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Pro-inflammatory cytokines among individuals with skin findings long-term after sulfur mustard exposure: Sardasht-Iran Cohort Study

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ABSTRACT

Sulfur mustard (SM) is a potent alkylation vesicant warfare chemical agent which causes severe damage to the interface organs, skin, lungs and eyes. The most common chronic skin lesions are mustard scars, xerosis, eczema, seborrhoeic dermatitis, cherry angioma and hyperpigmentation. This study is part of Sardasht-Iran Cohort Study (SICS) which was performed to compare the serum levels of inflammatory cytokines in SM-exposed individuals (n = 372) with long-term relevant skin findings versus unexposed controls (n = 128). Serum levels of pro-inflammatory cytokines including IL-1α, IL-1β, IL-1Ra, IL-6, and TNF (tumor necrosis factor) were titrated using ELISA method, 79.9% (n = 290) of the exposed group and 60.5% (n = 98) of the control group showed skin findings. In the exposed group, 52.1% (n = 180) had only skin findings (OSFE) and in the control group, 32% (n = 41) had no problem (NC, normal). Median serum levels of cytokines IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α in the OSFE group were: 1.077, 1.745, 25.640, 0.602 and 12.786 pg/ml, respectively. These values in normal controls were 1.889, 1.896, 32.190, 1.022 and 23.786 pg/ml, respectively which are higher than the corresponding values in the OSFE group, the differences were statistically significant only for IL-1α and TNF-α. This may be due to a damage incurred upon precursors of cytokine producing cells or failure of their functions, increase in suppressive mediators or other mechanisms which are not well known. More studies are needed in molecular dimensions of the immune and cytokine responses in the SM-exposed patients.

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1. Introduction

Sulfur mustard (SM) is a potent alkylation vesicant warfare chemical agent which had been used by Iraqi forces against Iranian army and civilians in Sardasht-Iran [1–4]. SM causes severe damages to the interface organs including the skin, lungs and eyes. The skin damages include acute and chronic lesions [5–8] with aberrant healing process leading to blister formation, ulceration and secondary infections. Upon contact with skin, about 80% of the SM evaporates and 20% is absorbed and accumulates in the skin tissues and distributes via circulation throughout the body which increases the possibility of damage to several organs [1,2]. SM-induced injuries are usually chronic and non-fatal [2]. The most common chronic skin lesions are mustard scars, xerosis, eczema, seborrhoeic dermatitis, cherry angioma and hyperpigmentation [4]. Despite various studies on long-term clinical manifestations of skin toxicity in SM individuals, there are a few reports on molecular nature of SM-induced skin disorders.

The skin is the largest organ of the body and a mechanical and also affects the immune responses through what is known as “skin associated lymphoid tissues (SALT)” [9]. The skin is composed of many cells including keratinocytes, Langerhans cells, melanocytes, dermal dendritic cells and migratory lymphocytes which are important immune response factors [10]. Keratinocytes respond to both external stimuli and endogenous trigger factors and secrete a large amount of cytokines, chemokines, peptides and growth factors. In the presence of a
robust stimulus, keratinocyte derived cytokines may enter the circulation and subsequently induce systemic effects [11].

Cytokines are soluble mediators which act as messengers of the immune system. They are critical in fundamental processes such as host defenses, cell cycle control, inflammation, cancer, fibrosis, wound healing and angiogenesis [12]. The predominant role of cytokines is to mediate inflammatory and immune responses. Cytokines determine the switching of the immune response from a pro- to anti-inflammatory state. While proinflammatory cytokines regulate the body’s response to the external or internal dangers, anti-inflammatory cytokines are capable of restoring tissue homeostasis by suppressing inflammatory processes [11]. Cytokines play a major role in both acute and chronic inflammatory processes, probably including those produced by SM.

This study is part of Sardasht-Iran Cohort Study (SICS) which was designed to evaluate long-term complications in SM exposed individuals [13]. Herewith, the serum levels of inflammatory cytokines among SM-exposed subjects with long-term skin complications 20 years after the exposure were titrated and compared with the unexposed control groups.

