[c]	0.05 ± 0.02
MMA-SS	11.99 ± 2.63	4.63 ± 0.23	6.99 ± 2.44[a]	0.36 ± 0.08
Day 3				
Saline	7.00 ± 0.65	6.97 ± 0.65	0.02 ± 0.01	0.00 ± 0.00
GMA-MS	13.64 ± 1.30[a]	11.54 ± 1.06[a]	2.00 ± 0.38	0.06 ± 0.02
GMA-SS	16.01 ± 2.58[a]	12.53 ± 2.03[a]	3.40 ± 0.66	0.00 ± 0.00
MMA-SS	23.08 ± 1.52[b]	16.49 ± 1.62[a]	3.27 ± 0.71	3.28 ± 0.60[c]
Day 6				
Saline	7.21 ± 0.52	7.19 ± 0.52	0.02 ± 0.01	0.01 ± 0.01
GMA-MS	9.65 ± 1.16	9.30 ± 1.22	0.35 ± 0.15	0.00 ± 0.00
GMA-SS	14.32 ± 1.46	12.55 ± 0.90	1.77 ± 0.65	0.00 ± 0.00
MMA-SS	21.45 ± 1.34[b]	17.45 ± 1.12[b]	2.50 ± 0.54	1.50 ± 0.30[c]

Note. Values are means ± SE; $N = 4$–9 per group.
[a]Significantly different than saline control group ($p < 0.05$).
[b]Significantly greater than GMA-MS and saline control groups ($p < 0.05$).
[c]Significantly greater than all other groups ($p < 0.05$).

albumin was increased by MMA-SS treatment at days 1, 3, and 6, with the maximum level found on day 3 (Fig. 3A). Further experiments indicated that damage from both the soluble and insoluble fractions were additive in producing the damage observed after treatment with the total fume (Fig. 3B). LDH, a constitutive cytosolic enzyme, can leak from cells that have lost membrane integrity and are usually dead or dying. While treatment with all fumes increased LDH activity in the first BAL fraction at day 3, MMA-SS treatment significantly increased BAL LDH activity on days 1, 3, and 6 (Fig. 4A). Again experiments with the various fractions of MMA-SS showed the effect to be additive between the soluble and insoluble fractions, especially during the peak response at day 3 (Fig. 4B).

To assess any potential oxidative damage occurring in the lungs following welding fume treatment, the lipid peroxidation by-products MDA and 4-HNE were measured in left lung homogenates at the various time points. Both of the SS fumes increased left lung LPO (Fig. 5A), but the maximal response followed the MMA-SS treatment at day 3. Additional investigation revealed that both the soluble and insoluble fractions of MMA-SS induced LPO at day 3, although the insoluble fraction appeared to be responsible for most of the response observed following the MMA-SS-Tot treatment (Fig. 5B).

To gain insight into the mechanisms underlying the acute inflammatory response to the different fumes, the cytokines TNF-α, IL-1β, IL-6, and IL-10 were assayed in the first BAL fraction at all time-points by ELISA. Significant differences from saline treatment were only seen at day 1 in TNF-α and IL-6 levels. Figure 6A shows that both SS fumes caused a significant elevation in TNF-α in the first BAL fraction. Interestingly, the MMA-SS-Sol fraction was also significantly elevated at day 1 (Fig. 6B). Only treatment with the MMA-SS fume caused a significant elevation in IL-6 in the first BAL fraction (Fig. 7A), with the data suggesting that the MMA-SS-Insol fraction was mostly responsible for the elevation (Fig. 7B). The MMA-SS-Insol treatment also caused the only significant elevation in IL-6 at 3 h, whereas the MMA-SS-Sol, MMA-SS-Tot, or the other fumes did not (data not shown). No significant differences were observed in IL-1β and IL-10 in the first BAL fraction at any time point following any welding fume treatment (data not shown).

DISCUSSION

This study compared the effects of three welding fumes generated from different processes using different materials on acute lung damage and inflammation. The fumes differed in both metal content and solubility. The GMA-MS fume was comprised mainly of Fe and Mn, whereas the GMA-SS and MMA-SS fumes contained Fe and Mn along with Cr and Ni.

