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B Data Collection
C Statistical Analysis
D Data Interpretation
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F Literature Search
G Funds Collection

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Summary

Background: Sulfur mustard is a chemical warfare agent that produces cellular damage via alkylation and protein cross-linking. Sulfur mustard affects the skin, lungs, and eyes, as well as the gastrointestinal, endocrinal, and hematologic systems. We studied the potential delayed toxic effects of sulfur mustard on white blood cells and some of its derivatives including polymorphonuclear lymphocytes and lymphocytes ($CD4^+$ and $CD8^+$) among Iranian veterans, approximately 25 years after exposure.

Material/Methods: One hundred thirteen sulfur mustard-poisoned veterans registered for this prospective study. Hematologic, immunophenotyping, and flow cytometric evaluations were done to samples from patients as well as 20 healthy age- and sex-matched control volunteers. Hematologic and immunologic variables were compared between both groups of subjects. Values for *P* less than.05 were considered statistically significant.

Results: Total white blood cell count and percentage of polymorphonuclear lymphocytes were significantly higher in sulfur-mustard–exposed veterans than in control subjects (P=0.008 and <0.001). The percentages of total and $CD4^+$ lymphocytes were significantly lower in sulfur-mustard–exposed veterans than they were in control subjects (P=0.008 and P<0.001). $CD8^+$ lymphocyte percentage and $CD4^+/CD8^+$ ratio were not significantly different between the 2 groups.

Conclusions: Severe exposure to sulfur mustard may cause long-term damage to the immune system in humans. $CD4^+$ T cells were significantly lower in persons exposed to sulfur mustard. However, there was no statistically significant between-group difference regarding $CD8^+$ T cells. Impaired immunity may be responsible for the increased risk of infections in these patients.

key words: sulfur mustard • lymphocyte • immunologic system • chemical warfare agent

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BACKGROUND

Sulfur mustard, chemically known as 2,2'-bis-chloroethyl-sulfide, is a colorless to amber, oily liquid with an oniony or garliclike fragrance. It is an electrophilic alkylating agent that has mutagenic, cytotoxic, carcinogenic, and vesicating effects on living tissues [1–3]. Sulfur mustard gained notoriety during World War I and was used most recently in the Iran-Iraq war (1980–1987). As a chemical warfare agent with massive vesicant properties, it produced more than 100 000 chemical casualties during that war [3].

The major targets of sulfur mustard exposure are the eyes, skin, and respiratory system [2]. Other things affected are the endocrine and gastrointestinal systems and circulatory organs. Sulfur mustard, when absorbed in large doses, can damage rapidly proliferating cells of the bone marrow and cause severe suppression of the immune system [4]. High-dose exposure has been shown to induce a cytotoxic effect on hematopoietic stem cells and to induce pancytopenia [5].

The influence of sulfur mustard exposure on the immune system has been the subject of previous research as follows: Severe suppression of the immune system can be a possible reason for lesion and blister formation, opportunistic infections, and septicemia leading ultimately to death [6]. Lymphocytes are reported to be especially susceptible to the cytotoxic effects of sulfur mustard [4]. Leukopenia is the first manifestation to appear, usually at 10 to 14 days after exposure, in patients with severe skin burns. Thrombocytopenia and anemia appear later among surviving patients [5]. Studies on the status of immune-competent cells in the blood of patients exposed to sulfur mustard showed that T-cell and monocyte counts dropped in 54% and 65% of the patients, respectively, from day 1 until the 7th week after exposure. Eosinophil counts dropped in 35%, and neutrophil numbers dropped in 60%, of the patients. B-lymphocyte counts were normal until the seventh week after exposure [6].

Owing to a lack of data on the long-term effects of sulfur mustard on the immune system, this study was done to determine the prolonged toxic effects of sulfur mustard on the white blood cell count and some of its derivatives including polymorphonuclear lymphocytes and lymphocytes ($CD4^+$ and $CD8^+$) in Iranian veterans exposed to this agent.

MATERIAL AND METHODS

Patients and study design

This prospective study included 113 sulfur-mustard – exposed veterans exposed to sulfur mustard during the Iran-Iraq war referred to Baqiyatallah General Hospital between April 2004 and December 2006. All patients in the experimental group were on the same drug protocol regarding their respiratory, skin, and other medical problems associated with sulfur mustard exposure. None of the volunteers in the experimental group had exacerbations of any medical problems such as respiratory diseases, and there were no signs or symptoms of any infectious problem.