2. Materials and methods

2.1. Study design and participants

This study is part of SICS and the methodology was previously reported in detail [13]. Briefly, 368 male, 20–60 year old subjects who were exposed to SM 20 years ago and 126 age/match unexposed individuals were included in the cohort. Since the SM-exposed participants had developed various organ complications, they were categorized in specific subgroups to make it easier to understand the relationship between serum cytokine levels and skin disorders. In this study 4 groups were included: 1 – exposed group with only skin findings (OSFE), 2 – exposed with eye and respiratory other than skin lesion findings (SERE), 3 – the health control group without any problem (NC, normal control) and 4 – the control with only skin findings (OSFC).

2.2. Ethical considerations

The study was approved by the ethical committee of the Board of Research Ethics of Janbazan Medical and Engineering Research Center (JMERC), the Board of Research of Ministry of Health, and Shahed University. Individuals who wish to participate in the study and sign an informed consent were recruited.

2.3. Clinical evaluation

All participants were interviewed and physically examined by a physician. Respiratory system, eyes and skin conditions were evaluated by a relevant specialist according to the study protocol [13].

2.4. Serum collection

Venous blood samples were collected and allowed to clot at room temperature for 1 h. The samples were then centrifuged for 20 min at 3000 g. Serum samples were collected, aliquoted and stored at –80 °C until used. Serum levels of proinflammatory cytokines including interleukin (IL)-1α, IL-1β, IL-1Ra, IL-6 and tumor necrosis factor α (TNF-α) was titrated using ELISA method.

2.5. Cytokine measurement

Human IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α DuoSet® ELISA Development Kits (R&D Systems) were used to titrate IL-1α, IL-1β, IL-1Ra, IL-6 and TNF-α levels in the serum samples. The catalog numbers of the kits are DY200, DY201, DY263, DY206, and DY210 respectively. All standards were diluted with 1% bovine serum albumin (BSA) in phosphate buffer saline (PBS). 0.05% Tween 20 in PBS was used as washing buffer, 1% BSA in PBS as blocking buffer and PBS was used to wash and block buffer containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4 and 1.5 mM KH2PO4.

2.6. Statistical analysis

All analyses were performed using SPSS 16 (SPSS Inc., Chicago, Illinois, USA). Clinical data is compared between the study groups using Chi-square test. For cytokine analysis, the data are presented as median and first and third quartiles (Q1, Q3) and are compared between the study groups using Mann–Whitney test. P<0.05 was considered significant.

3. Results

In the main cohort 79.9% (n=290) of the exposed group and 60.5% (n=98) of the control group showed skin findings. In the exposed group, 52.1% (n=189) only showed skin findings (OSFE) and 27.8% (n=101) showed also eye and respiratory other than skin findings (SERE). In the control group 32% (n=41) were healthy without any problem (NC, normal control) and 47.3% (n=61) showed only skin findings (OSFC).

Mean and standard deviation of age in OSFE group was 34.1 ± 11.0 years, in SERE group 44.8 ± 11.2 years, in NC group 41.6 ± 11.7 years and in OSFC group 40.5 ± 8.7 years (P=0.094). There were 25.4% in OSFE group, 25.7% in SERE group, 17.1% in NC group and 18.0% in OSFC group of participants were smokers (P=0.536).

3.1. Serum levels of IL-1α

Median serum levels of IL-1α were 1.077 (Q1=0.274, Q3=1.889) pg/ml in the OSFE group, 1.889 (Q1=0.808, Q3=3.812) pg/ml in the NC group (P=0.005) and 1.889 (Q1=0.274, Q3=3.536) pg/ml in the OSFC group (P=0.041). No statistically significant difference was seen between IL-1α levels in the OSFE and SERE groups in the SM exposed subjects (Table 1).

3.2. Serum levels of IL-1β

Median serum levels of IL-1β were 1.745 pg/ml in the OSFE group, 1.896 pg/ml in the NC group (P=0.216) and 1.815 pg/ml in the OSFC group (P=0.39). The difference between the OSFE and SERE groups was also not significant (Table 2).

