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Sulfur Mustard Induces Immune Sensitization in Hairless Guinea Pigs

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Abstract

Sulfur mustard (SM, bis-(2-chloroethyl) sulfide) is a well known chemical warfare agent that may cause long-term debilitating injury. Because of the ease of production and storage, it has a strong potential for chemical terrorism; however, the mechanism by which SM causes chronic tissue damage is essentially unknown. SM is a potent protein alkylating agent, and we tested the possibility that SM modifies cellular antigens, leading to an immunological response to “altered self” and a potential long-term injury. To that end, in this communication, we show that dermal exposure of euthymic hairless guinea pigs induced infiltration of both CD4⁺ and CD8⁺ T cells into the SM-exposed skin and strong upregulated expression of proinflammatory cytokines and chemokines (TNF- α , IFN- γ , and IL-8) in distal tissues such as the lung and the lymph nodes. Moreover, we present evidence for the first time that SM induces a specific delayed-type hypersensitivity response that is associated with splenomegaly, lymphadenopathy, and proliferation of cells in these tissues. These results clearly suggest that dermal exposure to SM leads to immune activation, infiltration of T cells into the SM-exposed skin, delayed-type hypersensitivity response, and molecular imprints of inflammation in tissues distal from the site of SM exposure. These immunological responses may contribute to the long-term sequelae of SM toxicity.

Keywords

Sulfur mustard; Delayed-type hypersensitivity; Immune sensitization; Cytokine; Inflammation

1. Introduction

Sulfur mustard (SM; bis-(2-chloroethyl) sulfide), the highly reactive alkylating agent, was used as a chemical warfare agent in World War I and the 1980s Iraq/Iran conflict. Due to the ease of production and storage, SM is a potential threat to both military and civilian targets [1]. Exposure to SM may cause ocular, respiratory, and cutaneous damage that is palpable only several hours after the exposure. Dermal contact can produce severe skin blisters that last for weeks to months [2], and inhaled SM can cause severe lung damage [3–5]. In addition to direct SM-induced tissue damage, secondary inflammatory responses are common and, in human

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survivors, pulmonary fibrosis with progressive lung dysfunction and death may occur [6]. The delayed effects may arise months to years after SM exposure and include chronic bronchitis, asthma, bronchiectasis, and pulmonary fibrosis [7–9]. In a recent report, in many SM victims, chronic laryngitis was observed nearly 20 years after SM-exposure [10]. In vitro and animal experiments suggest that SM induces infiltration of inflammatory cells and production of inflammatory cytokines and chemokines locally [11–17]. Moreover, guinea pigs exposed to SM via inhalation exhibit abnormal epithelial growth, cellular infiltration, and long-term inflammatory changes in the lung [18,19].

Despite decades of research the mechanism of SM-induced chronic lung injury is unclear. SM has a short half-life of 19–24 min in normal saline and 30–60 min in the blood [20] and, given the short half-life of SM, the development of long-term pulmonary effects after a single SM exposure is surprising. It is conceivable that following the initial inflammatory response (30 min–12 h) secondary proinflammatory events ensue to perpetuate the tissue injury [13,21 & 22]. SM is a strong alkylating agent and could potentially alter “self” antigens, leading to an allogenic-type of response to “altered self” antigens. In this communication, we present evidence that dermal exposure of euthymic hairless guinea pigs to SM, induces a delayed-type hypersensitivity (DTH) response associated with splenomegaly, lymphadenopathy, and inflammatory responses distal to the site of SM exposure.

2. Materials and Methods

2.1. Chemicals

Except where noted, all the chemicals and reagents were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO).

2.2. Animals

Male euthymic hairless guinea pigs, weighing 350–450 g were purchased from Charles River Laboratory (Raleigh, NC) and maintained in an animal care facility at Lovelace Respiratory Research Institute (LRRI) that is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Food and water were provided *ad libitum* throughout the experimental period. After release from quarantine, the animals underwent short periods of socialization by study personnel and acclimation. Animals were identified by BMDS microchip implants providing identity and temperature readout. All studies in conducting the research described in this report were approved by the Institutional Animal Care and Use Committee of LRRI.

