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RESEARCH ARTICLE

Relationship between levels of IFNγ, TNFα, and TGFβ and pruritus in sulfur mustard-exposed veterans

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Abstract

One of the foremost negative effects of sulfur mustard (SM) is chronic pruritus, which affects the quality-of-life. In the present study, pruritus was assessed in relation with inflammatory factors in the blood. **S**eventy-two blood samples were collected from SM-injured veterans of the Iran–Iraq War (Case Group; $n = 36$) and non-exposed patients (Control Group; $n = 36$) suffering from skin pruritus. Pruritus severity in all subjects was assessed, as were levels of IFNγ, TGFβ, and TNFα. The results indicated that total pruritus severity did not significantly differ between the two groups. While WBC counts in Control patients were significantly higher than among the exposed veterans, there were no significant differences in levels of any specific WBC sub-classes. Levels of serum IFNγ and TGFβ in the control subjects were significantly greater than those in the exposed veterans. In contrast, serum TNF*α* in the SM-exposed group appeared to be in the normal range, albeit significantly higher than that of the control group. A positive correlation between pruritus and each of the evaluated cytokines was noted in the Case Group. As for the non-SM-exposed veterans, correlations were significant only in the cases of IFNγ (stimulated) and TGFβ. The results of the present study suggested that there might be a relationship between cytokine alterations and pruritus in SM-exposed veterans. Based on these studies, designing of new treatments to modulate blood levels of mediators might be helpful to decrease the problem of SM-induced pruritus, thereby improving the quality-of-life in exposed veterans.

Keywords: Sulfur mustard, pruritus, tumor necrosis factor-α, transforming growth factor-β, interferon-γ

Introduction

Sulfur mustard (SM), commonly known as mustard gas, was one of the major chemical warfare agents developed and used during World War I (Namazi et al., 2009). The last military use of SM was in the Iran–Iraq war (from 1980–1988). Its use injured more than 100,000 Iranians, one-third of whom are still suffering from the effects (Namazi et al., 2009; Ghabili et al., 2010). SM toxicity can affect different organs, including the skin, eyes, and respiratory tract (Reid et al., 2000; Ghanei, 2004; Hefazi et al., 2005). After its absorption, SM undergoes intramolecular cyclization to form a sulfonium ion that, in turn, alkylates DNA and proteins, leading to disruption of DNA strands and eventually cell death (Simpson and Lindsay, 2005; Heinrich et al., 2009; Jowsey et al., 2009).

The effects of SM in various organs have previously been reported (Hassan and Ebtekar, 2001). The main acute pathological findings of SM exposure in humans include ocular and dermal injury, respiratory tract damage, immunological and neuropsychiatric changes, reproductive and developmental defects, gastrointestinal and hematological effects, and cancer (Korkmaz et al., 2008; Namazi et al., 2009; Rowell et al., 2009). Moreover, chronic consequences of SM exposure are now becoming apparent and leading to long-term social and economic problems for exposed individuals and their families (Hassan et al., 2006).

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One of the foremost negative effects of this agent is chronic pruritus. This disease plagues SM-injured veterans for life and down-grades their quality-of-life (Panahi et al., 2008). Thus, it is essential to identify effective strategies to mitigate chronic pruritus in SM-exposed veterans and other individuals. Recently, studies have been shown that SM provokes an acute inflammatory response in the skin (Blaha et al., 2000). This is associated with inflammatory cell accumulation and increased expression of tumor necrosis factor (TNF)-α and other pro-inflammatory cytokines, as well as reactive oxygen and nitrogen species (Wormser et al., 2005). It has been reported that there are significant variations in blood inflammatory mediators of patients with itchy skin lesions, especially interferon (IFN)-γ, interleukin (IL)-4, IL-10, IL-6, and IL-2 levels (Gao et al., 2007; Tewari-Singh et al., 2009). It has also been shown that SM victims who suffer from chronic pulmonary lesions have high levels of transforming growth factor (TGF)-β and low levels of IFNγ in their blood (Sabourin et al., 2000). Different medicines are applied regularly to the treatment of itchy skin lesions in non-SM-exposed patients; these seem to manifest their therapeutic effects by impacting upon inflammatory mediators. Still, the mechanisms by which SM induces skin pruritus in SM-exposed veterans are unknown. It seems one mechanism might be induction of a variation in the blood levels of key pro-inflammatory cytokines.

