TOXICOLOGICAL SCIENCES **114(1)**, 5–19 (2010)
doi:10.1093/toxsci/kfp253
Advance Access publication October 15, 2009

REVIEW

Mechanisms Mediating the Vesicant Actions of Sulfur Mustard after Cutaneous Exposure

Michael P. Shakarjian,*,† Diane E. Heck,* Joshua P. Gray,‡ Patrick J. Sinko,§ Marion K. Gordon,¶ Robert P. Casillas,‖ Ned D. Heindel,‖‖ Donald R. Gerecke,¶ Debra L. Laskin,¶ and Jeffrey D. Laskin‖‖‖,[1]

*Department of Environmental Health Science, School of Health Sciences and Practice, Institute of Public Health, New York Medical College, Valhalla, New York 10595; †Department of Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854; ‡Departments of Science, United States Coast Gaurd Academy, New London, Connecticut; §Pharmaceutics and ¶Pharmacology and Toxicology, Rutgers University, Ernest Mario School of Pharmacy, Piscataway, New Jersey 08854; ‖Biomedical Science & Technology Product Line, Battelle Memorial Institute, Columbus, Ohio 43201; ‖‖Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015; and ‖‖‖Environmental and Occupational Medicine, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

[1] To whom correspondence should be addressed at Department of Environmental and Occupational Medicine, University of Medicine Dentistry of New Jersey-Robert Wood Johnson Medical School, 170 Frelinghuysen Road, Piscataway, NJ 08854. Fax: 732-445-0119. E-mail: jlaskin@eohsi.rutgers.edu.

Received October 3, 2009; accepted October 11, 2009

Sulfur mustard (SM), a chemical weapon first employed during World War I, targets the skin, eyes, and lung. It remains a significant military and civilian threat. The characteristic response of human skin to SM involves erythema of delayed onset, followed by edema with inflammatory cell infiltration, the appearance of large blisters in the affected area, and a prolonged healing period. Several *in vivo* and *in vitro* models have been established to understand the pathology and investigate the mechanism of action of this vesicating agent in the skin. SM is a bifunctional alkylating agent which reacts with many targets including lipids, proteins, and DNA, forming both intra- and intermolecular cross-links. Despite the relatively nonselective chemical reactivity of this agent, basal keratinocytes are more sensitive, and blistering involves detachment of these cells from their basement membrane adherence zones. The sequence and manner in which these cells die and detach is still unresolved. Much has been discovered over the past two decades with respect to the mechanisms of SM-induced cytotoxicity and the intracellular and extracellular targets of this vesicant. In this review, the effects of SM exposure on the skin are described, as well as potential mechanisms mediating its actions. Successful therapy for SM poisoning will depend on following new mechanistic leads to develop drugs that target one or more of its sites of action.

Key Words: **alkylating agents; blistering; dermatotoxicity; skin; sulfur mustard.**

Sulfur mustard (SM), or mustard gas, is a bifunctional alkylating agent (bis[2-chloroethyl] sulfide) (see Fig. 1 for structure). It was first synthesized in the early 19th century by Despretz (1822) and later by Guthrie (1860) and Niemann (1860), during which time its distinctive mustard and garlic-like odor and blistering action on the skin were noted. Meyer (1886) subsequently devised a process for the synthesis of pure SM that was later used by the Germans for its preparation as a chemical warfare agent. Its first use on the battlefield was during the German attack near the Belgian town of Ypres, in 1917. As a consequence, the French named it Yperite (Dacre and Goldman, 1996). SM was also referred to as "lost," an acronym from the first letters of the chemists Lommel and Steinkopf, who investigated the military use of the compound (Kehe and Szinicz, 2005), "Yellow Cross," because of the markings on German munitions containing SM, and HD (from Hunstoffe, distilled), the designated acronym used by the U.S. military (Dacre and Goldman, 1996). It is distinguished from other chemical warfare agents by its persistence in the environment, latency of action, and debilitating effects which incapacitate its victims. Chemically related vesicants include 2-chlorethylethyl sulfide (CEES) or half-mustard, a monofunctional alkylating agent with attenuated activity, and methylbis (2-chlorethyl) amine or mechlorethamine (HN2), a nitrogen analog of SM that is used in the treatment of leukemia (Mustargen-Merck, 2002) (Fig. 1).

The primary targets of SM are the skin, cornea, and respiratory tissues. Signs of injury follow an asymptomatic period of variable length, depending on the level of exposure and the target organ. The asymptomatic period is briefest for the eye and is followed by redness and irritation which can progress to corneal damage with photophobia, blepharospasm, and temporary blindness (Papirmeister *et al*., 1991). Airway exposure primarily targets the nasal, laryngeal, and tracheobronchial mucosa, with early mild irritation giving rise to epistaxis, laryngeal injury, and bronchial edema and damage

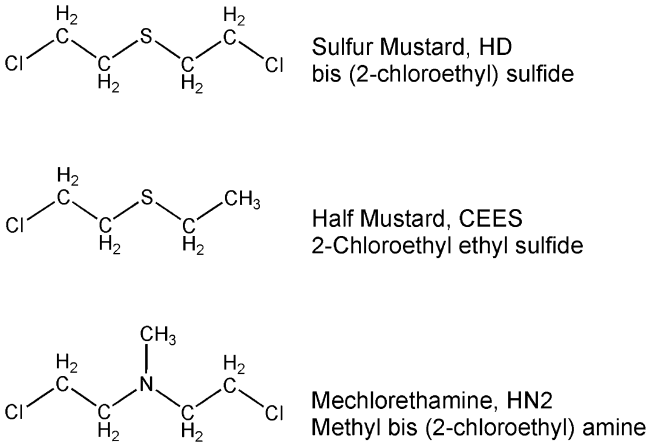

FIG. 1. Structures of SM and related analogs, chloroethyl ethyl sulfide (CEES, half-mustard), and mechlorethamine (nitrogen mustard, HN2). All three agents induce blistering of the skin. Sulfur mustard is the most potent of these agents followed by HN2 and CEES (Fox and Scott, 1980; Goldenberg and Begleiter, 1980; Goldenberg *et al*., 1971; Sharma *et al*., 2008).

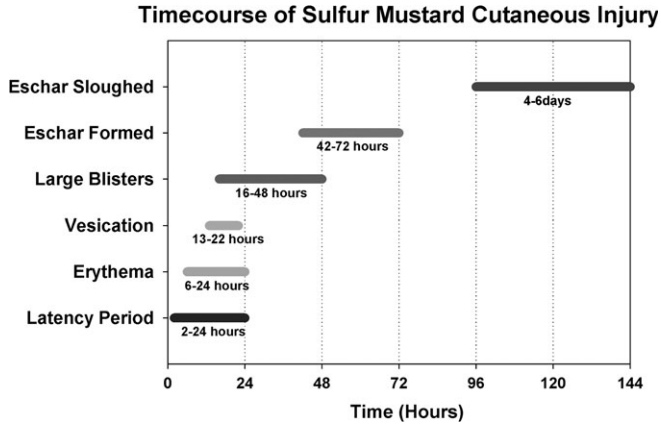

FIG. 2. Time course of pathophysiological changes that occur after dermal exposure to SM. A latency period of variable length precedes erythema, the first sign of injury. Vesiculation commences between 13 and 22 h after exposure and develops over the course of 48 h to form large blisters. A second round of blistering may also be observed. Necrosis and eschar formation occurs after blisters collapse. Healing is prolonged, taking 2–3 weeks for vesicating lesions and several weeks for full-thickness erosions (Balali-Mood and Hefazi, 2005; Papirmeister *et al*., 1991).

(Papirmeister *et al*., 1991). Respiratory barrier function is compromised, and at higher concentrations, the lower airway epithelium becomes involved, with corresponding impairment of gas exchange (Papirmeister *et al*., 1991). Initial contact with the skin is typically not associated with discomfort. Erythema, the first sign of exposure, develops after a latency of 2–24 h and may be associated with intense itching. Vesicles filled with a pale yellow fluid appear several hours later, and with time, they may coalesce to form pendulous blisters. Blisters subsequently burst, resulting in formation of a necrotic layer or eschar on the affected skin surface. Typically, the wounds resolve over the course of 10–50 days, leaving pigmentation changes that may persist for months or years (Balali-Mood and Hefazi, 2005; Chiesman, 1944). Figure 2 depicts the time course of pathophysiological responses following cutaneous SM exposure. Multiple rounds of blistering and healing in the same individual have also been reported (Balali-Mood and Hefazi, 2005).

Inflammation is thought to play a significant role in SM-induced pathology with initial exposure characterized by an accumulation of both granulocytes and macrophages at sites of injury (Dannenberg *et al*., 1985; Guignabert *et al*., 2005; McClintock *et al*., 2002; Millard *et al*., 1997; Papirmeister *et al*., 1991; Smith *et al*., 1995). Exposure to high levels of SM can also result in systemic toxicity; notably bone marrow depression, consequent immune suppression, and increased susceptibility to infection (Balali-Mood and Hefazi, 2005). Chronic conditions arising from acute or long-term low-level exposure to SM have been documented involving the skin, eyes, lung, nervous system, immune system, and gastrointestinal tract (Balali-Mood and Hefazi, 2005; Ghanei *et al*., 2006; Khateri *et al*., 2003; Sidell and Hurst, 1997). Occupational exposures to SM that have occurred during its manufacture have also been associated with various forms of cancer (Easton *et al*., 1988; Inada *et al*., 1978; Pechura and Rall, 1993; Wada *et al*., 1968; A. Weiss and B. Weiss, 1975; Yanagida *et al*., 1988). The present review surveys the current knowledge of the biological effects of SM in the skin, with a focus toward understanding mechanisms involved in cutaneous blister formation.

CHARACTERISTICS OF SM-INDUCED SKIN LESIONS

Insight into the cutaneous histopathological effects of SM comes from early scientific experiments of human subjects (Ginzler and Davis, 1943; Henriques *et al*., 1943; Pappenheimer, 1926; Renshaw, 1946; Warthin and Weller, 1919) and more recent observations of victims of the Iran-Iraq war (Mehzad, 1988; Requena *et al*., 1988). The earliest changes in the epidermis are noted during the erythematous phase of injury, during which time a few scattered basal keratinocytes were identified as undergoing degenerative alterations including nuclear swelling, dispersion of chromatin, and cytoplasmic vacuole formation. With sufficient exposure, the lesion progresses and involves greater numbers of keratinocytes located predominately in the basal layer. The appearance of pyknotic nuclei is followed by disintegration of the cytoplasmic membrane. Foci of necrotic cells then appear which coalesce to form microblisters and eventually to macroblisters. Reports are generally consistent with a locus of blistering at the epidermal-dermal junction (Papirmeister *et al*., 1991). In some instances, necrotic lesions have been identified where bulla formation was bypassed, and the full thickness of the epidermis was affected (Henriques *et al*., 1943).

Damage to the dermal layer of the skin has also been described in humans exposed to SM (Ginzler and Davis, 1943; Henriques *et al*., 1943; Warthin and Weller, 1919). Most

affected are cells of the adnexal structures, in particular, epithelial cells surrounding hair follicles. Capillary reactions are also observed, with hyperemia coinciding with the erythematous phase of SM-induced injury. At higher doses, SM causes more serious injury to the capillaries. Damage to individual dermal fibroblasts is noted at very early time points; however, the frequency of these effects varies in vesicating lesions. Inflammatory cells are also present in the skin of SM-exposed individuals. Mononuclear cells are detected very early in the erythematous phase of injury. This increases in intensity with time and is followed by an influx of neutrophils into the tissue prior to vesication.

MODELS TO ASSESS CUTANEOUS ACTIONS OF SM

Various animal models have been utilized to investigate mechanisms mediating SM-induced toxicity in the skin. Common species employed include rabbits, guinea pigs, pigs, and mice (Casillas *et al*., 1997; Isidore *et al*., 2007; Lindsay *et al*., 2004; Monteiro-Riviere and Inman, 1995; Smith *et al*., 1997a; Tewari-Singh *et al*., 2009). SM was applied to the skin as a vapor using a vapor cup or in liquid form, diluted in an appropriate vehicle, such as dichloromethane. In each of these models, four characteristic stages of SM-induced injury are observed: latency, erythema, vesiculation, and necrosis. Although gross vesiculation characteristic of human exposures is not evident in animal models, microblisters are detectable (Smith *et al*., 1997a). Several *in vitro* models, including biopsied human skin, various forms of engineered human skin equivalent (MatTek, LifeCell, Skin2, others) (Blaha *et al*., 2000; Greenberg *et al*., 2006; Hayden *et al*., 2005, 2009), and isolated human and mouse epidermal keratinocytes (Shakarjian *et al*., 2007; Smith *et al*., 1990), have also been established. These *in vivo* and *in vitro* models display varying degrees of utility in terms of recapitulating the pathophysiological stages of SM-induced injury and repair, understanding molecular mechanisms of toxicity, or screening for the discovery of useful countermeasures.

