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RESEARCH ARTICLE

Short-term inhalation of stainless steel welding fume causes sustained lung toxicity but no tumorigenesis in lung tumor susceptible A/J mice

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Abstract

Debate exists as to whether welding fume is carcinogenic, but epidemiological evidence suggests that welders are an at-risk population for development of lung cancer. Our objective was to expose, by inhalation, lung tumor susceptible (A/J) and resistant C57BL/6J (B6) mice to stainless steel (SS) welding fume containing carcinogenic metals and characterize the lung-inflammatory and tumorigenic response. Male mice were exposed to air or gas metal arc (GMA)-SS welding fume at 40 mg/m^3 × 3 h/day for 6 and 10 days. At 1, 4, 7, 10, 14, and 28 days after 10 days of exposure, bronchoalveolar lavage (BAL) was done. Lung cytotoxicity, permeability, inflammatory cytokines, and cell differentials were analyzed. For the lung tumor study, gross tumor counts and histopathological changes were assessed in A/J mice at 78 weeks after 6 and 10 days of exposure. Inhalation of GMA-SS fume caused an early, sustained macrophage and lymphocyte response followed by a gradual neutrophil influx and the magnitudes of these differed between the mouse strains. Monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α) were increased in both strains while the B6 also had increased interleukin-6 (IL-6) protein. BAL measures of cytotoxicity and damage were similar between the strains and significantly increased at all time points. Histopathology and tumorigenesis were unremarkable at 78 weeks. In conclusion, GMA-SS welding fume induced a significant and sustained inflammatory response in both mouse strains with no recovery by 28 days. Under our exposure conditions, GMA-SS exposure resulted in no significant tumor development in A/J mice.

Keywords: welding, chromium, lung cancer, strain A mice, bronchoalveolar lavage

Introduction

Electric arc welding joins pieces of metal that are rendered liquid by heat. Arc temperatures above 4000°C heat the base metal pieces to be joined and the consumable electrode wire that is continuously fed into the weld. The vaporized metals, derived primarily from the wire, react with air and form the fume, which consists of a complex mixture of metal oxides. Depending on the welding process employed, the electrode coating, shielding gases, fluxes, base metal, and paint or surface coatings may comprise the welding fume (Antonini, 2003).

Among the numerous types of welding processes, gas metal arc (GMA) welding is commonly used in the workplace. GMA welding with stainless steel (SS) wire produces fumes that contain carcinogenic metals such as Cr and Ni, which are primarily in an insoluble form. As a result, the potential carcinogenic nature of SS welding fume has emerged as a chief health concern for welders; this concern remains one of the most important unanswered questions in welding fume-related toxicological research. The harmful health effects of welding are well documented and epidemiological evidence

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generally supports the hypothesis that exposure to welding fume increases lung cancer risk, but confounders such as asbestos exposure and smoking obscure these findings (Sferlazza and Beckett, 1991; Moulin, 1997; Danielsen et al., 1998; Sorensen et al., 2007). At present, the International Agency for Research on Cancer (IARC) categorizes welding fume as possibly carcinogenic to humans (i.e. group 2B agent). This conclusion by the IARC, however, was based on limited evidence in humans and inadequate evidence in animals, which justifies our present study (IARC, 1990).

A/J mice are genetically predisposed to spontaneous and/or chemically-induced lung tumors while C57BL/6J (B6) mice are essentially resistant (Shimkin and Stoner, 1975). In a recent study, we found that exposure by pharyngeal aspiration to welding fume caused lung inflammation [polymorphonuclear leukocyte (PMN) influx] and increased lung cytotoxicity, permeability, and inflammatory cytokines found in the bronchoalveolar lavage (BAL) fluid of both A/J and B6 mice. The A/J strain, however, exhibited a significantly greater magnitude of lung response and an attenuated resolution of the response compared to the resistant B6 strain. We also found that the SS fumes, particularly those from GMA welding, provoked a mild chronic inflammation in the A/J lung and tended to cause the greatest, overall, lung toxicity compared to other types of welding fumes. Furthermore, we observed a trend for an increased lung tumor incidence in the GMA-SS welding fume-exposed A/J mice, which, when considered in conjunction with our other findings, suggested that a chronic lung response to GMA-SS welding fume may enhance tumorigenesis in the A/J model (Zeidler-Erdely et al., 2008). To expand on this work, our objectives in this study were to characterize the inflammatory lung response in both mouse strains and examine the lung tumorigenic response in A/J mice after short-term inhalation of GMA-SS welding fume. Inhalation is the preferred route for welding fume-related toxicity studies in animals because it best mimics an occupational exposure primarily with respect to the particle size and surface properties of the fume and the lung particle deposition.

Material and methods

Animals

Male A/J and B6 mice, 4 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited, specific pathogen-free, environmentally-controlled facility. All mice were free of endogenous viral pathogens, parasites, mycoplasmas, Helicobacter, and cilia-associated respiratory bacillus. Mice were individually housed in ventilated cages and provided high-efficiency particulate air-filtered air under a controlled light cycle (12 h light/12 h dark) at a standard temperature (22–24°C) and 30–70% relative humidity. Animals were acclimated to the animal facility for a minimum of 1 week and allowed access to a conventional diet (6% Irradiated NIH-31 Diet, Harlan Teklad, Madison, WI) and tap water *ad libitum*. All procedures were performed using protocols approved by the National Institute for Occupational Safety and Health (NIOSH) Institutional Animal Care and Use Committee.

