Progressive Lung Injury, Inflammation, and Fibrosis in Rats Following Inhalation of Sulfur Mustard

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ABSTRACT

Sulfur mustard (SM) inhalation causes debilitating pulmonary injury in humans which progresses to fibrosis. Herein, we developed a rat model of SM toxicity which parallels pathological changes in the respiratory tract observed in humans. SM vapor inhalation caused dose (0.2–0.6 mg/kg)-related damage to the respiratory tract within 3 days of exposure. At 0.4–0.6 mg/kg, ulceration of the proximal bronchioles, edema and inflammation were observed, along with a proteinaceous exudate containing inflammatory cells in alveolar regions. Time course studies revealed that the pathologic response was biphasic. Thus, changes observed at 3 days post-SM were reduced at 7–16 days; this was followed by more robust aberrations at 28 days, including epithelial necrosis and hyperplasia in the distal bronchioles, thickened alveolar walls, enlarged vacuolated macrophages, and interstitial fibrosis. Histopathologic changes were correlated with biphasic increases in bronchoalveolar lavage (BAL) cell and protein content and proliferating cell nuclear antigen expression. Proinflammatory proteins receptor for advanced glycation end product (RAGE), high-mobility group box protein (HMGB)-1, and matrix metalloproteinase (MMP)-9 also increased in a biphasic manner following SM inhalation, along with surfactant protein-D (SP-D). Tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS), inflammatory proteins implicated in mustard lung toxicity, and the proinflammatory/profibrotic protein, galectin (Gal)-3, were upregulated in alveolar macrophages and in bronchiolar regions at 3 and 28 days post-SM. Inflammatory changes in the lung were associated with oxidative stress, as reflected by increased expression of heme oxygenase (HO)-1. These data demonstrate a similar pathologic response to inhaled SM in rats and humans suggesting that this rodent model can be used for mechanistic studies and for the identification of efficacious therapeutics for mitigating toxicity.

Key words: sulfur mustard; lung; inflammation; oxidative stress; fibrosis.

Sulfur mustard (SM) is a cytotoxic vesicant developed as a chemical warfare agent during World War I. However, many recent incidents of intentional and accidental exposure to SM have been reported (Dacre and Goldman, 1996; Malaviya et al., 2020; Tang and Loke, 2012); thus, it remains a high-priority chemical threat agent. Exposure to SM causes injury to the skin, the eyes, and the respiratory tract, as well as the gastrointestinal and nervous systems (Smith et al., 1995; Somani and Babu, 1989). The severity of injury induced by SM varies with the route of exposure, proximity to the source, environmental conditions, and the use of protective equipment (Graham and Schoneboom, 2013). Pulmonary toxicity is responsible for most morbidity and mortality following SM exposure (Balali-Mood and Hefazi, 2005; Keyser et al., 2014; Razavi et al., 2013; Weinberger et al., 2011). In the proximal airways, this is characterized by rapid ulceration of airway epithelium, mucosal edema, and inflammation, whereas in the distal airways and alveolar regions, hemorrhage, vascular congestion, edema, and epithelial cell necrosis are observed (Borak and Sidell, 1992; Ghabili et al., 2010; Greenfield et al., 2002; Kehe et al., 2008; Urbanetti, 1988). After a period of mild alleviation of symptoms, injury progresses resulting in aberrant airway epithelial repair, scarring, mucosal thickening and stenosis, bronchitis and bronchiectasis, whereas parenchymal changes include atelectasis, interstitial lung disease, and pulmonary fibrosis (Emad and Rezaian, 1997; Ghabili et al., 2010; Malaviya et al., 2020; Weinberger et al., 2011).

Understanding the pathogenesis of acute injury and the progression to chronic disease is important as there are currently no therapeutics to treat SM poisoning in victims exposed to mild-to-moderate levels of this toxic vesicant. We hypothesized that the response of rodents to inhaled SM vapors would more accurately reflect human pathology following exposure than current experimental models of poisoning involving intratracheal administration of liquid nitrogen mustard, a related bifunctional alkylating agent (Malaviya et al., 2015). To test this, we developed a novel inhalation model in rats which provides direct delivery of SM vapor to the lung and airways, major human targets of this cytotoxic vesicant (Weinberger et al., 2016). Consistent with the histopathologic response of humans to inhaled SM, we observed early acute lung injury, oxidative stress, and inflammation, followed by a period of reduced toxicity, and then a secondary more prominent chronic response characterized by fibrosis. These findings demonstrate that our rodent inhalation model can be effectively used for mechanistic studies on SM poisoning in humans, and for preclinical studies to assess the ability of drugs to mitigate acute injury and chronic disease pathogenesis.

MATERIALS AND METHODS

Animals and treatments. Male Wistar rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, Massachusetts) or Envigo RMS, Inc. (Indianapolis, Indiana). Animals were housed in filter top microisolation cages and provided food and water *ad libitum*; they received humane care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. To ensure deposition of mustard vapor in the airways and lung, an intratracheal inhalation model was developed; this was important as nose-only and whole body exposures to SM vapor result in absorption mainly in the mucosal surfaces of nasal cavity causing extensive injury and distress to the rats (Weber et al., 2010). Rats were anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine

(10 mg/kg), intubated, and then exposed intratracheally to a vapor containing SM (0.06–0.6 mg/kg) or air control using a modified 5-port inhalation exposure system (CH Technologies, Westward, New Jersey; Supplementary Figure 1). Each port was fitted with a self-sealing silicone rubber dam which was modified for insertion of an intratracheal breathing tube during the exposure.

The flow rate through the chamber was maintained at 5 l/min for all exposures and monitored using calibrated mass flow meters. This flow rate delivered 1 l/min to each exposure port at approximately 10 times the minute respiration volume of an anesthetized rat. The flow displacement provided fresh replenishment and regeneration of SM vapor in the respiration zone of the animals, as well as removal of respiratory byproducts which eliminated potential rebreathing concerns. SM vapor and rat exhalation byproducts were exhausted through a scrubber system and activated carbon trap driven by a flow-controlled exhaust pump.