ULTRASTRUCTURAL STUDIES ON SKIN VESICATION INDUCED BY SM

A number of groups have investigated the effects of SM using the pig skin model, which shares structural and functional similarities to human skin, including density of hair follicles, and the presence of sweat glands (Bartek *et al*., 1972; Monteiro-Riviere and Inman, 1997; Monteiro-Riviere and Riviere, 1996; Sabourin *et al*., 2002). Using the isolated perfused porcine skin flap model, Monteiro-Riviere and Inman (1995, 1997) demonstrated dose-dependent gross blister formation in response to SM (0.04–2 mg in 200 μl). Epidermal changes induced by SM are generally localized to the *stratum basale*, the innermost keratinocyte layer, comprised of self-renewing cells which maintain attachments *via* hemidesmosomes to the basement membrane. Significant mitochondrial destruction and nuclear pyknosis are also observed in response to SM with occasional cytoplasmic vacuoles and lipid inclusion bodies in affected basal keratinocytes. These changes precede signs of vesication. SM-induced cellular changes are focal in nature and coincide with sites of microblister formation. Transmission electron microscopy has revealed separation between the laminin-rich *lamina lucida* and the collagen IV–rich *lamina densa* of the basement membrane. Further definition of the locus of disruption has been provided by immunohistochemical analysis. Antibodies against the hemidesmosomal marker, bullous pemphigoid antigen (BP230), stain the roof of blisters, while type IV collagen is evident at the base of the blister. Whereas most laminin immunoreactivity is localized at the base of the blister, a smaller amount is associated with the *stratum basale*. These findings suggest that the plane of SM-induced epidermal-dermal separation is beneath the hemidesmosomes, within the upper portion of the *lamina lucida* (Monteiro-Riviere and Inman, 1995).

A mouse ear model was initially used by Brinkley *et al.* (1989) to assess SM-induced dermal alterations and then further characterized and developed by Casillas *et al.* (1997). Termed the mouse ear vesicant model (MEVM), liquid SM (0.04–0.64 mg in 5 μl) is applied to the medial surface of one ear, with the other ear serving as the vehicle control. After a characteristic latency period, a dose-dependent increase in the frequency and severity of histopathological markers including edema, epidermal necrosis, and the formation of microblisters on medial and lateral surfaces is observed. Immunostaining and electron microscopy has revealed that epidermal-dermal separation occurs at the level of the upper *lamina lucida*, with focal cleavage into the lower portion of the *stratum basale* as indicated by the detection of hemidesmosomal components on both dermal and epidermal sides of the cleavage site (Monteiro-Riviere *et al*., 1999). This locus of separation is attributed to the unique anatomical complexity of the ear relative to other epidermal sites.

More recently, a newer method for assessing SM-induced skin injury using nude mice grafted with human skin has been described (Greenberg *et al*., 2006). Previous variations of this model used biopsied partial thickness human skin as graft tissue (Papirmeister *et al*., 1984; Vogt *et al*., 1984). In these systems, SM was found to induce edema and microvesication, but only at relatively high doses (Papirmeister *et al*., 1991). The newer method utilizes engineered human skin formed by growing keratinocytes at an air-liquid interface on a fibroblast-populated collagen matrix. Though devoid of adnexal structures, these grafts appear to retain many of the morphological and functional features of normal human skin (Kolodka *et al*., 1998; Smith *et al*., 1997a). Using this model, two distinct stages of SM-induced epithelial damage have been identified by Greenberg *et al.* (2006). The first, a prevesication stage, observed 6-h postexposure, is largely independent of dose. It is characterized by the appearance of discrete clusters of pyknotic

epithelial cells located exclusively in the *stratum basale*. The second stage, observed at 24 h, is dose dependent and involves significantly greater number of cells in the basal and suprabasal layers of the epidermis. Typical of this stage is the formation of microblisters through separation along the basement membrane zone. At moderate doses of SM, a polymorphonuclear cellular infiltrate is also evident in the dermis at 6 h, with focal infiltration into the epidermis at 24 h. Moreover, while the distribution of type VII collagen and laminin-332 immunoreactivity is unchanged during prevesication, a discontinuous pattern is noted after 24 h, a time coincident with microvesication.

In conclusion, application of SM to pig, mouse ear, and engineered human skin graft models reveals similar pathological sequelae and demonstrates a locus of vesication at the level of the lamina lucida of the basement membrane. These changes are consistent with those observed in humans after SM exposure; however, they do not precisely mimic clinical SM exposure, and thus, opportunities remain for improved methods development.

ROLE OF INFLAMMATION AND INFLAMMATORY MEDIATORS IN SM-INDUCED CUTANEOUS INURY

Because the prevesication stage of SM-induced injury is characterized by only mild-to-moderate inflammatory cell infiltration, and more pronounced inflammation is not observed until later in the pathologic process, it has been suggested that inflammation plays a minor role in the primary events mediating cutaneous injury and vesication (Papirmeister *et al*., 1991). In more recent studies, however, it has been argued that inflammation is in fact significant in the early vesication event and that inflammatory cells and mediators may actually contribute directly to the formation of the primary lesion (Cowan and Broomfield, 1993).

An influx of neutrophils into the forearm of skin of human subjects has been observed as early as 30 min after exposure to SM (Warthin and Weller, 1919). Similarly, in the rabbit model, increases in granulocytes and mononuclear cells have been reported within 2 h of SM administration, persisting for 24 h (Dannenberg *et al*., 1985). Leukocyte emigration into the papillary dermis and epidermis has also been reported in mice and in human skin explants after SM exposure (Lindsay and Rice, 1996; Wormser *et al*., 2005). Myeloperoxidase activity, an indicator of neutrophil influx, increases within 9 h, preceding macrophage migration into the skin of hairless mice after CEES exposure (Tewari-Singh *et al*., 2009). In the MEVM, pretreatment of mice with neutrophil-depleting monoclonal antibodies significantly reduces late-stage necrosis induced by SM, suggesting a role for these cells in skin toxicity (Levitt *et al*., 2004). In contrast, while there is evidence of dermal mast cell degranulation in SM-treated tissues and increases in dermal mast cell number after CEES treatment has been reported (Levitt *et al*., 2004; Rikimaru *et al*., 1991; Tewari-Singh *et al*., 2009), treatment of wild-type and mast cell–deficient (W/W^v) mice yielded similar changes in ear weight and extravasation, suggesting a lack of involvement of mast cells in the edematous stage of SM-induced skin pathology (Levitt *et al*., 2004).

Several *in vivo* studies have documented increased expression of proinflammatory cytokines in the skin following SM exposure. Using *in situ* hybridization techniques, increases in interleukin (IL)-1β, IL-8, monocyte chemoattractant protein (MCP)-1, and growth related gene mRNA were noted as early as 2 h after application of liquid SM to rabbit skin (Tsuruta *et al*., 1996). In mouse ear, IL-1β, IL-6, tumor necrosis factor (TNF)-α, and granulocyte monocyte-colony stimulating factor have been reported to be elevated within 6 h (Ricketts *et al*., 2000; Sabourin *et al*., 2000; Wormser *et al*., 2005). Increases in IL-1α protein and IL-1β, TNF-α, macrophage inflammatory protein, MIP-2, and MCP-1 mRNA have also been detected in the dorsal skin of hairless mice after exposure to SM vapor (Ricketts *et al*., 2000; Sabourin *et al*., 2003). Similarly, following vapor cup exposure of weanling pigs to SM, increases in relative mRNA levels of IL-1β, IL-6, IL-8, and TNF-α were noted (Sabourin *et al*., 2002). In cultured human keratinocytes, SM stimulates the release of IL-1β, IL-6, IL-8, and TNF-α at 100–300μM, doses relevant to *in vivo* exposure (Arroyo *et al*., 2000). Cultured skin fibroblasts have also been shown to express IL-6 in response to SM (Arroyo *et al*., 2001). These cytokines are thought to be key to inflammatory cell recruitment and activation at sites of injury, initiating a second phase of soluble mediator release. Cytokine expression is controlled by several signaling molecules, including the transcription factors nuclear factor-kappaB (NF-κB) (Ghosh *et al*., 1998) and activator protein-1 (AP-1) (Zenz *et al*., 2008). NF-κB has been reported to be activated after SM exposure (Atkins *et al*., 2000; Minsavage and Dillman, 2007; Rebholz *et al*., 2008) and both AP-1 and NF-κB after CEES exposure (Pal *et al*., 2009).

Arachidonic acid and its cyclooxygenase and lipooxygenase products are important inflammatory mediators that have also been observed in the skin after SM exposure (Blaha *et al*., 2000; Dachir *et al*., 2004; Lefkowitz and Smith, 2002; Rikimaru *et al*., 1991; Tanaka *et al*., 1997). Several of these mediators increase capillary permeability facilitating the influx of additional inflammatory substances including complement components, kinins, and fibrin into the dermal interstitium (Rikimaru *et al*., 1991). Cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin biosynthesis, has also been identified in the epidermis of SM-treated mice (Nyska *et al*., 2001). Findings that nonsteroidal anti-inflammatory agents (NSAIDs) reduce skin injury suggest that these mediators are important in SM toxicity (Casillas *et al*., 2000). That COX-2 is involved in toxicity is also supported by studies showing that the extent of ear swelling and histopathological signs of lesion severity are markedly reduced in COX-2 null mice treated with SM or in wild-type mice

treated with celecoxib, a COX-2–specific inhibitor (Wormser *et al*., 2004). In contrast, loss of COX-1, the constitutive isoform of the enzyme, has no effect on cutaneous injury induced by SM. Taken together, these studies suggest an involvement of inflammatory mediators in SM cutaneous pathology. However, it remains to be determined which of these are important in the vesication process.

Much has been theorized regarding the potential for SM and its analogs to produce oxidative and electrophilic stress, processes often associated with inflammation, and the role that this plays in toxicity (Dacre and Goldman, 1996; Paromov *et al*., 2007). Under homeostatic conditions, a net reducing environment is maintained in tissues by the presence of glutathione (GSH), which serves as a buffer against cytotoxic electrophiles and reactive oxygen species (ROS). The propensity of SM to react with sulfhydryls is thought to lead to a concentration-dependent depletion of reducing equivalents within cells. Recent findings also suggest that SM and related vesicants can interact with key intracellular reductases to generate mustard-free radicals (Brimfield *et al*., 2009). In addition, inflammatory cells, which infiltrate into the skin in response to SM-induced injury, generate additional ROS that contribute to oxidative stress (Dröge, 2002). This raises the possibility that the toxicity of mustard alkylating agents involves oxidative stress. In this regard, lipid peroxides formed by the reaction of ROS with membrane lipids, have been reported to be elevated systemically after percutaneous intoxication of rats with SM (Vijayaraghavan *et al*., 1991). There is also evidence of increases in lipid peroxidation in A431 cells after HN2 or CEES (Pino *et al*., 2007), in cultured human keratinocytes after SM (Steinritz *et al*., 2009), and on the dorsal skin of hairless mice after CEES exposure (Pal *et al*., 2009). Support for a role of superoxide anion in SM-induced injury comes from findings that administration of superoxide dismutase reduces cutaneous toxicity in a guinea pig model (Eldad *et al*., 1998). Human skin cell lines pretreated with the GSH-depleting agent, buthionine sulfoximine, display enhanced toxicity to SM (Simpson and Lindsay, 2005). Conversely, the cytotoxic actions of SM are reduced in primary keratinocytes pretreated with sulforaphane, a cytoprotective agent which increases intracellular GSH levels by activating the transcription nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Gross *et al*., 2006). Direct evidence for the GSH-depleting action of chloroethyl alkylating agents has been described in lymphocytes treated with CEES (Han *et al*., 2004). It has been suggested that GSH depletion by SM can lead to the production of quinone-generated free radicals in melanocytes (Smith, 1999). Findings that pharmacological inhibition of these quinone radicals protects G361 melanocytes against SM-induced toxicity provides support for this concept (Smith and Lindsay, 2001). Based on these findings, it is tempting to speculate that unregulated oxidative/electrophilic stress contributes significantly to the cutaneous vesicating action SM; however, this remains to be determined.