2.3. SM synthesis

SM was synthesized by proprietary methods. The identification and purity of SM was characterized by gas chromatography/mass spectroscopy (GC/MS, Agilent, Santa Clara, CA), proton nuclear magnetic resonance imaging (^1H NMR, Bruker, Billerica, MA), and MiniCAMS GC/flame ionization detector (FID) (O I Analytical, Pelham, AL). The GC/MS ionization pattern in the spectrum was consistent with the structure of SM. ^1H NMR analysis indicated a single, pure compound with chemical shifts consistent with the structure of SM. GC/FID also showed a single peak in an extended chromatogram intended to identify the presence of any reaction byproducts or SM breakdown products; none were detected. The purity of sulfur mustard was determined to be greater than 99%.

2.4. Dermal SM exposure of hairless guinea pigs

Guinea pigs were anesthetized by subcutaneous administration of a cocktail containing ketamine (10 mg/kg) and xylazine (2.5 mg/kg). Buprenorphine (0.01–0.05 mg/kg) was

subcutaneously administered before exposure and thereafter, every 8–12 h if needed. Upon anesthesia, the animals were placed in sternal recumbency on a nonabsorbent frame and secured with strips of Velcro near their shoulders and hips. Double-sided tape containing 2.5-cm diameter holes was placed on the guinea pig's back to secure Teflon exposure cups to the skin. The anesthetized guinea pigs were passed in to a glove box for exposure. SM was placed on a filter inside the exposure cup and allowed to vaporize freely, resulting in a dermal exposure. Animals were exposed to approximately 400 mg/m³ of SM vapor for 6 min, and monitored for respiration, heart rate, and temperature throughout the duration of exposure. At the conclusion of the exposure, the animals were placed on a heating pad in an off-gassing chamber until the SM levels reached at acceptable levels, and returned to their home cages.

2.5. Immunohistochemistry of skin sections

Control and SM-exposed animals were sacrificed at indicated times post-exposure. Skin samples were excised, central portions embedded in OCT, and frozen in liquid nitrogen. Cryosections of the skin were cut approximately 5- μ m thick and stained in a double-labeling immunohistochemistry (IHC) procedure for the T-lymphocyte cell-surface markers CD4 and CD8. Briefly, sections were fixed in cold acetone and washed in phosphate-buffered saline (PBS), endogenous peroxidase was blocked with hydrogen peroxide, and nonspecific sites were blocked with the buffer containing normal horse and guinea pig serums. For the first label, sections were incubated for 2 h at room temperature with a mouse anti-CD8 antibody at 1:800 (clone CT6, AbD Serotec, Raleigh, NC). The label was detected with kit reagents (Vector Laboratories; Burlingame, CA), including a peroxidase polymer-linked horse anti-mouse secondary antibody (ImmPRESSTM) and nickel-enhanced diaminobenzidine substrate. For the second label, the sections were washed and blocked for endogenous peroxidase as before, blocked with the buffer containing normal horse serum (Vector Laboratories), and incubated overnight at 4°C with a mouse anti-CD4 antibody at 1:400 (clone CT7, AbD Serotec). The second label was detected with the ImmPRESSTM reagent and NovaRED peroxidase substrate (Vector Laboratories), and the sections were counterstained with hematoxylin. Thus, CD8⁺ cells stained black, CD4⁺ cells stained red, and other cells stained pale blue. During the staining run, cryosections of guinea pig spleen were used as controls, where all reagents were included (positive control) or nonspecific mouse IgG₁ (clone GTX76632, GeneTex, Inc.; Irvine, CA) was included instead of the primary antibodies (negative control).