Welding fume generator and inhalation exposure system

Mice were exposed by whole-body inhalation, in individual steel mesh cages, to aerosols generated during GMA-SS welding for 6 or 10 days at a target concentration of 40 mg/m^3 for 3 h/day. This exposure regime was based on our prior inhalation data in rats that examined a dose near (15 mg/m^3) and slightly above (40 mg/m^3) the previous ACGIH® recommended threshold exposure limit value-time weighted average (TLV-TWA) of 5 mg/m^3 for 8 h/day, a level commonly exceeded in the workplace (Susi et al., 2000; ACGIH, 2001; Antonini et al., 2007).

The design and construction of the welding fume aerosol generator and the characterization of the fume were previously described (Antonini et al., 2006). Briefly, mice inhaled welding fume composed of the following metals (weight %): Fe (57), Cr (20.2), Mn (13.8), Ni (8.8), and Cu (0.2) with trace amounts of Si, Al, and V. The particle diameters ranged from ultrafine (0.01–0.1 μm) to coarse (1.0–10 μm) with the majority of particles in the fine size range (0.1–1.0 μm). The mass median aerodynamic diameter was 0.255 μm with a geometric standard deviation of 1.352.

Body weight determination

Prior to the inhalation exposure, the animals were tattooed and weight matched. All mice were weighed after the exposure period and at the 1, 4, 7, 10, 14, and 28-day sacrifices. All groups gained weight throughout the exposure and 28-day time course equally. A/J mice kept for 78 weeks post-exposure were also weighed at 50 weeks then again at sacrifice.

Whole lung metal analysis

Non-lavaged, whole lungs were excised, trimmed, and lyophilized. The freeze-dried tissue was weighed then acid digested. The amount of Cr, Cu, Fe, Mn, and Ni present in the lung was determined by inductively coupled argon plasma, atomic emission spectroscopy at NIOSH-Division of Applied Research and Technology (Cincinnati, OH) according to NIOSH method 7300 modified to accommodate the sample matrix (NIOSH, 1994).

Whole lung BAL

Whole lung BAL was used to assess the lung response in A/J and B6 mice at 1, 4, 7, 10, 14, and 28 days after exposure to GMA-SS welding fume or air. Mice were deeply anesthetized with Sleepaway [26% sodium pentobarbital, 7.8% isopropyl alcohol, and 20.7% propylene glycol] (Fort Dodge Animal Health, Fort Dodge, IA) then weighed.

Once unresponsive, the abdomen was opened and the vena cava was cut to exsanguinate the mouse. For BAL, the trachea was cannulated with a blunted 22-gauge needle and 0.6 mL of cold Ca^{+2} and Mg^{+2}-free phosphate buffered saline (PBS) was slowly instilled into the lung then withdrawn and placed on ice. This constituted the first fraction of BAL fluid. Two subsequent lavages (1.0 mL/instillate) were collected into a separate tube which represented the second fraction. The BAL fluid was kept on ice; then the samples were centrifuged (500*g*, 10 min, 4°C).

Aliquots of the first fraction BAL supernatant were used for biochemical measurements or frozen at –80°C for later analysis. The supernatant of the second fraction was discarded. The cell pellets from both fractions were combined and centrifuged (500*g*, 6 min, 4°C) and the supernatant discarded. The final cell pellet was suspended in 1.0 mL PBS and used for cell enumeration and differential staining.

Total cell numbers were determined using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, FL). For cell differential analysis, BAL cells were plated onto glass slides using a Cytospin 3 centrifuge (Shandon Life Sciences International, Cheshire, England) set at 800 rpm for 5 min. Slides were stained using Leukostat stain (Fisher Scientific, Pittsburgh, PA) then coverslipped. A minimum of 300 cells/slide were identified using light microscopy. Slides from shams consisted typically of >99% alveolar macrophages.

Lung-inflammatory cytokine analysis

Concentrations of cytokines from the first fraction BAL supernatant were measured using a mouse inflammation cytometric bead array kit (BD Biosciences, San Diego, CA) and analyzed on a FACSCalibur flow cytometer as previously described (Young et al., 2008). The following cytokines were measured: interleukin-6 (IL-6), IL-10, monocyte chemotactic protein-1 (MCP-1), interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-12p70 (IL-12p70). Standard curves with a range of 20–5000 pg/mL were determined for each cytokine. The sensitivity of the assay for each protein ranged from 2.5 to 52.7 pg/mL. Because BAL protein levels for some analytes were at or below assay sensitivity, which limited statistical analysis, a scatter plot was chosen with the mean values indicated for each group to appropriately represent the protein data. The concentration of macrophage inflammatory protein-2 (MIP-2) was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Biochemical measurements

Albumin, a measure of damage to the lung alveolar epithelial barrier, and lactate dehydrogenase (LDH) activity, indicative of lung cell death, were measured in the first fraction BAL fluid supernatant. The albumin concentration was determined colorimetrically at 628 nm based on albumin binding to bromocresol green (BCG), using an albumin BCG diagnostic kit (Sigma Chemical Co., St. Louis, MO). LDH activity was determined by measuring the oxidation of lactate to pyruvate coupled with the formation of NADH (nicotinamide adenine dinucleotide) at 340 nm. Both measurements were performed with a COBAS MIRA Plus auto-analyzer (Roche Diagnostic Systems, Montclair, NJ).