Evidence is also accumulating that reactive nitrogen species (RNS) including nitric oxide (NO), may also contribute to SM-induced toxicity. NO is generated from L-arginine via the enzyme, nitric oxide synthase (NOS). Three isoforms of the enzyme have been identified, including two constitutive isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), and an inducible NOS isoform, (iNOS). NO is a potent oxidizing agent. It can also react rapidly with superoxide anion generating a more long-lived RNS, peroxynitrite (Virág *et al*., 2002). iNOS has been reported to be upregulated by SM *in vivo*, in the guinea pig skin back model (Nyska *et al*., 2001). Using an *in vitro* scratch wound model, Ishida *et al*. (2008) found that iNOS induction is accompanied by wound closure in human keratinocytes. Moreover, knockdown of iNOS by a small interfering RNA inhibits wound closure. A noncytotoxic concentration of SM (20μM) acted similarly to the iNOS knockdown, inhibiting iNOS induction in the scratched monolayer while also blocking reepithelialization. These data suggest that SM may delay wound healing by blocking iNOS induction. Using 100 and 300μM concentrations, Steinritz *et al*. (2009) demonstrated that SM induces expression of iNOS, as well as eNOS within 6 h in HaCaT cell monolayers. These changes coincided with nitrotyrosine modifications of cellular proteins, which is a biochemical marker for peroxynitrite generation. Further studies are necessary to explore the precise role of RNS in the cutaneous actions of SM.

TARGETS OF SM IN THE SKIN

Aqueous solutions of SM are highly reactive, displaying a half-life of only 24 min in physiological solutions at room temperature (Bartlett and Swain, 1949). Rates of hydrolysis increase with increasing temperature and decreasing chloride ion concentration, resulting in its conversion to thiodiglycol and HCl. The initial reaction involves the formation of a cyclic ethylene sulfonium ion intermediate followed by electrophilic attack on the target molecule (Fig. 3). One mole equivalent of H^+ and Cl^- are liberated in each reaction. Reactive groups on target molecules include sulfhydryls, phosphates, ring nitrogens, and carboxyl groups. Thus, DNA, RNA, proteins, carbohydrates, and lipids are targets for alkylation by SM (Debouzy *et al*., 2002; Mol *et al*., 2008; Noort *et al*., 2002; Papirmeister *et al*., 1991). Because it is a bifunctional alkylating agent, SM can form not only monofunctional adducts but also intra- and intermolecular cross-links.

The most extensively investigated molecular target of SM alkylation is DNA. Its significance was initially established in bacteria where inhibition of DNA replication was noted at doses relevant to *in vivo* mammalian toxicity (Papirmeister *et al*., 1991). Subsequent use of ^{35}S-labeled SM led to the identification of alkylation sites on DNA. Approximately 65% and 17% of the DNA alkylation products are monofunctional adducts on the N^7 of guanine and the N^3 of adenine,

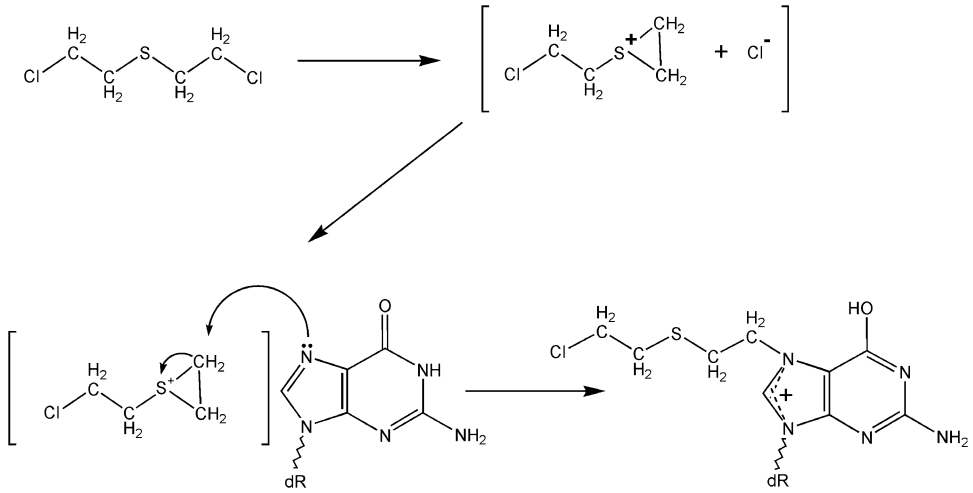

FIG. 3. Mechanism of mustard-induced alkylation. Example of an alkylation reaction between SM and a 2-deoxyguanosine base. One chloroethyl side chain undergoes a first-order (SN1) intramolecular cyclization, releasing chloride and forming a positively charged ethylsulfonium ring. This intermediate reacts rapidly (through carbonium ion or formation of a transition complex intermediate) with nucleophilic groups, such as the N7 of 2-deoxyguanosine. The remaining choloroethyl side chain will then also cyclize and react with another nearby nucleophilic group or with water (Calabresi and Chabner, 1990).

respectively, and approximately 17%, N^7 guanine bifunctional cross-links. In mammalian cells, it has been estimated that 25% of the DNA cross-links occur between complementary DNA strands, while the remainder are intrastrand (Walker, 1971). The fact that monofunctional analogs such as CEES exhibit reduced potency relative to SM suggests that DNA cross-linking is important in toxicity. However, the observation that CEES is a biologically active vesicant suggests that bifunctional cross-links are not required for skin injury.

Evidence suggests that the extent of SM-induced cytotoxicity due to DNA alkylation and cross-linking is influenced not only by the capacity of cells to repair DNA but also by the specific repair mechanism activated. Studies using *Escherichia coli* and mouse embryo fibroblasts have revealed that the nucleotide excision repair (NER) and base excision repair (BER) pathways play contrasting roles in SM toxicity (Matijasevic and Volkert, 2007). Thus, while *E. coli* lacking NER capability exhibit greater sensitivity to the cytotoxic actions of SM than wild-type *E. coli*, their susceptibility to CEES is minimally altered, underscoring the importance of NER in repairing cross-linked DNA. Both *E. coli* and mouse embryo fibroblasts deficient in BER are also less susceptible to SM. In contrast, an intact BER pathway protects cells from the monofunctional methylating agent, methyl methanesulfonate, and pharmacological inhibition of BER increases the sensitivity of skin cells to CEES (Jowsey *et al.*, 2009; Matijasevic and Volkert, 2007). Interestingly, hypothermia protects the skin and keratinocytes, as well as mouse embryo fibroblasts, from the damaging effects of SM, a response markedly reduced in cells deficient in BER (Matijasevic and Volkert, 2007; Mi *et al.*, 2003; Sawyer and Risk, 1999; Sawyer *et al.*, 2002). These findings suggest that BER may play a distinct role in the response to mono- and bifunctional alkylating agents.

MECHANISMS MEDIATING SM-INDUCED CYTOTOXICITY

Poly(ADP-Ribose) Polymerase

DNA alkylation, and the formation of apurinic sites during repair processes, can result in single- and double-strand DNA breaks. This leads to activation of poly(ADP-ribose) polymerase (PARP), a family of nuclear cell signaling enzymes involved in poly-ADP ribosylation of DNA-binding proteins (Shall and de Murcia, 2000). While low levels of PARP activation signal repair, excessive activity can deplete cells of NAD^+ and adenine triphosphate (ATP) resulting in cytotoxicity. Whether this results in apoptosis or necrosis depends on the cell type and other factors (Nicotera and Melino, 2004; Rosenthal *et al.*, 2001). Using a hairless guinea pig model, Kan *et al.* (2003) observed the appearance of apoptotic basal keratinocytes within 6 h of SM exposure, which was followed by necrosis 24 h later, a time when there is significant cell lysis and neutrophilic infiltration. This suggests that in the skin, SM produces a temporal continuum of apoptosis followed by necrosis. The role of PARP in SM-induced cytotoxicity has also been addressed using a transgenic mouse model (Rosenthal *et al.*, 2001). Fibroblasts isolated from mice lacking PARP-1, the most abundant PARP isoform, are much more likely to undergo apoptosis, when compared to wild-type cells, which preferentially undergo necrotic cell death. Unlike results in fibroblasts, immortalized keratinocytes derived from wild-type or PARP-$1^{-/-}$ mice only exhibit apoptosis following SM treatment. These data suggest that while PARP may determine

the mode of SM-induced death in some cell types, apoptosis appears to predominate in mouse keratinocytes. Using the HaCaT human keratinocyte cell line, Kehe *et al.* (2008) demonstrated that SM readily stimulates PARP-1 activity and produces a dose-dependent continuum of cell death from apoptosis to necrosis. Furthermore, treatment of the cells with 3-aminobenzamide, an inhibitor of PARP, causes a discernible inhibition of necrosis. These results contrast to the observations of Rosenthal *et al.* (2001) described above and suggest that PARP may contribute, at least in part, to necrosis in keratinocytes. While reasons for this discrepancy are unclear, cell type, culture conditions, and rates of proliferation may all be factors (Paromov *et al*., 2007). Differences may also be due to the use of immortalized cells which exhibit altered responses to DNA-damaging agents when compared to primary cells (Petit-Frère *et al*., 2000).

SM-Induced Apoptosis in Keratinocytes

Mechanisms underlying SM-induced apoptosis have been explored using primary cultures of human keratinocytes. An increase in the pro-apoptotic protein p53, and a decrease in the anti-apoptotic protein Bcl-2 have been observed in keratinocytes after SM exposure (Rosenthal *et al*., 1998, 2000). These findings are consistent with previous reports in an *in vivo* weanling pig skin model (Smith *et al*., 1997b). Treatment of human keratinocytes with 100–300μM SM also leads to activation of caspase-8, which initiates the Fas-dependent death receptor pathway, as well as caspase-9, which initiates the mitochondrial apoptotic pathway (Rosenthal *et al*., 2003). These two pathways converge to activate caspase-3, the central executioner protease (Zimmermann *et al*., 2001). Transfection of immortalized keratinocytes with a dominant-negative Fas-associated death domain results in a blunted caspase response following SM treatment (Rosenthal *et al*., 2003). Microvesication and tissue injury produced by SM treatment of transfected cells after grafting on to athymic nude mice are also reduced. A recent report has also demonstrated protective effects of inhibitors of caspase-8 or caspase-9 on SM–induced apoptosis in cultured human skin (Mol *et al*., 2009).

Changes in intracellular calcium levels are known to activate the mitochondrial pathway of apoptosis. A key regulator of calcium-dependent proteins is calmodulin. SM causes a time-dependent induction of calmodulin in human keratinocytes (Simbulan-Rosenthal *et al*., 2006). Moreover, depletion of keratinocyte calmodulin using antisense probes attenuates SM-induced activation of caspases and nuclear fragmentation. Bad, a pro-apoptotic Bcl-2 family member present in an inactive phosphorylated form in viable cells, is also activated by SM. Furthermore, cyclosporine A, a selective inhibitor of calcineurin, a Bad phosphatase, inhibits SM-induced apoptosis. These results suggest that calcium-dependent activation of Bad may be a mechanism by which SM induces apoptosis in keratinocytes.

One form of cellular demise common to epithelial cells is detachment-initiated apoptosis, also referred to as anoikis (Chiarugi and Giannoni, 2008; Frisch and Francis, 1994). Keratinocytes rely on signals derived from the surrounding extracellular matrix (ECM) for survival. For instance, matrix proteins, such as laminin-332 interact directly with integrins α3β1 and α6β4, essential components of hemidesmosomes found on the basolateral keratinocyte surface (Schneider *et al*., 2006). Integrin-associated molecules including paxillin, caveolin, Shc, integrin signaling kinase (ILK), focal adhesion kinase (FAK), and various growth factor receptors are thought to transduce anchorage-dependent survival signals (Chiarugi and Giannoni, 2008; Frisch and Screaton, 2001). In the absence of these signals, keratinocytes undergo anoikis via Fas-dependent or mitochondrial pathways of apoptosis (Chiarugi and Giannoni, 2008). Evidence suggests that loss of survival signals contributes to SM-induced epidermal injury and that cell detachment from the basal lamina precedes cytotoxicity. SM can alter the dynamics of cytosolic proteins that control the attachment of cells to the basement membrane. For example, SM has been reported to modify intracellular actin microfilaments and keratin intermediate filaments which are known to be important in maintaining epithelial cell connections with the basal lamina (Hinshaw *et al*., 1999). In fact, modifications of actin microfilament architecture and cell morphology have been observed within 3 h of exposure of human keratinocytes to SM. These changes are associated with a significant decrease in keratinocyte adherence without evidence of cytotoxicity (Hinshaw *et al*., 1999). In addition, SM has been reported to cause rapid decreases in expression of keratins 5 and 14, intermediate filaments present in undifferentiated keratinocytes (Werrlein and Madren-Whalley, 2000). Experiments with animals (Gunhan *et al*., 2004), keratinocyte cultures (Dillman *et al*., 2003; Mol *et al*., 2008), and purified proteins (Hess and FitzGerald, 2007) have all demonstrated that keratins 5 and 14 become alkylated by SM and its analogs, HN2 and CEES. Sites of alkylation may be similar to the dominantly acting mutations in keratins 5 and 14 that are responsible for the human blistering disorder, epidermolysis bullosa simplex, in which basal epidermal cells are also targeted (Fuchs, 1997). The keratin cytoskeleton of basal keratinocytes is linked to the hemidesmosome and, through plectin, makes connections with the β4 cytoplasmic tail of integrin α6β4, thereby strengthening adhesion to the basement membrane via laminin-332 (Giancotti and Tarone, 2003). Alkylation of keratins 5 and 14 by SM may cause aggregation of the intermediate filament network resulting in basal cell separation from the basement membrane.

