Current status of the acquired immune system of Iranian patients with long-term complications of sulfur mustard poisoning

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Abstract

Background Sulfur mustard (SM) is a powerful blistering chemical warfare agent that has genotoxic effects. Cells with excessive proliferation such as lymphocytes may inherit this cellular toxicity which can lead to their malfunctions in the long-term. This study was designed to evaluate the status of acquired immunity among SM poisoned veterans around three decades after exposure.

Methods Thirty five male Iranian veterans having at least 25% disability due to SM poisoning with long-term complications in the respiratory system, skin or eyes were investigated. Non-functional/functional tests including hematological parameters, immunostaining analysis, lymphocyte proliferation assay, cytokine profile, and levels of total serum IgM, IgG and IgA were performed.

Results The results showed that most of the parameters of adaptive immune system of the veterans were currently within the normal ranges. However, changes in the proliferation index (PI) of lymphocytes showed problems with the lymphocytes which cannot be proliferated appropriately. PI values for PBMCs (peripheral blood mononuclear cells) in presence of PHA (Phytohemagglutinin-A) and LPS (lipopolysaccharide) mitogens were 1.16 ± 0.14 and 1.13 ± 0.07 , respectively which are less than expected.

Conclusions Based on the results gathered in this study, most of the parameters of acquired immunity were normal. However, the observed failure of lymphocyte functions may disrupt physiological activity of whole immune system leading to long-term complications; including recurrent respiratory tract infections. Indeed, further cellular and molecular studies with regard to lymphocytes function are required to better understand the status of adaptive immunity in these patients.

Keywords Sulfur mustard · Acquired immunity · Veterans · Adaptive immune system · Lymphocytes

Introduction

Sulfur mustard (SM) is a vesicant chemical warfare agent (CWA) that causes blisters on contact with the skin and mucous membrane [1]. Its last confirmed military use in a large scale was during 1980–1988 Iran-Iraq war that many of victims are still suffering from its delayed toxic effects [2, 3]. SM

toxicity can affect different organs, including the skin, the eyes, and respiratory tracts [4, 5]. After absorption, SM forms sulfonium ion which alkylates DNA and disrupt its strands [6–8]. Acute SM poisoning results in respiratory, ocular and dermal disorders. However, other organs particularly the immune system are also affected [2, 9, 10]. After 2 decades of SM exposure, chronic outcomes occurred among the veterans [11]. There are reports on the prolonged toxicity and complications of SM exposure in Iranian veterans describing such disorders [5, 12–17].

Among various body organs, the immune system has not been studied well. The adaptive immunity is the most important arm of defense against infection of pathogens. Functionally, suppressed immune system increases the risk of infectious diseases and formation of certain cancers. Therefore, it is crucial to have an efficient immune apparatus functioning in well accordance with the other body organs.

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Functionally, suppressed immune system increases the risk of infectious diseases and formation of certain cancers [18]. On the other hand, the prolonged effects of SM poisoning have been mostly reported in the organs which act as barriers against foreign agents. Hence, evaluating the status of the immune system as the pivotal helper complex of those barriers is of high priority. Moreover, few research have been carried out on the immune system [12]. Duration after exposure as a characteristic of the effects of time on the integrity of human immune system was considered in the current study. Based on mutagenic effects of SM, time effect on the lymphocytes as highly proliferative cells plays a significant role. With view of the common complications of SM poisoning which get worsen over time, the hypothesis of immune dysfunction of veterans is justified [1]. Therefore, in this study, to understand the degree to which the immune system has been influenced by SM exposure and to discuss a possible association between long-term SM complications and the status of the immune system, we aimed at screening the status of the immune system, specifically focusing on some important indices of acquired immunity of the veterans. Parameters such as serum immunoglobulins, cell blood counts and acquired immune cells population as well as lymphoproliferation assay and cytokine production in SM-intoxicated Iranian veterans were investigated.