To the best of our knowledge, the relationship between chronic pruritus and variations in levels of cytokines—especially IFNγ, TGFβ, and TNFα—in SM-exposed veterans has not been studied. Therefore, in this study whose ultimate goal was to help derive an effective treatment, serum IFNγ, TNFα, and TGFβ levels in SM-exposed veterans and non-exposed veterans with skin pruritus were compared and their relationship to chronic pruritus determined.

Materials and methods

Study populations

This study was conducted from April 2009 to January 2010 in an out-patient Dermatology clinic in the Baqiyatallah Hospital in Tehran, Iran. This hospital provides medical care for SM-exposed Iraq–Iran war veterans and maintains medical records from such patients. The Baqiyatallah Medical Sciences University Ethics Committee approved the study protocol; all patients were required to provide written informed consent prior to enrolment.

The sample populations consisted of males (30–65-years-old) suffering from pruritus. At the onset of study, each patient completed a questionnaire that collected information about age, pruritus score, history of treatment, leukopenia, history of lung function disturbances, adrenal dysfunction, etc. The inclusion criteria applied for the chemically-injured group were male gender, being chemically injured (or not as in the case of the controls), and having established previous documentation of skin lesions with resistance to routine treatments (including oral anti-histamine/topical cortico-steroids) (Panahi et al., 2007). Exclusion criteria used in this study was other concomitant dermatologic or medical disorders that might cause pruritus and/or and itching that resulted from systemic or cutaneous non-chemical diseases. The control group was populated with patients without any history of mustard exposure and chemical injury but who suffered from pruritus due to other reasons, most frequently dermatitis. In accordance with the decision of the Ethics Committee of Baqiyatallah Medical Sciences University as well as of the Ministry of Health, the study was not able to enrol control healthy subjects who had no signs of pruritus.

Ultimately, the study was comprised of 72 subjects, i.e., 36 chemically-injured veterans (Case Group) and 36 non-exposed patients (Control Group). A pruritic score was determined for each patient. The severity, distribution, and frequency of pruritus and pruritus-related sleep disturbance were determined and total pruritus severity was calculated from these data (see Table 1). Pruritus scores could range from 0–48 points, with higher scores indicating a more severe pruritus. The severity of pruritus was graded and placed in three categories, i.e., mild (1–16), moderate (17–32), and severe (33–48 points).

Blood sampling and analyses

Venous blood samples were collected from each study subject. Thereafter, the number of white blood cells (WBC) was measured using an electronic automatic counter (Technicon, France H1, Fontenay-le-Fleury, France). Differential counts of the WBC were determined via microscopic examination of smears prepared from the collected samples.

The remainder of each subject's sampled blood was then used for analysis of cytokine levels. Equal volumes (i.e., 1000 μl across subjects) of each sample were placed in culture tubes and then received either a 100 μl aliquot of saline (nil [negative] control) or phytohemagglutinin (mitogen-positive control; 5 μg PHA/ml, final concentration). After overnight incubation at 37°C, the serum in each sample was separated and its IFNγ concentration then quantified using an ELISA kit (Biosource, Camarillo, CA). Both the basal and stimulated levels—as well as the net change from basal formation—were then calculated. The serum concentrations of TGFβ and TNFα in the 24-h nil samples were also measured using ELISA kits (Biosource). The sensitivity for IFNγ, TGFβ, and TNFα in their respective kits was < 0.35, < 15.6, and < 0.17 pg/ml.