In addition to targeting epidermal cells, SM can directly alkylate ECM proteins in the skin, a process that can interfere with the ability of basal keratinocytes to maintain vital connections with the basement membrane. This is supported by findings that SM reduces the ability of naive human keratinocytes to deposit laminin at the dermal-epidermal interface (Gentilhomme *et al*., 1998). Moreover, treatment of fibronectin and laminin with SM interferes with adherence of keratinocytes to these matrix proteins, a response blocked

by SM scavengers (Zhang *et al.*, 1995a). Similarly, experiments in our laboratories have demonstrated reduced adherence of keratinocytes to HN2- or CEES-treated laminin-332 (Shakarjian *et al.*, 2008). SM and HN2 also reduces tissue immunreactivity of laminin-332, as well as integrin α6β4 and BP230, two hemidesmosomal components that are critical for keratinocyte adherence (Kan *et al.*, 2003; Smith *et al.*, 1997b, 1998; Werrlein and Madren-Whalley, 2000; Zhang and Monteiro-Riviere, 1997). Interestingly, each of these ECM proteins, like keratins, has been implicated in human blistering disorders involving separation of the epidermis at the dermal-epidermal junction (Pulkkinen *et al.*, 1998; Yancey, 2005). These findings suggest that SM can alter the interaction of basal cells with matrix proteins that are critical for basement membrane adherence.

Role of Matrix-Degrading Proteases in SM-Induced Toxicity

Although the precise sequence of events leading to blister formation after dermal exposure to SM have not been established, an involvement of proteases is considered important in the vesication process (Papirmeister *et al.*, 1985). Proteases have been implicated in several subepidermal blistering diseases in humans including epidermolysis bullosa aquisita (Shimanovich *et al.*, 2004), dermatitis herpetiformis (Airola *et al.*, 1995; Oikarinen *et al.*, 1983), pemphigus vulgaris (Koch *et al.*, 1997), and bullous pemphigoid (Liu *et al.*, 1998). A number of animal models have been utilized to investigate the contribution of proteases to SM toxicity. SM has been reported to increase protease and antiprotease activities within 24 h in rabbit skin explants (Higuchi *et al.*, 1988). Matrix metalloproteinase (MMP) activity has also been detected in culture fluids from SM-treated rabbit skin (Woessner *et al.*, 1990). Plasminogen activator, as well as histamine, increases in an organ culture of full-thickness human skin after treatment with SM or HN2 (Rikimaru *et al.*, 1991). Histamine is a major product of mast cells and a marker of mast cell degranulation. Mast cell degranulation also releases proteases including chymase and tryptase (Prussin and Metcalfe, 2006). Elastase, tryptase, calpain, and gelatinase (MMP-2 and MMP-9) activity is increased within 24 h after SM treatment of mouse ears (Powers *et al.*, 2000). Increases in gelatinases are of particular interest because of their ability to cleave basement membrane components and disrupt the dermal-epidermal junction (Malemud, 2006). Using a weanling pig model, Sabourin *et al.* (2002) found increases in relative mRNA of MMP-9, but not MMP-2, 24 h post-SM treatment. Similarly, our laboratories found that latent gelatinase activity is rapidly increased after SM exposure and remains elevated for at least 7 days in the mouse ear model (Shakarjian *et al.*, 2006). This increase in activity appears to be due to a rise in MMP-9, but not MMP-2, protein levels. Likely sources of gelatinases include not only infiltrating neutrophils but also epidermal keratinocytes and dermal fibroblasts. A recent report by Ries *et al.* (2008) suggests that epidermal-dermal cross talk may contribute to the increase in cutaneous MMP-9 in response to SM.

A critical role of MMP-9 has also been described in a murine model of bullous pemphigoid initiated by administration of antibodies to the hemidesmosomal protein bullous pemphigoid antigen 180 (BP180, also known as collagen XVII) (Liu *et al.*, 1998, 2000, 2005). Mice with a targeted deletion in the gene for MMP-9 fail to develop blistering in this model (Liu *et al.*, 1998). By destroying α1 protease inhibitor (α1PI) and allowing elastase to degrade BP180, MMP-9 plays an essential role in disease pathology in this model (Liu *et al.*, 2000). The importance of neutrophil elastase and MMP-9 in bullous pemphigoid, as well as epidermolysis bullosa acquisita, has been confirmed experimentally using human tissue (Shimanovich *et al.*, 2004). Thus, early elevations of elastase and MMP-9 after cutaneous exposure to SM, combined with their ability to cause dermal-epidermal separation, suggest that these proteases are potential effectors of SM-induced vesication.

Laminin-332, like BP180, is a matrix protein essential for junctional integrity; laminin-332 mutations are associated with severe forms of junctional epidermolysis bullosa (Igoucheva *et al.*, 2007). As described above, laminin-332 immunoreactivity is reduced in SM-induced skin lesions, and evidence indicates that this matrix protein is a target of degradation. Lindsay and Rice (1995) discovered partially degraded laminin in extracts of SM-treated minipig skin, suggesting an involvement of matrix-degrading proteases in blister formation. Subsequently, Chakrabarti *et al.* (1998) isolated a serine protease from SM-treated human epidermal keratinocytes with laminin-cleaving activity. Further investigation revealed that SM and HN2, in contrast to nonvesicating alkylating agents, induce multiple laminin-332 cleaving activities in these cells. The use of class-specific protease inhibitors has demonstrated that these consisted of serine proteases and MMP's (Jin *et al.*, 2004, 2008). Although neutrophil elastase and MMP-2 degrade the gamma2 chain of laminin-332, a lack of species matching has made these results inconclusive (Giannelli *et al.*, 1997; Mydel *et al.*, 2008). Current knowledge indicates that mammalian tolloid metalloproteinase (Veitch *et al.*, 2003), BMP-1 (Amano *et al.*, 2000), and MMP-19 (Sadowski *et al.*, 2005) cleave the gamma2 chain, while MMP-2 and MMP-14 cleave the α3 chain, of laminin-332 (Veitch *et al.*, 2003). Keratinocytes are known sources of these enzymes. The potential role of membrane-type metalloproteinases, such as MMP-14, in vesication induced by SM has been investigated in cultured human skin explants (Mol *et al.*, 2009). Findings that selective inhibitors of TNF-α–converting enzyme (TACE) and furin, proteins essential for the processing of membrane-type MMPs and A Disintegrin and Metalloproteinases into their catalytic forms, protect cultured human skin against injury, suggest an involvement of these proteases in SM toxicity. It is hypothesized that proteases such as MMP-14 and TACE/ADAM17 participate

in the vesication response to SM through their actions on BP180 and laminin-332. Examination of the involvement of these and other proteolytic enzymes should provide further insight into the role of active matrix degradation in the pathogenesis of SM-induced dermal-epidermal separation.

CONTRIBUTION OF MICROARRAYS AND PROTEOMICS TO VESICANT RESEARCH

Several recent studies have applied differential gene expression profiling techniques to ascertain a more genome-wide view of the skin's response to SM. Using the MEVM, Rogers *et al.* (2004) identified 19 genes specifically upregulated in mouse skin 24 h after exposure to SM. Major gene categories activated include apoptosis, transcription factors, cell cycle, oncogenes, and inflammation. An effect of SM dose is observed in terms of the number and the category of genes upregulated. Mouse ears exposed to SM alone or in combination with the antivesicants dimercaprol, octyl homovanillamide, or indomethacin have also been analyzed (Dillman *et al.*, 2006). Genes categories altered by SM include cell cycle and growth, inflammatory and immune response, cytoskeletal and cell adhesion, and signal transduction. A correlation has been reported between the changes in several of these genes and the ability of antivesicants to reduce SM-induced ear edema. Significant changes in gene expression have also been reported up to 7 days following exposure of mice to SM (Gerecke *et al.*, 2009). Gene ontology analysis suggests that the most significantly altered biological processes are immune and inflammatory responses and that the specific genes expressed differ as a function of time after exposure. Transcriptional responses of porcine skin to SM and thermal burns have also been compared (Price *et al.*, 2009; Rogers *et al.*, 2008). Several overlapping biological functions have been identified between the two types of skin injury, suggesting the potential for applying pharmacological agents known to be effective against burns to SM-injured skin. Clearly, there is potential for biomarker identification and high throughput analytical tools to be developed for further microarray studies. The significance of individual genes and pathways will become more apparent as PCR and protein expression validation methods are applied. However, at the present time, microarray studies have yet to provide a precise understanding of the complex mechanisms involved in the vesication process.

To elucidate molecular changes that occur after SM exposure, Mol *et al.* (2008) treated human keratinocytes with ^{14}C-SM and followed adducted proteins by two-dimensional electrophoresis. Nineteen modified proteins, the majority, variants of keratins 5, 6, 14, 16, and 17, have been identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Two high–molecular weight cross-linked aggregates of keratins were also identified. Three additional proteins identified as SM alkylation targets were actin, stratifin, an adaptor protein that can interact with BP180 (Li *et al.*, 2007), and galectin-7, a protein involved in cell adhesion and proliferation (Klíma *et al.*, 2008). Differential protein expression analysis using two-dimensional electrophoresis demonstrated significant changes in patterns between 8- and 42-h posttreatment. A number of protein species unique to SM-treated keratinocytes were detected, the majority being fragments of keratins 14, 16, and 17. Findings that the appearance of these fragments is mitigated by preincubating keratinocytes with caspase inhibitors suggests an active apoptotic process. Two additional proteins were identified as a phosphorylated variant of HSP27 and ribosomal protein P0. HSP27 may be involved in activation of the p38 MAP kinase pathway and has been implicated in pemphigus vulgaris (Berkowitz *et al.*, 2008). Ribosomal protein P0, essential for proper protein synthesis, was found in its inactive, unphosphorylated form, suggesting that SM impairs ribosomal function. Thus, the proteomic approach has shown value by illuminating known and novel targets of SM that may be important in the cell's response to this agent.

CONCLUSIONS AND FUTURE DIRECTIONS

SM remains a significant threat to the military and civilian populations. Despite extensive research aimed at understanding the molecular mechanisms of SM action, the pathways leading from exposure to vesication remains unclear, and effective medical countermeasures have yet to be developed. Human cutaneous exposures have defined four stages of injury from SM: latency, erythema, vesiculation, and necrosis. Although SM is a relatively nonselective alkylating agent, keratinocytes of the *stratum basale* appear to be the most sensitive to its cytotoxic actions, and blistering involves the detachment of these cells from the supporting basal lamina of the basement membrane of the epidermal-dermal junction. The reason for the sensitivity of this epidermal layer remains unresolved. It has been established that basal keratinocytes differ from neighboring epidermal cells in their self-renewing nature and greater capacity for proliferation. A different spectrum of transcription factors also predominate in these cells which direct expression of a unique protein repertoire including cytoskeletal proteins, such as keratins 5 and 14 (as compared to the keratins 1 and 10 found in differentiating keratinocytes), and the protein constituents of the hemidesmosome, a structure unique to basal cells, and includes BP180, BP230, and the α6 and ß4 integrin chains (Koster, 2009; Xu *et al.*, 2004). It is also possible that basal keratinocytes respond to DNA damage differently than their more differentiated counterparts in terms of capacity for repair. Residing on the basement membrane, basal keratinocytes may also be more susceptible to ECM-degrading enzymes. Further examination of the unique characteristics of basal keratinocytes will likely provide clues for the selectivity of SM.