A heated diffusion cell containing SM (purity >99% as determined by gas chromatography-mass spectrometry [GC-MS]) was used to generate SM vapor into a converging ultrapure carrier air flow for mixing and dilution. The concentration of delivered SM was verified by GC-MS prior to each animal exposure. Six mm outer diameter Tenax-TA tubes were used to collect samples from the exposure system at a metered and constant flow rate and time. Samples were extracted with 10 ml of hexane using a Rainin Rabbit-Plus peristaltic pump. Approximately 1 ml of the extract was transferred to an autosampler vial for analysis by GC-MS. Controls, prepared by spiking the Tenax-TA tubes with 190 µg of SM under a flow of air, were extracted in the same manner as the animal exposure samples.

Since the rats were anesthetized and intubated with intratracheal breathing tubes during exposures, the traditional Guyton's formula could not be utilized to calculate respiratory minute volume as this is based on an at rest natural breathing pattern. To calculate the respired dose, an estimated inhalation minute volume of 0.1 l/min was used. Inhalation exposure concentrations were based on the recovered SM concentration in the sample tube using the following equation:

$$\text{mg/l} = \text{SM concentration (mg)} / \text{sample flow rate (l/min)} \\ \times \text{total sample time (min)}$$

Exposure times were targeted to be in the range of 10–15 min with the delivered SM vapor concentrations obtained using a preset variation of diffusion cell temperature to regulate the SM vaporization rate, and diffusion cell vapor generation flow rate to regulate vapor delivery rate. Variations in these parameters (fixed during exposures and characterization) were used to verify exposure operating parameters in pre-exposure characterization sham tests. The exposure inhalation concentrations assumed 100% lung retention of respired SM vapor (mg/l) and were defined by fixed and monitored sample tube flows and sample times, and concentration analyzed via calibrated GC-MS. SM inhalation exposure concentrations were calculated based on the weight (mg/kg) of each rat using the following equation:

$$\text{SM dose (mg/kg)} = (C \times T \times MV \times DF) / BW,$$

where C, SM concentration (mg/l); T, exposure duration (min); MV, minute volume (l/min) = 0.1; DF, pulmonary deposition factor = 1; and BW, body weight (kg).

Figure 1. Dose-related histopathological changes in rat lung following sulfur mustard (SM) inhalation. Lung sections were prepared 3 days after exposure of rats to SM (0.06–0.6 mg/kg) or control (CTL), and stained with hematoxylin and eosin. Proximal bronchiolar region (magnification 40 $\times$), left panels; insets shown in middle panels (magnification 200 $\times$); alveolar region, right panels (magnification 200 $\times$). Abbreviations: am, alveolar macrophages; d, luminal debris; e, peribronchial edema; p, alveolar proteinaceous exudate; u, bronchial epithelium ulceration. Representative sections from CTL ($n=9$), 0.06 mg/kg SM ($n=4$), 0.2 mg/kg SM ($n=10$), 0.4 mg/kg SM ($n=9$), and 0.6 mg/kg SM ($n=9$) are shown.

The individual weight of each rat was used to define the exposure time to obtain the same mg/kg inhalation exposure concentration based on the estimated 0.1 ml/min respiration minute volume of all animals in the exposure group. The actual lung deposition or retention of SM vapor was not investigated in this study and was reported as 100% based on the presented SM vapor concentration.

Sample collection. Animals were euthanized 3, 7, 16, or 28 days after administration of SM or control by i.p. injection of ketamine (125 mg/kg) and xylazine (12.5 mg/kg). For collection of bronchoalveolar lavage (BAL) fluid, the trachea was cannulated with a 16-gauge blunted needle and instilled with 10 ml of PBS. BAL was collected by gently withdrawing the fluid using a syringe. In previous studies, we demonstrated that this procedure had

no significant effect on macrophage populations in the lung (Venosa et al., 2016). BAL cell and protein content were assayed as previously described (Malaviya et al., 2012). For preparation of histological sections, following lung lavage, the left lobe of the lung was perfused via the trachea with 3% paraformaldehyde in PBS, removed and fixed in 3% paraformaldehyde. After overnight incubation at 4°C, the tissue was rinsed and transferred to 50% ethanol. The remaining lung lobes were excised and frozen at –80°C.

Histology. A total of 10–15 longitudinally cut tissue sections (5 μ m) were prepared from each sample. One random section from each rat was stained with hematoxylin and eosin or Masson's trichrome stain and examined by light microscopy. Images were acquired using a VS 120 slide scanner (Olympus

Figure 2. Effects of sulfur mustard (SM) on bronchoalveolar lavage (BAL) cell and protein content. BAL collected 3 days after exposure of rats to SM (0.06–0.6 mg/kg) or control (CTL; A) or 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or CTL (B) was analyzed for protein (upper panels) and cell content (lower panels). Bars, mean $\pm$ SE; CTL ($n = 14$ –36), 0.06 mg/kg SM ($n = 4$), 0.2 mg/kg SM ($n = 9$), 0.4 mg/kg SM ($n = 9$), 0.6 mg/kg SM ($n = 9$), 3 days post-SM 0.04 mg/kg ($n = 29$), 7 days post-SM 0.04 mg/kg ($n = 6$), 16 days post-SM 0.04 mg/kg ($n = 7$), 28 days post-SM 0.04 mg/kg ($n = 5$). ^aSignificantly ($p \leq .05$) different from CTL.

Corporation, Center Valley, Pennsylvania). Histopathologic changes in the lung were assessed by a board-certified veterinary pathologist (Daniel Weinstock, DVM, PhD). Lung sections were analyzed for bronchiolar epithelial degeneration/necrosis, epithelial attenuation, metaplasia/regeneration, peribronchial edema, peribronchial inflammation, alveolar septal defects, alveolar inflammatory cells, alveolar proteinaceous fluid, fibrin, and fibrosis. Semiquantitative grades (0–4) were assigned with grade 0 indicating no changes; grade 1, minimal or small changes present in $\leq 10\%$ of the tissue; grade 2, mild changes present in 10–30% of the tissue; grade 3, moderate changes present in 30–50% of the tissue; grade 4, extensive or severe changes present in $> 50\%$ of the tissue, relative to controls.