Gross lung tumor counts and histopathology

At 78 weeks post-exposure, A/J mice were euthanized by carbon dioxide asphyxiation, weighed, then the abdomen was opened and the vena cava was cut to exsanguinate the mouse. The whole lung was excised and gross tumor counts and size measurements were recorded for each lung lobe. Apparent merged tumors, defined as a single tumor pattern in double-nodule form or an apparent collision of two different tumors, were counted as one because this was impossible to distinguish at necropsy. The lungs were then inflated and fixed with 10% neutral buffered formalin for a minimum of 24 h. Lungs were embedded in paraffin then a 5 μm standardized section was cut. Slides were stained with hematoxylin and eosin and interpreted by a contracted board certified veterinary pathologist in a blinded fashion for morphological changes and proliferative/neoplastic lesions. If abnormal changes were found, severity was scored as follows: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe. The final severity score reflects the average of the right and left lung lobe scores. Proliferative/neoplastic changes were scored as P = preneoplastic epithelial proliferation, AP = adenoma arising within a proliferation, A = adenoma, CA = carcinoma arising within an adenoma, C = carcinoma, or MC = microcarcinoma according to Belinksy et al. (1992). Since examination of a single histological section per lung underestimates the total number of lesions per lung (Rehm and Ward, 1989), the gross count at necropsy is more representative of the response.

Statistical comparisons and analysis

All analyses were performed either using JMP version 5.0.1, or the SAS system for Windows version 9.1 (SAS Institute, Cary, NC). Factorial analysis of variance (ANOVA) was utilized on continuous variables to incorporate strain and treatment into each analysis. For some variables a log transformation was performed on the data to reduce heterogeneous variance and meet the assumptions of the ANOVA. All post-hoc comparisons were carried out using Fishers Least Significant Difference Test. For all analyses, the criterion of significance was $p < 0.05$. Gross tumor counts and histopathology counts from sections were analyzed similarly. Tumor incidence (presence or absence of tumors) was analyzed using a chi-square test in SAS 'Proc Freq', while tumor multiplicity (number of tumors/lung) was analyzed using Poisson regression in SAS 'Proc Genmod.' All analyses on tumor data utilized only those animals surviving to 78 weeks.

Results

Effects of GMA-SS welding fume inhalation on body weight and survival: 78-week study

The mean starting body weights (±SE) for the 6-day air and GMA-SS-exposed groups were 17.7 ± 0.47 g and 17.8 ± 0.56 g, respectively. Air controls weighed 28.14 ± 0.21 g and GMA-SS-exposed mice were 28.01 ± 0.39 g at 50 weeks post-exposure. The mean starting body weights for the 10-day air and GMA-SS-exposed groups were 18.0 ± 0.28 g and 17.6 ± 0.24 g, respectively. Air controls weighed 27.71 ± 0.38 g and GMA-SS-exposed mice were 27.28 ± 0.44 g at 50 weeks post-exposure. At 78 weeks, body weight had decreased from 50 weeks (1–3 g) in all groups, but there was no significant effect on final body weight due to welding fume exposure (data not shown). Also, compared to air controls, exposure to GMA-SS welding fume for 6 or 10 days had no effect on survival. At 78 weeks post-exposure, survival was >73% for all groups.

Lung metal deposition after GMA-SS welding fume inhalation

Metal analysis was done on whole lungs from A/J and B6 mice after air or GMA-SS exposure. At 1 day after the 10-day exposure, concentrations of Cr, Cu, Fe, Mn, and Ni were increased similarly in the lungs of both mouse strains (Figure 1). As predicted by the metal profile of the generated SS fume alone, reported previously by Antonini et al., 2006, Fe was the most abundant species followed by Cr, Mn, Ni then Cu. By 1-day post-exposure, 111 µg of these metals remained in the lung. To determine the daily deposition, lungs from B6 mice were harvested immediately after a single 3-h exposure to GMA-SS welding fume and analyzed for metals. After 3 h, approximately 11.8 µg were initially deposited in the B6 mouse lung. These data indicate minimal clearance of the total deposited dose 1 day after the cessation of exposure. Also, assuming a 15% lung deposition, a TLV-TWA of 5 mg/m³ for 8 h/day and a daily deposition of 11.8 µg, our 6- and 10-day exposure regime is equivalent to approximately 30 and 50 days of exposure, respectively, in a 75 kg welder.

Lung cytotoxicity and alveolar epithelial barrier damage

Lung cytotoxicity (measured as LDH activity) and alveolar epithelial barrier damage (measured as albumin) were significantly increased throughout the time course in both A/J (Figure 2A) and B6 (Figure 2B) mice compared to the corresponding air control. Similar degrees of lung cytotoxicity and damage were found between the strains.

BAL cell profile

The recovered BAL cells consisted of macrophages, lymphocytes, and PMN in both mouse strains exposed to GMA-SS welding fume (Figure 3A and 3B). Both A/J and B6 mice had a significant and sustained increase in macrophages from days 1 through 28 with no resolution of the response. PMN gradually increased from day 1 and peaked later in the time course for both mouse strains. Cell

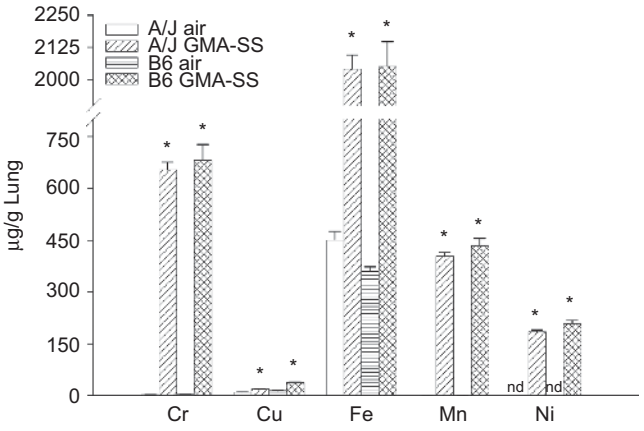

Figure 1. Metal deposition in the lungs of A/J and B6 mice 1 day after 10 days of exposure (3 h/day) to air or 40 mg/m³ gas metal arc-stainless steel (GMA-SS) welding fume. *Indicates a significant increase versus corresponding air control ($p < 0.05$; $n = 3$); nd, below limit of detection.

