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Freshly generated stainless steel welding fume induces greater lung inflammation in rats as compared to aged fume

James M. Antonini [a,*], Robert W. Clarke [a], G.G. Krishna Murthy [a], Pradeep Sreekanthan [b], Neil Jenkins [b], Thomas W. Eagar [b], Joseph D. Brain [a]

[a] *Physiology Program, Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115, USA*
[b] *Department of Materials Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

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Abstract

It has been previously reported that both short- and long-lived reactive oxygen species (ROS) are present on the surface of freshly generated fumes. The objective of this study was to determine if freshly formed welding fume induces greater lung inflammation and injury in rats due to the presence of reactive oxygen species than aged welding fume. Fume was collected during gas metal arc welding using a stainless steel consumable electrode and found to be of respirable size with a mean diameter of 0.77 μm $\pm$ 0.48. Male CD/VAF rats were dosed intratracheally with the welding fume 30 min (fresh) and 1 and 7 days (aged) after fume collection at a dose of 1.0 mg/100 g b wt. Bronchoalveolar lavage (BAL) was performed 24 h post-instillation. Lung injury and inflammation were assessed by measuring the concentration of neutrophils, albumin, lactate dehydrogenase (LDH), and glucosaminidase (GLU) in the recovered BAL fluid. More neutrophils and enhanced GLU activity were observed for the 'fresh' group as compared to both 'aged' groups ($P < 0.05$). Slight, but not significant, elevations were seen in albumin content and LDH activity for the 'fresh' group as compared to the 'aged' groups. No significant differences were observed for any of the parameters when fume aged for 1 and 7 days were compared. When the 'fresh' and 'aged' fumes (12.5, 25, and 50 μg/ml) were suspended in dichlorofluorescin (15 μM), a probe which becomes fluorescent when oxidized, the concentration-dependent increases in fluorescence were greater for the 'fresh' fume versus the 'aged' fumes. We have demonstrated that freshly generated stainless steel welding fume induces greater lung inflammation than 'aged' fume. This is likely due to a higher concentration of ROS on fresh fume surfaces.

Keywords: Welding fumes; Reactive oxygen species; Free radicals; Lung inflammation

* Corresponding author. Present address: Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Mail Stop 2015, 1095 Willowdale Drive, Morgantown, WV 26505, USA. Tel.: +1 304 2856244; fax: +1 304 2855938.

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1. Introduction

Oxygen radicals and their metabolites, collectively described as reactive oxygen species (ROS), have been shown to play a major role in pulmonary toxicity caused by the inhalation of different particles and fumes (Vallyathan and Shi, 1997). It has been well documented that freshly fractured silica has a higher concentration of surface radicals and is more biologically reactive to lung cells and tissue when compared to aged silica (Vallyathan et al., 1988, 1995). Several laboratories have shown that ROS with different lifetimes and chemical characteristics are present on the surfaces of different types of fumes (Pryor, 1992; Shusterman, 1993). In the assessment of lung toxicity induced by perfluoropolymer (PFP) fume, Warheit et al. (1990) demonstrated that the pulmonary response to aged PFP fume was diminished in a time-dependent manner when compared with freshly generated PFP fumes.

Arc welding joins pieces of metal that have been made liquid by the heat produced as electricity passes from one conductor to another (Howden, 1988). The extremely high temperatures (>4000°C) of this process heat both the base metal pieces to be joined and a consumable electrode fed into the weld. Fumes are formed by the evaporation of the metals primarily at the tip of the electrode. The metal vapors are oxidized on contact with the air and form small particulates of different complexes of metal oxides.

Respiratory effects seen in full-time welders have included bronchitis, airway irritation, metal fume fever, chemical pneumonitis, lung function changes, and a possible increase in the incidence of lung cancer (Sferlazza and Beckett, 1991). Acute upper and lower respiratory tract infections are increased in terms of severity, duration, and frequency among welders as compared to the general population (Howden, 1988). In recent animal studies, we have shown that differences exist in the extent of pulmonary inflammation and injury in rats after treatment with fumes generated using different welding processes and materials (Antonini et al., 1996, 1997). In these studies, the fumes were collected and stored for varying lengths of time until tested in animals. Since welders are exposed to freshly generated fumes at work sites, and it is possible that fresh fumes may be more biologically reactive, the goal of this current investigation was to compare the potential of fresh versus aged stainless steel (SS) welding fumes to induce lung damage.