3.3. Serum levels of IL-1Ra

As shown in Table 3 median serum levels of IL-1Ra were 25.640 pg/ml in the OSFE group, 32.190 pg/ml in the NC group (P=0.106) and 32.140 pg/ml in the OSFC group (P=0.135). The difference between the OSFE and SERE groups was statistically significant (P=0.0001).

3.4. Serum levels of IL-6

Median serum levels of IL-6 were 0.602 pg/ml in the OSFE group, 1.022 pg/ml in the NC group (P=0.295) and 1.533 pg/ml in the OSFC group (P=0.016). There was no significant difference between the OSFE and SERE groups (Table 4).

3.5. Serum levels of TNF-α

Median serum levels of TNF-α were 12.768 pg/ml in the OSFE group, 23.786 in the NC group (P=0.045) and 17.416 pg/ml in the
OSFC group ($P = 0.209$). Median serum value of the TNF-α level in the SERE group was the lowest (10.249 pg/ml) but the difference was not significant compared to the OSFC group (Table 5).

### 4. Discussion

SM, as an exogenous factor is a strong alkylation agent with multiple targets including DNA, proteins, and small molecules. DNA damage may lead to cytotoxicity, trigger other changes, result in cellular dysfunction, cell death or disrupt tissue repair [14,15]. Impairment of both hemoral and cellular immune responses following SM exposure is reported [16–20]. SM up-regulates the inflammatory mediators such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α, and other cytokines in acute phase of exposure [14]. Down-regulation of serum concentrations of inflammatory cytokines such as IL-1α, IL-1β, IL-1Ra and TNF is reported by the same group [21] 20 years after SM exposure in the SCS.

The purpose of this study was to titrate the serum levels of proinflammatory cytokines in SM exposed subjects with long-term dermatological skin complications. As shown in the Results section, 79.9% ($n = 290$) of the exposed group and 60.5% ($n = 98$) of the control group showed chronic skin findings. In this work we focused on serum levels of IL-1α, IL-1β, IL-1Ra and TNF-α in SM-exposed individuals with only skin findings (OSFE) compared to normal controls (NC), controls with only skin findings (OSFC) and those SM-exposed subjects who had eye or respiratory complications other than dermatologic problems. Similar to our previous report [21], decreased serum concentration of these pro-inflammatory cytokines was observed in patients with only skin findings (OSFE) 20 years after SM exposure.

In this study the serum level of IL-1α and IL-1β in the OSFC group was lower than those of normal controls as well as those in the OSFE group. There was no statistically significant difference between OSFE and SERE groups in this regard (Tables 1 and 2). Median serum level of the OSFE group was lower as compared to the NC or OSFC group but the differences were not statistically significant. There was no statistically significant difference between the OSFE and SERE subgroups in the exposed group in terms of IL-1β levels (Table 2).

Despite of many reports indicating an increased level of IL-1 in acute phase of SM toxicity, in the current study down-regulation of IL-1β but not IL-1α in SM-exposed subjects with long-term skin disorders is seen. IL-1α is biologically active but IL-1β is actively weakly cleaved by caspase 1. In general, IL-1β appears to be the dominant form of IL-1 produced by monocytes, macrophages, Langerhans cells, and dendritic cells, whereas IL-1α predominates in epithelial cells, including keratinocytes. Keratinocytes, when injured, release biologically active IL-1α and may initiate inflammation. Studies on mice with IL-1α and IL-1β gene deficiencies suggested that both molecules are important in contact hypersensitivity and that IL-1α is more crucial [23–25]. In the present study we can conclude that skin findings indicate impairment in pro-inflammatory cytokine levels in people who have been exposed to SM a long time ago. Although the local levels of these cytokines are more important and should be investigated.