Western blot analysis. BAL protein was reduced with dithiothreitol, denatured, and equal volumes fractionated on 4–12% Novex Bis-Tris gels (Invitrogen, San Diego, California). This was necessary as the total amount of protein in BAL varies with the dose of SM and time after exposure due to changes in the epithelial barrier, thus increasing the proportion of albumin and other plasma proteins. Proteins were then transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of the blots with 10% nonfat dry milk in Tris-buffer (20 mM Tris Base, pH 7.6, 137 mM NaCl, and 0.5% Tween-20) for 1 h at room temperature (RT). The blots were incubated overnight at 4°C with rabbit polyclonal antibody against receptor for advanced glycation end products (RAGE; Abcam Inc., Cambridge, Massachusetts; 1:1,000), high-mobility group box protein-1 (HMGB-1; Abcam; 1:1,000), fibrinogen/fibrin (DAKO, Carpinteria, California, 1:1,000), or surfactant protein-D (SP-D; DU117, gift of Dr. A. Pastva, Duke University, North Carolina; 1:10,000), or

rabbit monoclonal antibody against matrix metalloproteinase-9 (MMP-9; Abcam; 1:1,000) in Tris-buffer containing 1% nonfat dry milk. After washing 3 times, the blots were incubated at RT for 1 h with horseradish peroxidase-conjugated secondary antibody (1:5,000) diluted in Tris-buffer containing milk. Immunoreactive bands were visualized using an ECL detection system (GE Healthcare Biosciences, Piscataway, New Jersey) and analyzed by densitometry using Image J software (National Institutes of Health, Bethesda, Maryland).

Immunohistochemistry. Tissue sections were deparaffinized and endogenous peroxidase quenched using 3% hydrogen peroxide diluted in methanol. Antigen retrieval was performed by warming the specimens for 20–30 min in 10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween-20. To block nonspecific binding, sections were incubated for 2 h at RT in Tris-buffer containing 5–100% horse, goat, or rabbit serum. Sections were then incubated overnight at 4°C in a humidified chamber with mouse monoclonal antibody against tumor necrosis factor alpha (TNF- α ; Abcam; 1:100), or rabbit polyclonal antibodies against heme oxygenase (HO)-1 (Enzo Life Sciences, Farmingdale, New York; 1:1,000), inducible nitric oxide synthase (iNOS; Abcam; 1:200), proliferating cell nuclear antigen (PCNA; Abcam; 1:500), fibrinogen/fibrin (DAKO, 1:5,000), or goat polyclonal antibody against MMP-9 (R&D Systems, Minneapolis, Minnesota; 1:4,000) or galectin-3 (Gal-3; R&D Systems, 1:8,000), or appropriate immunoglobulin G controls diluted in blocking buffer. Sections were then rinsed and incubated at RT for 30 min with biotinylated secondary antibody (Vectastain Elite ABC Kit, Vector Labs, Burlingame, California). Binding was visualized using an avidin-biotinylated enzyme complex with 3, 3'-diaminobenzidine as

Figure 3. Time-related effects of SM on histopathology. Sections prepared 3, 7, 16, and 28 days after exposure of rats to sulfur mustard (SM) (0.4 mg/kg) or control (CTL) were stained with hematoxylin and eosin. Original magnification, 100 $\times$ (bronchiolar regions) and 200 $\times$ (alveolar region). Abbreviations: a, aberrant repair; am, alveolar macrophages; d, luminal debris; e, edema; f, fibrosis; h, honeycombing; m, mucus; u, bronchial epithelium ulceration. Representative sections from CTL ($n=6$), 3 days post-SM ($n=5$), 7 days post-SM ($n=6$), 16 days post-SM ($n=7$), 28 days post-SM ($n=5$) are shown.

the substrate. Random sections from 3 to 6 animals/treatment group were evaluated for each antibody and semiquantitative grades (04) assigned based on the intensity of staining, with grade 0 indicating no staining; grade 1, minimal staining; grade 2, medium staining; grade 3, moderate staining; and grade 4, extensive staining. An average staining score was calculated for each treatment group. Based on this average, representative images were selected for presentation in the figures.

Statistical analysis. Data were analyzed by 1- or 2-way (with no matching between groups) ANOVA using Dunnett's multiple comparison test with a single-pooled variance. A p value of $\leq .05$ was considered statistically significant.

RESULTS

Inhaled SM Causes Progressive Dose-Related Pulmonary Injury and Inflammation in Rats

Treatment of rats with SM resulted in dose-related multifocal histopathological changes in the respiratory tract which were evident within 3 days (Figure 1). Although no significant changes in lung pathology were observed at the lowest dose of SM (0.06 mg/kg) relative to control (Figs. 1A–F), at 0.2 mg/kg, mild-to-moderate inflammatory changes were evident including increases in proximal peribronchiolar edema and in perivascular inflammatory cells (Figs. 1G and 1H). Focal increases in interstitial thickening and in numbers of macrophages in