Rats were intratracheally instilled with fresh and aged welding fumes, and a variety of biochemical and cellular parameters of damage were measured in bronchoalveolar lavage (BAL) fluid to assess injury and inflammation. Previous studies have shown that analysis of the BAL fluid is a sensitive means of characterizing lung inflammation and injury (Beck et al., 1982; Henderson, 1984; Khan and Gupta, 1991; Antonini et al., 1996). To examine the possible mechanism by which fresh welding fumes may be more reactive than aged fume, we treated the collected fumes with dichlorofluorescin (DCFH), a non-fluorescent probe which becomes fluorescent when oxidized. DCFH fluorescence was measured to determine if there was an increase in ROS on fresh fume surfaces when compared with aged fume surfaces.

2. Materials and methods

2.1. Welding process and fume collection

Welding was performed with a gas metal arc welder (GMA; Hobart Arc-Master 500, Hobart Brothers, Troy, OH) using a stainless steel 308

Table 1
Size and composition of the GMA-SS welding fume

Count mean diameter (μm)[a]	Bulk analysis (% weight)[b]	Surface analysis (% weight)
0.77 ± 0.48	18.5% Fe, 12.7% Mn, 11.1% Cr, 1.51% Ni, 0.20% Cu, 0.02% Co, 0.02% Mo	54.7% O, 27.8% C, 6.01% Fe, 4.83% Mn, 3.41% Si, 3.21% Cr

[a] Diameter values are means + S.E.; $n = 200$ particles/sample.
[b] Chemical analysis for metals only.

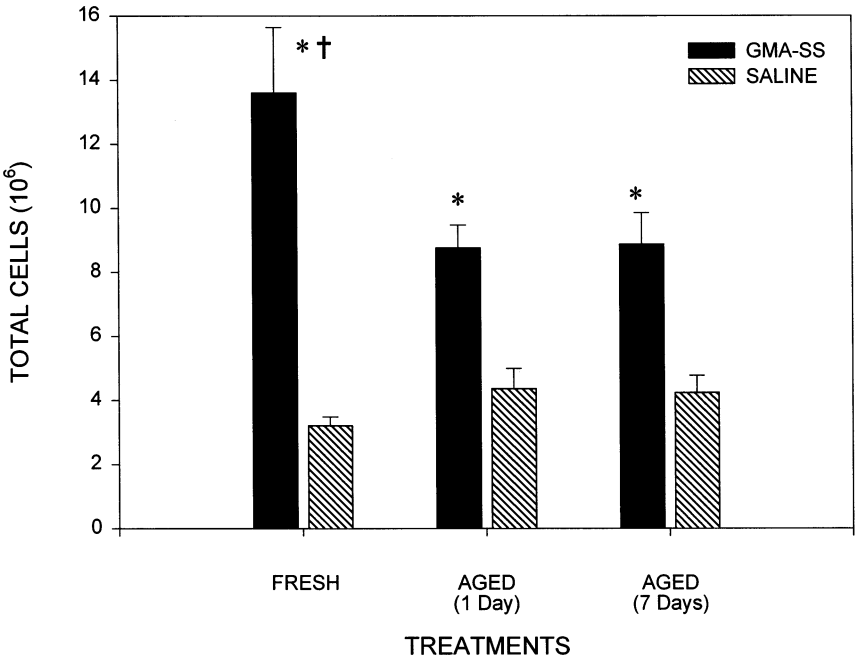

Fig. 1. Total cells recovered from the lungs of rats 24 h after intratracheal instillation of 'fresh' and 'aged' (1 and 7 days) GMA-SS welding fume. The animals received a single dose of 1.0 mg in 150 μl of sterile saline/100 g body weight. Saline was used as the vehicle control. Values are means $\pm$ S.E. (* significantly greater than saline controls; † significantly greater than GMA-SS 'aged' groups ($P < 0.05$)).