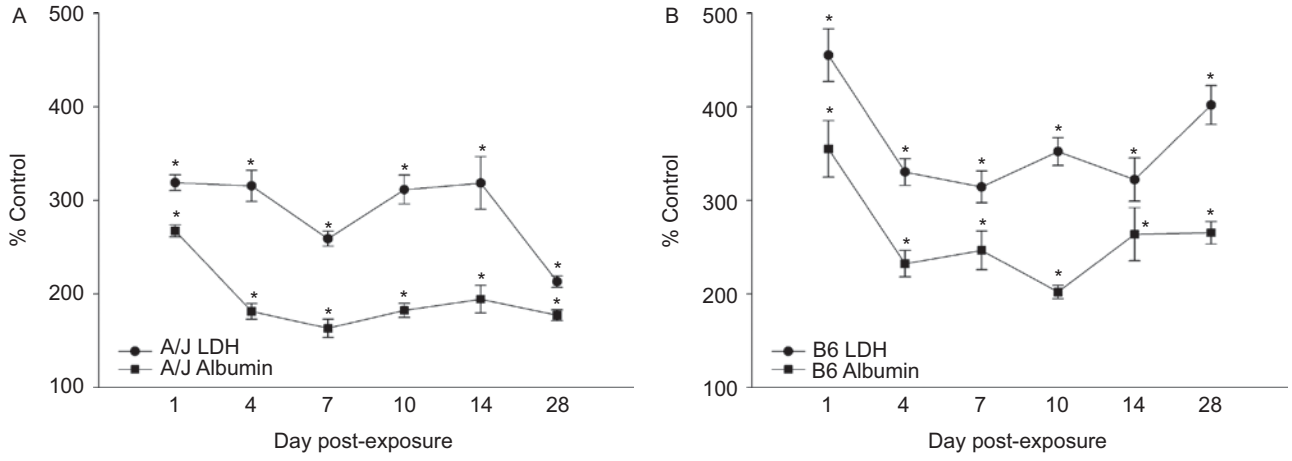

Figure 2. Effect of gas metal arc-stainless steel welding fume on bronchoalveolar lavage levels of lactate dehydrogenase (LDH) and albumin in A/J (A) and B6 mice (B) at 1, 4, 7, 10, 14, and 28 days post-exposure. Data are presented as percent control and values are mean ± SE ($n = 3$–7 per group). *Indicates a significant increase versus air control ($p < 0.05$).

differential analysis revealed a greater percentage of PMN in the BAL of the A/J compared to the B6 mouse strain. At 1, 4, 7, 10, 14, and 28 days post-exposure to GMA-SS welding fume, PMNs were 5, 11, 35, 53, 51, and 38% and 4, 3, 4, 6, 18, and 11% of the total BAL cells recovered in the A/J and B6 mice, respectively. Compared to the A/J strain, macrophages and lymphocytes dominated the B6 lung response. For example, at 7 days, lymphocytes reached 10% in the B6 compared to 1.3% in the A/J strain.

Lung-inflammatory cytokine analysis

The first fraction BAL supernatant was used to determine the protein levels of IFN-γ, IL-6, IL-10, IL-12p70, MCP-1, MIP-2, and TNF-α throughout the 28-day time course. Protein levels of IL-10 and IL-12p70 were not increased after GMA-SS exposure in either strain at any time point.

Both mouse strains had significant BAL protein levels of MIP-2 in response to GMA-SS welding fume throughout the 28-day time course. The B6 strain had consistent levels through 10 days post-exposure, but then the levels further increased at days 14 and 28. MIP-2 protein levels in the A/J were consistent through all post-exposure time points and even though levels were greater in the B6, this did not directly reflect the PMN response in the lung of this strain (Figure 4A and 4B).

MCP-1 and TNF-α protein levels were increased on post-exposure days 10 through 28 in the BAL of the A/J; a similar cytokine profile for TNF-α was found in the B6 mouse strain (Figure 5A, 5C, and 5D). In contrast, MCP-1 protein was increased at all time points post-exposure in the B6 and reached the highest level at day 28 (Figure 5B). IL-6 and IFN-γ proteins were not detected at any time point post-exposure in the A/J strain; however, the B6 had increased levels of IL-6 at all time points post-exposure and a mild increase in IFN-γ at days 14 and 28 only (Figure 6A and 6B).