TABLE 3
Bronchoalveolar Lavage Cell Profiles: MMA-SS-Sol, Insol, and Tot

Treatment	Cell number (10^6)			
	Total cells	Macrophages	Neutrophils	Eosinophils
Naive	6.52 ± 0.81	6.48 ± 0.82	0.04 ± 0.02	0.00 ± 0.00
3 h				
Saline	6.74 ± 0.52	6.64 ± 0.52	0.10 ± 0.02	0.01 ± 0.01
MMA-SS-Sol	4.62 ± 0.22	4.39 ± 0.25	0.19 ± 0.05	0.05 ± 0.02
MMA-SS-Insol	7.06 ± 0.39	6.72 ± 0.35	0.30 ± 0.10	0.05 ± 0.03
MMA-SS-Tot	4.63 ± 0.29	4.38 ± 0.29	0.20 ± 0.03	0.06 ± 0.02
Day 1				
Saline	6.91 ± 0.38	6.81 ± 0.39	0.09 ± 0.02	0.01 ± 0.00
MMA-SS-Sol	7.36 ± 0.46	4.65 ± 0.34	2.19 ± 0.40	0.51 ± 0.12
MMA-SS-Insol	13.00 ± 1.34[a]	6.53 ± 0.46	6.29 ± 1.16[c]	0.17 ± 0.08
MMA-SS-Tot	8.36 ± 0.63	4.04 ± 0.27	3.87 ± 0.48[b]	0.45 ± 0.06
Day 3				
Saline	6.35 ± 0.44	6.32 ± 0.44	0.02 ± 0.01	0.01 ± 0.01
MMA-SS-Sol	10.37 ± 1.12	7.73 ± 0.93	1.08 ± 0.24	1.47 ± 0.36[d]
MMA-SS-Insol	13.18 ± 1.47[a]	10.22 ± 1.39	2.95 ± 0.56[a]	0.27 ± 0.07
MMA-SS-Tot	17.39 ± 1.66[b]	12.42 ± 1.36[b]	2.99 ± 0.35[a]	1.98 ± 0.40[d]
Day 6				
Saline	6.35 ± 1.65	6.33 ± 1.66	0.02 ± 0.01	0.00 ± 0.00
MMA-SS-Sol	14.69 ± 1.46[a]	13.31 ± 1.44[a]	0.33 ± 0.05	1.05 ± 0.48
MMA-SS-Insol	19.17 ± 2.96[a]	14.86 ± 2.50[a]	4.17 ± 1.14[b]	0.14 ± 0.04
MMA-SS-Tot	20.89 ± 1.13[b]	17.09 ± 0.87[a]	2.28 ± 0.40	1.51 ± 0.19[d]

Note. Values are means ± SE; $N = 5$–11 per group.
[a]Significantly different than saline control group ($p < 0.05$).
[b]Significantly greater than MMA-SS-Sol and saline control groups ($p < 0.05$).
[c]Significantly greater than all other groups ($p < 0.05$).
[d]Significantly greater than MMA-SS-Insol and saline control ($p < 0.05$).

The MMA-SS fume was the most soluble, with the water-soluble fraction containing mostly Cr.

Particle size can play a role in determining the toxicity of welding fumes. Individual fume particles are first formed near the arc in the submicron ultrafine size range (0.01–0.10 μm) (Voitkevich, 1995). Due to the turbulent conditions resulting from the extreme heat generation at the arc, the particles quickly aggregate together in the air to form longer chains of primary particles (Clapp and Owen, 1977; Zimmer and Biswas, 2001). In the atmosphere of the welders′ breathing zone, welding fume particles have been observed to be 0.50–2.0 μm in aerodynamic diameter (Villaume *et al*., 1979; Voitkevich, 1995). This is very similar to the size of the particles used in this study, which, after collection, resuspention, and sonication, were GMA-MS, 0.83 ± 0.15; GMA-SS, 0.77 ± 0.48; and MMA-SS, 0.92 ± 0.11 (Antonini *et al*., 1997, 1998). Therefore, it was concluded that the collection by filtration and subsequent resuspension used in this study did not significantly alter the particle size as compared to what is observed in the welders' breathing zone.

