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Modified immunoslotblot assay to detect hemi and sulfur mustard DNA adducts

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ABSTRACT

Sulfur mustard (SM) is an old chemical warfare agent causing blisters (vesicant). Skin toxicity is thought to be partly caused by SM induced DNA damage. SM and the hemi mustard 2-chloroethyl ethyl sulfide (CEES) are bi- and monofunctional DNA alkylating agents, respectively. Both chemicals react especially with N_7 guanine. The most abundant adducts are 7-hydroxyethylthioethylguanine for SM (61%) and 7-ethyl thioethylguanine for CEES. Thus, DNA alkylation should serve as a biomarker of SM exposure. A specific monoclonal antibody (2F8) was previously developed to detect SM and CEES adducts at N_7 position by means of immunoslotblot (ISB) technique (van der Schans et al. (2004) [16]). Nitrogen mustards (HN-1, HN-2, HN-3) are alkylating agents with structural similarities, which can form DNA adducts with N_7 guanine. The aim of the presented work was to modify the van der Schans protocol for use in a field laboratory and to test the cross reactivity of the 2F8 antibody against nitrogen mustards. Briefly, human keratinocytes were exposed to SM and CEES (0–300 μM, 60 min) or HN-1, HN-2, HN-3 (120 min). After exposure, cells were scraped and DNA was isolated and normalized. 1 μg DNA was transferred to a nitrocellulose membrane using a slotblot technique. After incubation with 2F8 antibody, the DNA adducts were visualized with chromogen staining (3,3′-diaminobenzidine (DAB), SeramunGrün). Blots were photographed and signal intensity was quantified. In general, DAB was superior to SeramunGrün stain. A staining was seen from 30 nM to 300 μM of SM or CEES, respectively. However, statistically significant DNA adducts were detected after CEES and SM exposure above 30 μM which is below the vesicant threshold. No signal was observed after HN-1, HN-2, HN-3 exposure. The total hands-on time to complete the assay was about 36 h. Further studies are necessary to validate SM or CEES exposure in blister roofs of exposed patients.

1. Introduction

Sulfur mustard was used as a chemical warfare agent since 1917. Due to its strong vesicating properties on skin, it is classified as a blistering agent [1]. Sulfur mustard, either as liquid or gas, is a lipophilic compound that can easily penetrate in the body. Exposure to sulfur mustard does not cause immediate signs of intoxication. A symptomless or -free period of several hours was frequently reported. The first symptoms are observed on the eyes, skin and in the bronchial tract [2]. SM is classified as a vesicant agent because of its strong capability to induce blistering on the skin. The epidermis detaches from the dermis. Keratinocyte cell death is detectable within the stratum basale [3]. Eye symptoms involve redness of the eyes, swollen eyelids, tearing, pain, sensitivity to light and severe blepharospasm [4]. Inhalation of sulfur mustard vapor damages epithelial surfaces of the laryngeal and bronchial mucosa. Acute symptoms and signs are hoarseness, sneezing, lacrimation, hacking cough and in severe cases pseudomembrane formation in the bronchial tract [5,6]. Nevertheless, exposure to sulfur mustard results often in systemic poisoning with nausea, vomiting and leucopenia at a later stage. The clinical picture has been extensively reviewed in the past [6–8].

The continuous threat of poisoning has prompted a huge research program in the last century. Despite this effort, the pathophysiology of sulfur mustard poisoning is still not completely understood. It is widely accepted that the reaction of SM with the DNA is one if not the main pathophysiological event that triggers the cell death cascade. SM is a highly reactive bifunctional alkylating agent. It hydrolyzes in water to form an ethylene sulfonium ion intermediate. A highly reactive carbenium ion is formed,

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which is highly reactive. Cell constituents like DNA, RNA, proteins, and other molecules are rapidly alkylated [1]. SM reactions with DNA lead to mono- or bifunctional adducts with guanine and adenosine. Reactions with thymine or uracil were not detected under physiological conditions. SM preferentially reacts with ring nitrogen atoms of the DNA in decreasing order of reactivity with N_7 of guanine, N_3 of adenine, N_1 of adenine and N_1 of cytosine [9]. Thus, 61% of all alkylations occur at the N_7 of guanine [10]. Nearly 17% of total alkylations produce intra- or interstrand-crosslinks [11]. The genotoxic properties of SM have been recognized to be the most important trigger of cell death [12]. The strong cytotoxic properties of SM against rapidly dividing cells were used against cancer cells. Several mustards were synthesized and screened for their usefulness as chemotherapeutic drugs. Initially, several N-mustards were introduced in anti-cancer therapy and used as starting substance for other chemotherapeutic drugs [13]. Nitrogen mustards (HN-1, HN-2, HN-3) were also synthesized as chemical warfare agents.