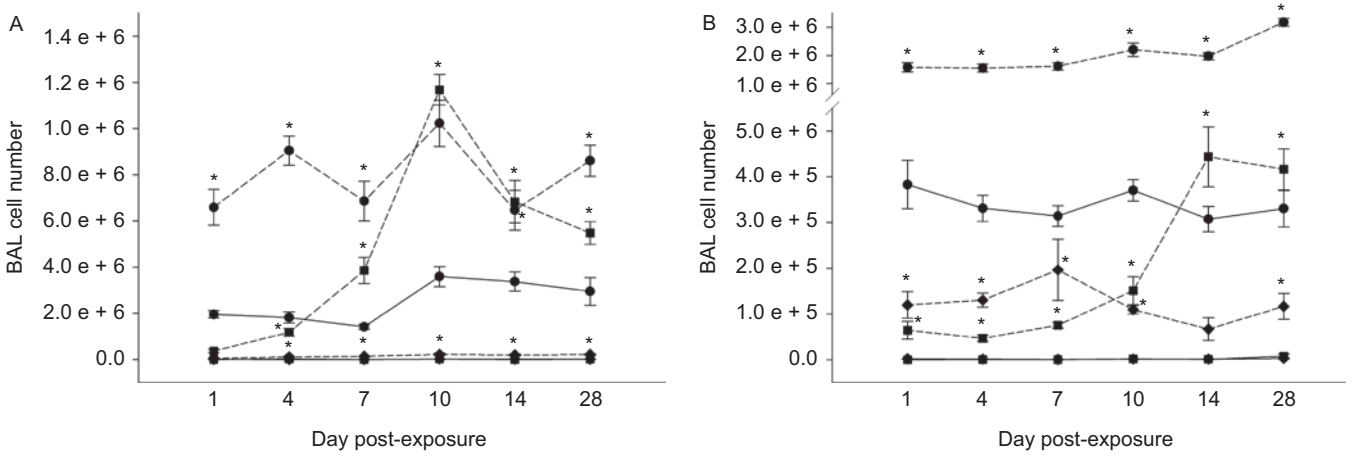

Figure 3. Effect of gas metal arc-stainless steel (GMA-SS) welding fume on recovered alveolar macrophages (closed circle), polymorphonuclear leukocytes (closed squares) and lymphocytes (closed diamonds) by bronchoalveolar lavage (BAL) at 1, 4, 7, 10, 14, and 28 days post-exposure in A/J (A) and B6 mice (B). Solid lines represent air controls and dotted lines are GMA-SS-exposed mice. Values are mean ± SE ($n = 3$–7 per group). *Indicates a significant increase versus air control.

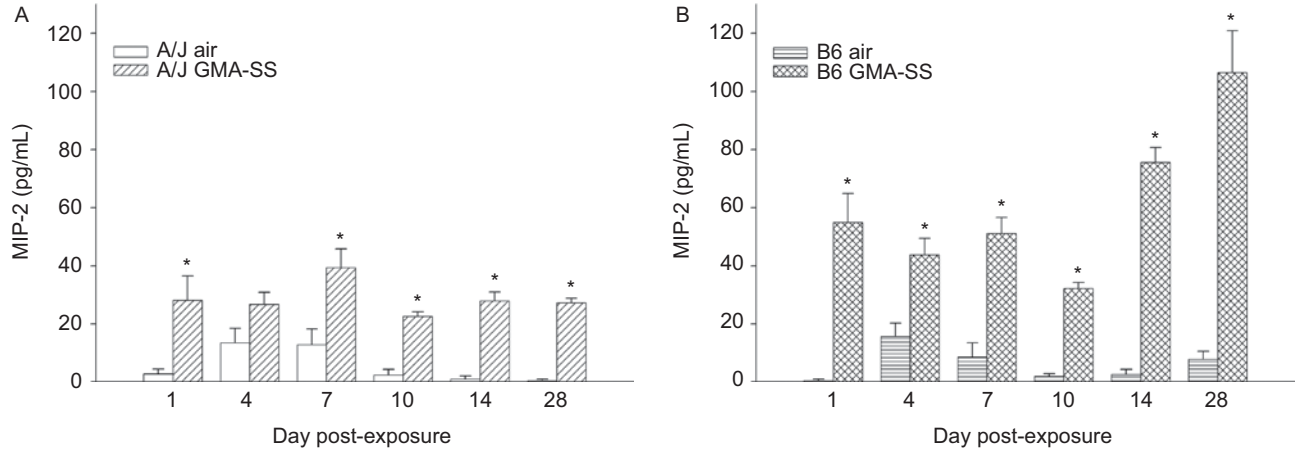

Figure 4. Effect of gas metal arc-stainless steel (GMA-SS) welding fume on macrophage inflammatory protein-2 (MIP-2) bronchoalveolar lavage protein in A/J (A) and B6 (B) mice at 1, 4, 7, 10, 14, and 28 days post-exposure. Values are mean ± SE ($n = 3$–7 per group). *Indicates a significant increase versus air control ($p < 0.05$).