Table 1. Time-Related Effects of SM Inhalation on Histopathology in Rats

		Days Post-SM			
	CTL	3	7	16	28
Proximal bronchioles					
Epithelial degeneration/necrosis	0	3.8 ± 0.2 ^a	2.0 ± 0.9 ^a	2.3 ± 0.7 ^a	2.6 ± 0.4 ^a
Epithelial attenuation	0	0	2.0 ± 0.9 ^a	2.1 ± 0.7 ^a	2.9 ± 0.5 ^a
Epithelial metaplasia/regeneration	0	0	0.3 ± 0.3	1.1 ± 0.3	2.4 ± 0.5 ^a
Peribronchial inflammation	0	2.6 ± 0.2 ^a	1.3 ± 0.7 ^a	1.7 ± 0.4 ^a	2.1 ± 0.4 ^a
Peribronchial edema	0	2.2 ± 0.5 ^a	0.3 ± 0.2	0.7 ± 0.4	0.8 ± 0.2
Distal bronchioles					
Epithelial degeneration/necrosis	0	1.2 ± 0.4	0.3 ± 0.2	1.4 ± 0.2 ^a	1.0 ± 0.3
Epithelial hyperplasia/metaplasia	0	0	0	0.4 ± 0.2	1.0 ± 0.7
Peribronchial inflammation	0	0	0.5 ± 0.3	0.7 ± 0.4	1.4 ± 0.4 ^a
Peribronchial edema	0	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.4 ± 0.4
Respiratory bronchiole					
Epithelial degeneration/necrosis	0	0.4 ± 0.2	0.7 ± 0.3	1.3 ± 0.3 ^a	0.5 ± 0.4
Inflammation	0	0	0.5 ± 0.3	0.4 ± 0.2	0.2 ± 0.2
Septal defects	0	0	0.7 ± 0.3	1.4 ± 0.4 ^a	0.9 ± 0.4
Alveoli					
Alveolar macrophages	0	1.3 ± 0.6 ^a	0.7 ± 0.5	1.0 ± 0.3	1.2 ± 0.3
Fibrin	0	0.8 ± 0.7	0	0.3 ± 0.2	1.0 ± 0.7
Proteinaceous fluid	0	1.0 ± 0.6	0	0	0.8 ± 0.6
Perivascular edema and inflammatory cells	0	1.6 ± 0.5 ^a	1.2 ± 0.7 ^a	1.7 ± 0.7 ^a	1.5 ± 0.3 ^a
Thickening of alveolar wall	0	0.8 ± 0.6	0.3 ± 0.2	0.9 ± 0.4	1.9 ± 0.7 ^a
Honeycomb formation	0	0	0	0	1.2 ± 0.8
Fibrosis	0	0	0	0	1.0 ± 0.7

Data are mean ± SE (n = 5–7 rats/time point).

^aSignificantly ($p \leq .05$) different from control (CTL).

alveolar spaces were also noted (Figure 1I). At higher doses of SM (0.4–0.6 mg/kg), ulceration of the proximal bronchioles was apparent, along with varying levels of perivascular and peribronchiolar edema and inflammation (Figs. 1J, 1K, 1M, and 1N). Fibrillar membranes occluding the proximal bronchiole lumen were also observed (Figs. 1M and 1N). At 3 days post-SM exposure, pathologic changes were relatively less severe and more variable in distal bronchioles (data not shown), whereas in alveolar regions, focal inflammatory cell accumulation was observed that included enlarged vacuolated macrophages (Figure 1L), and a proteinaceous alveolar exudate containing entrapped inflammatory cells (Figure 1O). SM-induced histopathological alterations in the lower lung were associated with increases in BAL protein and cell content at doses of 0.2 mg/kg and greater (Figure 2A).

A time course study was next performed using 0.4 mg/kg SM (Figure 3). As described above, at 3 days postexposure, extensive necrosis of the proximal bronchiolar epithelium, minimal to moderate peribronchial edema and moderate increases in mononuclear cells were evident (Figure 3D and Table 1). In distal bronchioles, mild multifocal degeneration of the epithelium was noted, along with minimal perivascular edema (Figure 3E and Table 1). Foamy macrophages and fibrin or proteinaceous fluid were present in the alveolar spaces; this was accompanied by mild-to-moderate perivascular edema and areas of thickened alveolar walls (Figure 3F and Table 1). At 7 days post-SM exposure, peribronchiolar edema and inflammation declined, whereas epithelial necrosis and debris persisted in the proximal bronchioles (Figure 3G and Table 1). At this time, distal and respiratory bronchioles exhibited mild epithelial damage and peribronchiolar neutrophilic inflammation. Alveolar changes included reduced perivascular edema and inflammatory cells

(Figure 3H), decreased numbers of macrophages, fibrin and proteinaceous fluid, and interstitial thickening (Figure 3I and Table 1). Epithelial degeneration and necrosis progressed with time in proximal, distal, and respiratory bronchioles (Figure 3K and Table 1); at 16 days post-SM exposure, there were also signs of aberrant repair in proximal bronchiolar epithelium as reflected by hyperplasia and squamous metaplasia (Figure 3J and Table 1). Multifocal vacuolated alveolar macrophages were also present (Figure 3L). At 28 days post-SM, epithelial necrosis was evident, along with moderate regeneration and metaplasia in the proximal airways, whereas in the distal airways mild hyperplasia was evident (Figs. 3M and 3N and Table 1). Mucoid secretions were noted in proximal and distal bronchioles (Figs. 3M and 3N). Mild perivascular edema was also apparent in the alveolar parenchyma, as well as moderately thickened septal walls and increases in vacuolated macrophages (Table 1). In addition, at 28 days post-SM, there was evidence of alveolar interstitial fibrosis and a multifocal honeycombing pattern within the tissue, characterized by altered alveolar epithelium, severely thickened alveolar walls containing elongated fusiform fibrocytes/fibroblasts, alveolar fibrin and amorphous, proteinaceous fluid entrapping cells of varying morphology including eosinophils, macrophages, epithelial cells, and debris (Figure 3O and Table 1). Pathologic changes in the lung 3 days post-SM exposure were associated with trichrome staining in the peribronchiolar, perivascular, and alveolar walls, which may be due to edema and mucoidal substances that pick up trichrome (Figs. 4D–F); this was reduced at 7 days (Figs. 4G–I). At 16 and 28 days, frank peribronchial fibrosis was apparent, along with focal alveolar interstitial collagen deposits (Figs. 4J and 4M–O and Table 1). Consistent with the histopathologic evidence of lung injury, BAL protein content was increased 3 days after SM

Figure 4. Effects of sulfur mustard (SM) on collagen deposition. Sections prepared 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) were stained with Masson's trichrome. Original magnification, 100 $\times$ (bronchiolar regions) and 200 $\times$ (alveolar region). Representative sections from CTL ($n=6$), 3 days post-SM ($n=5$), 7 days post-SM ($n=6$), 16 days post-SM ($n=7$), and 28 days post-SM ($n=5$) are shown.

exposure; this was followed by a decline at 7 days and then a secondary increase at 16 and 28 days (Figure 2B, upper panel).