Retrospective diagnosis and verification of SM poisoning may be difficult. The portion of SM that does not penetrate very rapidly through the skin evaporates quickly from the surface [14]. Off-gassing of SM vapor has been detected up to 24 h after exposure in pig models, and a reservoir of agent has been noted in the upper levels of the stratum corneum of pigs [15]. Rapid methods for reliable and fast detection of SM poisoning are needed to initiate appropriate clinical treatment. Sophisticated analytical methods may be useful to confirm a possible SM intoxication with high evidence. Recently, an immunochemical method was described to detect the monofunctional SM adduct at the N_7 position of guanine [15]. A substantial portion of SM that has penetrated the skin is fixed at the DNA mainly within the epidermis. It is hypothesized that extracted DNA of blister roofs or affected skin contain sufficient amounts of reacted SM to be detectable with the above mentioned antibody. The aim of this study was to modify the immunoslotblot technique and to test the cross-reactivity of the 2F8 antibody against SM, semi mustard (CEES) and nitrogen mustards (HN-1, HN-2, HN-3).

2. Materials and methods

2.1. Chemicals

SM was obtained from TNO, Rijswijk, The Netherlands. All other chemicals used were reagent grade products obtained from Sigma (Deisenhofen, Germany). SeramunGrün was from Seramun Diagnostica, Heidesee, Germany.

The tested sulfur or nitrogen mustards are listed in Table 1.

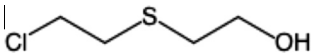
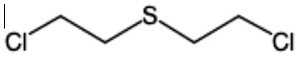
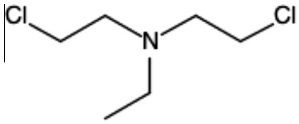
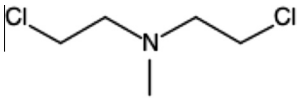
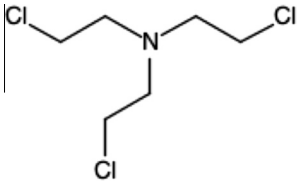
2.2. Cell cultures

HaCaT cells, a spontaneously transformed human keratinocyte cell line [16,17], was kindly provided by Prof. Dr. N. Fusenig (German Cancer Research Center, Heidelberg, FRG). HaCaT cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F12 (DMEM/F12) supplemented with 2.45 mmol/l glutamine, 10% fetal calf serum (Life Technologies, Eggenstein, FRG) using standard cell culture flasks (75 cm^2, Falcon, Heidelberg, FRG). Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Cell doubling was 22 h. Cells were seeded with 10^5 cells/cm^2 and experiments were performed with subconfluent cultures on the 1st day after seeding.

2.3. Exposure protocol

HaCaT cells were washed with PBS and incubated at 25 °C for 60 min with various concentrations of SM or CEES, or for 120 min with various concentrations of HN-1, HN-2, or HN-3 in Modified Eagle's Medium (MEM). SM was dissolved first in ethanol. The final concentration of the solvent in the exposure fluids was below 1%. Control cultures were exposed to the same amount of ethanol. The obtained stock solutions of SM, CEES and nitrogen mustards in MEM were used immediately after dilution to minimize hydrolysis. After exposure, HaCaT cells were washed with PBS and subjected to analysis. Every experiment was repeated three times and each immunoslotblot consists of three replicate experiments ($n = 9$).

Table 1
Structures of tested chemical compounds.

chemical compound	structure
CEES CAS-Nr. 693-07-2	
sulfur mustard CAS-Nr. 505-60-2	
HN-1 (Chlormethine) CAS-Nr. 51-75-2	
HN-2 (Mustine, Mustargen) CAS-Nr. 107-99-3	
HN-3 (Trimustine) CAS-Nr. 817-09-4	

2.4. Cell isolation

HaCaT cells were treated with 2 ml of 0.05%-Trypsin–EDTA (GIBCO® invitrogen) and incubated at 37 °C until trypsination was complete. The HaCaT cells were diluted with 13 ml of Dulbecco's Modified Eagle Medium (DMEM) containing FKS and transferred to 50 ml tubes, from which 100 µl samples were taken for cell count (CASY Modell TTC, Innovatis Systems, Reutlingen). The tubes were centrifuged for 5 min at 1350 g (ROTINA35 R, Hettich Zentrifugen, Tübingen) and the supernatant removed. The remaining HaCaT cells were re-suspended in DMEM to a concentration of 5–10 × 10^6 cells/ml. From each sample 200 µl were transferred to 1.5 ml sample tubes and centrifuged for 5 s at 16.1 g (Centrifuge 5415 R, Eppendorf, Hamburg). The supernatant medium was removed and the sample tubes were vortexed.

2.5. DNA isolation

DNA was isolated from the HaCaT cells using the Puregene Core Kit A (QIAGEN, Hilden, Germany). Cell lysis solution (300 µl) was added to the isolated cells and vortexed for 10 s. 1.5 µl RNase A (50 mg/ml) was added, the mixture was incubated for 15 min at 37 °C and cooled at 0 °C for 1 min. Protein precipitation solution (100 µl) was added to the mixture, vortexed for 20 s and centrifuged for 5 min at 16.1 g. The supernatant was carefully decanted from theformed pellet into isopropanol (300 µl). The obtained solution was mixed by mild shaking, and centrifuged for 5 min at 16.1 g. The supernatant was decanted from the formed pellet, ethanol (300 µl, 70%) was added to the pellet and again centrifuged for 5 min at 16.1 g. After decantation of the supernatant the samples were dried for 15 min at room temperature, DNA Hydration Solution (50 µl) was added, the mixture was incubated for 1 h at