In this study, the pulmonary responses to different welding fumes were compared following intratracheal instillation. Thus a bolus of particles in liquid was given at one time, as opposed to a more gradual accumulation of particles that occurs during inhalation (Driscoll *et al*, 2000). To minimize the effects of this route of administration, a dose of fume was chosen that produced measurable effects without causing massive toxicity based on a dose–response curve constructed during a previous study (Antonini *et al*., 1997). Henderson *et al.* (1995) compared the inflammatory response of the lungs to particulates of high and low toxicity by intratracheal instillation and inhalation. Their results indicated that the degree of pulmonary inflammation caused by various doses of different particulates could be evaluated appropriately using either exposure method. A recent study by Reasor and Antonini (2001) found similar results when intratracheally administering the same total dose of silica at one time or spread across five daily separate instillations. Thus the effect of the large bolus of particles seems to be minimal as long as the dose is not overly large.

While the intratracheal route of administration is less physiological, there are advantages to its use in this study as well. The actual dose of fume to each animal is very uniform and can be delivered accurately, without concern for any particles being removed nasally. Additionally, aspects of this study examining the results of the soluble and insoluble fractions of the MMA-SS fume would be impossible to ascertain via inhalation. Furthermore, by comparing these fumes by intratracheal instillation, experiments using inhalational exposure of partic-

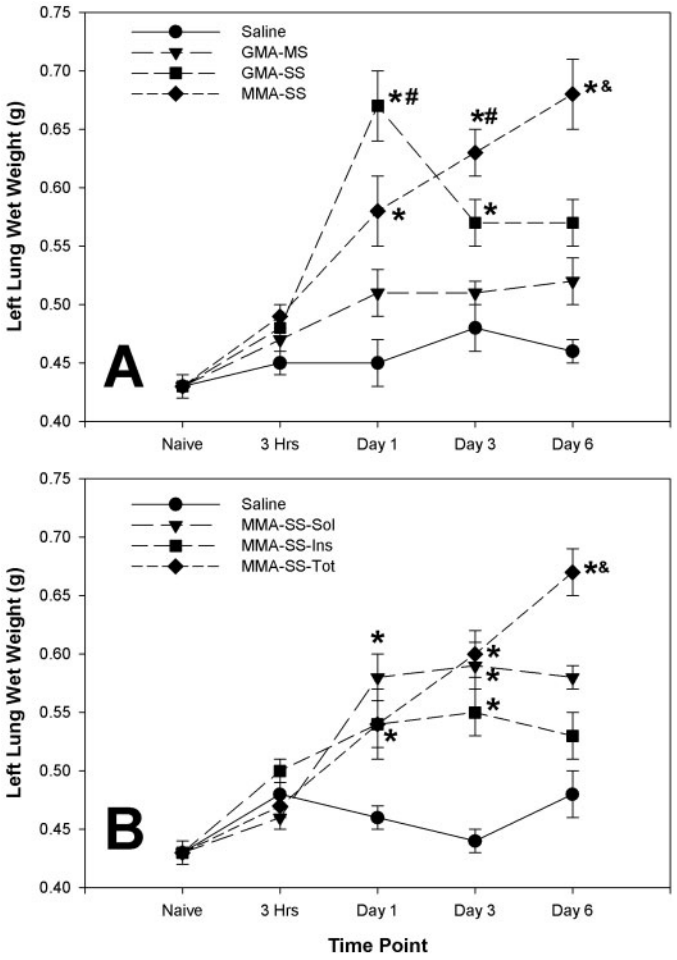

FIG. 2. Effect of welding fume treatment on left lung wet weight. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, or MMA-SS-Tot (B). Their left lungs were removed and weighed 3 h or 1, 3, or 6 days following treatment. Values are means ± SE (n = 5–11 per group). *Significantly different from saline. #Significantly different than GMA-MS. &Significantly different than other welding fume groups.

ular fumes of interest can be planned, and possible mechanisms underlying the toxicity can be explored.