electrode (SS). The voltage used for the welding was 30 V set at a current level of 200 A. During the welding process, oxygen-free shielding gases are used to produce a shielded gas environment which excludes oxygen, thus protecting the weld from immediate weakening by oxidation of the welded metal (Sferlazza and Beckett, 1991). The mixture of hot gases produced during welding is less dense than the surrounding air, causing them to rise out of the shielded environment and carry upward fine metal particulates which are oxidized on contact with the air. A standard shielding gas combination of 95% argon and 5% CO_2 was used. GMA-SS fumes were collected for 2 min onto 0.2 μm Nuclepore filters (Corning-Costar, Cambridge, MA) during the welding process. Collection of fumes continued for an additional 2 min after the welding had been completed. Collected fumes were used within 30 min (fresh) or allowed to age in air for 4 h, 1, 7 and 30 days (aged) for the animals studies.

2.2. Welding fume characterization

The metal analysis of the collected fume was performed by the Trace Metals Laboratory at the Harvard School of Public Health using a Perkin-Elmer ELAN 500 Inductively Coupled Plasma-Mass Spectrometer. The elements of interest were Fe, Cr, Ni, Mn, Cu, Co, and Mo. X-ray photoelectron spectroscopy was conducted at the Gordon-McKay Labs at Harvard University, Cambridge, MA to obtain information about the surface composition of the fume.

The GMA-SS fume was suspended in 0.9% saline, sonicated for 1 min, dispersed onto glass slides in microwell chambers, and then sized with a Sarastro 2000 (Molecular Dynamics, Sunnyvale CA) laser scanning confocal microscope (Optiphot-2, Nikon, Melville, PA) fitted with an argon laser as we described earlier (Antonini et al., 1996).

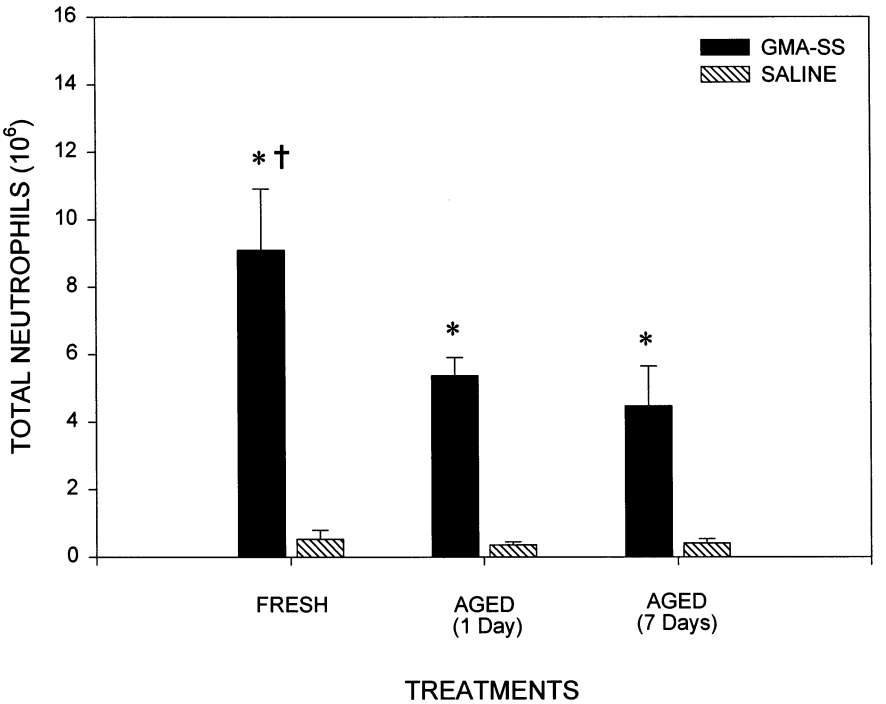

Fig. 2. The number of neutrophils recovered from the lungs of rats 24 h after intratracheal instillation of 'fresh' and 'aged' (1 and 7 days) GMA-SS welding fume. The animals received a single dose of 1.0 mg in 150 μl of sterile saline/100 g body weight. Saline was used as the vehicle control. Values are means $\pm$ S.E. (* significantly greater than saline controls; † significantly greater than GMA-SS 'aged' groups ($P < 0.05$)).