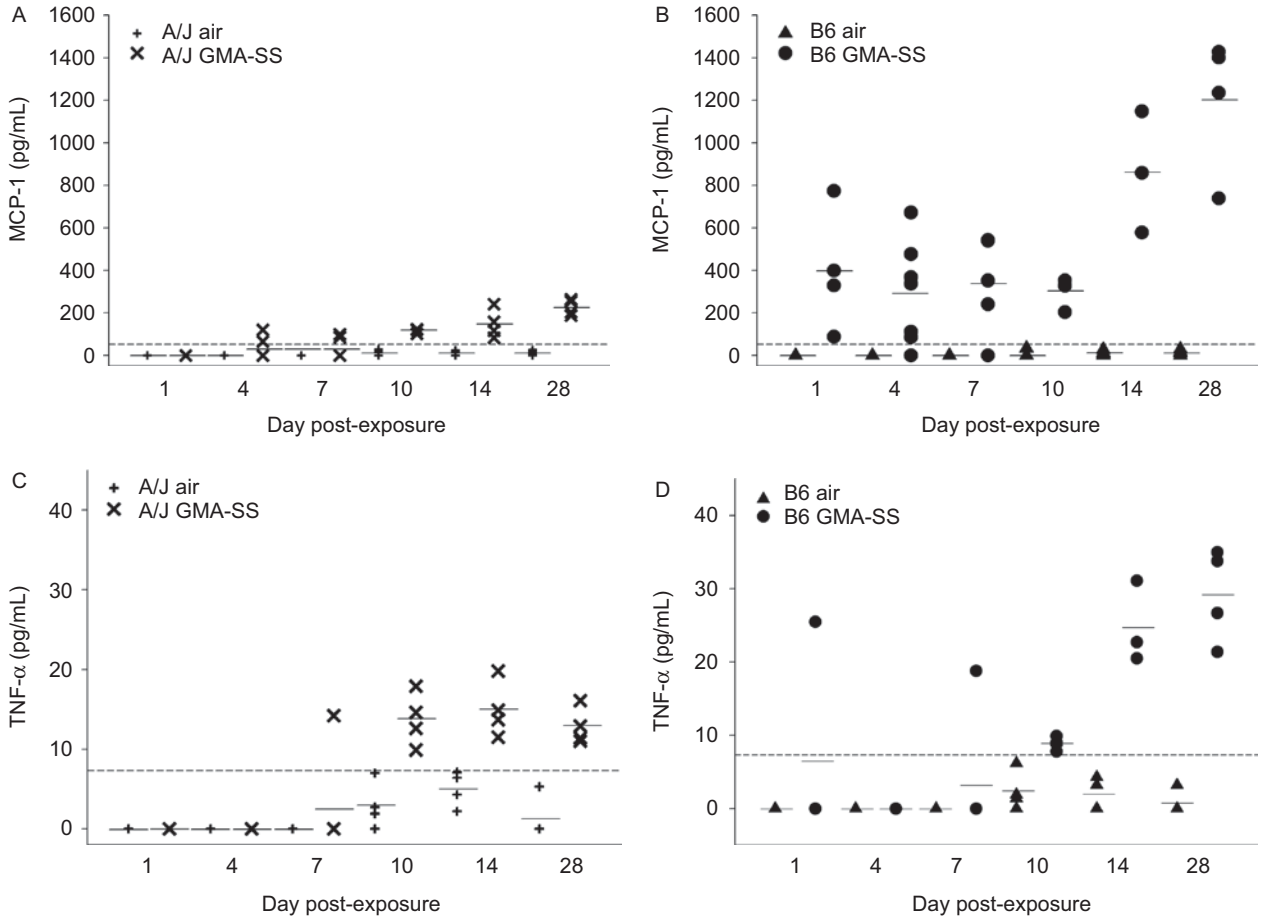

Figure 5. Effect of gas metal arc-stainless steel (GMA-SS) welding fume on bronchoalveolar lavage protein levels of monocyte chemotactic protein-1 (MCP-1) (A) and (B) and tumor necrosis factor-α (TNF-α) (C) and (D) at 1, 4, 7, 10, 14, and 28 days post-exposure in A/J and B6 mice. The dotted line represents the assay sensitivity for each protein and mean lines (solid line) are shown for each group (n = 3–7).

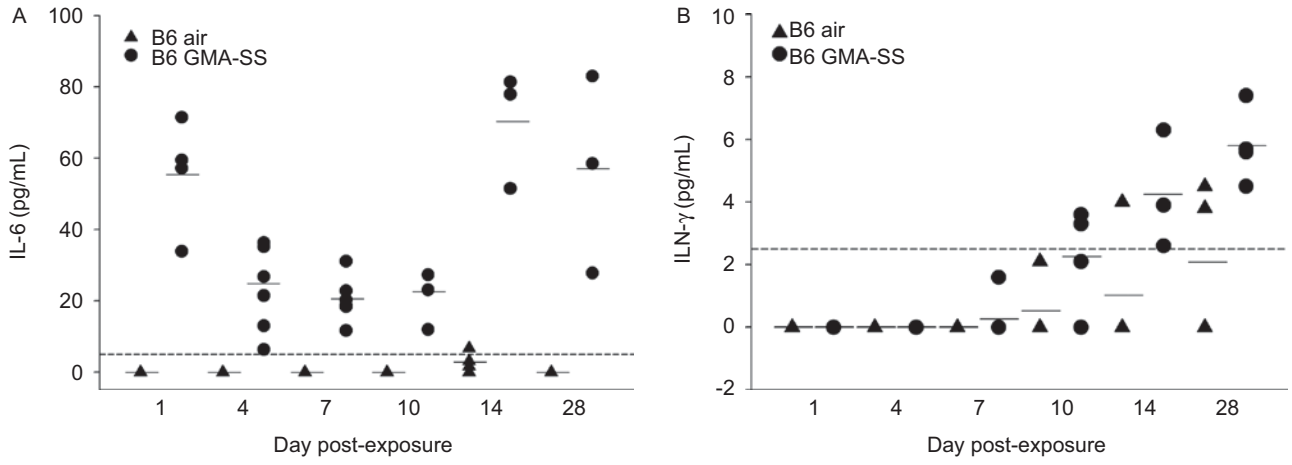

Figure 6. Effect of gas metal arc-stainless steel (GMA-SS) welding fume on bronchoalveolar lavage protein levels of interleukin-6 (IL-6) (A) and interferon-γ (IFN-γ) (B) at 1, 4, 7, 10, 14, and 28 days post-exposure in B6 mice. The dotted line represents the assay sensitivity for each inflammatory protein and mean lines (solid line) are shown for each group (n = 3–7).

Gross lung tumor findings in A/J mice at 78 weeks after GMA-SS welding fume inhalation

At 78 weeks post-exposure, lung tumor multiplicity, and incidence were unremarkable in mice exposed to GMA-SS welding fume for 6 or 10 days (Table 1). After 6 days of exposure, tumor incidence was 73 and 51% in the air and GMA-SS-exposed groups, respectively. Air controls had a 77% tumor incidence compared to 62% in the GMA-SS-exposed group after 10 days of exposure. Welding fume exposure had no effect on tumor size. Average tumor size was approximately 3 mm for the 6- and 10-day air and welding fume groups.