It is well documented that free radical generation is a significant contributor to the pathogenesis of lung disease caused by particulate inhalation (Vallyathan and Shi, 1997). Several metals present in the welding fumes have been shown to produce free radicals under various conditions, including Cr (Leonard *et al*., 2000; Ye *et al*., 1999) and Ni (Huang *et al*., 2001). Antonini *et al*. (1998) also reported indirect evidence of reactive species involvement in welding fume-induced pneumotoxicity when they found fresh fume to be more pneumotoxic to rats than aged fume of the same composition. Thus, the ability of these fumes to produce free radicals was examined by ESR in the current study. The MMA-SS fume was the only fume to produce hydroxyl radicals when reacted with H_2O_2. When analyzed separately, the Insol fraction produced a greater signal than the Sol fraction, and both were less than Tot. Thus the lung damage from each fraction could be attributed to possible free radical production. Furthermore, the MMA-SS was the only fume to contain reactive Cr(VI), evident from the characteristic Cr(V) signal. In this instance, the Sol fraction produced a significantly stronger signal intensity as compared to Insol. Thus the ESR experiments demonstrated that the MMA-SS was the most reactive fume, with each fraction able to produce free radicals in various conditions. The implied reactivity of Cr(VI) in the Sol fraction correlates with the amount of soluble Cr present in that fume as compared to the other fumes. This result corroborates a previous study (White *et al*., 1982) in which animals were administered a single i.t. instillation of soluble and insoluble fractions of a stainless-steel welding fume or potassium dichromate containing the same Cr(VI) concentrations found in the fumes. They

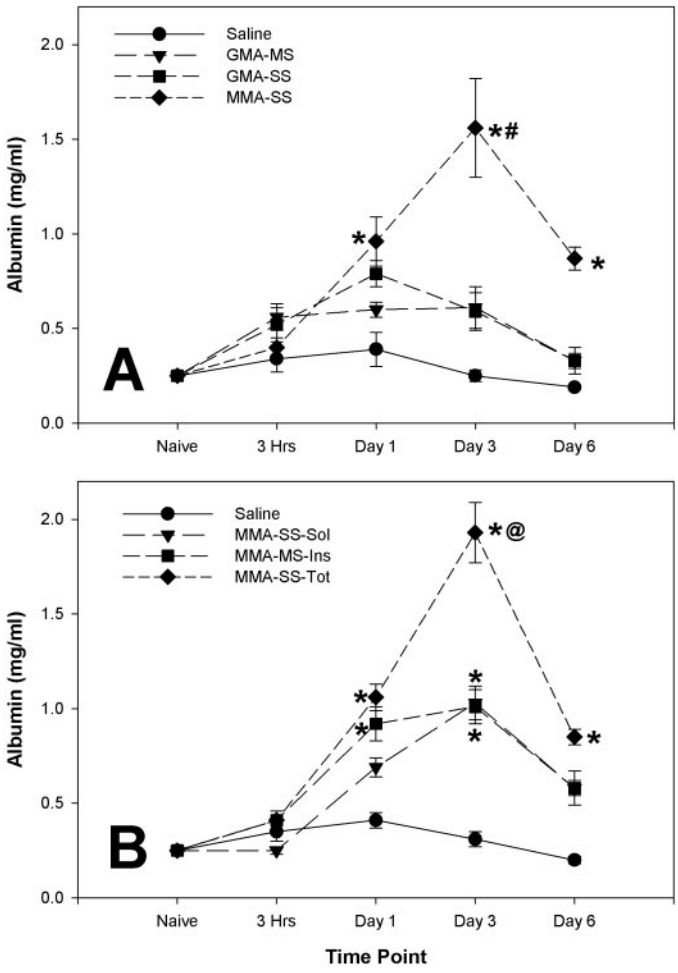

FIG. 3. Effect of welding fume treatment on albumin in the first BAL fraction. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, MMA-SS-Tot, or saline (B) and subjected to BAL 3 h or 1, 3, or 6 days following treatment. Albumin levels were measured in the first BAL fraction. Values are means ± SE (n = 5–11 per group). *Significantly different from saline. #Significantly different than GMA-MS. @Significantly different than MMA-SS-Insol.