2.3. Animals

Male CD/VAF rats weighing 200–250 g were used for all experiments (Charles River Laboratories, Wilmington, MA). They were given a conventional laboratory diet and tap water ad libitum and were housed in a clean air, viral- and antigen-free room with restricted access.

2.4. Intratracheal Instillation

Rats ($n = 8$–9/treatment group) were intratracheally instilled with 1.0 mg suspended in 150 μl of 0.9% sterile saline/100 g body weight of the GMA-SS fume 30 min (fresh) and 1 and 7 days (aged) after fume collection. Animals in the vehicle control group were intratracheally dosed with 150 μl of 0.9% sterile saline/100 g body weight.

Before the intratracheal instillation procedure, the rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of 1% sodium methohexital (Brevital, Eli Lilly, Indianapolis, IN). The instillations were performed according to Brain et al. (1976). Before instillation, the fume samples were sonicated for 1 min.

2.5. Bronchoalveolar lavage

BAL was performed on rats from each group 24 h post-instillation. The rats were deeply anesthetized with an overdose of sodium pentobarbital and then exsanguinated by severing the abdominal aorta. Their lungs were first lavaged with two separate 3 ml aliquots of warm, calcium- and magnesium-free phosphate buffer solution (PBS), pH 7.4. These two BALF samples were centrifuged at $500 \times g$ for 7 min, and the resultant cell-free supernatant was analyzed for various biochemical parameters. Then, the lungs were lavaged 12 more times with 3-ml aliquots of PBS.

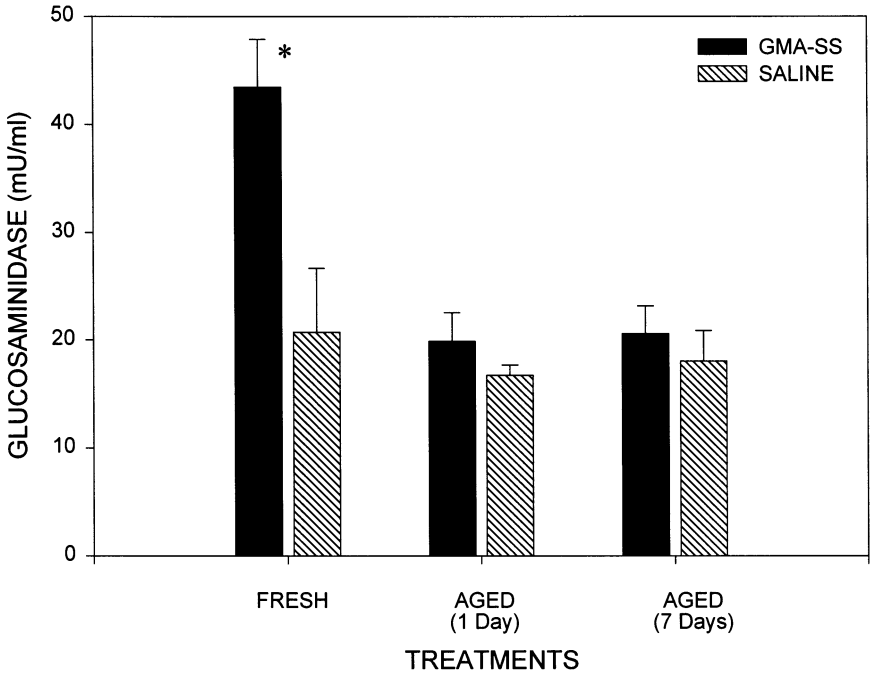

Fig. 3. Glucosaminidase activity of the cell-free bronchoalveolar lavage fluid recovered from the lungs of rats 24 h after intratracheal instillation of 'fresh' and 'aged' (1 and 7 days) GMA-SS welding fume. The animals received a single dose of 1.0 mg in 150 μl of sterile saline/100 g body weight. Saline was used as the vehicle control. Values are means $\pm$ S.E. (* significantly greater than all groups ($P < 0.05$)).