Sections were examined by light microscopy, and numbers of positively stained cells were estimated using a 40x objective to encompass microscopic fields within the superficial dermis (approximately 250–300 μ m deep to the epidermal basement membrane) where compression, folding, and fragmentation artifacts were minimal. Average numbers ($\pm$ standard deviation [SD]) of fields examined per group were as follows: controls at 24 h post-exposure, 10.2 $\pm$ 4.3 (n = 4 exposure sites); controls at 48 h, 5.7 $\pm$ 2.3 (n = 3); controls at 2 weeks, 10.3 $\pm$ 2.8 (n = 4); SM at 24 h, 8.5 $\pm$ 2.4 (n = 6); SM at 48 h, 11.8 $\pm$ 4.2 (n = 6); and SM at 2 weeks, 10.2 $\pm$ 2.1 (n = 6). Each field was categorized into one of four grades where (1) $\leq$ 5, (2) 6–15, (3) 16–45, or (4) $>$ 45 positively stained cells were estimated per field. Because of the variability in the number of fields assessed per specimen, data were analyzed statistically as the proportion (percentage) of fields in each of the grade categories. Proportions within a grade were transformed by ranking and compared by two-way analysis of variance (ANOVA) with exposure (control or SM) and the duration post-exposure as the independent variables.

2.6. Spleen and lymph node cell preparation

Animals were euthanized by isoflurane inhalation. The thoracic cavity was opened, and the trachea was exposed. Spleens and lymph nodes (cervical and axillary) were quickly collected. Spleen and lymph node cells were prepared as described elsewhere [23] with some minor modifications. Briefly, spleens and lymph nodes were pressed through stainless steel mesh,

and red blood cells were lysed by NH_4Cl treatment. After centrifugation, cells were washed three times with cold PBS and counted on a hemocytometer in 0.1% eosin in PBS. Cells were resuspended in complete tissue culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum [FBS], 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1% pen/strep) for further analysis.

2.7. Immune cell proliferation assay

All cell culture experiments were performed in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS (Sigma-Aldrich Co., St. Louis, MO) and 1% penicillin-streptomycin (Life Technologies). Lymph node or spleen cells (2×10^5 cells) were cultured in 0.2 ml of complete medium in microtiter wells. The cultures were incubated at 37°C in the presence of 5% CO_2 . After 5 days of culture, the proliferation rate was determined by quantification of DNA synthesis after incubation with 1 μCi [^3H]thymidine (ICN Pharmaceuticals, Irvine, CA) for the last 8 h of culture. Cells were harvested by a Skatron cell harvester (Skatron Instruments Inc., Sterling, VA) onto glass-fiber filters (FilterMAT, Skatron Inc.), and [^3H]thymidine incorporation was measured using a liquid scintillation counter. The results were expressed as the mean counts per minute (cpm) $\pm$ SD of triplicate analysis.

2.8. Real-time PCR (qPCR)

Total RNA was isolated from lymph node cells and lung tissues using TRI reagent (Molecular Research Center, Cincinnati, OH) as described elsewhere [24]. Briefly, lung tissues were homogenized in 1 ml Tri-Reagent and, after addition of 100 μl BCP (Molecular Research Center), the homogenates were centrifuged at $13,000 \times g$ for 10 min at 4°C . The aqueous layer was collected and mixed with 600 μl of isopropanol. After 15 min at room temperature, samples were centrifuged ($13,000 \times g$; 10 min) and the pellet was resuspended in 75% ethanol, centrifuged as above, and then air dried. The samples were resuspended in diethylpyrocarbonate (DEPC)-treated water (55°C for 10 min to dissolve RNA) and quantified spectrophotometrically. qPCR analysis was performed on the ABI PRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix (Applied Biosystems, Foster City, CA). The relative expression of each mRNA was calculated by the method described earlier [24]. Normalizing mRNA levels using 18S rRNA, and GAPDH showed similar results. All specific labeled primer/probe sets for IL-8, IFN- γ , TNF- α , 18S, and GAPDH were purchased from Applied Biosystems.

2.9. Determination of DTH response

On day 7 after the initial dermal sensitization, animals were challenged on the ears with SM or the vehicle. Briefly, 5 μl of SM (2 $\mu\text{g}/\mu\text{l}$ in ethanol) was applied to the right ear while the left ear received 5 μl of ethanol (vehicle). Ear thickness was measured by Verner caliper at 24 and 48 h after the SM challenge.