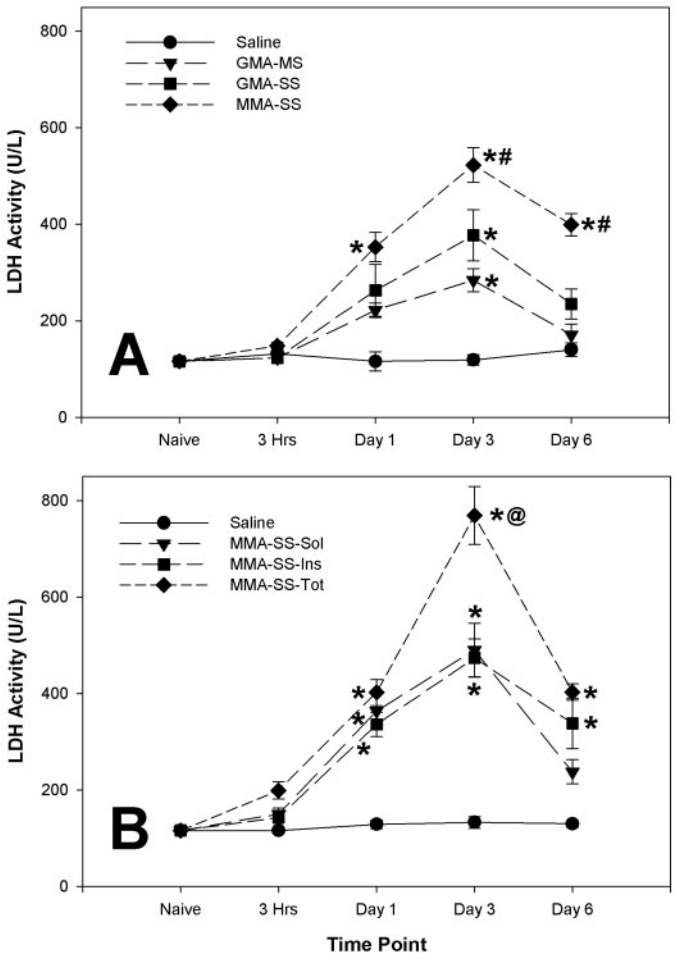

FIG. 4. Effect of welding fume treatment on LDH activity in the first BAL fraction. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, MMA-SS-Tot, or saline (B) and subjected to BAL 3 h or 1, 3, or 6 days following treatment. LDH activity was measured in the first BAL fraction. Values are means ± SE (n = 5–11 per group). *Significantly different from saline. #Significantly different than GMA-MS. @Significantly different than MMA-SS-Insol.

observed that most of the toxicity observed 7 days following i.t. treatment was attributable to the soluble Cr(VI) in the fume. The inflammation subsided over time, leading them to conclude that this was due to the removal of the soluble Cr(VI) from the lungs.

To study the ability of the fumes to produce lung inflammation and damage, the same amount of each fume, including the Sol and Insol fractions of the MMA-SS, was intratracheally administered to the rats. Parameters of damage and inflammation were then assessed at 3 h and 1, 3, and 6 days following treatment. The administration of the various fumes produced differing inflammatory profiles. All of the fumes eventually led to an increase in macrophages in all of the treatment groups, with the MMA-SS–treated group tending to be higher. Both the Sol and Insol fractions of the MMA-SS were required for the maximal response. Treatment with the relatively insoluble GMA-SS fume produced an early neutrophil influx while only the MMA-SS fume treatment led to eosinophil recruitment. Further experiments with MMA-SS demonstrated that the Sol fraction was responsible for the eosinophil recruitment, whereas the Insol fraction led to the recruitment of neutrophils. This indicates a differential inflammatory response, with neutrophils responding to the insoluble particulates of the MMA-SS fume and eosinophils being recruited following treatment with the soluble constituents. While soluble metals are most likely the cause, additional experiments are required to determine the exact metal or metals responsible and the recruitment pathways involved.

The increased number of eosinophils in the BAL fluid following the MMA-SS treatment indicates a possible immune reaction following fume treatment. It has been reported that