Materials and methods

A thorough list of SM poisoned veterans still living in Khorasan Razavi after three decades of exposure was prepared based on the medical documents of the Veterans' Foundation. At the start, 43 veterans with more than 25% disability due to complications of SM poisoning were volunteered, but 35 of them were completely investigated. The other 8 patients were excluded due to their critical health condition or lack of compliances. Demographic data of patients including age, height, weight and percentage of disability due to SM exposure were recorded in this regard. Since finding an appropriate matched control group (veterans of the similar ages and war physical disabilities with no chemical exposure) was impossible, we compared the patients' laboratory findings with the standard range of each test. The current study was carried out in accordance with the Declaration of Helsinki and guidelines on Good Clinical Practice. After approval of the medical research ethics committee of Mashhad University of Medical Sciences and obtaining written informed consents of the patients, peripheral blood samples were taken from cases using the standard protocols. A volume of 10 mL blood samples were taken from brachial vein of each patient. Each blood sample was transferred into two tubes; one containing K_3EDTA anticoagulant for hematological parameters and immunostaining analysis and the other containing sodium heparin for lymphocyte

proliferation assay. Besides, supernatant from proliferation of lymphocytes were collected and used for determination of cytokines IFN- γ (interferon gamma), IL-4 (interleukin 4) and IL-10 (interleukin 10) levels. Moreover, serum was separated to measure the levels of total IgM, IgG and IgA.

Hematological tests

Complete blood count (CBC) was performed with a fully automated hematology cell counter. Routine hematological indices including WBC, RBC, Hct, Hb, Plt as well as MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration) and MCV (mean corpuscular volume) were determined. In addition, a blood smear stained with Giemsa dye was prepared in order to determine differentiation of the white blood cells and evaluate cells morphology.

Immunophenotyping analysis

Immunofluorescence staining assay was carried out to determine peripheral blood lymphocyte subsets using flow cytometer (FACSCalibur, BD, San Jose, CA) equipped with the Cellquest software. Blood was processed within 3 h of collecting while tubes kept at 4 °C. For phenotypic analysis a three-color reagents of anti- $CD_3^+/CD_4^+/CD_8^+$ (CYQ- $CD_3^+/FITC-CD_4^+/PE-CD_8^+$; IQ; USA) and anti- $CD_3^+/CD_{19}^+/CD_{45}^+$ (FITC- $CD_3^+/PE-CD_{19}^+/CYQ-CD_{45}^+$; IQ; USA) was used. In brief, whole blood (100 μ L) of each sample was incubated with related monoclonal antibodies (20 μ L) for 20 min at room temperature for immunostaining. After labeling, cells were fixed in paraformaldehyde 1%. Then, 1 mL of FACS Lysing Solution was added to lyse red blood cells for 5 min at room temperature. After centrifugation at $300\times g$ for 5 min, white blood cells were washed twice by 2 mL phosphate buffered saline (PBS) solution (pH = 7.2). Before flow cytometry analysis, the final pellet was resuspended in 500 μ L PBS. The aforementioned flow cytometer analyzed 10,000 events per sample. Phenotypes were expressed as percentages and absolute counts of cells stained with specific antibodies.

PBMC proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated prior to performing lymphocyte proliferation test. Cells were separated by the standard procedure of Ficoll-Hypaque 1077 centrifugation. Mononuclear cells that were mostly comprised of lymphocytes were collected from the interface. Then, 100 μ L aliquots of PBMCs - standardized at 2 million cells per mL - were pipetted into a 96-well microtiter plate in triplicates. Triplicate wells for each sample received no mitogen, Phytohemagglutinin-A (PHA) or lipopolysaccharide (LPS), respectively. The final concentration of PHA and LPS per well were 5 and 1 μ g/mL, respectively. After incubation for 48 h at

37 °C and 5% CO₂ in humid incubator, cell proliferation was determined by MTT-based assay. Afterwards, 15 µL MTT [3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium] solution 10% (5 mg/mL) was added to each well and incubated at 37 °C in 5% CO₂ humid incubator for 4 h. Formed blue formazan precipitate was then dissolved in 100 µL DMSO (dimethyl sulfoxide) and its optical density was measured at 570 nm using Stat-Fax™ ELISA Reader. Proliferation index (PI) was calculated as absorbance of stimulated cells divided by absorbance of unstimulated cells.