2.10. Statistical analysis

The data were analyzed by GraphPad Prism Software 3.0 (GraphPad Software, Inc., San Diego, CA) using Student's t-test, or by two-way ANOVA. The data are shown as the mean $\pm$ standard error (SE) of the combined experiments. The differences with a p value of ≤ 0.05 were considered significant.

3. Results

3.1. Dermal exposure to SM promotes T cell accumulation in the skin

To ascertain whether dermal exposure promotes local inflammation, histochemically stained cryosections of skin from vehicle-treated control and SM-exposed animals were examined by

light microscopically for the presence of CD4⁺ and CD8⁺ cells in the superficial dermis at different times post exposure. Results presented in Figure 1 show that compared to vehicle-treated control animals, increased numbers of CD4⁺ (red) and CD8⁺ (black) cells are present in the dermis of SM-treated animal at the site of SM application at 2 weeks post exposure. The number of CD4⁺ and CD8⁺ cells were quantified at 24 h, 48 h, and 2 weeks after the exposure. Results presented in Figure 2 indicate that, compared to control, SM promotes a significant accumulation of CD4⁺ cells in the skin (i.e., presence of higher percentages of cells in the microscopic fields encompassing grades 3 and 4 with a proportional decrease in cell numbers in grade 2) within 24 h of SM exposure. Accumulation of CD8⁺ cells became prominent at 2 weeks after SM exposure. A mild to moderate increase in the accumulation of heterophils was accompanied with mononuclear leukocytic infiltration in these tissues.. These results suggest that SM causes immunological activation and accumulation of leukocytes, including T cell, at the site of exposure.

3.2. SM induces splenomegaly and lymphadenopathy

Many lymphoproliferative diseases such as the graft versus host disease, some autoimmune diseases, and DTH are associated with increased proliferation of lymphocytes in the lymphoid tissues, leading to splenomegaly and/or lymphadenopathy [25–27]. To ascertain whether the dermal exposure to SM, perhaps through SM-generated auto-antigens, caused lymphoproliferation, we determined the cellularity of the spleens and lymph nodes (cervical and axillary) 4 days after SM treatment. Surprisingly, within a relatively short time period (4 days), the average spleen size had increased by about 50% after SM exposure. Moreover, in SM-treated guinea pigs the cellularity of the spleen was increased by over 80% (Figure 3A). Similarly, in SM-treated animals, the lymph nodes appeared larger with approximately three times as many cells (Figure 3B) and incorporated nearly twice as much ³H-thymidine than control lymph node cells after a 6-h incubation in the culture medium (Figure 3C). These results clearly suggest that SM treatment stimulates lymphoproliferation.

3.3. SM induces the expression of proinflammatory cytokines/chemokines in lymph nodes and lungs

Victims of SM exposure during Iran-Iraq war have developed pulmonary complications after established dermal exposure [28]; however, in these subjects the possibility of SM exposure through inhalation has not been ruled out. Therefore, we investigated whether dermal exposure to SM might also affect lung responses and determined the expression of proinflammatory cytokines/chemokines by qPCR analysis in lymph node cells and lung tissues 4 days after SM exposure. Figure 4 shows that SM markedly increased the expression of the neutrophil chemokine IL-8 (CXCL-8) and the inflammatory cytokines TNF- α and IFN- γ in lymph nodes (Figure 4A) as well as the lung (Figure 4B). These results suggest that SM can induce inflammatory responses in tissues that are distal to the site of application.

3.4. SM induces DTH

SM is a powerful alkylating agent and, because it induces lymphoid proliferation and migration of T cells into the site of inflammation, we determined whether SM induced a DTH reactivity in response to potentially modified self antigens. Animals were dermally exposed to SM and after 2 weeks challenged with a small amount of SM on the right ear and the vehicle on the left ear. The ear thickness was measured at 24 and 48 h after the challenge. Figure 5A shows that increased ear thickness is evident in the SM-sensitized animals, and the increase is quantitatively significant (Figure 5B). These data suggest that SM induces a strong DTH response, and might have implications in understanding the chronic effects of SM exposure.