A scheme summarizing current understanding of the potential mechanisms contributing to the dermal toxicity of

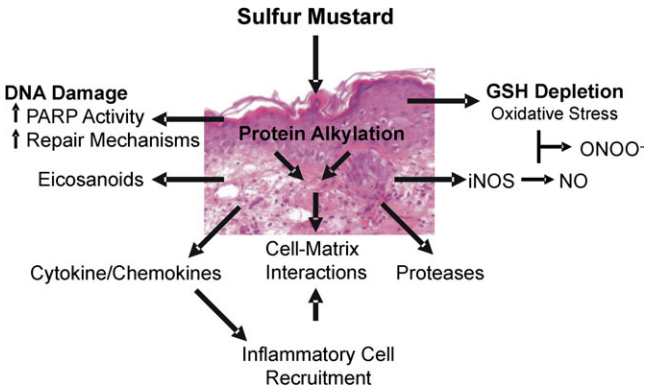

FIG. 4. Mechanisms of cutaneous injury induced by SM. SM alkylates DNA, proteins, and small molecules in the skin. DNA damage can lead directly to cell death or activate PARP and other repair enzymes; cells may be rescued or progress to death by apoptosis or necrosis. Protein alkylation of cytoskeletal, hemidesmosomal, and ECM proteins can impair anchoring of basal keratinocytes to the basement membrane, leading to cell detachment and anoikis. Alkylation of intracellular GSH increases tissue susceptibility to oxidative stress. SM exposure also results in increased expression of a number of proinflammatory proteins in the skin, including iNOS, matrix-degrading proteases, COX-2, cytokines, and chemokines. NO generated by iNOS can combine with other oxidants generating more long-lived and cytotoxic species such as peroxynitrite. Proteases can destroy epidermal-basement membrane connections. Prostaglandins generated via COX-2 increase membrane permeability and together with cytokines and chemokines participate in recruitment of circulating leukocytes, thereby amplifying inflammation and tissue injury. Weakening of the connections between basal keratinocytes and their basement membrane connections through these various mechanisms will result in epidermal-dermal separation and frank vesication.

SM is depicted in Figure 4. Because of its ability to alkylate a wide variety of small and large molecules, SM is likely to have multiple targets, including DNA, protein, and small molecules that contribute to its vesicating action. DNA damage can lead directly to cytotoxicity, or it may trigger other changes resulting in cellular dysfunction, cell death or tissue repair. For example, whereas overactivation of PARP following exposure to high concentrations of SM can trigger apoptotic or necrotic cell death, depending on the extent of ATP depletion, milder activation can trigger DNA repair mechanisms that may prove to be either beneficial or detrimental to cell survival. Although less well studied, intracellular and extracellular protein alkylation by SM might also contribute to vesication. Current data suggest that alkylation of cytoskeletal, cell anchoring–related, and ECM proteins may weaken keratinocyte-basement membrane interactions, ultimately leading to epidermal-dermal separation and anoikis. However, one cannot exclude the possibility that less abundant protein targets are also significant to the pathogenic process. Finally, intracellular GSH likely exemplifies a small molecule target and the depletion of this important reducing agent following exposure to SM can initiate oxidative stress leading to lipid peroxidation and other oxidative cellular damage.

A variety of proinflammatory proteins are induced following dermal exposure to SM including enzymes that produce RNS and ROS, chemokines, cytokines, and proteases. These most likely act in concert to promote tissue injury and amplify the inflammatory response. At present, the precise role of these different inflammatory mediators and cells in the toxicity of SM is unknown. Evidence suggests that disruption of cell-matrix interactions is key to the process, and it remains to be determined if inflammatory cells participate in this pathogenic step.

Studies on the mechanism of action of SM have suggested several potential therapeutic approaches. For example, the observation that SM induces inflammation and the release of cytotoxic/proinflammatory mediators suggests that anti-inflammatory drugs may be efficacious in mitigating the cytotoxic actions of SM. In this regard, both administrations of NSAIDs including indomethacin as well as COX-2 inhibitors have proven to be efficacious against SM-induced injury in a variety of *in vivo* models (Babin *et al*., 2000; Casillas *et al*., 2000; Yourick *et al*., 1995; Zhang *et al*., 1995b). Neurogenic inflammation is also thought to be an important component of tissue damage induced by SM. Nonmyelinated sensory C-fibers function as dual sensory afferents, transmitting sensory information to the central nervous system and also releasing nociceptive and inflammatory neuropeptides, such as substance P (Szallasi and Blumberg, 1999). Many of these actions are mediated by transient receptor potential V1 (TRPV1) in the skin. Capsaicin, a constituent of hot pepper, has been reported to exert its actions via TRPV1, and several capsaicin analogs significantly reduce SM-induced inflammation in the mouse ear model (Casillas *et al*., 2000; Sabourin *et al*., 2003). Targeting specific proinflammatory cytokines and chemokines may also prove clinically efficacious in treating SM poisoning. This is supported by findings that antibodies to TNF-α inhibit SM damage in the MEVM (Wormser *et al*., 2005).

A variety of enzyme inhibitors have also been tested as SM therapeutics. For example, recent studies have shown that PARP inhibitors abrogate SM toxicity in the MEVM (Smith, 2009). Similarly, inhibition of caspases that mediate apoptosis have been shown to suppress the toxic and vesicating actions of SM (Mol *et al*., 2009; Rosenthal *et al*., 2003). Moreover, blocking the activity of matrix-degrading enzymes reduces injury from SM exposure in human skin organ cultures (Mol *et al*., 2009).

Another interesting approach to treating SM toxicity is the use of iodine. Wormser and colleagues recently isolated a peptide factor from iodine-treated burns that may partially mediate iodine's actions (Brodsky *et al*., 2008). The peptide, an 11 amino acid fragment of histone H2A, reduces the severity of SM-induced ear swelling in the MEVM, and transfection of a plasmid encoding this factor into HaCaT cells significantly increases their resistance to the cytotoxic effects of SM.

Vesicants such as SM remain a significant health threat. Thus, it is imperative to identify targets for therapeutic intervention in the skin, as well as the cornea and lung. This can best be accomplished by further investigation into the mechanism of cytotoxic actions of SM. Of particular importance is elucidating the roles of oxidative and nitrosative

stress in toxicity and the specific contribution of inflammatory mediators. Leads derived from microarray analyses and proteomics are likely to be useful in identifying new targets for countermeasure development.

FUNDING

National Institutes of Health (AR055073, CA100994, CA132624, CA093798, ES004738, GM034310, EY009056, AI051214, AI084137, and ES005022); National Institutes of Health Counter ACT Program through the National Institute of Arthritis and Musculoskeletal and Skin Diseases (Award No. U54AR055073). This work's contents are solely the responsibility of the authors and do not necessarily represent the official views of the federal government.

REFERENCES

Airola, K., Vaalamo, M., Reunala, T., and Saarialho-Kere, U. K. (1995). Enhanced expression of interstitial collagenase, stromelysin-1, and urokinas1 plasminogen activator in lesions of dermatitis herpetiformis. *J. Invest. Dermatol.* **105,** 184–189.

Amano, S., Scott, I. C., Takahara, K., Koch, M., Champliaud, M. F., Gerecke, D. R., Keene, D. R., Hudson, D. L., Nishiyama, T., Lee, S., *et al.* (2000). Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin 5 gamma 2 chain. *J. Biol. Chem.* **275,** 22728–22735.

Arroyo, C. M., Broomfield, C. A., and Hackley, B. E., Jr. (2001). The role of interleukin-6 (IL-6) in human sulfur mustard (HD) toxicology. *Int. J. Toxicol.* **20,** 281–296.

Arroyo, C. M., Schafer, R. J., Kurt, E. M., Broomfield, C. A., and Carmichael, A. J. (2000). Response of normal human keratinocytes to sulfur mustard: Cytokine release. *J. Appl. Toxicol.* **20**(Suppl. 1), S63–S72.

Atkins, K. B., Lodhi, I. J., Hurley, L. L., and Hinshaw, D. B. (2000). *N*-acetylcysteine and endothelial cell injury by sulfur mustard. *J. Appl. Toxicol.* **20**(Suppl. 1), S125–S128.

Babin, M. C., Ricketts, K., Skvorak, J. P., Gazaway, M., Mitcheltree, L. W., and Casillas, R. P. (2000). Systemic administration of candidate antivesicants to protect against topically applied sulfur mustard in the mouse ear vesicant model (MEVM). *J. Appl. Toxicol.* **20**(Suppl. 1), S141–S144.

Balali-Mood, M., and Hefazi, M. (2005). The pharmacology, toxicology, and medical treatment of sulphur mustard poisoning. *Fundam. Clin. Pharmacol.* **19,** 297–315.

Bartek, M. J., LaBudde, J. A., and Maibach, H. I. (1972). Skin permeability in vivo: Comparison in rat, rabbit, pig and man. *J. Invest. Dermatol.* **58,** 114–123.

Bartlett, P. D., and Swain, C. G. (1949). Kinetics of hydrolysis and displacement reactions of β,β'-dichlorodiethyl sulfide (mustard gas) and of β-chloro-β'-hydroxydiethyl sulfide (mustard chlorohydrin). *J. Am. Chem. Soc.* **71,** 1406–1415.

Berkowitz, P., Diaz, L., Hall, R., and Rubenstein, D. (2008). Induction of p38MAPK and HSP27 phosphorylation in pemphigus patient skin. *J. Invest. Dermatol.* **128,** 738–740.

Blaha, M., Bowers, W., Jr, Kohl, J., DuBose, D., Walker, J., Alkhyyat, A., and Wong, G. (2000). Effects of CEES on inflammatory mediators, heat shock protein 70A, histology and ultrastructure in two skin models. *J. Appl. Toxicol.* **20**(Suppl. 1), S101–S108.

Brimfield, A., Mancebo, A., Mason, R., Jiang, J., Siraki, A., and Novak, M. (2009). Free radical production from the interaction of 2-chloroethyl vesicants (mustard gas) with pyridine nucleotide-driven flavoprotein electron transport systems. *Toxicol. Appl. Pharmacol.* **234,** 128–134.

Brinkley, F. B., Mershon, M. M., Yaverbaum, S., Doxzon, B. F., and Wade, J. V. (1989). The mouse ear model as an in vivo bioassay for the assessment of topical mustard (HD) injury. In *1989 Medical Defense Bioscience Review,* 15–17 August 1989, Aberdeen Proving Ground, MD, pp. 595–602.

Brodsky, B., Erlanger-Rosengarten, A., Proscura, E., Shapira, E., and Wormser, U. (2008). From topical antidote against skin irritants to a novel counter-irritating and anti-inflammatory peptide. *Toxicol. Appl. Pharmacol.* **229,** 342–350.

Calabresi, P., and Chabner, B. (1990). Antineoplastic Agents. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (A. Gilman, T. Rall, A. Nies, and P. Taylor, Eds.), pp. 1209–1219. McGraw-Hill, New York.

Casillas, R. P., Kiser, R. C., Truxall, J. A., Singer, A. W., Shumaker, S. M., Niemuth, N. A., Ricketts, K. M., Mitcheltree, L. W., Castrejon, L. R., and Blank, J. A. (2000). Therapeutic approaches to dermatotoxicity by sulfur mustard. I. Modulaton of sulfur mustard-induced cutaneous injury in the mouse ear vesicant model. *J. Appl. Toxicol.* **20**(Suppl. 1), S145–S151.

Casillas, R. P., Mitcheltree, L. W., and Stemler, F. W. (1997). The mouse ear model of cutaneous sulfur mustard injury. *Toxicol. Methods* **7,** 381–397.

Chakrabarti, A. K., Ray, P., Broomfield, C. A., and Ray, R. (1998). Purification and characterization of protease activated by sulfur mustard in normal human epidermal keratinocytes. *Biochem. Pharmacol.* **56,** 467–472.

Chiarugi, P., and Giannoni, E. (2008). Anoikis: A necessary death program for anchorage-dependent cells. *Biochem. Pharmacol.* **76,** 1352–1374.

Chiesman, W. (1944). Diagnosis and treatment of lesions due to vesicants. *Br. Med. J.* **2,** 109–112.

Cowan, F. M., and Broomfield, C. A. (1993). Putative roles of inflammation in the dermatopathology of sulfur mustard. *Cell Biol. Toxicol.* **9,** 201–213.

Dachir, S., Fishbeine, E., Meshulam, Y., Sahar, R., Chapman, S., Amir, A., and Kadar, T. (2004). Amelioration of sulfur mustard skin injury following a topical treatment with a mixture of a steroid and a NSAID. *J. Appl. Toxicol.* **24,** 107–113.

Dacre, J. C., and Goldman, M. (1996). Toxicology and pharmacology of the chemical warfare agent sulfur mustard. *Pharmacol. Rev.* **48,** 289–326.

Dannenberg, A. M., Jr, Pula, P. J., Liu, L. H., Harada, S., Tanaka, F., Vogt, R. F., Jr., Kajiki, A., and Higuchi, K. (1985). Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced in vivo by sulfur mustard. I. Quantitative histopathology; PMN, basophil, and mononuclear cell survival; and unbound (serum) protein content. *Am. J. Pathol.* **121,** 15–27.

Debouzy, J. C., Aous, S., Dabouis, V., Neveux, Y., and Gentilhomme, E. (2002). Phospholipid matrix as a target for sulfur mustard (HD): NMR study in model membrane systems. *Cell Biol. Toxicol.* **18,** 397–408.

Despretz, M. (1822). Des composes triples du chlore. *Ann. Chem. Phys.* **21,** 438.