PCNA is a nuclear marker of cell proliferation, a characteristic response to tissue injury (Park et al., 2016). PCNA expression was increased in both proximal and distal bronchiolar epithelium 3 days after SM administration (Figure 5). In proximal bronchioles, some subepithelial cells also expressed PCNA. Although at 7 days post-SM exposure PCNA expression was reduced, at 16 and 28 days, it increased again; moreover, expression was more extensive. PCNA was also evident in alveolar macrophages and epithelial cells at all post-SM exposure time analyzed, suggesting that these cells are also targets of SM. At 28 days post-SM, PCNA was localized mainly in alveolar epithelial cells and interstitial cells, and in focal areas exhibiting honeycombing and/or fibrosis.

Fibrinogen is an acute phase protein released by the liver in response to injury and inflammation; it is converted into fibrin in tissues by thrombin (Luyendyk et al., 2019). In accord with our histopathologic findings, SM exposure resulted in increases in fibrin in both bronchiolar and alveolar regions of the lung (Figure 6). Although at 3 days fibrin was observed in proximal bronchiolar walls, alveolar spaces and in macrophages, at 7 days it was mainly in the alveolar epithelium. At 16 and 28 days, fibrin was evident in proximal bronchiolar walls and alveolar epithelium. At 28 days post-SM exposure, fibrin was also noted in airways lumen, alveolar spaces and in macrophages. A significant increase in levels of soluble fibrinogen was also observed in BAL at 3 and 28 days post-SM exposure (Figure 7). This was identified by increases in the appearance of α , β , and γ fibrinogen chains (Mosesson, 2005).

Figure 5. Effects of sulfur mustard (SM) on proliferating cell nuclear antigen (PCNA) expression. Sections prepared 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) were stained with antibody to PCNA. Binding was visualized using a Vectastain kit. Original magnification, 100 $\times$ (bronchiolar regions) and 600 $\times$ (alveolar region). Representative sections from CTL ($n=5$), 3 days post-SM ($n=5$), 7 days post-SM ($n=5$), 16 days post-SM ($n=5$), and 28 days post-SM ($n=5$) are shown.

In line with SM-induced lung inflammation, BAL cell content increased 3 days after SM exposure (Figure 2B, lower panel). At 7 and 16 days, BAL cell content was at control levels; however, it was significantly increased again at 28 days. Similarly, soluble SP-D, a marker of lung inflammation (Sorensen, 2018), was increased in BAL at 3 and at 28 days post-SM, when compared with control, and to the response at 7 and 16 days post-SM (Figure 8).

Effects of SM on Inflammatory Proteins in the Lung

We next determined if histologic and biochemical markers of inflammation were correlated with increases in inflammatory proteins in the lung. RAGE is a membrane receptor expressed on alveolar epithelial cells and macrophages that promotes proinflammatory signaling (Hudson and Lippman, 2018; Sanders et al., 2019). It is cleaved to soluble receptor for advanced glycation end products (sRAGE) by proteinases, including MMP-9 generated following injury (Oczypok et al., 2017).

Within 3 days of SM exposure, increases in levels of sRAGE, as well as the RAGE ligand, HMGB-1 (Yang et al., 2005), were observed in BAL; MMP-9 levels were also increased at this time (Figure 8). Subsequently, levels of these proinflammatory proteins declined; this was followed by a secondary increase at 28 days post-SM exposure. MMP-9 was also upregulated in lung tissue following SM exposure (Figure 9). This was evident in epithelial cells and some subepithelial cells in proximal bronchioles, distal bronchiolar epithelium, and in alveolar epithelial cells and macrophages at 3 and 7 days post-SM. A decrease in alveolar MMP-9 was noted at 16 days, followed by an increase at 28 days.

In further studies, we analyzed the effects of SM on expression of 2 macrophage-derived inflammatory proteins implicated in lung injury, TNF- α and iNOS (Laskin et al., 2019). Treatment of rats with SM resulted in increases in enlarged TNF- α ⁺ and iNOS⁺ alveolar macrophages in the lung at 3 days post-SM exposure (Figure 10). Whereas the number and size of

Figure 6. Effects of SM on expression of fibrin. Sections prepared 3, 7, 16, and 28 days after exposure of rats to sulfur mustard (SM; 0.4 mg/kg) or control (CTL) were stained with antibody to fibrin/fibrinogen. Binding was visualized using a Vectastain kit. Original magnification, 100 $\times$ (bronchiolar regions) and 600 $\times$ (alveolar region). Representative sections from CTL ($n = 5$), 3 days post-SM ($n = 5$), 7 days post-SM ($n = 5$), 16 days post-SM ($n = 5$), and 28 days post-SM ($n = 5$) are shown.

positively staining cells declined at 7 and 16 days, both positively staining macrophage populations increased again at 28 days post-SM. However, at this time, TNF- α ⁺ macrophages were fewer in number and smaller in size than at 3 days post-SM. TNF- α and iNOS were also upregulated in bronchiolar regions of the lung (Supplementary Figs. 2 and 3). Although iNOS was expressed in both proximal and distal bronchiolar epithelial cells, TNF- α was mainly evident in proximal bronchiolar epithelium.