Median serum level of IL-1Ra in the OSFE group was lower than that of the NC and OSFC groups but the difference was not statistically significant. The difference was however significant when compared with the SERE group ($P = 0.0001$) (Table 3). IL-1Ra exerts potent anti-inflammatory effects. The important function of IL-1Ra is to potentially inhibit the deleterious effects of IL-1 during inflammation [11]. IL-1Ra deficient mice showed an exaggerated and persistent inflammatory response [25]. IL-1Ra is produced by hepatocytes with the characteristics of an acute phase protein. Endogenous IL-1Ra is produced in numerous diseases in experimental animal models as well as in human autoimmune and chronic inflammatory diseases. Patients with rheumatoid arthritis treated with IL-1Ra for six months showed clinical improvements with radiographic evidences of joint damage [26,27]. Our result suggests a role for IL-1Ra lacking in late skin complications induced by SM. More studies are needed to clarify the relationship between IL-1Ra and SM induced late skin complications.

SM provokes acute inflammatory response in the skin which is regulated by keratinocytes with a higher release of IL-1Ra which may provide a useful marker for cytotoxicity. The high level of IL-1Ra and increased release with injury induced by 2-chloroethyl ethyl sulfide (CEES) suggested a primary function for IL-1Ra in down-regulating the IL-1 inflammatory responses in the skin [28].

### Table 1

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Subgroups</th>
<th>N</th>
<th>Median (pg/ml)</th>
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<th>Q3</th>
<th>P1*</th>
<th>P2*</th>
<th>P3*</th>
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</table>

Bold data shows significant differences with $p < 0.05$.

* Mann-Whitney test. OSFE: exposed with only skin findings; SERE: exposed with skin plus eye or respiratory findings; NC: normal control without any problem; OSFC: control with only skin findings; $P_1$: P-value for comparing OSFE and NC groups; $P_2$: P-value for comparing OSFE and SERE groups; $P_3$: P-value for comparing OSFE and SERE groups.

### Table 2

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<th>Study groups</th>
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<th>Q3</th>
<th>P1*</th>
<th>P2*</th>
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* Mann-Whitney test. OSFE: exposed with only skin findings; SERE: exposed with skin plus eye or respiratory findings; NC: normal control without any problem; OSFC: control with only skin findings; $P_1$: P-value for comparing OSFE and NC groups; $P_2$: P-value for comparing OSFE and SERE groups; $P_3$: P-value for comparing OSFE and SERE groups.

### Table 3

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Bold data shows significant differences with $p < 0.05$.

* Mann-Whitney test. OSFE: exposed with only skin findings; SERE: exposed with skin plus eye or respiratory findings; NC: normal control without any problem; OSFC: control with only skin findings; $P_1$: P-value for comparing OSFE and NC groups; $P_2$: P-value for comparing OSFE and SERE groups; $P_3$: P-value for comparing OSFE and SERE groups.

### Table 4

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<th>Q3</th>
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* Mann-Whitney test. OSFE: exposed with only skin findings; SERE: exposed with skin plus eye or respiratory findings; NC: normal control without any problem; OSFC: control with only skin findings; $P_1$: P-value for comparing OSFE and NC groups; $P_2$: P-value for comparing OSFE and SERE groups; $P_3$: P-value for comparing OSFE and SERE groups.
Table 5

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Bold data shows significant differences with p-value <0.05.

* Mann-Whitney test. OSF: exposed with only skin findings; SERE: exposed with skin plus eye or respiratory findings; NC: normal control without any problem; OSFC: control with only skin findings; P1: P-value for comparing OSF and NC groups; P2: P-value for comparing OSF and OSFC groups; P3: P-value for comparing OSF, SERE, and OSFC groups.

IL-6 is produced by different cell types, including monocytes, bone marrow cells, fibroblasts, endothelial cells, some T cells, B cells and keratinocytes [22,29]. IL-6 functions in both innate and adaptive immunity. In the innate immunity IL-6 functions as an ‘early’ pro-inflammatory cytokine which stimulates the synthesis of acute phase proteins and therefore contributes to the acute phase response. The increased formation of IL-6 by fibroblasts, endothelial cells and keratinocytes stimulated by IL-1 and TNF-α may represent a significant amplifying process in the inflammation. IL-6 also stimulates production of neutrophils from bone marrow progenitors and promotes cell-mediated immune reactions and mediates activation, growth and differentiation of T cells [22,30,31].