Dillman, J. F., III, Hege, A. I., Phillips, C. S., Orzolek, L. D., Sylvester, A. J., Bossone, C., Henemyre-Harris, C., Kiser, R. C., Choi, Y. W., Schlager, J. J., *et al.* (2006). Microarray analysis of mouse ear tissue exposed to bis-(2-chloroethyl) sulfide: Gene expression profiles correlate with treatment efficacy and an established clinical endpoint. *J. Pharmacol. Exp. Ther.* **317,** 76–87.

Dillman, J. F., III, McGary, K. L., and Schlager, J. J. (2003). Sulfur mustard induces the formation of keratin aggregates in human epidermal keratinocytes. *Toxicol. Appl. Pharmacol.* **193,** 228–236.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiol. Rev.* **82,** 47–95.

Easton, D. F., Peto, J., and Doll, R. (1988). Cancers of the respiratory tract in mustard gas workers. *Br. J. Ind. Med.* **45,** 652–659.

Eldad, A., Ben Meir, P., Breiterman, S., Chaouat, M., Shafran, A., and Ben-Bassat, H. (1998). Superoxide dismutase (SOD) for mustard gas burns. *Burns* **24,** 114–119.

Fox, M., and Scott, D. (1980). The genetic toxicology of nitrogen and sulphur mustard. *Mutat. Res.* **75,** 131–168.

Frisch, S., and Screaton, R. (2001). Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13,** 555–562.

Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124,** 619–626.

Fuchs, E. (1997). Keith R. Porter Lecture, 1996. Of mice and men: Genetic disorders of the cytoskeleton. *Mol. Biol. Cell* **8,** 189–203.

Gentilhomme, E., Reano, A., Pradel, D., Bergier, J., Schmitt, D., and Neveux, Y. (1998). In vitro dermal intoxication by bis(chloroethyl)sulfide. Effect on secondary epidermization. *Cell Biol. Toxicol.* **14,** 1–11.

Gerecke, D., Chen, M., Isukapalli, S., Gordon, M., Chang, Y., Tong, W., Androulakis, I., and Georgopoulos, P. (2009). Differential gene expression profiling of mouse skin after sulfur mustard exposure: Extended time response and inhibitor effect. *Toxicol. Appl. Pharmacol.* **234,** 156–165.

Ghanei, M., Panahi, Y., Mojtahedzadeh, M., Khalili, A. R. H., and Aslani, J. (2006). Effect of gamma interferon on lung function of mustard gas exposed patients, after 15 years. *Pulm. Pharmacol. Ther.* **19,** 148–153.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-κB AND REL PROTEINS: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.

Giancotti, F. G., and Tarone, G. (2003). Positional control of cell fate through joint integrin/receptor protein kinase signaling. *Annu. Rev. Cell Dev. Biol.* **19,** 173–206.

Giannelli, G., Falk-Marzillier, J. O. S., Stetler-Stevenson, W. G., and Quaranta, V. (1997). Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* **277,** 225–228.

Ginzler, A. M., and Davis, M. I. J. (1943). The pathology of mustard burns of human skin. U.S. Army Medical Research Laboratory, Edgewood Arsenal, MD.

Goldenberg, G., Vanstone, C., and Bihler, I. (1971). Transport of nitrogen mustard on the transport-carrier for choline in L5178Y lymphoblasts. *Science* **172,** 1148–1149.

Goldenberg, G. J., and Begleiter, A. (1980). Membrane transport of alkylating agents. *Pharmacol. Ther.* **8,** 237–274.

Greenberg, S., Kamath, P., Petrali, J., Hamilton, T., Garfield, J., and Garlick, J. A. (2006). Characterization of the initial response of engineered human skin to sulfur mustard. *Toxicol. Sci.* **90,** 549–557.

Gross, C. L., Nealley, E. W., Nipwoda, M. T., and Smith, W. J. (2006). Pretreatment of human epidermal keratinocytes with D,L-sulforaphane protects against sulfur mustard cytotoxicity. *Cutan. Ocul. Toxicol.* **25,** 155–163.

Guignabert, C., Taysse, L., Calvet, J. H., Planus, E., Delamanche, S., Galiacy, S., and d'Ortho, M. P. (2005). Effect of doxycycline on sulfur mustard-induced respiratory lesions in guinea pigs. *Am. J. Physiol. Lung Cell Mol. Physiol.* **289,** L67–L74.

Gunhan, O., Kurt, B., Karayilanoglu, T., Kenar, L., and Celasun, B. (2004). Morphological and immunohistochemical changes on rat skin exposed to nitrogen mustard. *Mil. Med.* **169,** 7–10.

Guthrie, F. (1860). Uber einige Derivate der Kohlenwaserstoffe CnHn. *Ann. Chem. Pharm.* **113,** 266–288.

Han, S., Espinoza, L. A., Liao, H., Boulares, A. H., and Smulson, M. E. (2004). Protection by antioxidants against toxicity and apoptosis induced by the sulphur mustard analog 2-chloroethylethyl sulphide (CEES) in Jurkat T cells and normal human lymphocytes. *Br. J. Pharmacol.* **141,** 795–802.

Hayden, P., Petrali, J., Stolper, G., Hamilton, T., Jackson, G. J., Wertz, P., Ito, S., Smith, W., and Klausner, M. (2009). Microvesicating effects of sulfur mustard on an in vitro human skin model. *Toxicol. In Vitro* **23,** 1396–1405.

Hayden, P. J., Petrali, J. P., Hamilton, T. A., Kubilus, J., Smith, W. J., and Klausner, M. (2005). Development of a full thickness *in vitro* human skin equivalent (Epiderm-FT) for sulfur mustard research. *U.S. Army Medical Research Institute of Chemical Defense*. Aberdeen Proving Ground, MD. Presented at Society of Investigative Dermatology, St. Louis, MO, 4–7 May, 2005.

Henriques, F. C., Jr, Moritz, A. R., Breyfogle, H. S., and Patterson, L. A. (1943). In *The Mechanism of Cutaneous Injury by Mustard Gas. An Experimental Study Using Mustard Prepared with Radioactive Sulfur*. Division 9, National Defense Research Committee of the Office of Scientific Research and Development, Washington, DC.

Hess, J. F., and FitzGerald, P. G. (2007). Treatment of keratin intermediate filaments with sulfur mustard analogs. *Biochem. Biophys. Res. Commun.* **359,** 616–621.

Higuchi, K., Kajiki, A., Nakamura, M., Harada, S., Pula, P. J., Scott, A. L., and Dannenberg, A. M., Jr. (1988). Proteases released in organ culture by acute dermal inflammatory lesions produced in vivo in rabbit skin by sulfur mustard: Hydrolysis of synthetic peptide substrates for trypsin-like and chymotrypsin-like enzymes. *Inflammation* **12,** 311–334.

Hinshaw, D. B., Lodhi, I. J., Hurley, L. L., Atkins, K. B., and Dabrowska, M. I. (1999). Activation of poly [ADP-ribose] polymerase in endothelial cells and keratinocytes: Role in an in vitro model of sulfur mustard-mediated vesication. *Toxicol. Appl. Pharmacol.* **156,** 17–29.

Igoucheva, O., Kelly, A., Uitto, J., and Alexeev, V. (2007). Protein therapeutics for junctional epidermolysis bullosa: Incorporation of recombinant beta3 chain into laminin 332 in beta3−/− keratinocytes in vitro. *J. Invest. Dermatol.* **128,** 1476–1486.

Inada, S., Hiragun, K., and Seo, K. (1978). Multiple Bowens disease observed in former workers of a poison gas factory in Japan with special reference to mustard gas exposure. *J. Dermatol.* **5,** 49–60.

Ishida, H., Ray, R., and Ray, P. (2008). Sulfur mustard downregulates iNOS expression to inhibit wound healing in a human keratinocyte model. *J. Dermatol Sci.* 207–216.

Isidore, M., Castagna, M., Steele, K., Gordon, R., and Nambiar, M. (2007). A dorsal model for cutaneous vesicant injury by 2-chloroethyl ethyl sulfide using C57BL/6 mice. *Cutan. Ocul. Toxicol.* **26,** 265–276.

Jin, X., Ray, R., Leng, Y., and Ray, P. (2008). Molecular determination of laminin-5 degradation: A biomarker for mustard gas exposure diagnosis and its mechanism of action. *Exp. Dermatol.* **17,** 49–56.

Jin, X., Ray, R., Xu, G., and Ray, P. (2004). Studies on mustard-stimulated proteases and inhibitors in human epidermal keratinocytes (HEK): Development of antivesicant drugs. In *Proceedings of the U.S. Army Medical Defense Bioscience Review,* 16–21 May 2004, Hunt Valley, MD, pp. 1–10.

Jowsey, P., Williams, F., and Blain, P. (2009). DNA damage, signalling and repair after exposure of cells to the sulphur mustard analogue 2-chloroethyl ethyl sulphide. *Toxicology* **257,** 105–112.

Kan, R. K., Pleva, C. M., Hamilton, T. A., Anderson, D. R., and Petrali, J. P. (2003). Sulfur mustard-induced apoptosis in hairless guinea pig skin. *Toxicol. Pathol.* **31,** 185–190.

Kehe, K., Raithel, K., Kreppel, H., Jochum, M., Worek, F., and Thiermann, H. (2008). Inhibition of poly(ADP-ribose) polymerase (PARP) influences the mode of sulfur mustard (SM)-induced cell death in HaCaT cells. *Arch. Toxicol.* **82,** 461–470.

Kehe, K., and Szinicz, L. (2005). Medical aspects of sulphur mustard poisoning. *Toxicology* **214,** 198–209.

Khateri, S., Ghanei, M., Keshavarz, S., Soroush, M., and Haines, D. (2003). Incidence of lung, eye, and skin lesions as late complications in 34,000 Iranians with wartime exposure to mustart agent. *J. Occup. Environ. Med.* **45,** 1136–1143.

Klíma, J., Lacina, L., Dvořánková, B., Herrmann, D., Carnwath, J. W., Niemann, H., Kaltner, H., André, S., Motlík, J., Gabius, H. J., *et al.* (2008). Differential regulation of galectin expression/reactivity during wound healing in porcine skin and in cultures of epidermal cells with functional impact on migration. *Physiol. Res.* Advance Access published on June 2009.

Koch, P. J., Mahoney, M. G., Ishikawa, H., Pulkkinen, L., Uitto, J., Shultz, L., Murphy, G. F., Whitaker-Menezes, D., and Stanley, J. R. (1997). Targeted

disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J. Cell. Biol.* **137,** 1091–1102.

Kolodka, T. M., Garlick, J. A., and Taichman, L. B. (1998). Evidence for keratinocyte stem cells in vitro: Long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 4356–4361.

Koster, M. (2009). Making an epidermis. *Ann. N. Y. Acad. Sci.* **1170,** 7–10.

Lefkowitz, L. J., and Smith, W. J. (2002). Sulfur mustard-induced arachidonic acid release is mediated by phospholipase D in human keratinocytes. *Biochem. Biophys. Res. Commun.* **295,** 1062–1067.

Levitt, J. M., Vavra, A. K., Laurent, C. J., and Sweeney, J. F. (2004). The presence of polymorphonuclear leukocytes (PMN) affect the severity of sulfur mustard injury in the mouse ear model. In *Proceedings of the U.S. Army Medical Defense Bioscience Review,* 16–21 May 2004, Hunt Valley, MD, pp. 1–11.

Li, Y., Lin, X., Kilani, R., Jones, J., and Ghahary, A. (2007). 14-3-3 Sigma isoform interacts with the cytoplasmic domain of the transmembrane BP180 in keratinocytes. *J. Cell. Physiol.* **212,** 675–681.

Lindsay, C. D., Hambrook, J. L., Brown, R. F., Platt, J. C., Knight, R., and Rice, P. (2004). Examination of changes in connective tissue macromolecular components of large white pig skin following application of Lewisite vapour. *J. Appl. Toxicol.* **24,** 37–46.

Lindsay, C. D., and Rice, P. (1995). Changes in connective tissue macromolecular components of Yucatan mini-pig skin following application of sulphur mustard vapour. *Hum. Exp. Toxicol.* **14,** 341–348.

Lindsay, C. D., and Rice, P. (1996). Assessment of the biochemical effects of percutaneous exposure of sulphur mustard in an in vitro human skin system. *Hum. Exp. Toxicol.* **15,** 237–244.

Liu, Z., Li, N., Diaz, L. A., Shipley, M., Senior, R. M., and Werb, Z. (2005). Synergy between a plasminogen cascade and MMP-9 in autoimmune disease. *J. Clin. Invest.* **115,** 879–887.

Liu, Z., Shipley, J. M., Vu, T. H., Zhou, X., Diaz, L. A., Werb, Z., and Senior, R. M. (1998). Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid. *J. Exp. Med.* **188,** 475–482.