Cytokine profile

For measuring cytokines produced from stimulated PBMCs, 48 h after cultivation in presence of PHA mitogen as described above, supernatants were harvested and kept at −70 °C until testing. IFN-γ (eBioscience, Santa Clara, USA), IL-4 (eBioscience, Santa Clara, USA) and IL-10 (eBioscience, Santa Clara, USA) levels were determined using commercially available ELISA kits according to the manufacturer's protocol.

Serum levels of immunoglobulin isotypes

Finally, total immunoglobulin isotypes including IgM (eBioscience, Santa Clara, USA), IgG (eBioscience, Santa Clara, USA) and IgA (eBioscience, Santa Clara, USA) were evaluated by commercial ELISA kits according to the manufacturer's instructions.

Results

Demographic information

The sulfur mustard poisoned veterans aged 46 to 78 years old. According to information in the medical records of veterans, they had 25–70% of disability due to SM exposure with a mean of $49.2\% \pm 14.13$. Medical history of the patients revealed that the SM intoxicated veterans were poisoned during 1983 and 1988. Most cases of poisoning occurred in 1984 and 1985. Therefore, according to the time of incidence, 28–33 years was passed after poisoning. The average time passed was 30.0 ± 1.4 years. Veterans were 182–157 cm in height, with the mean of 168.6 ± 6.0 . Their weight was in the range of 58–122 kg with the mean weight of 77.06 ± 13.31 kg. Accordingly, body mass index (BMI) varied from 21.2 to 36.8 kg/m² with the mean BMI which was 27.0 ± 3.82 kg/m².

Hematological examination

All hematological findings of patients (Table 1) were within the normal ranges. Abnormal findings were not observed in this regard.

Table 1 Hematological profile of SM poisoned patients three decades after exposure

Parameter per µL	SM poisoned veterans	Normal ranges
WBC ($\times 10^3$)	7.3 ± 1.69	4–10
RBC ($\times 10^6$)	5.1 ± 0.61	3.5–5.5
Hb (g/dL)	14.5 ± 1.77	11–16
Hct (%)	44.7 ± 5.62	37–54
MCH (pg)	28.4 ± 1.48	27–34
MCHC (g/dL)	32.4 ± 0.92	32–36
MCV (fL)	87.8 ± 4.28	80–100
Platelet ($\times 10^3$)	257 ± 58	150–450
Neutrophil (%)	57.3 ± 11.46	50–70
Lymphocyte (%)	31.7 ± 10.49	20–40
Monocyte (%)	7.68 ± 1.72	3–12
Eosinophil (%)	2.8 ± 1.68	0.5–5
Basophil (%)	0.46 ± 0.19	0–1
Neutrophil ($\times 10^3$)	4.3 ± 1.47	2–7
Lymphocyte ($\times 10^3$)	2.2 ± 0.60	0.8–4
Monocyte ($\times 10^3$)	0.5 ± 0.16	0.12–1.2
Eosinophil ($\times 10^3$)	0.2 ± 0.14	0.02–0.5
Basophil ($\times 10^3$)	0.03 ± 0.01	0–0.1

Data shown as Mean $\pm$ SD

Immunophenotyping analysis

The data of immunofluorescence analysis of immune white blood cells related to veterans were summarized in Table 2. There were no abnormal findings in T cell, B cell and natural killer cell percentages and counts of PBMCs when compared to their related reference values. Moreover, CD3⁺CD4⁺/CD3⁺CD8⁺ ratio was also within the standard limits.

Table 2 Immunostaining analysis of blood lymphocytes of SM poisoned veterans three decades after exposure

Parameter	SM poisoned veterans	Standard ranges
CD3 ⁺ (%)	66.1 ± 8.10	55–83
CD3 ⁺ CD4 ⁺ (%)	39.9 ± 8.82	23–51
CD3 ⁺ CD8 ⁺ (%)	20.5 ± 1.62	18–48
CD19 ⁺ (%)	10.9 ± 4.56	5–21
CD3 ⁺ ($\times 10^3$)	1.46 ± 0.39	0.68–2.03
CD3 ⁺ CD4 ⁺ ($\times 10^3$)	0.88 ± 0.26	0.35–1.21
CD3 ⁺ CD8 ⁺ ($\times 10^3$)	0.45 ± 0.20	0.19–0.75
CD19 ⁺ ($\times 10^3$)	0.25 ± 0.15	0.08–0.5
CD3 ⁺ CD4 ⁺ /CD3 ⁺ CD8 ⁺ ratio	2.29 ± 1.26	0.6–2.5