4. Discussion

SM was extensively used during World War I and more recently during the Iran-Iraq war of 1980s [29]. In addition to its military use, it is a potential weapon of mass destruction against civilian targets and causes both acute and chronic health effects. Respiratory, ocular, and cutaneous injuries are well-documented acute effects of SM exposure. Diverse chronic effects have also been reported, and respiratory complications are the major cause of disability and mortality in SM victims [29–31]. The clinical manifestations of SM exposure result from multiple mechanisms, although the exact pathways are not yet elucidated. The recurrent exacerbation of the injuries suggests an autoimmune-like etiology; however, so far experimental evidence to support this possibility has been lacking.

SM is a potent alkylating agent [1,32] and some of the highly reactive chemicals are known to cause immunologic sensitization and activation of T cells in vitro and in vivo. Thus natural and synthetic compounds in food, medicines, and cosmetic products can cause skin inflammation, allergic/asthma symptoms, and autoimmunity [33–35]. Chemicals such as dinitrofluorobenzene, D-penicillamine, and diphenylhydantoin induce autoimmune responses that resemble graft-versus-host like disease [36,37]. Multiple reports suggest that the T-cell responses, including the DTH response to many chemical sensitizers reflect the alloantigen-like response to modified “self” [38,39] and may involve chemical modification of the major histocompatibility complex (MHC) antigens [38–40]. SM being a highly reactive alkylating agent, it was likely that it modified self cellular antigens to yield a specific DTH-like response. Indeed, our data clearly shows that dermal application of subclinical doses of SM (i.e., the concentrations that did not cause overt blistering in hairless guinea pigs) induced a significant DTH response. SM sensitization is also associated with lymphoproliferation, leading to splenomegaly and lymphadenopathy of the draining lymph nodes. While it is likely that the DTH represents the T-cell response against SM-modified self MHC antigens, at this time we have no direct experiments (e.g., autologous mixed lymphocyte reaction, adoptive transfer of DTH by sensitized T cells) to prove this possibility.

At this time it is unclear how the development of SM-induced DTH would produce the delayed SM-induced sequelae. Drug-induced hypersensitivity has been correlated with the development of autoimmunity, sequential activation of herpesviruses, and loss of Treg function [40,41], the latter may promote a hyperimmune response to various pathological agents. Infiltration of both CD4⁺ and CD8⁺ T cells was seen in the skin; however, their individual contribution to potential disease is not known. DTH is primarily contributed by CD4⁺ T cells [38]; however, there is evidence that in humans intraepidermal CD8⁺ T cells may persist after drug-induced hypersensitivity, even in the absence of antigenic stimuli, for more than 4 years [39]. Thus, there are a number of potential immunological pathways through which chemical hypersensitivity might lead to autoimmune-like responses and chronic tissue injury.

The clinical and histopathological aspects of SM cutaneous exposure have been well studied, and a variety of in vivo and in vitro experiments show induction of inflammatory mediators after exposure to SM. TNF- α , a prototype proinflammatory cytokine, is produced by a number of cell types, predominantly by macrophages and monocytes [39,40], and is strongly upregulated in hairless guinea pigs by SM after dermal exposure. TNF- α stimulates the production of the neutrophil chemokine IL-8 by alveolar macrophages [39,40] and, along with IFN- γ , it plays a critical role in the experimental models of lymphoproliferation [38]. Thus, it is conceivable that increased expression of TNF- α and IFN- γ contribute to the lymphoproliferation observed in SM-treated animals. IFN- γ also promotes Th1 polarization [39]; however, we have no evidence that the CD8⁺ T cells, seen at the site of inflammation, develop into cytotoxic T cells. To a lesser extent, SM also increased the expression of IL-8 in the lymph nodes and might contribute to the neutrophilic infiltration in the skin of SM-exposed

animals. Qabar et al. [42] have shown that the expression of TNF- α altered keratinocyte sensitivity to SM-induced cell death, and it is likely that SM stimulates the expression of other proinflammatory cytokine/chemokines, but the lack of guinea pig-specific reagents prevented us to determine their expression. However, the manners through which inflammatory factors or other hitherto unidentified factors produced in response to SM exposure, lead to tissue injury is not clear at present and remain to be delineated.