IL-6 plays an important role in the early and late courses of inflammation, trauma, and wound healing caused by sulfur mustard [32–34]. Our work revealed a lowering of IL-6 levels in the SM-exposed groups. Median serum level of IL-6 in OSF group was lower than that of the NC group but the difference was not statistically significant. Its level in the OSFC group was significantly lower than that of the OSF group (P = 0.016). There was no significant difference between the OSF and SERE groups in terms of IL-6 levels (Table 4). IL-6 levels in controls with skin findings were 2.5 times more than those in the exposed group with skin findings. Due to the important role of IL-6, particularly in the early stages of the immune response, a lowering in its levels could adversely affect the whole network of cytokines involved in humoral and cellular responses as well as tissue repairing and healing processes.

Another inflammatory cytokine which was evaluated in this study was TNF-α. TNF-α is secreted by monocytes, macrophages, mast cells, fibroblasts, smooth muscle cells, endothelial cells, keratinocytes, T cells, and certain NK cells. TNF-α has multiple pro- and anti-inflammatory activities and also plays a role as a growth factor in normal physiological regeneration and wound healing [35,36]. Upon stimulation by exogenous and endogenous factors, large amounts of TNF are released within minutes and synthesis of TNF-α is rapidly increased. The rate of synthesis is controlled by interferon-γ, but several other cytokines may antagonize and influence further release (e.g. Transforming Growth Factor-β IL-6, PGE2 or vitamin D3). TNF has many biological properties similar to those of IL-1 therefore IL-1 may synergistically enhance TNF activity [11].

TNF-α has proinflammatory effects and induces apoptosis. It is an important mediator of cutaneous inflammation and its expression in the skin is induced in the course of almost all inflammatory responses. Toxins, hapten as well as ultraviolet light are potent inducers of TNF-α release from keratinocytes. Cutaneous inflammation stimulated by irritants and contact sensitizers is associated with strong induction of TNF-α production by keratinocytes [11,25,37]. Epidermal keratinocytes exposed to SM release high levels of TNF-α [38–40]. In this work, median serum TNF-α level in the OSF group was significantly (P = 0.0045) lower than that in the NC group, it was also lower than that of OSFC group but not significant. The lowest median serum level of TNF-α was seen in the SERE group, however this difference between the SERE and OSF groups was not statistically significant (Table 5).

TNF-α induces intense expression of ICAM-1 in keratinocytes. Via activation of NF-κB and C/EBPα, TNF-α regulates the synthesis of cytokkeletal proteins participating in epidural responses to inflammation or wound healing. In contrast to its tissue-damaging properties, TNF is also a growth factor, stimulating fibroblast proliferation and synthesis, and exhibits synergy with EGF by increasing the EGF receptor, PDGF and insulin. Therefore lower levels of TNF-α in the SM-exposed patients could predispose them to growth factor deficiency like disorders. Also a decrease in TNF-α level could lead to disruption of the healing processes in these patients. TNF-α has also been identified as one of the cytokines required for the maturation of DC and therefore plays a role in the initiation of an immune response, alterations in the levels of this cytokine could lead to modifications or deficiencies in the cellular immune response. The pathological relevance of TNF-α has been demonstrated in various models of infection, inflammation and autoimmune disease [11].

5. Conclusion

In sulfur mustard exposed individual with skin findings after 20 years, serum levels of pro-inflammatory cytokines (IL-1α, IL-1Ra, IL-6 and TNF-α) were lower as compared to the controls of healthy volunteers and those controls that have skin findings. This might be related to the damages incurred upon cytokines producing, or failure of their functions, or unknown mechanism(s). Our findings also indicate a role for IL-1Ra down-regulation in SM induced late skin lesions. The results showed that SM-exposed patients especially years after exposure do not display known inflammatory profile and therefore do not need to be treated with anti-inflammatory and steroid drugs. This study revealed that this is not true for long-term problems and that is needed to revise conventional theories and practices when dealing with long-term complications of SM exposure. There is definitely need to further studies on cellular and molecular immunology parameters in SM-exposed patients.

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