Gal-3 is a carbohydrate-binding lectin released by macrophages; it promotes inflammation by activating neutrophils and by stimulating the production of reactive oxygen species; Gal-3 also acts on macrophages to promote an M2 profibrotic phenotype, contributing to the development and progression of fibrosis (McLeod et al., 2018). Low numbers of Gal-3⁺ alveolar

macrophages were observed in lungs of control animals (Figure 10). Following SM administration, Gal-3⁺ macrophages increased in the alveoli; this was most notable at 3 and 28 days post-exposure; a few interstitial macrophages also expressed Gal-3. At these times, Gal-3⁺ macrophages were generally larger than positively staining cells in control lungs and in lungs at 7 and 16 days post-SM exposure. Gal-3 was also expressed in bronchiolar walls of control animals (Supplementary Figure 4). Exposure to SM was associated with increases in Gal-3 expression in both proximal and distal bronchiolar epithelial cells at 3 days. Some subepithelial cells also expressed Gal-3 at this time. Although expression decreased thereafter in distal bronchioles; in proximal bronchioles, a transient depression in Gal-3⁺ in

Figure 7. Effects of sulfur mustard (SM) on fibrinogen levels in bronchoalveolar lavage (BAL). Upper panel: BAL was collected 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) and analyzed for fibrinogen by western blotting. Lower panel: Combined band intensity of α , β , and γ fibrinogen chains was quantified by densitometry for each sample. Data are presented as fold change over control. Bars, mean $\pm$ SE; CTL ($n=10$), 3 days post-SM ($n=5$), 7 days post-SM ($n=6$), 16 days post-SM ($n=6$), and 28 days post-SM ($n=5$). ^aSignificantly ($p \leq .05$) different from CTL.

epithelial cells at 7 days was followed by an increase at 16 and 28 days.

SM Induces Oxidative Stress in the Lung

Structural and inflammatory changes in the lung following SM exposure were associated with oxidative stress, as reflected by increases in expression of the antioxidant HO-1 (Laskin et al., 2010; Ryter and Choi, 2005). Thus, at 3 days following SM exposure, HO-1 was upregulated in proximal bronchiolar epithelial and subepithelial cells and distal bronchiolar epithelium (Figure 11). Although in proximal bronchiolar epithelial cells HO-1 remained upregulated for at least 28 days post-SM exposure, in distal bronchioles, expression declined. SM exposure also resulted in a persistent increase in numbers of HO-1⁺ alveolar macrophages in the lung. These cells were enlarged and vacuolated at 3, 16, and 28 days, but at 7 days, they were smaller and staining intensity was reduced. At 28 days post-SM exposure, macrophages staining for HO-1 were observed occluding the alveoli, mainly in fibrotic foci.

DISCUSSION

Previously, we described a rat lung injury model induced by intratracheal instillation of nitrogen mustard (Malaviya et al., 2012, 2015). In this model, acute injury was largely evident in lung parenchyma and was characterized by severe multifocal alveolar accumulation of inflammatory cells, blood vessel hemorrhage, and thickening and bronchiolization of the alveolar

walls. Moderate bronchiolar changes were also noted including peribronchial edema and bronchiolar epithelial and goblet cell hyperplasia and hypertrophy which culminated in multiple parenchymal fibrotic foci (Malaviya et al., 2012, 2015). By comparison, in humans, inhalation of mild-to-moderate doses of SM is associated with acute necrosis of airway mucosa, sloughing of respiratory epithelial cells, pulmonary edema, and leukocytosis, which progresses to bronchial wall thickening, bronchitis, bronchiectasis, emphysema, atelectasis, and pulmonary fibrosis (Bagheri et al., 2003; Graham and Schoneboom, 2013; Khateri et al., 2003). In these studies, we developed and characterized a rodent inhalation exposure model that more closely reflects pathological pulmonary responses of humans to SM.

Inhaled SM in the range of 0.2–0.6 mg/kg caused rapid (within 3 days), dose-related pathological changes in the respiratory tract of rats. At higher doses (0.4 and 0.6 mg/kg), necrosis of the proximal bronchiolar epithelium, mucosal damage, and ulceration were observed, as well as perivascular and peribronchiolar edema and inflammation. Similarly, in victims of SM poisoning, necrosis of the bronchiolar epithelium, inflammation, and edema occur relatively quickly (4–18 days) after exposure (Dacre and Goldman, 1996; Kilic et al., 2018; Wolfe et al., 2019). Our time course studies with SM in rats revealed that the response was biphasic. Thus, the acute injury response noted at 3 days post-SM exposure was followed by a transient reduction in histopathology, edema and inflammation at 7 days, and then a secondary, more robust increase at 16–28 days post-exposure, which culminated in fibrosis, as evidenced by focal alveolar collagen deposits, along with extensive alterations in alveolar architecture (McKleroy et al., 2013; Wynn and Ramalingam, 2012). An analogous biphasic response to mustard poisoning has been described in humans, characterized by a transient decrease in acute pathology and symptoms during the first several months after exposure, and then over time, bronchial wall thickening, airway stenosis, emphysema, and fibrosis (Tang and Loke, 2012; Zarchi et al., 2004). In this context, it has been reported that the number of annual pulmonary complications (APC) per 10,000 person-years in mustard war victims is lowest during the first year after mustard exposure; thereafter, numbers of APC increased (Zarchi et al., 2004). Similarly, the frequency and severity of SM-induced pulmonary complications escalated dramatically in victims 18 years postexposure, relative to levels observed after 4–7 years (Hefazi et al., 2005). In rat lung, epithelial cell necrosis and sloughing of proximal airways, which were evident 3 days post-SM exposure, progressed with time into the smaller distal and respiratory bronchioles. At 16 days postexposure, multifocal increases in enlarged alveolar macrophages were evident, along with interstitial wall thickening. Increases in alveolar macrophages and interstitial thickening with mononuclear cell accumulation have also been described in human lungs up to 14 years after SM inhalation (Beheshti et al., 2006). Injury to the proximal bronchiolar epithelium in rats persisted for at least 28 days following SM inhalation; signs of abnormal tissue regeneration and diffuse metaplasia and mucoid secretion were also evident. Abnormal recovery of airway epithelia and squamous metaplasia have similarly been described in rats and guinea pigs up to 35 days after SM exposure (Alves et al., 2015; Calvet et al., 1996; Malaviya et al., 2020). This is in contrast to normal repair of the epithelium observed following pulmonary injury induced by toxicants such as methyl isocyanate, toluene diisocyanate, or ozone (Calvet et al., 1996). It is possible that SM damages cells important in epithelial regeneration; alternatively, SM may directly impair the regenerative response of epithelial cells. Further studies are required to explore these