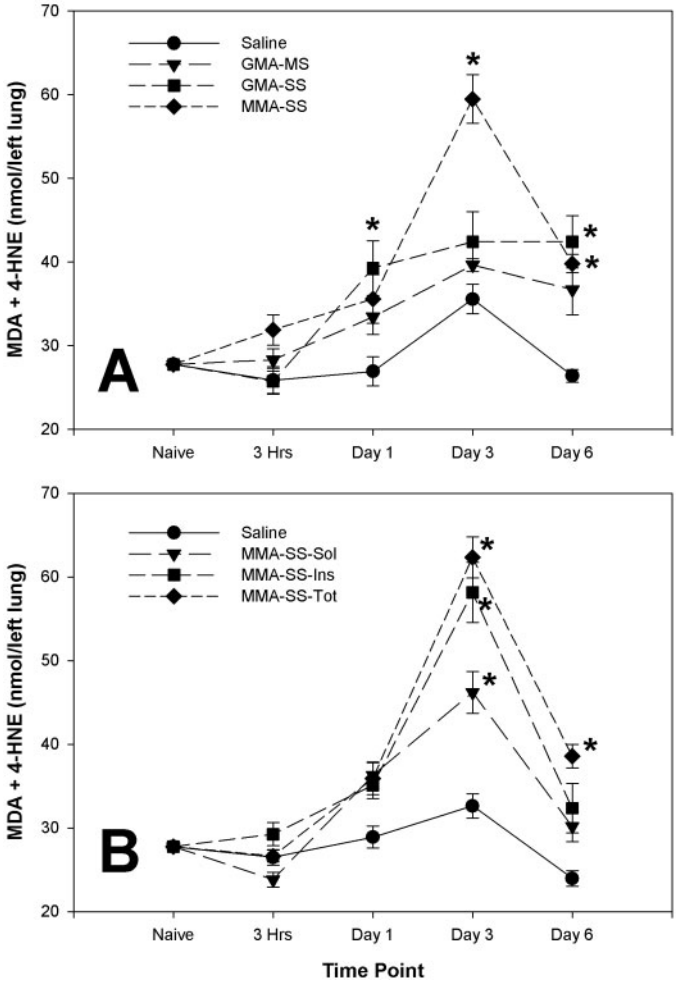

FIG. 5. Effect of welding fume treatment on lipid peroxidation. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, MMA-SS-Tot, or saline (B). Their left lungs were removed and assayed for the lipid peroxidation products malondialdehyde (MDA) and 4-hydroxyalkenal (4-HNE) at 3 h or 1, 3, or 6 days following treatment. Values are means ± SE (n = 5–11 per group). *Significantly different from saline.

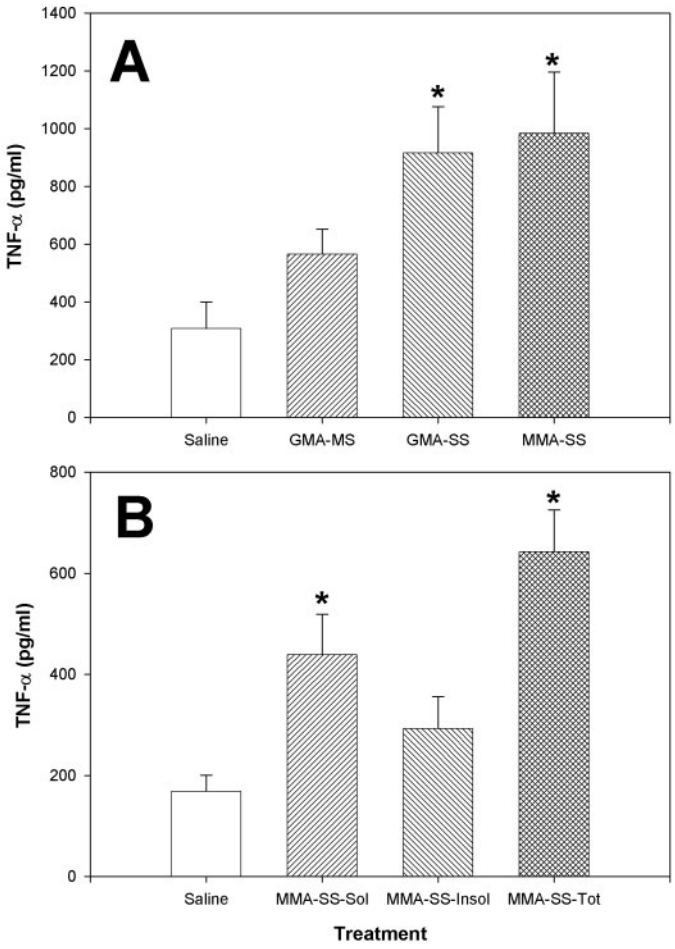

FIG. 6. Effect of welding fume treatment on TNF-α levels in the first BAL fraction at day 1. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, MMA-SS-Tot, or saline (B) and subjected to BAL 1 day following treatment. TNF-α levels were measured in the first BAL fraction. Values are means $\pm$ SE (n = 5–11 per group). *Significantly different from saline.

respiratory infections are increased in severity, duration, and frequency among welders (Howden *et al*., 1988). Chemical irritation of the airways caused by metal fumes is a suspected cause of increased incidence of lung infection (Coggen *et al*., 1994). Wergeland and Iverson (2001) have discussed the potentially lethal risk association of pneumonia with the inhalation of metal fumes, such as those generated in welding. Thus the recruitment of eosinophils after MMA-SS treatment in rats may also be a marker for the occurrence of an immune dysfunction. Chromium has been implicated in the development of respiratory sensitization leading to asthma (Park *et al*., 1994), indicating that it is capable of causing abnormal immune reactions. Thus chromium may be responsible for the abnormal recruitment of eosinophils to the lungs following MMA-SS-Sol treatment.