Increasing evidence suggests that noninhalation exposures of SM might produce pulmonary pathology, which is the major cause of morbidity and mortality among human SM victims [30]; however, the mechanism of pulmonary toxicity under these conditions is unclear. Interestingly, guinea pigs exposed to SM through the cutaneous route also exhibit increased expression of TNF- α and IFN- γ in the lung. SM is a highly reactive compound and preliminary results suggest that very little if any enters circulation after the dermal exposure (Benson et al., unpublished observation). Whether SM promotes the emigration of activated leukocytes, particularly T cells, is not known, and might be critical for understanding the mechanism of lung injury following dermal exposure to SM.

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Figure 1A **Figure 1B** **Figure 1.**

Dermal exposure to SM promotes T cell emigration into the skin. Guinea pig skin from a site exposed to air (control) (A) and 2 weeks post exposure of SM (B). Cryosections of the skin were stained for the T-lymphocyte cell-surface markers, CD4 and CD8 as detailed in Materials and Methods. Red, CD4⁺ cells (black arrowhead) and black, CD8⁺ cells (white arrowhead) are shown in periadnexal superficial dermis of control animals (grade 2 = 6–15 cells per 40 × field) and more numerous in this focus of superficial dermis of SM-exposed animals after 2 weeks (grade 3 = 16–45 cells per 40 × field) than in most microscopic fields of control specimens. Other indicators: k, keratin; e, epidermis; f, follicle. Bar = 25 μm.

Figure 2.

Kinetics of T cell accumulation in the skin after SM exposure. Animals were sacrificed at 24 h, 48 h, and 2 weeks post SM exposure and skin cryosections quantitated for T cells by immunohistochemistry. Statistically, SM significantly increased the percentage of microscopic fields with grades 3 and 4 CD4⁺ staining (16–45 and > 45 cells per field, respectively) and the percentage of grade 3 CD8⁺ staining, while the percentage of grade 2 staining (6–15 cells per field) for both CD4⁺ and CD8⁺ was proportionately and significantly reduced in comparison to controls ($P < 0.05$, ANOVA).

Figure 3A **Figure 3B**

Figure 3C

Figure 3.

SM induces splenomegaly and lymphadenopathy. The hairless guinea pigs were exposed SM as described in Materials and Methods. Animals were euthanized by isoflurane inhalation and spleens and lymph nodes (cervical and axillary) were quickly collected. Spleen (A) and lymph node cells (B) were prepared as described in Materials and Methods and counted on a hemocytometer in 0.1% eosin in PBS. After 5 days of cell culture, the proliferation rate was determined by quantification of DNA synthesis after incubation with 1 μ Ci [³H]thymidine for the last 8 h of culture. Cells were harvested and [³H]thymidine incorporation was measured using a liquid scintillation counter (C). The results were expressed as the mean counts per minute (cpm) $\pm$ SD of triplicate analysis.

Figure 4A **Figure 4B** **Figure 4.**

SM induces the expression of proinflammatory cytokines/chemokines in lymph nodes and lungs. Animals were exposed to SM or vehicle, and total RNA was isolated from lymph node cells and lung tissues as described in Materials and Methods. The qPCR analysis was performed on the ABI PRISM 7900HT Real-Time PCR System using the One-Step RT-PCR Master Mix. The relative expression of each mRNA was calculated in lymph node cells (A) and lungs (B).

Figure 5A

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Figure 5B **Figure 5.**

SM induces delayed-type hypersensitivity response in hairless guinea pigs. Animals were exposed to air or SM, and challenged on the ear with SM or vehicle 7 d post sensitization. A, Visual changes in the ear thickness: (a) control (air exposed), (b) SM-exposed (vehicle control), (c and d) SM-exposed (SM challenge). B, Quantitation of the ear thicknesses measured at 24 h and 48 h after SM challenge and represented as fold stimulation over control. Bars represents mean $\pm$ SD of triplicate values of 4 animals per group.