These samples were also centrifuged for 7 min at $500 \times g$ and the cell-free BALF discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 1 ml of PBS buffer and evaluated as described below.

2.6. Cellular evaluation

Total cell numbers were determined using a hemacytometer. Viability of recovered cells was assessed by trypan blue exclusion and found to be $> 95\%$ for all cell types. Using a cytospin centrifuge (Shandon Southern Products, Cheshire, UK), 1×10^5 cells were spun for 5 min at 800 rpm and pelleted onto a slide. Cells (200/rat) were identified on cytocentrifuge-prepared slides after staining with Wright Giemsa Sure Stain (Fisher Scientific, Pittsburgh, PA).

2.7. Biochemical Assays

Within the acellular supernatant BAL fluid, three indicators of pulmonary damage were assessed: (1) albumin to quantitate increased permeability of the bronchoalveolar-capillary barrier; (2) the activity of the lysosomal enzyme, β-*n*-acetyl glucosaminidase (GLU), to detect the release of enzyme from activated or lysed phagocytes; and (3) the activity of the cytosolic enzyme, lactate dehydrogenase (LDH), to detect general cytotoxicity. Albumin content was determined by the method of Doumas and Biggs, (1972), and the GLU and LDH activities were assayed by the methods of Sellinger et al. (1960) and Pesce et al. (1964), respectively. Enzyme reagents were from Sigma (St. Louis, MO), while the other chemicals used were from Fisher (Pittsburgh, PA).

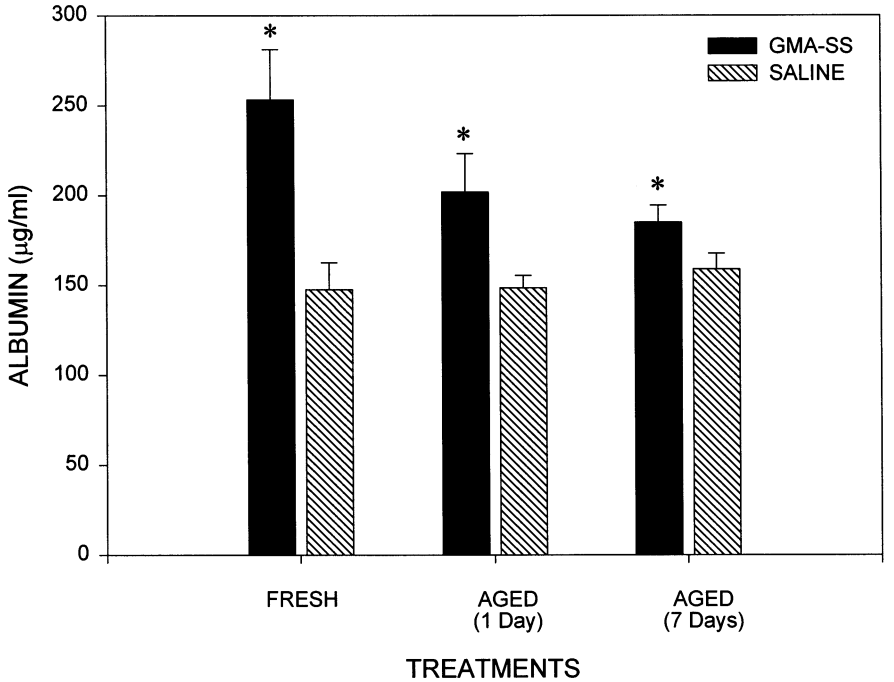

Fig. 4. Total albumin of the cell-free bronchoalveolar lavage fluid recovered from the lungs of rats 24 h after intratracheal instillation of 'fresh' and 'aged' (1 and 7 days) GMA-SS welding fume. The animals received a single dose of 1.0 mg in 150 μl of sterile saline/100 g body weight. Saline was used as the vehicle control. Values are means $\pm$ S.E. (* significantly greater than saline controls ($P < 0.05$)).