Differential responses in lung damage parameters were observed after treatment with the various welding fumes, with the MMA-SS fume being the most toxic. The left lung weight of animals treated with GMA-SS peaked at day 1, while following MMA-SS treatment the weight continued to increase until day 6. This indicates a continuing increase in edema and/or damage, which corresponds to the increase in cellularity of the BAL fluid. The MMA-SS-Sol and Insol fractions produced additive effects at this time point, indicating that both are necessary for the total response.

Albumin, a protein normally found in the blood, is used as an indicator of alveolar–capillary barrier damage when increased amounts are found in the airspace. The MMA-SS fume treatment caused maximal albumin leakage at day 3 with the MMA-SS-Sol and Insol fraction responses being equal and additive. LDH activity in the BAL fluid, an indicator of cellular damage and death, was increased following all three fume treatments, but it was highest and more sustained following

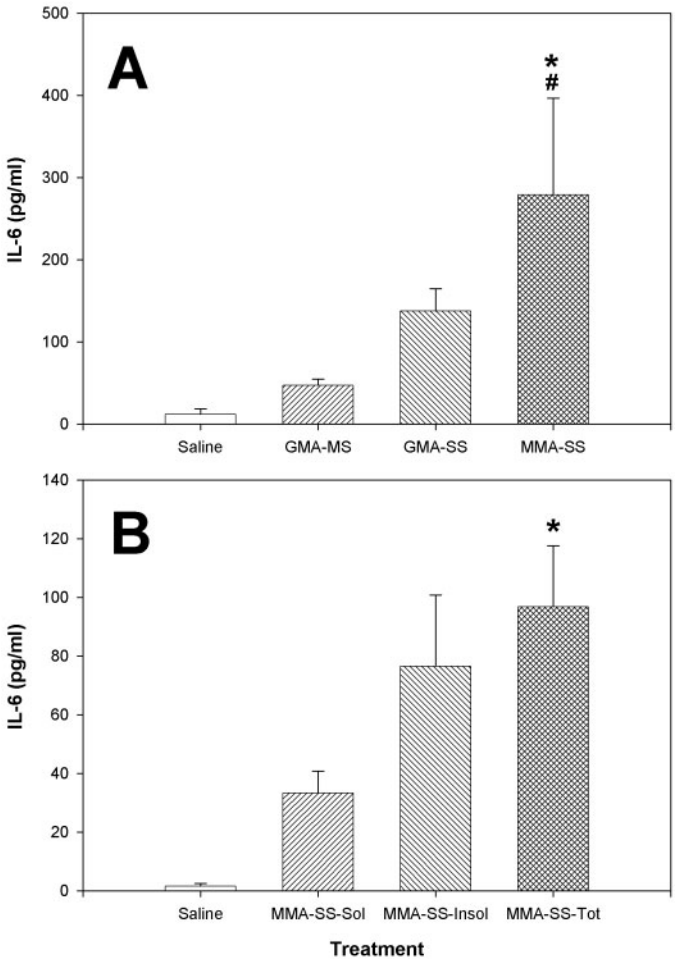

FIG. 7. Effect of welding fume treatment on IL-6 levels in the first BAL fraction at day 1. Rats were intratracheally treated with GMA-MS, GMA-SS, MMA-SS, or the saline vehicle (A), or MMA-SS-Sol, MMA-SS-Insol, MMA-SS-Tot, or saline (B) and subjected to BAL 1 day following treatment. IL-6 levels were measured in the first BAL fraction. Values are means $\pm$ SE (n = 5–11 per group). *Significantly different from saline. #Significantly different than GMA-MS.