Statistical analyses

All data are reported as means (± SD). An independent *t*-test was utilized to compare the scores of each of the measures and mean of the parameters data between the two groups. The correlation between total pruritus severity and serum levels of each cytokine was determined by Spearman rank correlation. A Mann-Whitney U-test was applied to

Table 1. Calculation of total pruritus scores from detailed related variables.

	Score	Description	Total score
Severity	1	Slight itching sensation without necessity of scratching	5
	2	Slight itching sensation with necessity to scratch, but without excoriations	
	4	Scratching accompanied by excoriation	
	5	Pruritus causing total restlessness	
Distribution	1	For each region (arms, trunk or legs)	5
	5	Generalized itching	
Frequency	0.5	Two periods of less than 10 minutes	5
	1	For each period more than 10 minutes	
	5	Maximally	
The score of severity, distribution and frequency of pruritus was recorded separately for the morning and the afternoon, so that a maximum of 30 points could be achieved[a]			15 + 15 = 30
Sleep disturbance	1	Each scratching episode leading to excoriation during the night; max = 5	5
	2	Each episode of waking up due to itching; max = 10	10
For each time, i.e., morning, evening, and night with itching, 1 point added to score. Max = 1 + 1 + 1 = 3[b]			3
Total score			48

[a]For example; in patients who showed maximal frequency both in the morning and afternoon their score for frequency was calculated as 5 + 5 = 10. [b]For example; if a patient had an itching experience through the morning and evening that affected his sleep, 2 points were added to the total score.

determine the difference between the two groups percentages of patients with mild, moderate, or severe pruritus. A p-value < 0.05 was considered significant. Data were analyzed using SPSS, Version 11.5 (SPSS Inc, Chicago, IL).

Results

The mean ages of Case (SM-exposed) and Control (SM-unexposed) participants with verified pruritis were 44.77 (± 10.97) and 41.25 (± 7.62) years, respectively. The mean total pruritus score in the SM and control group subjects were, respectively, 34.1 (± 12.3) and 30.1 (± 12.3) (Table 2). There was no difference between the groups with respect to the mean total pruritus severity ($p = 0.77$), or the percentage of patients with mild, moderate, or severe pruritus ($p = 0.80$).

The results regarding the blood cells analysis in both groups are shown in Table 3. As shown, the mean WBC count in the control subjects was significantly higher than that of the SM-exposed veterans ($p < 0.05$). In contrast, the mean percentages of specific cell types, i.e., neutrophils, lymphocytes, monocytes, eosinophils, and basophils in the blood did not significantly differ between the members of these two groups.

Table 2. Pruritus severity based on pruritic score.

Treatment status		Chemical	Non-chemical	p-value
Pruritus severity (total score)		34.1 ± 12.3	30.1 ± 12.3	> 0.05
Pruritus score	Mild	6 (16.68)[a]	9 (25)	> 0.05
	Moderate	11 (30.55)	12 (33.33)	
	Severe	19 (52.77)	15 (41.67)	

[a]Values shown are number of patients with designation; percentage of the total group with that designation is in parenthese.

Table 3. Blood cells analysis in the two study groups.

Parameters	Chemical	Non-chemical	p-value
WBC count[a]	7126 ± 211	8228 ± 234	< 0.05
Neutrophil (%)	62.4 ± 6.9	62.5 ± 6.2	> 0.05
Lymphocytes (%)	33.6 ± 6.7	33.3 ± 6.2	> 0.05
Monocytes (%)	2.6 ± 1.2	3.1 ± 1.2	> 0.05
Eosinophil (%)	1.3 ± 1.4	1.1 ± 1.5	> 0.05
Basophil (%)	0.0 ± 0.0	0.03 ± 0.2	> 0.05

[a]Value in terms of the calculated number of white blood cells/μl blood.