2.8. Measurement of reactive oxygen species on fume surfaces

Dichlorofluorescin (DCFH) is a probe which becomes fluorescent when oxidized in the presence of reactive oxygen species. At a stock concentration of 1 mg/ml, the 'fresh' and 'aged' fumes were suspended in the non-fluorescent precursor DCFH diacetate (15 μM) and incubated for 15 min at 37°C. After the incubation period, the fume samples were added to a 96-well plate (Corning Costar, Cambridge, MA) at concentrations of 12.5, 25, and 50 μg/ml and incubated for 30 min at 37°C. The oxidation of DCFH to the fluorescent form dichlorofluorescein (DCF) was measured in a Cytofluor 2300 microplate reader (Millipore, Bedford, MA).

2.9. Statistical analysis

Results are expressed as means $\pm$ standard error of measurement (S.E.). Statistical analyses were carried out with Statview statistical analysis software (Abacus Concepts, Berkeley, CA). For all parameters, an analysis of variance (ANOVA) was performed. If a significant interaction was present, the significance between each of the individual groups was analyzed using the Fischer's Least Significance Difference post-hoc test. For all analyses, the criterion of significance was $P < 0.05$.

3. Results

The chemical composition and size of the GMA-SS welding fume were determined (Table 1). Many of the individual particles were of the ultrafine size (0.1 μm in diameter), but had quickly aggregated together during the generation of the fume and collection of the particles onto filters. The collected particle agglomerates had a

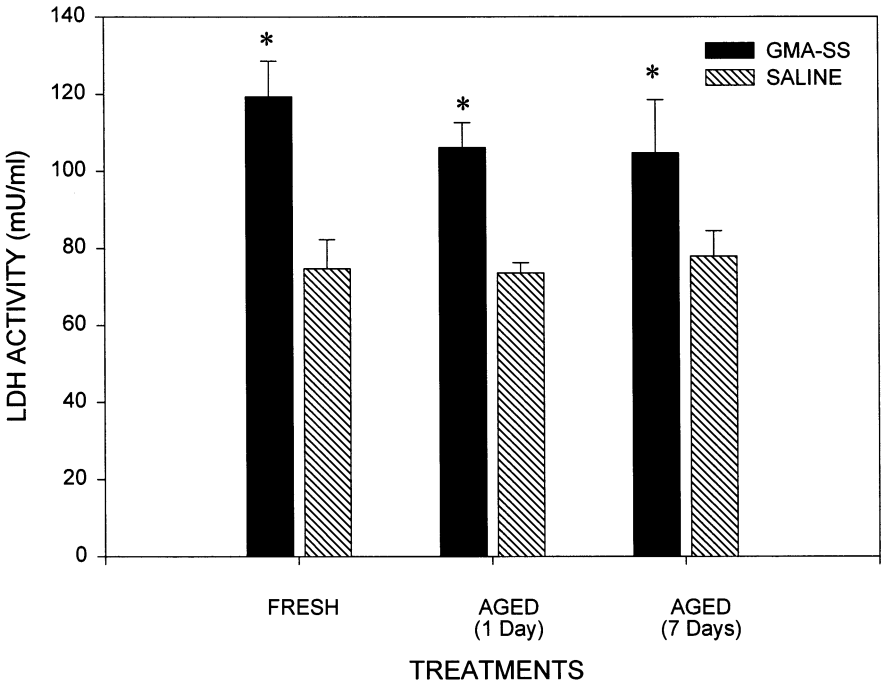

Fig. 5. Lactate dehydrogenase (LDH) activity of the cell-free bronchoalveolar lavage fluid recovered from the lungs of rats 24 h after intratracheal instillation of 'fresh' and 'aged' (1 and 7 days) GMA-SS welding fume. The animals received a single dose of 1.0 mg in 150 μl of sterile saline/100 g body weight. Saline was used as the vehicle control. Values are means $\pm$ S.E. (* significantly greater than saline controls ($P < 0.05$)).

mean diameter of 0.77 μm which was of respirable size. The bulk of the particles were comprised predominately of three metals—Fe, Mn, and Cr. These metals, along with Si, were also found on the surface of the particles. It is of interest to note that the Si detected was of the amorphous form and not the highly pneumotoxic, crystalline form.