Liu, Z., Zhou, X., Shapiro, S. D., Shipley, J. M., Twining, S. S., Diaz, L. A., Senior, R. M., and Werb, Z. (2000). The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo. *Cell* **102,** 647–655.

Malemud, C. J. (2006). Matrix metalloproteinases (MMPs) in health and disease: An overview. *Front. Biosci.* **11,** 1696–1701.

Matijasevic, Z., and Volkert, M. R. (2007). Base excision repair sensitizes cells to sulfur mustard and chloroethyl ethyl sulfide. *DNA Repair* **6,** 733–741.

McClintock, S. D., Till, G. O., Smith, M. G., and Ward, P. A. (2002). Protection from half-mustard-gas-induced acute lung injury in the rat. *J. Appl. Toxicol.* **22,** 257–262.

Mehzad, M. (1988). Pathological study of skin lesions in chemical casualties. In *First International Medical Congress on Chemical Warfare Agents in Iran,* 13–16 June 1988, Mashhad, Iran.

Meyer, V. (1886). Ueber thiodiglykolverbindungen. *Ber. d. Dtsch. Chem. Ges.* **19,** 3259–3266.

Mi, L., Gong, W., Nelson, P., Martin, L., and Sawyer, T. W. (2003). Hypothermia reduces sulphur mustard toxicity. *Toxicol. Appl. Pharmacol.* **193,** 73–83.

Millard, C. B., Bongiovanni, R., and Broomfield, C. A. (1997). Cutaneous exposure to bis-(2-chloroethyl)sulfide results in neutrophil infiltration and increased solubility of 180,000 Mr subepidermal collagens. *Biochem. Pharmacol.* **53,** 1405–1412.

Minsavage, G. D., and Dillman, J. F. I. (2007). Bifunctional alkylating agent-induced p53 and nonclassical nuclear factor-kappa B (NF-{kappa}B) responses and cell death are altered by caffeic acid phenethyl ester (CAPE): A potential role for antioxidant/electrophilic response element (ARE/EpRE) signaling. *J. Pharmacol. Exp. Ther.* **321,** 202–212.

Mol, M. A., van den Berg, R. M., and Benschop, H. P. (2008). Proteomic assessment of sulfur mustard-induced protein adducts and other protein modifications in human epidermal keratinocytes. *Toxicol. Appl. Pharmacol.* **230,** 97–108.

Mol, M. A., van den Berg, R. M., and Benschop, H. P. (2009). Involvement of caspases and transmembrane metalloproteases in sulphur mustard-induced microvesication in adult human skin in organ culture: Directions for therapy. *Toxicology* **258,** 39–46.

Monteiro-Riviere, N. A., and Inman, A. O. (1995). Indirect immunohistochemistry and immunoelectron microscopy distribution of eight epidermal-dermal junction epitopes in the pig and in isolated perfused skin treated with bis(2-chloroethyl) sulfide. *Toxicol. Pathol.* **23,** 313–325.

Monteiro-Riviere, N. A., and Inman, A. O. (1997). Ultrastructural characterization of sulfur mustard-induced vesication in isolated perfused porcine skin. *Microsc. Res. Tech.* **37,** 229–241.

Monteiro-Riviere, N. A., Inman, A. O., Babin, M. C., and Casillas, R. P. (1999). Immunohistochemical characterization of the basement membrane epitopes in bis (2-chloroethyl) sulfide-induced toxicity in mouse ear skin. *J. Appl. Toxicol.* **19,** 313–328.

Monteiro-Riviere, N. A., and Riviere, J. E. (1996). The pig as a model for cutaneous pharmacology and toxicology research. *Cutan. Pharmacol. Toxicol. Res.* **38,** 425–458.

Mustargen-Merck. (2002). *Physicians' Desk Reference*. Thomson Medical Economics, Montvale, NJ.

Mydel, P., Shipley, J. M., Adair-Kirk, T. L., Kelley, D. G., Broekelmann, T. J., Mecham, R. P., and Senior, R. M. (2008). Neutrophil elastase cleaves laminin-332 (laminin-5) generating peptides that are chemotactic for neutrophils. *J. Biol. Chem.* **283,** 9513–9522.

Nicotera, P., and Melino, G. (2004). Regulation of the apoptosis-necrosis switch. *Oncogene* **23,** 2757–2765.

Niemann, A. (1860). Ueber die einwirkung des brauen chlorschwefels auf elaygas. *Ann. d. Chem. u. Pharm.* **113,** 288–292.

Noort, D., Benschop, H. P., and Black, R. M. (2002). Biomonitoring of exposure to chemical warfare agents: A review. *Toxicol. Appl. Pharmacol.* **184,** 116–126.

Nyska, A., Lomnitski, L., Maronpot, R., Moomaw, C., Brodsky, B., Sintov, A., and Wormser, U. (2001). Effects of iodine on inducible nitric oxide synthase and cyclooxygenase-2 expression in sulfur mustard-induced skin. *Arch. Toxicol.* **74,** 768–774.

Oikarinen, A. I., Zone, J. J., Ahmed, A. R., Kiistala, U., and Uitto, J. (1983). Demonstration of collagenase and elastase activities in the blister fluids from bullous skin diseases. Comparison between dermatitis herpetiformis and bullous pemphigoid. *J. Invest. Dermatol.* **81,** 261–266.

Pal, A., Tewari-Singh, N., Gu, M., Agarwal, C., Huang, J., Day, B. J., White, C. W., and Agarwal, R. (2009). Sulfur mustard analog induces oxidative stress and activates signaling cascades in the skin of SKH-1 hairless mice. *Free Radic. Biol. Med.* **47,** 1640–1651.

Papirmeister, B., Feister, A. J., Robinson, S. I., and Ford, R. D. (1991). In *Medical Defense against Mustard Gas: Toxic Mechanisms and Pharmacological Implications*. CRC Press, Boca Raton, FL.

Papirmeister, B., Gross, C. L., Meier, H. L., Petrali, J. P., and Johnson, J. B. (1985). Molecular basis for mustard-induced vesication. *Fundam. Appl. Toxicol.* **5,** S134–S149.

Papirmeister, B., Gross, C. L., and Petrali, J. P. (1984). Pathology produced by sulfur mustard in human skin grafts on athymic nude mice: 2. Ultastructural changes. *J. Toxicol. Cutaneous Ocul. Toxicol.* **3,** 393–408.

Pappenheimer, A. M. (1926). Medical aspects of gas warfare. In *The Medical Department of the United States Army in the World War* (C. Lynch, Ed.). U.S. Government Printing Office, Washington, DC.

Paromov, V., Suntres, Z., Smith, M., and Stone, W. L. (2007). Sulfur mustard toxicity following dermal exposure: Role of oxidative stress and antioxidant therapy. *J. Burns Wounds* **7,** e7.

Pechura C. M., and Rall, D. P., Eds. (1993). *Veterans At Risk: The Health Effects of Mustard Gas and Lewisite*. National Academy Press, Washington, DC.

Petit-Frère, C., Capulas, E., Lyon, D., Norbury, C., Lowe, J., Clingen, P., Riballo, E., Green, M., and Arlett, C. (2000). Apoptosis and cytokine release induced by ionizing or ultraviolet B radiation in primary and immortalized human keratinocytes. *Carcinogenesis* 1087–1095.

Pino, M. A., Hardej, D., and Billack, B. (2007). Mechlorethamine toxicity in skin cells is inhibited by butylated hydroxyanisole. *The Toxicologist*. 96, 33, 25–27 March 2007.

Powers, J. C., Kam, C. M., Ricketts, K. M., and Casillas, R. P. (2000). Cutaneous protease activity in the mouse ear vesicant model. *J. Appl. Toxicol.* **20**(Suppl. 1), S177–S182.

Price, J., Rogers, J., McDougal, J., Shaw, M., Reid, F., and Graham, J. (2009). Transcriptional changes in porcine skin at 7 days following sulfur mustard and thermal burn injury. *Cutan. Ocul. Toxicol.* **28,** 129–140.

Prussin, C., and Metcalfe, D. D. (2006). IgE, mast cells, basophils, and eosinophils. *J. Allergy Clin. Immunol.* **117,** S450–S456.

Pulkkinen, L., Rouan, F., Bruckner-Tuderman, L., Wallerstein, R., Garzon, M., Brown, T., Smith, L., Carter, W., and Uitto, J. (1998). Novel ITGB4 mutations in lethal and nonlethal variants of epidermolysis bullosa with pyloric atresia: Missense versus nonsense. *Am. J. Hum. Genet.* **63,** 1376–1387.

Rebholz, B., Kehe, K., Ruzicka, T., and Rupec, R. (2008). Role of NF-kappaB/RelA and MAPK pathways in keratinocytes in response to sulfur mustard. *J. Invest. Dermatol.* **128,** 1626–1632.

Renshaw, B. (1946). Mechanisms in production of cutaneous injuries by sulfur and nitrogen mustards. In *Chemical Warfare Agents and Related Chemical Problems* U.S. Office of Scientific Research and Development, National Defense Research Commmittee Washington, DC, 479–518

Requena, L., Requena, C., Sanchez, M., Jaqueti, G., Aguilar, A., Sanchez-Yus, E., and Hernandez-Moro, B. (1988). Chemical warfare. Cutaneous lesions from mustard gas. *J. Am. Acad. Dermatol.* **19,** 529–536.

Ricketts, K. M., Santai, C. T., France, J. A., Graziosi, A. M., Doyel, T. D., Gazaway, M. Y., and Casillas, R. P. (2000). Inflammatory cytokine response in sulfur mustard-exposed mouse skin. *J. Appl. Toxicol.* **20**(Suppl. 1), S73–S76.

Ries, C., Popp, T., Egea, V., Kehe, K., and Jochum, M. (2008). Matrix metalloproteinase-9 expression and release from skin fibroblasts interacting with keratinocytes: Upregulation in response to sulphur mustard. *Toxicology* **263,** 26–31.

Rikimaru, T., Nakamura, M., Yano, T., Beck, G., Habicht, G. S., Rennie, L. L., Widra, M., Hirshman, C. A., Boulay, M. G., Spannhake, E. W., *et al.* (1991). Mediators, initiating the inflammatory response, released in organ culture by full-thickness human skin explants exposed to the irritant, sulfur mustard. *J. Invest. Dermatol.* **96,** 888–897.

Rogers, J., Choi, Y., Kiser, R., Babin, M., Casillas, R., Schlager, J., and Sabourin, C. (2004). Microarray analysis of gene expression in murine skin exposed to sulfur mustard. *J. Biochem. Mol. Toxicol.* **18,** 289–299.

Rogers, J., McDougal, J., Price, J., Reid, F., and Graham, J. (2008). Transcriptional responses associated with sulfur mustard and thermal burns in porcine skin. *Cutan. Ocul. Toxicol.* **27,** 135–160.

Rosenthal, D. S., Simbulan-Rosenthal, C. M., Iyer, S., Smith, W. J., Ray, R., and Smulson, M. E. (2000). Calmodulin, poly(ADP-ribose) polymerase and p53 are targets for modulating the effects of sulfur mustard. *J. Appl. Toxicol.* **20**(Suppl. 1), S43–S49.

Rosenthal, D. S., Simbulan-Rosenthal, C. M., Iyer, S., Spoonde, A., Smith, W., Ray, R., and Smulson, M. E. (1998). Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca^{2+}-calmodulin and caspase-dependent pathway. *J. Invest. Dermatol.* **111,** 64–71.

Rosenthal, D. S., Simbulan-Rosenthal, C. M., Liu, W. F., Velena, A., Anderson, D., Benton, B., Wang, Z. Q., Smith, W., Ray, R., and Smulson, M. E. (2001). PARP determines the mode of cell death in skin fibroblasts, but not keratinocytes, exposed to sulfur mustard. *J. Invest. Dermatol.* **117,** 1566–1573.

Rosenthal, D. S., Velena, A., Chou, F. P., Schlegel, R., Ray, R., Benton, B., Anderson, D., Smith, W. J., and Simbulan-Rosenthal, C. M. (2003). Expression of dominant-negative Fas-associated death domain blocks human keratinocyte apoptosis and vesication induced by sulfur mustard. *J. Biol. Chem.* **278,** 8531–8540.

Sabourin, C. L. K., Danne, M. M., Buxton, K. L., Casillas, R. P., and Schlager, J. J. (2002). Cytokine, chemokine, and matrix metalloproteinase response after sulfur mustard injury to weanling pig skin. *J. Biochem. Mol. Toxicol.* **16,** 263–272.

Sabourin, C. L. K., Danne, M. M., Buxton, K. L., Rogers, J. V., Niemuth, N. A., Blank, J. A., Babin, M. C., and Casillas, R. P. (2003). Modulation of sulfur mustard-induced inflammation and gene expression by olvanil in the hairless mouse vesicant model. *J. Toxicol. Cutaneous Ocul. Toxicol.* **22,** 125–136.