Table 1. Gross lung tumor findings for A/J mice 78 weeks post-exposure.

	Tumor multiplicity*		Tumor incidence†	
Exposure	6 Days	10 Days	6 Days	10 Days
Air	1.36 ± 0.21 (33)	0.93 ± 0.11 (43)	73% (24/33)	77% (33/43)
GMA-SS	0.84 ± 0.16 (37)	0.86 ± 0.14 (42)	51% (19/37)	62% (26/42)

*Average number of tumors per lung (±SE) and includes mice with no tumors. Parentheses indicate total animal number.
†Percentage of tumor-bearing mice out of the total. Parentheses indicate tumor-bearing /total animal number.
GMA-SS, gas metal arc-stainless steel.

Lung histopathological findings in A/J mice 78 weeks after welding fume inhalation

In addition to the gross lung tumor evaluation at necropsy, histopathological analysis was done from selected mice in the study to evaluate lung morphological changes (Table 2). There were no significant findings related to GMA-SS welding fume exposure found at 78 weeks post-exposure in the 6-day group. In the 10-day group, all welding fume-exposed mice had minimal amounts of welding fume in the lung at 78 weeks (Table 2).

Table 2. Lung histopathology for A/J mice 78 weeks post-exposure.

Group	Exposure	*n*	Lymphoid infiltrates*	Welding fume-laden cells
6 Days	Air	6	1.25 ± 0.17	0.00 ± 0.00
	GMA-SS	14	1.25 ± 0.10	0.04 ± 0.04
10 Days	Air	4	1.00 ± 0.20	0.00 ± 0.00
	GMA-SS	14	0.61 ± 0.15	1.25 ± 0.10†

Data are mean ± SE.
If abnormal changes were found, severity was scored as follows: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe. The final severity score reflects the average of the right and left lung lobe scores.
*Perivascular/peribronchial associated lymphocytes, macrophages, and plasma cells. †Significantly increased from air ($p < 0.05$).
GMA-SS, gas metal arc-stainless steel.

Discussion

Recently, our laboratory initiated a multipart study to investigate the possibility of welding fume as a lung carcinogen. This important area of welding fume toxicology has, until present, been essentially unexamined in an animal model. Our prior work utilized pharyngeal aspiration as an exposure method in lung tumor susceptible (A/J) and resistant (B6) mice. We established that welding fume, primarily SS, caused a greater and prolonged inflammatory lung response in the A/J compared to the B6 mouse strain. This was accompanied by a mild chronic lung immune response and a trend for increased tumor incidence (i.e. mice with tumors), not multiplicity (i.e. number of tumors), in GMA-SS-exposed A/J mice. Unfortunately, due to the spontaneous tumor rate and mortality in the A/J model, no definitive conclusions on the carcinogenic nature of GMA-SS fume compared to the other welding fumes could be made (Zeidler-Erdely et al., 2008). To expand on our previous work, we therefore focused on GMA-SS welding fume and conducted an inhalation study with this fume. We subsequently characterized the lung response in both mouse strains over a 28-day recovery period and examined tumorigenesis in the A/J strain 78 weeks after 6 and 10 days of exposure.

Comparison of the inhalation and previous aspiration study showed that GMA-SS welding fume exposure causes lung inflammation and toxicity in both mouse strains. However, the major differences between the strains after the aspiration protocol were the time course of recovery and the magnitude of the response. In both strains, welding fume aspiration caused an early peak in the measured inflammatory parameters that was greater in the A/J mice. In this susceptible strain, some indices of the lung response remained elevated at 28 days post-exposure, whereas the response in the B6 strain had resolved by 7 days. Inhalation of GMA-SS fume, on the other hand, caused a later peak in inflammatory cell influx and the lung response (cytotoxicity and permeability) remained unresolved at 28 days after cessation of the exposure in both mouse strains. Except for the cellular profile in the BAL, the overall magnitude of the lung response was not remarkably different between the strains. Overall, these results agree with and confirm our previous data in rats that showed a differential lung response to inhaled versus instilled GMA-SS welding fume. In rats, inhalation of GMA-SS welding fume was associated with a late-peaking inflammatory cell influx, similar to the profile observed here (Antonini et al., 1996; Antonini et al., 2007; Antonini et al., 2009). Other poorly soluble particles such as titanium dioxide and quartz have also been shown to elicit different lung responses depending upon the animal exposure method. In studies by Driscoll et al., using similar deep lung particle burdens, adverse lung effects of quartz were not observed until 63 days post-inhalation compared to 7 days post-instillation in rats. In addition, instillation of titanium dioxide in rats resulted in a transient lung response while inhalation showed no significant effect (Driscoll et al., 1990a; Driscoll et al., 1990b; Driscoll et al., 1991).

Welding fume generated during our inhalation protocol, and in the workplace, is a complex and unique mixture of gaseous and aerosol byproducts. Aged welding fume, such as those used for instillation or aspiration protocols, which is collected onto filters then suspended in solution, has been shown to be less inflammatory because of decreased reactive oxygen species generation in comparison to the fresh fume (Antonini et al., 1998). Clearly, differences exist in the rate and distribution pattern of particle deposition in the lung among the various

exposure methods. Inhalation achieves a lighter, more even distribution of particles compared to the heavier, more centralized deposits observed with instillation (Brain et al., 1976; Driscoll et al., 2000). For these reasons, it was not unexpected that different lung responses were found in this study despite attempts to circumvent bolus effects by repeated aspiration exposures in our previous study (Zeidler-Erdely et al., 2008).