Intratracheal instillation of the 'fresh' and 'aged' GMA-SS fumes induced a dramatic infiltration of cells (primarily neutrophils) into the lungs that was significantly elevated compared to the saline control groups (Figs. 1 and 2). A significant increase in total cells and neutrophils was observed for the 'fresh' group as compared to both 'aged' groups.

An elevation in GLU activity was observed for the 'fresh' group as compared to both 'aged' groups (Fig. 3). No differences were seen between the two 'aged' groups and their saline controls. Slight, but not significant, elevations were seen in albumin content and LDH activity for the 'fresh' group as compared to the 'aged' groups (Figs. 4 and 5). Significant elevations in both parameters were observed for the 'fresh' and 'aged' fume groups as compared to their saline control groups.

When the 'fresh' and 'aged' fumes (12.5, 25, and 50 μg/ml) were suspended in DCFH, the concentration-dependent increases in fluorescence were greater for the 'fresh' fume collected 30 min after welding as compared to the fumes 'aged' for 4 h, 1, 7, and 30 days (Fig. 6). Slight differences in fluorescence were observed among the fumes 'aged' for 4 h, 1 and 7 days. By 30 days, only small levels of fluorescence could be detected in the fume sample.

4. Discussion

We evaluated the relative ability of freshly gen-

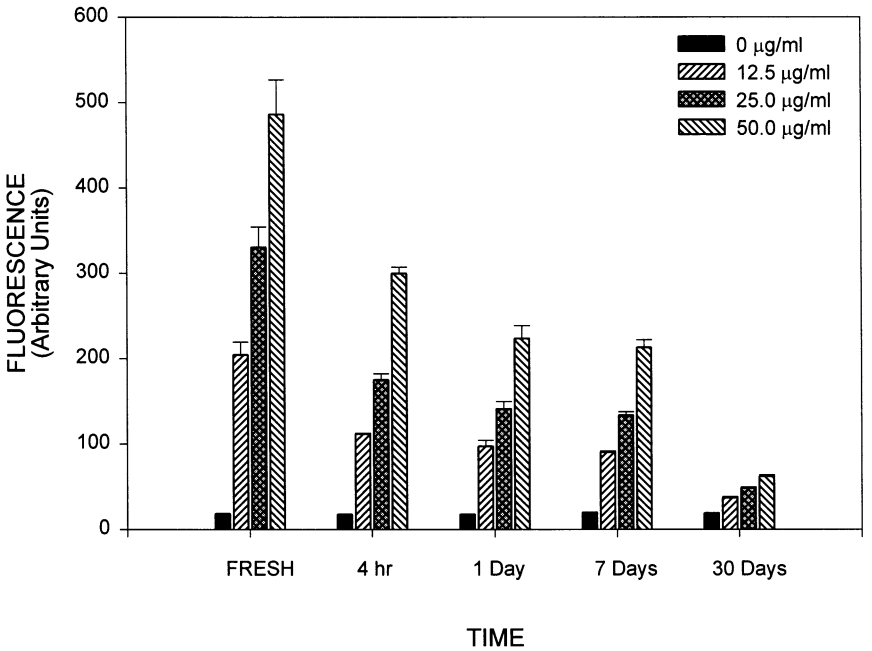

Fig. 6. Fluorescence of 'fresh' and 'aged' GMA-SS welding fume after incubation with dichlorofluorescin. Fluorescence of fume at concentrations of 12.5, 25, and 50 μg/ml was measured 30 min (fresh) and 4 h, 1, 7, and 30 days (aged) after fume collection.

erated and aged welding fumes to elicit lung injury and inflammation. An in vivo rat bioassay was used that measures a variety of markers of pulmonary damage within the BAL fluid. Fume was collected during gas metal arc welding using a stainless steel consumable electrode. The collected particles had a mean diameter of 0.77 μm, giving the fume a high probability of being deposited in the deep airspaces of the lungs. It has been shown previously by our group and others that most particles collected during welding have a mean diameter of <1 μm (Jarnuskiewicz et al., 1966; Aksellsson et al., 1976; Antonini et al., 1997).