Figure 8. Effects of sulfur mustard (SM) on bronchoalveolar lavage (BAL) levels of surfactant protein-D (SP-D), soluble receptor for glycation end products (sRAGE), high-mobility group box protein-1 (HMGB-1), and matrix metalloproteinase-9 (MMP-9). Left panels: BAL was collected 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) and analyzed for SP-D, sRAGE, HMGB-1, and MMP-9 content by western blotting. Right panels: Band intensity was quantified by densitometry for each sample. Data are presented as fold change over control. Bars, mean $\pm$ SE; CTL ($n=8-9$), 3 days post-SM ($n=5$), 7 days post-SM ($n=6$), 16 days post-SM ($n=7$), and 28 days post-SM ($n=5$). ^aSignificantly ($p \leq .05$) different from CTL.

possibilities. Prolonged pathologic responses of rodents to SM are consistent with reports of aberrant epithelial repair, abnormal cell differentiation, scarring and stenosis of the airways in long-term survivors of mustard gas exposure (Balali-Mood and Hefazi, 2005; Freitag et al., 1991; Graham and Schoneboom, 2013). In humans, aberrant repair of the epithelium is associated with airway hyperreactivity and impaired pulmonary function (Balali-Mood et al., 2011; Emad and Rezaian, 1997; Hefazi et al., 2005; Weinberger et al., 2011). We previously reported functional alterations in lungs of mice 14 days after exposure to nitrogen mustard (Sunil et al., 2020). As nitrogen mustard and SM induce generally similar pathologic alterations, we predict equivalent changes in pulmonary function after SM exposure in

rats and this is currently under investigation. This is supported by findings of bronchoconstriction, emphysema, and bronchiectasis in rodents up to 35 days following SM exposure (Malaviya et al., 2020). Longer term effects of inhaled SM in rats (28 days) were also characterized by parenchymal inflammation, multifocal honeycombing, alveolar fibrin accumulation, vacuolated alveolar macrophages, multifocal alveolar interstitial fibrosis, and peribronchial fibrosis. Similar pathogenic features have been described in human lungs 10–20 years after SM exposure (Beheshti et al., 2006; Emad and Rezaian, 1997; Khateri et al., 2003; Nishimura et al., 2016; Taghaddosinejad et al., 2011). Taken together, these histopathologic findings provide evidence that our rat model of mustard lung toxicity closely reflects the time-

Figure 9. Effects of sulfur mustard (SM) on matrix metalloproteinase-9 (MMP-9) expression. Sections prepared 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) were stained with antibody to MMP-9. Binding was visualized using a Vectastain kit. Original magnification, 100 $\times$ (bronchiolar regions) and 600 $\times$ (alveolar region). Representative sections from CTL ($n = 4$), 3 days post-SM ($n = 5$), 7 days post-SM ($n = 4$), 16 days post-SM ($n = 5$), and 28 days post-SM ($n = 4$) are shown.

related adverse responses observed in humans exposed to SM gas.

In our experimental model of SM lung toxicity, we also observed biphasic increases in biochemical markers of injury. Thus, increases in BAL protein, PCNA expression in the bronchioles, and fibrin/fibrinogen at 3 days post-SM exposure were followed by a decrease and then, a generally more robust increase at 28 days. Fibrinogen has been reported to be released within hours of exposure to mustard gas and is thought to contribute to the formation of obstructive fibrin containing casts observed in severely poisoned victims (Freitag *et al.*, 1991; Weinberger *et al.*, 2016). Fibrinogen has also been reported to suppress surfactant protein functioning; this may contribute to enhanced macrophage activation in the lung and prolonged inflammation (Seeger *et al.*, 1993; Wygrecka *et al.*, 2008). Chronic

respiratory diseases in victims of SM poisoning including tracheal/bronchial stenosis, chronic bronchitis and bronchiolitis obliterans are generally attributable to airway lesions that persist months to years after exposure (Ghanei *et al.*, 2008). Increases in fibrinogen in the lung after SM exposure may contribute to lung remodeling related to these pathologies (MacNee, 2013; Wedzicha *et al.*, 2000; Wygrecka *et al.*, 2008).

The biphasic accumulation of inflammatory cells in the lung following SM inhalation in rats was paralleled by a similar pattern of increases in proinflammatory proteins. Thus, increases in RAGE, HMGB-1, MMP-9, iNOS, and TNF- α in BAL and/or lung tissue at 3 days post-SM exposure were followed by a decline and then a secondary more prominent increase at 28 days. A comparable trend was observed in BAL levels of SP-D, which promotes proinflammatory macrophage activation following

Figure 10. Effects of sulfur mustard (SM) on inflammatory protein expression. Sections prepared 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) were stained with antibody to tumor necrosis factor- α , inducible nitric oxide synthase, or galectin-3. Binding was visualized using a Vectastain kit. Original magnification, 600 $\times$. Representative sections from CTL ($n=4$), 3 days post-SM ($n=4-5$), 7 days post-SM ($n=4$), 16 days post-SM ($n=4$), and 28 days post-SM ($n=4-5$) are shown.

oxidant-induced posttranslational modification (Starosta and Griese, 2006). Whereas RAGE and HMGB-1 augment oxidative stress, macrophage activation, and proinflammatory mediator release, MMP-9 degrades extracellular matrix components (Chakrabarti and Patel, 2005; Sanders et al., 2019; Sims et al., 2010). These activities have been linked to mustard-induced acute lung injury and the development of chronic lung diseases including asthma, acute respiratory distress syndrome, fibrosis, and chronic obstructive pulmonary disease in exposed victims (Craig et al., 2015; Ferhani et al., 2010; Gangemi et al., 2015; Hamada et al., 2008; Imbalzano et al., 2017; Reynolds et al., 2010; Sanders et al., 2019; Stogsdiill et al., 2013; Uchida et al., 2006). Findings that RAGE, HMGB-1, and MMP-9 are upregulated in the lung at early and at late times following SM inhalation suggest that they are involved in both the pathogenesis of acute injury and in chronic disease induced by this vesicant. It remains to be determined if changes in levels of these inflammatory proteins

are specific to SM toxicity or a reflection of persistent injury and/or inadequate repair in the lung.