Data shown as Mean $\pm$ SD

Table 3 Proliferation index (PI) of peripheral blood mononuclear cells among the SM poisoned veterans

Parameter	SM poisoned veterans
PI of PBMCs to PHA	1.16 ± 0.14
PI of PBMCs to LPS	1.13 ± 0.07

Data shown as Mean ± SD

PI proliferation index, PBMC peripheral blood mononuclear cells, PHA phytohemagglutinin-A mitogen, LPS lipopolysaccharide mitogen

PBMC proliferation assay

As can be seen in Table 3, although PBMCs were proliferated in presence of PHA or LPS, it seems that there are trivial proliferation indexes.

Cytokine profile

Production of cytokines is a necessary step in immune responses and immune regulation. The possible long-term effects of SM in the production of IFN- γ , IL-4 and IL-10 in non-PHA or PHA-stimulated PBMCs were presented in Table 4. There were 437%, 37% and 136% fold increase in levels of IFN- γ , IL-4 and IL-10 in presence of PHA relative to non-treated PBMCs.

Serum levels of immunoglobulin isotypes

As can be seen in Table 5, measures of mean of serum IgM, IgG and IgA concentrations were within the normal ranges.

Discussion

Long-term health effects of SM poisoning on the respiratory system, the eyes and the skin have been largely documented. The first report on long-term effects of SM exposure in Iranian victims was made in 1986. Several papers reporting the delayed toxic effects of SM in Iranian veterans have been published [12–18]. As the toxic effects of SM on cells, especially on cellular

Table 5 Immunoglobulin concentrations in SM poisoned veterans three decades after exposure

Parameter	SM poisoned veterans (mg/dL)	Standard ranges (mg/dL)
IgM	70.1 ± 49.93	40–283
IgG	705 ± 313.86	636–1518
IgA	164 ± 68.23	72–375

Data shown as Mean ± SD

DNA, are progressive and the complication of veterans can be exacerbated over time, follow-up studies are crucial to determine these side effects. With reference to these effects of SM, our study aimed to evaluate the current status of adaptive immune system of SM poisoned veterans three decades after exposure.

To our knowledge, some immunological studies in this field have been performed using control groups that were not appropriately matched with the patients. These studies may suffer a few setbacks in presenting the significant differences of immunological parameters between the case and control groups. In other words, reporting of comparison a determined parameter in the veterans with subjects of the control group with a significant difference while both results may still lie within the normal range, is questionable [19–24]. Since it was not possible to find the right control subjects whom experienced the war stress and have similar ages and disability percentages, but not exposed to SM, in the current study the patients' data were compared with the related laboratory normal ranges. Therefore, in our study unlike the previous studies, our gathered findings lied within the normal ranges of the tests.

Cellular and molecular components of acquired immune system both functionally and non-functionally were investigated. In the current study, the amounts of immunoglobulins, hematological parameters, percentages and count of lymphocyte subtypes were within the normal range. On the other hand, proliferation indices of PBMCs were somehow lower than other similar studies [25, 26]. This result may be due to a mild dysfunction of lymphocytes to proliferate. As a result, since lymphocytes play a critical role in protecting human body against foreign agents, some immunotoxic effects of

Table 4 Cytokine production from 35 SM poisoned veteran's peripheral blood mononuclear cells with or without PHA treatment

Cytokine	SM poisoned veterans	SM poisoned veterans (Increased percentage of released cytokine)
IFN- γ (non PHA) (pg/mL)	847 ± 1219	437
IFN- γ (PHA) (pg/mL)	4553 ± 1503	
IL-4 (non PHA) (pg/mL)	19 ± 17	37
IL-4 (PHA) (pg/mL)	26 ± 30	
IL-10 (non PHA) (pg/mL)	246 ± 214	136
IL-10 (PHA) (pg/mL)	581 ± 150	