Intratracheal instillation of the 'fresh' fume generated more lung inflammation as evidenced by significant elevations in the number of recovered neutrophils when compared to the fume when aged for 1 and 7 days. Enhanced GLU activity, an indication of a greater number of activated and dying cells, was also observed for the 'fresh' group as compared to the 'aged' groups. While the 'fresh' fume induced a greater inflammatory response as compared to the 'aged' fumes, only slight, but not significant, increases were observed when lung injury was assessed by measuring LDH activity and albumin content.

The differences observed in lung inflammation and GLU activity for the 'fresh' fume as compared to the 'aged' fumes were not surprising. Warheit et al. (1990) demonstrated that the toxicity of aged PFP fumes when compared with fresh PFP fumes was significantly diminished in a time-dependent manner. LDH and protein values in BAL fluid of animals exposed to aged PFP fumes were significantly less than those seen with fresh PFP fumes, suggesting the toxicant was unstable. More detailed studies have shown that free radicals are present in the particulate phase of PFP fumes. Direct electron spin resonance (ESR) analysis indicated that PFP particles contain highly reactive peroxy radicals that decay about 20% per day (Pryor et al., 1990). Metcalfe et al. (1991) reported two populations of ROS, one on

the surface that decays within 1–2 h and an internal radical that decays over days.

In related toxicology studies, Vallyathan et al. (1995) observed that the inhalation of freshly fractured silica increased total cells, neutrophils, and GLU activity by 119, 96, and 211% above the levels for aged silica, respectively. These differences were attributed to significantly higher concentrations of ROS associated with the freshly fractured silica as compared to the aged silica. It was shown that the surface radicals decayed over time, increased significantly with extended grinding, and was a reflection of the generation of greater number of surface radicals (Vallyathan et al., 1988; Shi et al., 1988). The concentration of ROS decreased following first order kinetics with a half-life of 30 h in air.

To determine whether ROS were responsible for the differences seen in the lung responses of the 'fresh' and 'aged' welding fumes in this current study, we treated the collected fumes with DCFH. DCFH has been used extensively to study the formation of ROS in a variety of cell culture and other aqueous systems (Scott et al., 1988; Murphy et al., 1989; Sanchez-Ferrer et al., 1990; LeBel and Bondy, 1990; Kobzik et al., 1990; Gunasekar et al., 1995). In the presence of iron and H_2O_2, DCFH is oxidized to the fluorescent DCF, and the potent chelator desferoxamine abolishes this H_2O_2-mediated DCFH oxidation (LeBel et al., 1992). Zhu et al. (1994) concluded that hydroxyl radical and perhaps a ferryl species, but not superoxide, may be responsible for the iron/H_2O_2-mediated DCFH oxidation.

We observed a concentration-dependent increase in DCFH-fluorescence for the 'fresh' fume as compared to the 'aged' fumes. No significant differences in DCFH-fluorescence were observed among the fumes 'aged' for 4 h, 1, and 7 days. By 30 days, ROS were still detected, but only in small amounts. When the data was fitted to an exponential decay model, the half-life of the ROS was 10 days when aged in air. Since the production of ROS was similar for the fumes 'aged' for 1 and 7 days, it wasn't surprising that no differences were observed for any of the lung toxicity parameters when comparing the two 'aged' groups.

In this study, we have demonstrated that freshly generated stainless steel welding fume induces greater lung inflammation than aged fume. Workers exposed to freshly formed fumes at active welding sites may be at a greater risk in developing lung inflammation. The increase in reactivity of the fresh fume as compared to the aged fume is most likely due to higher concentrations of ROS on fresh fume surfaces. To determine which ROS may be involved, ESR spin-trap techniques will be utilized in future studies to detect and identify radicals on the surfaces of fresh and aged welding fumes.

We have shown previously that different fluxes and shielding gases used during the welding process may affect the oxidation state of the generated fumes (Eagar et al., 1997), and the pulmonary responses of welding fumes varies according to the materials and processes used (Antonini et al., 1996, 1997). We will try to assess what effect altering the welding conditions (i.e. using different fluxes, electrodes, and shielding gases) will have on the development of lung inflammation after exposure to freshly generated welding fumes.

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