In previous studies, we demonstrated a role of macrophages in early acute lung injury and in the chronic fibrotic response to nitrogen mustard (Malaviya et al., 2015; Venosa et al., 2016). This activity is mediated by proinflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages, respectively. iNOS and TNF- α are prototypical markers of activated M1 macrophages (Laskin et al., 2019). Following inhalation of SM, iNOS⁺ and TNF- α ⁺ macrophages appeared in the lung within 3 days of exposure. The fact that this correlated with increases in RAGE, HMGB-1, and MMP-9 in the lung suggests that proinflammatory M1 macrophages are a significant source of these proteins. iNOS and TNF- α were also expressed by cells within the distal and proximal bronchioles; this indicates that cells other than alveolar macrophages contribute to pulmonary inflammation following SM exposure. TNF- α and nitric oxide

Figure 11. Effects of sulfur mustard (SM) on heme oxygenase-1 (HO-1) expression. Sections prepared 3, 7, 16, and 28 days after exposure of rats to SM (0.4 mg/kg) or control (CTL) were stained with antibody to HO-1. Binding was visualized using a Vectastain kit. Original magnification, 100 $\times$ (bronchiolar regions) and 600 $\times$ (alveolar region). Representative sections from CTL ($n=6$), 3 days post-SM ($n=5$), 7 days post-SM ($n=5$), 16 days post-SM ($n=7$), and 28 days post-SM ($n=5$) are shown.

generated via iNOS, released by M1 macrophages, have been implicated in acute lung injury induced by nitrogen mustard (Malaviya *et al.*, 2012, 2015). Both mediators promote oxidative stress and inflammation, which are keys, not only in the pathogenesis of acute injury but also in chronic disease pathogenesis. TNF- α also stimulates fibroblast production of transforming growth factor- β , which is important in fibrogenesis (Malaviya *et al.*, 2017; Sullivan *et al.*, 2005). These findings indicate that targeting TNF- α and iNOS may be an effective strategy for mitigating morbidity associated with exposure to inhaled SM. In this regard, we have previously demonstrated that inhibitors of TNF- α and iNOS reduce lung injury induced by nitrogen mustard (Malaviya *et al.*, 2016). Their ability to mitigate SM-induced lung injury is currently under investigation.

Gal-3 $^{+}$ macrophages also appeared in the lung rapidly after SM exposure and persisted at increased levels up to 28 days,

although at 7 and 16 days, they were relatively smaller in size and fewer in number. The role of Gal-3 $^{+}$ macrophages in SM-induced lung injury is unknown. While considered a profibrotic marker of M2 macrophages, Gal-3 has also been shown to promote oxidative stress and neutrophilic inflammation (Henderson and Sethi, 2009; Ou *et al.*, 2019). It may be that Gal-3 $^{+}$ macrophages play distinct roles in SM lung injury at early and later times after SM exposure. Gal-3 was also upregulated in bronchiolar walls at 3, 16, and 28 days post-SM exposure. Gal-3 has been reported to stimulate collagen production by fibroblasts (Henderson and Sethi, 2009). This suggests that Gal-3 may contribute to early epithelial cell degeneration by inducing oxidative stress, and later remodeling of the airways following SM exposure. This is supported by findings of high levels of Gal-3 in BAL from patients with idiopathic pulmonary fibrosis (MacKinnon *et al.*, 2012; Nishi *et al.*, 2007).

SM-induced inflammation was associated with oxidative stress, as reflected by increases in HO-1 in alveolar macrophages and in the bronchioles. Whereas in macrophages the response was biphasic, peaking at 3 and 28 days post-SM, in bronchiolar walls expression of HO-1 persisted for 28 days, although at relatively lower levels. Mustard-induced pulmonary toxicity is known to be due, in part, to oxidative stress (Laskin et al., 2010; Tahmasbpour et al., 2015). In humans, this is associated with increases in SM-glutathione metabolites in urine following exposure which is indicative of reduced intracellular antioxidant capacity (Paromov et al., 2007). Expression of HMOX1 and several other antioxidant genes including sulfiredoxin, peroxiredoxins, and oxidative stress-responsive-1, 20–30 years after SM exposure are consistent with chronic oxidative stress in the lungs of mustard gas victims (Tahmasbpour et al., 2016; Tahmasbpour Marzony et al., 2016). We speculate that acute oxidative stress is due to SM-induced generation of cytotoxic reactive intermediates, whereas the secondary response is an indirect consequence of persistent inflammation.

The present studies provide evidence for a biphasic lung injury, inflammatory and oxidative stress response in rats following SM vapor inhalation which is in accord with findings in exposed humans and consistent with acute and long-term pathologies (Graham and Schoneboom, 2013; Tahmasbpour et al., 2016; Weinberger et al., 2016). The biphasic response to SM in animals and humans most likely reflects a transition from proinflammatory to anti-inflammatory processes in the lung, as the microenvironment changes during the progression to chronic pulmonary disease (Laskin et al., 2019). The fact that some inflammatory proteins (eg, RAGE, HMGB-1, MMP-9, iNOS, Gal-3) are present in both the acute and chronic disease phases suggests that they may play distinct roles in the pathogenic response to SM at different times following exposure. For example, while early in the pathogenic response to SM, MMP-9 may function to initiate lung inflammation by cleaving RAGE to sRAGE, during the chronic phase, it may promote tissue remodeling during fibrogenesis. Taken together, results of our studies demonstrate that our rat model of SM inhalation closely resembles the response of humans to mustard gas, making it appropriate for use in studies aimed at identifying mechanistic targets for drug development and for testing therapeutics to mitigate mustard toxicity and disease pathogenesis.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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DECLARATION OF CONFLICTING INTERESTS

D.W. is an employee of Janssen R&D, Inc. and owns stock in Johnson and Johnson, the parent company of Janssen R&D, Inc.

R.C.R. and R.M. also own stock in Johnson and Johnson, the parent company of Janssen R&D, Inc. E.V.A., V.R.S., M.N., C.R.C., J.R., R.T., E.P., R.P.C., J.D.L., and D.L.L. declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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