Sabourin, C. L. K., Petrali, J. P., and Casillas, R. P. (2000). Alterations in inflammatory cytokine gene expression in sulfur mustard-exposed mouse skin. *J. Biochem. Mol. Toxicol.* **14,** 291–302.

Sadowski, T., Dietrich, S., Koschinsky, F., Ludwig, A., Proksch, E., Titz, B., and Sedlacek, R. (2005). Matrix metalloproteinase 19 processes the laminin 5 gamma 2 chain and induces epithelial cell migration. *Cell. Mol. Life Sci.* **62,** 870–880.

Sawyer, T. W., Nelson, P., Hill, I., Conley, J. D., Blohm, K., Davidson, C., and Sawyer, T. W. (2002). Therapeutic effects of cooling swine skin exposed to sulfur mustard. *Mil. Med.* **167,** 939–943.

Sawyer, T. W., and Risk, D. (1999). Effect of lowered temperature on the toxicity of sulphur mustard in vitro and in vivo. *Toxicology* **134,** 27–37.

Schneider, H., Mühle, C., and Pacho, F. (2006). Biological function of laminin-5 and pathogenic impact of its deficiency. *Eur. J. Cell Biol.* **86,** 701–717.

Shakarjian, M. P., Ajibade, D. V., Black, A. T., Chang, Y.-C., Gordon, M. K., Heck, D. E., Laskin, J. D., Riley, D. J., and Gerecke, D. R. (2007). Nitrogen mustard - induced alterations of matrix metalloproteinase-9 in mouse keratinocytes. *Toxicologist* **96,** 158.

Shakarjian, M. P., Bhatt, P., Gordon, M. K., Chang, Y. C., Casbohm, S. L., Rudge, T. L., Kiser, R. C., Sabourin, C. L. K., Casillas, R. P., Ohman-Strickland, P., *et al.* (2006). Preferential expression of matrix metalloproteinase-9 in mouse skin after sulfur mustard exposure. *J. Appl. Toxicol.* **26,** 239–246.

Shakarjian, M. P., Vetrano, A. M., Gray, J. P., DeSantis, A. S., Riley, D. J., Laskin, J. D., Chang, Y.-C., Gerecke, D. R., and Heck, D. E. (2008). Cell adhesion and migration changes in response to alkylation of laminin-332. In *Proceedings of the U.S. Army Medical Defense Bioscience Review*. 1–6 June 2008, Hunt Valley, MD, p. A138.

Shall, S., and de Murcia, G. (2000). Poly(ADP-ribose) polymerase-1: What have we learned from the deficient mouse model? *Mutat. Res.* **460,** 1–15.

Sharma, M., Vijayaraghavan, R., and Ganesan, K. (2008). Comparison of toxicity of selected mustard agents by percutaneous and subcutaneous routes. *Indian J. Exp. Biol.* **46,** 822–830.

Shimanovich, I., Mihai, S., Oostingh, G. J., Ilenchuk, T. T., Brocker, E. B., Opdenakker, G., Zillikens, D., and Sitaru, C. (2004). Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. *J. Pathol.* **204,** 519–527.

Sidell, F. R., and Hurst, C. G. (1997). Long-term health effects of nerve agents and mustard. In *Textbook of Military Medicine—Medical Aspects of Chemical and Biological Warfare* (R. Zaitchuk and R. F. Bellamy, Eds.), pp. 229–246. Office of the Surgeon General, Department of the Army, Washington, DC.

Simbulan-Rosenthal, C. M., Ray, R., Benton, B., Soeda, E., Daher, A., Anderson, D., Smith, W. J., and Rosenthal, D. S. (2006). Calmodulin

mediates sulfur mustard toxicity in human keratinocytes. *Toxicology* **227,** 21–35.

Simpson, R., and Lindsay, C. D. (2005). Effect of sulphur mustard on human skin cell lines with differential agent sensitivity. *J. Appl. Toxicol.* **25,** 115–128.

Smith, C. N., and Lindsay, C. D. (2001). Kojic acid reduces the cytotoxic effects of sulfur mustard on cultures containing human melanoma cells in vitro. *J. Appl. Toxicol.* **21,** 435–440.

Smith, K. J. (1999). The prevention and treatment of cutaneous injury secondary to chemical warfare agents. Application of these finding to other dermatologic conditions and wound healing. *Dermatol. Clin.* **17,** 41–60.

Smith, K. J., Casillas, R., Graham, J., Skelton, H. G., Stemler, F., and Hackley, B. E., Jr. (1997a). Histopathologic features seen with different animal models following cutaneous sulfur mustard exposure. *J. Dermatol. Sci.* **14,** 126–135.

Smith, K. J., Graham, J. S., Hamilton, T. A., Skelton, H. G., Petrali, J. P., and Hurst, C. G. (1997b). Immunohistochemical studies of basement membrane proteins and proliferation and apoptosis markers in sulfur mustard induced cutaneous lesions in weanling pigs. *J. Dermatol. Sci.* **15,** 173–182.

Smith, K. J., Graham, J. S., Moeller, R. B., Okerberg, C. V., Skelton, H., and Hurst, C. G. (1995). Histopathologic features seen in sulfur mustard induced cutaneous lesions in hairless guinea pigs. *J. Cutan. Pathol.* **22,** 260–268.

Smith, K. J., Smith, W. J., Hamilton, T., Skelton, H. G., Graham, J. S., Okerberg, C., Moeller, R., and Hackley, B. E., Jr. (1998). Histopathologic and immunohistochemical features in human skin after exposure to nitrogen and sulfur mustard. *Am. J. Dermatopathol.* **20,** 22–28.

Smith, W. J. (2009). Therapeutic options to treat sulfur mustard poisoning—The road ahead. *Toxicology* **263,** 70–73.

Smith, W. J., Gross, C. L., Chan, P., and Meier, H. L. (1990). The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. *Cell Biol. Toxicol.* **6,** 285–291.

Steinritz, D., Elischer, A., Balszuweit, F., Gonder, S., Heinrich, A., Bloch, W., Thiermann, H., and Kehe, K. (2009). Sulphur mustard induces time- and concentration-dependent regulation of NO-synthesizing enzymes. *Toxicol. Lett.* **188,** 263–269.

Szallasi, A., and Blumberg, P. M. (1999). Vanilloid (Capsaicin) receptors and mechanisms. *Pharmacol. Rev.* **51,** 159–212.

Tanaka, F., Dannenberg, A. M., Jr, Higuchi, K., Nakamura, M., Pula, P. J., Hugli, T. E., Discipio, R. G., and Kreutzer, D. L. (1997). Chemotactic factors released in culture by intact developing and healing skin lesions produced in rabbits by the irritant sulfur mustard. *Inflammation* **21,** 251–267.

Tewari-Singh, N., Rana, S., Gu, M., Pal, A., Orlicky, D. J., White, C. W., and Agarwal, R. (2009). Inflammatory biomarkers of sulfur mustard analog 2-chloroethyl ethyl sulfide-induced skin injury in SKH-1 hairless mice. *Toxicol. Sci.* **108,** 194–206.

Tsuruta, J., Sugisaki, K., Dannenberg, A. M., Jr, Yoshimura, T., Abe, Y., and Mounts, P. (1996). The cytokines NAP-1 (IL-8), MCP-1, IL-1 beta, and GRO in rabbit inflammatory skin lesions produced by the chemical irritant sulfur mustard. *Inflammation* **20,** 293–318.

Veitch, D. P., Nokelainen, P., McGowan, K. A., Nguyen, T. T., Nguyen, N. E., Stephenson, R., Pappano, W. N., Keene, D. R., Spong, S. M., Greenspan, D. S., *et al.* (2003). Mammalian tolloid metalloproteinase, and not matrix metalloprotease 2 or membrane type 1 metalloprotease, processes laminin-5 in keratinocytes and skin. *J. Biol. Chem.* **278,** 15661–15668.

Vijayaraghavan, R., Sugendran, K., Pant, S. C., Husain, K., and Malhotra, R. C. (1991). Dermal intoxication of mice with bis(2-chloroethyl)sulphide and the protective effect of flavonoids. *Toxicology* **69,** 35–42.

Virág, L., Szabó, E., Bakondi, E., Bai, P., Gergely, P., Hunyadi, J., and Szabó, C. (2002). Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the skin. *Exp. Dermatol.* **11,** 189–202.

Vogt, R. F., Jr., Dannenberg, A. M., Jr, Schofield, B. H., Hynes, N. A., and Papirmeister, B. (1984). Pathogenesis of skin lesions caused by sulfur mustard. *Fundam. Appl. Toxicol.* **4,** S71–S83.

Wada, S., Miyanishi, M., Nishimoto, Y., Kambe, S., and Miller, R. W. (1968). Mustard gas as a cause of respiratory neoplasia in man. *Lancet* **1,** 1161–1163.

Walker, I. G. (1971). Intrastrand bifunctional alkylation of DNA in mammalian cells treated with mustard gas. *Can. J. Biochem.* **49,** 332–336.

Warthin, A. S., and Weller, D. V. (1919). In *The Medical Aspects of Mustard Gas Poisoning*. C.V. Mosby Co., St Louis, MO.

Weiss, A., and Weiss, B. (1975). Carcinogenesis due to mustard gas exposure in man, important sign for therapy with alkylating agents. *Dtsch. Med. Wochenschr.* **100,** 919–923.

Werrlein, R. J., and Madren-Whalley, J. S. (2000). Effects of sulfur mustard on the basal cell adhesion complex. *J. Appl. Toxicol.* **20**(Suppl. 1), S115–S123.

Woessner, J. F., Jr., Dannenberg, A. M., Jr., Pula, P. J., Selzer, M. G., Ruppert, C. L., Higuchi, K., Kajiki, A., Nakamura, M., Dahms, N. M., Kerr, J. S., *et al.* (1990). Extracellular collagenase, proteoglycanase and products of their activity, released in organ culture by intact dermal inflammatory lesions produced by sulfur mustard. *J. Invest. Dermatol.* **95,** 717–726.

Wormser, U., Brodsky, B., Proscura, E., Foley, J. F., Jones, T., and Nyska, A. (2005). Involvement of tumor necrosis factor-alpha in sulfur mustard-induced skin lesion; effect of topical iodine. *Arch. Toxicol.* **79,** 660–670.

Wormser, U., Langenbach, R., Peddada, S., Sintov, A., Brodsky, B., and Nyska, A. (2004). Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice. *Toxicol. Appl. Pharmacol.* **200,** 40–47.

Xu, X., Kawachi, Y., Nakamura, Y., Sakurai, H., Hirota, A., Banno, T., Takahashi, T., Roop, D., and Otsuka, F. (2004). Yin-yang 1 negatively regulates the differentiation-specific transcription of mouse loricrin gene in undifferentiated keratinocytes. *J. Invest. Dermatol.* 1120–1126.

Yanagida, J., Hozawa, S., Ishioka, S., Maeda, H., Takahashi, K., Oyama, T., Takaishi, M., Hakoda, M., Akiyama, M., and Yamakido, M. (1988). Somatic mutation in peripheral lymphocytes of former workers at the Okunojima poison gas factory. *Jpn. J. Cancer Res.* **79,** 1276–1283.

Yancey, K. B. (2005). The pathophysiology of autoimmune blistering diseases. *J. Clin. Invest.* **115,** 825–828.

Yourick, J. J., Dawson, J. S., and Mitcheltree, L. W. (1995). Reduction of erythema in hairless guinea pigs after cutaneous sulfur mustard vapor exposure by pretreatment with niacinamide, promethazine and indomethacin. *J. Appl. Toxicol.* **15,** 133–138.

Zenz, R., Eferl, R., Scheinecker, C., Redlich, K., Smolen, J., Schonthaler, H., Kenner, L., Tschachler, E., and Wagner, E. (2008). Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Res. Ther.* **10,** 201–211.

Zhang, Z., and Monteiro-Riviere, N. A. (1997). Comparison of integrins in human skin, pig skin, and perfused skin: An in vitro skin toxicology model. *J. Appl. Toxicol.* **17,** 247–253.

Zhang, Z., Peters, B. P., and Monteiro-Riviere, N. A. (1995a). Assessment of sulfur mustard interaction with basement membrane components. *Cell Biol. Toxicol.* **11,** 89–101.

Zhang, Z., Riviere, J. E., and Monteiro-Riviere, N. A. (1995b). Evaluation of protective effects of sodium thiosulfate, cysteine, niacinamide and indomethacin on sulfur mustard-treated isolated perfused porcine skin. *Chem. Biol. Interact.* **96,** 249–262.

Zimmermann, K. C., Bonzon, C., and Green, D. R. (2001). The machinery of programmed cell death. *Pharmacol. Ther.* **92,** 57–70.