The mean IFNγ, TGFβ, and TNFα levels in the serum of individuals in both the SM and control groups are shown in Table 4. IFNγ was measured both in Mitogen (PHA-containing) and Nil (saline-containing) media. In the former medium, the mean IFNγ concentration in control subjects was significantly higher than that in the SM-exposed veterans ($p < 0.01$). In contrast, basal (unstimulated) IFNγ levels for the SM subjects samples were significantly higher than those for the controls ($p < 0.05$). Thus, the net mean increase in serum IFNγ levels for the control subjects was significantly higher than that in samples from the SM-exposed veterans ($p < 0.001$). Basal serum concentrations of TGFβ in the SM-exposed veterans were also significantly lower than those in samples from control subjects ($p < 0.05$). In stark contrast, the basal serum TNFα levels in the SM subjects were highly significantly greater than those in samples from their control counterparts ($p < 0.001$).

The potential relationship (correlation) between blood cytokine levels and total pruritus severity scores for both groups of patients was also determined (Table 5). These analyses indicated that, among the SM-exposed veterans, the correlation between purities score and TNFα level was the greatest of all the measured end-points ($r = 0.66$); the corresponding r-value for the non-exposed patients was only 0.31. Even though their respective levels differed significantly (see above), both the values for TNFβ and IFNγ had correlation values with pruritis scores that were more-or-less similar in both the SM-exposed and control pruritis patients.

Discussion

Sulfur mustard (SM) causes serious pathological effects upon contact with human skin. Skin exposed to SM

Table 4. Cytokine concentration in the two groups.

Cytokines	Chemical	Non-chemical	*p*-value
IFNγ (pg/ml)			
Mitogen[a]	57.4 ± 32.4	101.7 ± 25.1	< 0.01
Nil[b]	33.5 ± 6.3	15.4 ± 4.1	< 0.05
[Mitogen] – [Nil]	23.9 ± 26.1	86.3 ± 21.0	< 0.001
TGF-β (pg/ml)[b]	206.1 ± 28.7	417.9 ± 34.5	< 0.05
TNFα (pg/ml)[b]	15.9 ± 6.9	0.5 ± 0.1	< 0.001

Serum isolated from [a]PHA-stimulated or [b]unstimulated cells were measured.

Table 5. Correlation between concentration of each cytokine and pruritus severity.

	Chemical		Non-chemical	
Cytokines	*r*	*p*-value	*r*	*p*-value
IFNγ (pg/ml)				
Mitogen[a]	0.31	< 0.05	0.45	< 0.01
Nil[b]	0.49	< 0.010	0.33	> 0.05
TGFβ (pg/ml)[b]	0.33	< 0.05	0.34	> 0.05
TNFα (pg/ml)[b]	0.66	< 0.001	0.31	> 0.05

Serum isolated from [a]PHA-stimulated or [b]unstimulated cells were measured.

develops an inflammatory response that manifests as erythema followed by edema, and subsequently progresses to skin itching, blister formation, ulceration, necrosis, and ultimately desquamation (Wormser et al., 2005; Hefazi et al., 2006; Firooz et al., 2011).

The results of the present study demonstrated that TNFα levels were significantly increased in the blood of SM-exposed veterans with skin pruritus as compared to non-exposed patients. On the other hand, the mean levels of TGFβ and IFNγ (in mitogen medium) were found to be significantly decreased in the veterans. These results suggest that TNFα, TGFβ, and IFNγ may play a major role in the mediation of the inflammation, immune responses, and skin pruritus that are known to evolve after SM inhalation.