Data shown as Mean ± SD

PHA phytohemagglutinin-A mitogen

SM may happen similarly. Surprisingly, despite low PI, high levels of cytokines were observed. With regard to the much elevation of IFN- γ (437%) relative to the levels of IL-4 (37%) in presence of PHA, it seems that the immune system of SM poisoned veterans has shifted the TH1/TH2 equilibrium toward TH1 pathway. This somehow confirms that subsequent to antigen contact, the immune system of veterans sustains a pathway in which cellular components are more activated relative to molecular constituents to destroy pathogens. According to low PI and considerable cytokines secretion, this contradiction could justify the failure of veterans' lymphocytes to survive against proliferative/toxic effects of PHA during the time of cell culture relative to findings from healthy individuals reported earlier [25, 26]. In other words, at the early phase of culture, PHA has successfully acted as proliferative as expected, whereas in the late phase it becomes toxic and vulnerable for lymphocytes due to dysfunction of these cells. Nevertheless, based on low PI (in presence of PHA or LPS) demonstrated here (Table 3), the existence of recurrent infectious respiratory disorders could be explained. Therefore, disability of lymphocytes to be proliferated and differentiated in order to confront foreign micro-organisms may lead to the available respiratory complications observed among veterans. This hypothesis needs further studies to be approved.

Several studies on cellular and humoral components of veterans' immune system have been reported. In a study by Mohammadhoseiniakbari et al. (2008), potential delayed effects of SM was evaluated on leukocytes of 113 Iranian veterans more than two decades after exposure. White blood cells and also percentages of peripheral blood polymorphonuclear cells were found to be higher in exposed veterans. On the other hand, T-helper percentages were lower in exposed individuals whereas, cytotoxic T-lymphocytes percentages and also CD4⁺/CD8⁺ ratio remained unchanged [21]. Similarly, it was proposed that T lymphocytes may be involved in the immune reactions of the SM delayed respiratory complications. Results of the study on 372 victims 20 years after SM exposure showed T lymphocytes decrease, and a correlation between absolute numbers of T lymphocytes and respiratory functional parameters [20]. Another study at a 10-year after exposure on 75 SM poisoned patients demonstrated that leukocytes percentages in all patients were normal, while T lymphocyte subsets showed decrease in percentage when the severely poisoned group compared with the mild intoxicated group [27]. Serum cytokines profile of 43 veterans with disabilities due to SM poisoning; 23 years after their exposure revealed levels of ICAM-1 were higher, whereas serum IL-1 β , IL-8 and TNF- α were lower than normal. However, measurement of other cytokines such as IL-2, IL-4, IL-10 and IFN- γ were within the normal ranges and no abnormal findings were observed [28].

Disorders in cellular level due to SM exposure may contribute to the observed prolonged health problems of these veterans. SM acts as DNA alkylating and hence as a genotoxic

chemical, its cellular toxicity is mediated by covalent addition to guanine base of DNA [29, 30]. SM is able to form adducts and cross-links in genome; consequently this genotoxicity and cellular impairment may be inherited via cells with excessive proliferation such as lymphocytes; in a way that lymphocytes might be deprived of the proper function.

Conclusion

According to the results of our study, most indices of acquired immune system among the veterans around three decades after SM poisoning were within the reference ranges. However, the PI changes showed problems with the lymphocytes which cannot be proliferated appropriately. Despite the percentages and counts of lymphocyte subtypes, proliferation indices of PBMCs were abnormal. Lower PI resembles mild dysfunction of lymphocytes to proliferate. This can lead to immunotoxic effects of SM exposure. Contrary to low PI, high levels of cytokines were secreted. This again manifests abnormal behavior of lymphocytes to survive against toxic effects of PHA which in return results in observed low PI. Perhaps these problems may affect the body protection and inflict veterans' current symptoms such as recurrent respiratory complications. This hypothesis needs further studies to be approved. If this was the case, immunotherapy might be considered as an alternative choice for the management of long-term complications of SM poisoning.

Limitations

1. Lack of a matched control group was the first limitation of this study, but finding an appropriate matched control group of similar ages and war physical disabilities with no chemical exposure was impossible.
2. Low number of the studied cases was the second limitation. However, we called all SM veterans of Khorasan Razavi, 43 were first volunteered to enroll in the study, but 8 of them could not complete the study due to their critical health condition or lack of compliances.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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