After aspiration of GMA-SS welding fume, lymphocytes were negligible, PMNs were the majority and macrophage infiltration was not different between the A/J and B6 mouse strains (Zeidler-Erdely et al., 2008). In this study, inhalation of GMA-SS welding fume caused the B6 strain to respond with a sustained influx of primarily macrophages with increased lymphocytes. Although PMNs were present, these cells were initially the minority in this strain with a delayed increase. The A/J strain also had significantly elevated macrophages throughout, mildly elevated lymphocytes and similar to the B6, a delayed rise in PMN. For further elucidation of the BAL findings, key cytokines, reportedly involved in welding fume-induced lung inflammation, were evaluated (Antonini et al., 2007). Aspiration of GMA-SS welding fume induced cytokines such as IL-6, MCP-1, and TNF-α in both mouse strains with the greatest levels measured early after exposure. These levels subsequently declined and thereby the cytokine profile mirrored other BAL parameters (Zeidler-Erdely et al., 2008). Inhalation, on the other hand, resulted in a more complex cytokine profile in both strains. After exposure, common cytokines between the mouse strains were MCP-1, MIP-2, and TNF-α. MIP-2 levels were significantly increased from 1 through 28 days post-exposure in both strains. It is likely that these sustained levels resulted in the gradual accumulation of PMN in the lung. Previously in rats, it was found that MIP-2 levels were not increased until day 4 which immediately preceded PMNs on day 11 post-inhalation of GMA-SS fume. It was suggested that macrophage function and signaling could have been suppressed during the inhalation exposure which thereby caused the delayed PMN influx. This observation was further supported by an attenuated clearance of *Listeria monocytogenes* from the lung and a decreased reactive species generation from harvested alveolar macrophages stimulated *ex vivo* (Antonini et al., 2007). PMNs were increased early in the mice, as would be predicted with increased BAL MIP-2 levels found at all time points. Qualitatively, however, the response was similar to the rats with marked increases at 10 days. We predict that longer post-exposure time points in mice would reveal a concomitant decrease in MIP-2 levels and PMN similar to that previously observed in rats (Antonini et al., 2007). In addition, TNF-α was not increased until 10 days post-exposure which further strengthens the argument that macrophage suppression may be occurring during GMA-SS exposure.

Surprisingly, the A/J mice lacked an increase in IL-6 and MCP-1 levels were not increased until 10 days post-exposure. In contrast, because there was an early, sustained overproduction of IL-6 and MCP-1 in the B6 mice, these two cytokines may have driven the macrophage and lymphocyte-dominated response we observed post-inhalation (Kishimoto, 2005). It is possible that the time-frame of IL-6 production differed between the strains and increased levels of this acute phase protein may have occurred earlier than measured in the A/J mice. The discordance between the MCP-1 levels and the early, sustained macrophage influx, however, is unknown but does suggest that a different monocyte chemoattractant could play a role. Regardless, the persistent inflammatory cytokine levels in the BAL up to 28 days after exposure confirmed that the lung response to inhaled welding fume increases incrementally over time in both mouse strains. These results contrast with aspiration and instillation exposure responses to welding fume (Antonini et al., 1996; Taylor et al., 2003; Zeidler-Erdely et al., 2008).

Our aspiration protocol delivered four bolus doses (85 μg/exposure) of GMA-SS welding fume over a 10-day time period (~340 μg total; Zeidler-Erdely et al., 2008). In this study, inhalation exposure deposited ~12 μg per day (~120 μg total) over 10 days. Interestingly, despite this lower total lung burden, a sustained and, in some aspects, greater lung toxicity profile was found for inhalation of the fume compared to aspiration. Indeed, the reactivity of the freshly generated fume and possible noxious gas exposure during inhalation contributed to this outcome (Antonini et al., 1998). Although welding fume was still present in the A/J lung at 78 weeks after 10 days of exposure, we did not observe any indication of a tumorigenic or chronic lung response following either 6 or 10 days of inhalation exposure. Therefore, it appears that total lung burden, and not level of inflammation, is the more critical determinant of chronic lung or possible tumorigenic effects of GMA-SS fume. Magnetopneumatography data in shipyard arc welders, welding iron-rich MS (mild steel), showed that the net rate of alveolar deposition of iron particles per year was estimated at ~70 mg. After 10 years of welding, the average burden of particles in the lungs was ~1 g (Kalliomaki et al., 1983). Because our inhalation exposure regime was only equivalent to ~50 days (8 h shift/day) of exposure in a human at the previously recommended TLV-TWA, we believe that inhalation studies that achieve lung burdens more reflective of a lifetime occupational exposure are needed. To this end, our inhalation exposure team is implementing longer, sub-chronic animal exposure protocols.

In conclusion, the strain-dependent differences in the magnitude and resolution found after aspiration of GMA-SS welding fume were not confirmed in this inhalation study. Although some strain-dependent differences were evident, these were not as predicted from our previous study. Of importance, this study further justifies the use of inhalation, when possible, as the primary means of animal exposures in welding fume toxicological studies. We also conclude that inhalation of GMA-SS fume at this exposure level and duration does not appear to cause lung tumorigenesis in the A/J mouse model. This result provides more evidence on the weak nature of GMA-SS

fume as a tumor initiator in A/J mice and supports the results of our previous aspiration study. Although, taken together, the exposure levels used in the current and previous work may help to clarify those levels at which a worker's risk are the greatest. In the future, long-term inhalation exposures to achieve greater lung burdens are warranted to fully evaluate the tumorigenic potential of welding fume. In addition, BAL post-exposure time points greater than 28 days are needed to elucidate possible strain-dependent differences in the complete resolution of the lung response to inhaled GMA-SS welding fume.

Declaration of interest

The authors report no declarations of interest. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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