MMA-SS treatment. Analysis following treatment with the MMA-SS fractions again revealed an equal and additive response following MMA-SS-Sol and Insol treatment. MMA-SS fume treatment caused the only significant increase in LPO at day 3, indicating oxidative damage to lung tissue. The increase in LPO was caused mainly by the Insol fraction of the MMA-SS fume. Several mechanisms may be involved in this response. The oxidative damage could be caused by direct free radical production, as the insoluble fume particles were shown to generate $^{\bullet}OH$ and would tend to remain in the lungs longer than soluble metals. Also, the recruitment and activation of phagocytes capable of producing reactive oxygen and nitrogen species, as indicated by increased neutrophil and macrophage numbers and the presence of pro-inflammatory cytokines, discussed below, could be responsible. Finally, a combination of both factors could have been involved in the oxidative tissue damage observed after MMA-SS treatment.

The toxicity profile of these fumes correlates with a previous study (Antonini *et al*., 1996) that examined later time points. They reported that fumes from the welding of SS materials produced more lung injury and inflammation than fumes produced from MS welding at 14 and 35 days post–i.t., with the toxicity decreasing at the latest time point.

Both the GMA-SS and MMA-SS fumes caused an increase in the amount of TNF-α in the acellular first BAL fluid fraction at day 1. TNF-α is a major pro-inflammatory cytokine produced in response to a variety of exposures (Luster *et al*., 1999). Interestingly, the MMA-SS-Sol fraction was responsible for most of the TNF-α increase at this time point, although the responses to both fractions were additive in relation to the response from treatment with the MMA-SS-Tot fume. IL-6 in the first BAL fraction was increased only by the MMA-SS treatment, with the MMA-SS-Insol fraction being responsible for most of the response. IL-6 activates specific immune responses, indicating that differential inflammatory pathways are activated following MMA-SS treatment. This is further indicated by the presence of eosinophils in the BAL fluid, which were recruited mainly by the MMA-SS Sol fraction. Overall, the MMA-SS caused a greater inflammatory response, indicated by the eventual increase in total cells recovered by BAL. This reaction, however, may not indicate a stimulated immune response in the lungs. In a separate study (Antonini *et al*., 2001), it was found that MMA-SS fume pre-treatment slowed the clearance of a bacterial pathogen from the lungs whereas the GMA-MS and GMA-SS fumes had no effects. Taken together, these data suggest that MMA-SS fumes are capable of causing immune dysfunction and possible abnormal immune responses.

The effects of MMA-SS proved in most cases to be dependent on both the soluble and insoluble fractions of the fume. This is unique in that studies of urban air particulates and residual oil fly ash (ROFA) particles have attributed many of their pneumotoxic effects to the soluble metals associated with the particulates (Dreher *et al*., 1997; Gavett *et al*., 1997; Kodavanti *et al*, 1998; Pritchard *et al*., 1996). Dreher *et al*. (1997) demonstrated that transition metals are the causative agents of ROFA-induced acute lung injury. A leachate prepared from the ROFA, as well as a surrogate transition metal sulfate solution, largely reproduced the lung injury caused by the entire ROFA sample. Another study (Gavett *et al*., 1997) reinforced these results by comparing two different ROFA samples with differing soluble metals. Kodavanti *et al*. (1998) analyzed ten ROFA samples from different parts of a power plant and with differing leachable metal compositions. They were able to attribute different aspects of the toxicity to different transitional metals, namely Ni and V, by different pathways. The mechanisms of acute lung damage by welding fumes are less understood, but it was hypothesized that the soluble metals of the relatively soluble welding fume MMA-SS would largely be responsible for the toxicity of the fumes based on the above ROFA studies. This was not the case, as both fume fractions were often required to produce the maximal response. Further studies will be necessary to identify the components responsible for the damage from each fraction.

The results of the current study indicate that welding fumes of differing metal composition produce differential acute lung toxicities. Furthermore, the fume that produced the most free radicals in two acellular systems proved to be the most toxic, indicating free radical production as a possible mechanism for the toxicity. The effects of the MMA-SS proved in most cases to be dependent on both the soluble and insoluble fractions of the fume, which is different from ROFA particles, in which the soluble metals are associated with their pneumotoxic effects. The mechanisms of lung toxicity from welding fumes are less well characterized, but the evidence provided in this study suggests that both the soluble and insoluble fractions are involved with various aspects of the acute lung damage and inflammation following MMA-SS welding fume treatment.

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