Several studies in animal models have provided valuable data regarding SM-induced inflammatory reactions. Levels of inflammatory factors, such as prostaglandins, have been reported to increase upon exposure of skin to SM (Zhang and Monteiro-Riviere, 1997). *In vitro* and *in vivo* studies have provided evidence on the elevated production and release of interleukins (Arroyo et al., 2000; Ricketts et al., 2000) and phospholipase D-mediated arachi-donic acid (Lefkowitz and Smith, 2002) from SM-exposed normal human epidermal keratinocytes. Furthermore, a high expression of inflammatory mediators in rabbit skin exposed to SM has been reported previously (Tsuruta et al., 1996; Nyska et al., 2001). Recent studies have shown that cyclooxygenae (COX)-2 actively participates in the acute phase of inflammation caused by SM (Wormser et al., 2004). In the mouse ear model, SM exposure induced production of pro-inflammatory cytokines, including interleukins (i.e., IL-1β) as well as of TNFα (Sabourin et al., 2000; Wormser et al., 2005). In an *in vivo* study by Ricketts et al. (2000), cutaneous responses and inflammatory cytokines (e.g., IL-6, IL-1α, IL-1β, and TNFα) were studied in SM-exposed mice; in this case, there were no significant increases in IL-1β or TNFα levels. However, in more recent research by Gao et al. (2007), *in vitro* expression of pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNFα were examined in human respiratory epithelial cells. Those analyses showed that SM stimulated over-production of each of those cytokines.

Among various mediators, TNFα plays a crucial role in the development of inflammation and tissue damage caused by SM or its derivatives (Wormser et al., 2005). However, the mechanism by which TNFα, TGFβ, and IFNγ levels in the blood of SM-exposed veterans with skin pruritus varied is in question. Studies of the peripheral WBC of the people highly exposed to SM showed a significant decrease in the normal WBC level as compared to among un-exposed populations (Ghanei, 2004). The results of our study are comparable with that research. Our findings showed that the total number of WBC in the blood of SM-exposed veterans was significantly lower than in non-exposed patients; there were no significant differences in the mean percentages of sub-classes of WBC.

WBC are the main source of reactive oxygen species (ROS) *in situ* (Kimura et al., 2005). Recently, studies have shown that TNFα induction is associated with WBC ROS production. Studies of alveolar macrophages exposed to asbestos revealed that treatment with hydroxyl-radical scavengers (such as anti-oxidants) decreased this induction (Wormser et al., 2005). Although the mechanism underlying TNFα induction following skin itching remains unclear, there is evidence for the involvement of ROS in this induction. The inflammatory response may be associated with the induction of TNFα in the SM-exposed skin of the mouse ear, as was observed by Wormser et al. (2005). The higher blood concentrations of TNFα in the SM-exposed group compared to the control patients, and its positive correlation with pruritus severity in chemical-exposed veterans (but not in the control group), suggested to us that the increased TNFα concentration could be an important factor in induction of pruritic skin lesions in SM-exposed patients.

In conclusion, findings of the present study suggested the role of inflammatory response associated with the induction of inflammatory mediators that cause skin pruritus in SM-exposed veterans. It should be noted that one key limitation of this study is that subjects in both the chemical and non-chemical groups were *not* controlled for in any present or past history of immune stimulation (e.g., vaccinations, allergies, etc.). This matter could have an important impact on the levels of cytokines and so serve as a potential source of potential data mis-interpretation. Accordingly, it is highly recommended that future studies compare the levels of cytokines between SM-injured patients and normal healthy subjects who do not have any known allergic background and/or are matched for vaccination histories.

New studies in patients with atopic dermatitis have revealed that modulation of involved mediators (via the use of recombinant IFNγ and TNFα inhibitors) can

decrease pruritus (Pua and Barnetson, 2006; Jung et al., 2010; Misery, 2010; Panahi et al., 2011, 2012). Treatment with recombinant IFNγ has shown great success in improving both atopic dermatitis and related pruritus in animal and human subjects (Chang and Stevens, 2002). With regard to the present findings, IFNγ might play a major role in the development of pruritus in non-chemical subjects, whereas the role of TNFα in SM-exposed subjects is probably much more striking. Hence, the designing of new treatments to modulate levels of TNFα and other mediators is worth being investigated for the management of SM-induced pruritus.

Declaration of interest

All funds for this study were from the Baqiyatallah Medical Sciences University and there was no conflict of interest to other institutes. The authors alone are responsible for the content and writing of the paper.

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