The control group was composed of 20 age- and sex-matched volunteers. Patients with a history of blood diathesis were excluded. After approval by the medical ethics committee at the university and after written, informed consent had been obtained, blood samples were collected from the control and experimental groups, and hematologic and flow cytometric evaluations as well as immunophenotyping were done on these samples.

Hematologic studies

Blood samples were taken using standard phlebotomy procedures. Total white blood cells and polymorphonuclear and lymphocyte counts were measured using the Technicon H1 autoanalyzer (Bayer Medical Systems, New York, NY, USA). For lymphocyte subsets, 3 mL of blood was collected into EDTA tubes and kept at room temperature. Blood was evaluated within 3 hours of collection.

Immunophenotyping and flow cytometric analyses

Briefly, 100 µL of each blood samples was immunostained with 10 µL of monoclonal mouse antihuman antibody (DAKO A/S, Denmark) [7], directly conjugated with fluorescein isothiocyanate (FITC) or R-phycoerythrin, and then incubated for 30 minutes in 4°C.

After labeling, cells were fixed in 1% paraformaldehyde. Red blood cells were lysed with 100 µL of fluorescence-activated cell sorter lysing solution for 10 minutes. White blood cells were then centrifuged at 300 × g for 5 minutes, washed twice by adding 2 mL of phosphate-buffered saline (pH 7.2) solution to each tube, and then centrifuged again at 300 × g for 5 minutes. Next, the supernatant was discarded, and the sample was stored on ice in the dark for 6 hours. Before flow cytometry analysis, each sample was resuspended in 500 µL phosphate-buffered saline.

Two-color immunofluorescence analyses were done using a Coulter flow cytometer (Coulter, Epics-Profile II, Coulter Electronics, Inc, Hialeah, FL, USA) equipped with Cellquest software. In each tube, 10.000 events were analyzed. Isotypic controls (γ1-FITC/γ2a-PE) were used for each assay to determine nonspecific staining. Blood samples were gated on forward scatter versus side scatter to exclude debris and cell aggregates. We used a combination of anti-CD4 and anti-CD8 antibodies to establish precisely the lymphocyte gate and to measure the proportion of leukocytes in each sample. Phenotypes were expressed as percentages of cells stained with specific antibodies.

Statistical analyses

All data are expressed as means ±SD unless otherwise indicated. The data distribution was assessed with the Kolmogorov-Smirnov test. Intergroup comparisons were analyzed using the Mann-Whitney *U* test. Data were analyzed with SPSS software (Statistical Product and Services Solutions, version 11.5, SPSS Inc, Chicago, IL, USA), and values for *P* less than.05 were considered statistically significant. Power analysis was implicated using NCSS and PASS software (copyright 2000 by Jerry Hintze).

RESULTS

The age range of patients in the experimental group was 36.6±6.8 years; the age range for patients in the control

Table 1. Hematologic and flow cytometric variables in patients and control subjects.

Power (%)	P value	Controls (n=20)	Patients (n=113)	Variable
9.6	0.008	6370±1079	6555±1807	White blood cells (1000/mL)
68.9	<0.001	55±6.2	59.3±11.4	Polymorphonuclear (%)
97.4	0.008	42.8±6.4	35.9±10.9	Lymph (%)
96.5	<0.001	44.9±7.6	36.8±9.3	CD4 (%)
7.2	0.757	25.8±5	25.2±8.3	CD8 (%)
36.1	0.109	1.78±0.39	1.61±0.64	CD4/CD8

Lymph – lymphocyte; P<0.05 was considered statistically significant.

group was 40.3±9.3 years (P=0.39). Patients were studied 20 to 25 years after their initial exposure. A review of the patients' medical records revealed no previous blood diathesis in the study population.

Hematologic findings

The hematologic and flow cytometric comparison of 113 patients and 20 control subjects and the power analysis results are summarized in Table 1. Total white blood cell counts and the percentage of polymorphonuclear cells were significantly higher in sulfur-mustard–exposed veterans than they were in control subjects (P=0.008 and P<0.001). The percentage of lymphocytes was significantly lower in sulfur-mustard–exposed veterans than it was in control subjects (P=0.008).

Flow cytometric findings

Flow cytometric variables of sulfur-mustard–exposed veterans and controls and the power analysis results are summarized in Table 1. The percentages of $CD4^+$ lymphocytes were significantly lower in sulfur-mustard–exposed veterans than they were in control subjects (P<0.001). The $CD8^+$ lymphocyte percentage and the $CD4^+/CD8^+$ ratio were not significantly different between sulfur-mustard–exposed veterans and control subjects.

Discussion

After this investigation, we discovered that blood diathesis is still present in sulfur-mustard–exposed veterans 25 years after exposure. The percentage of $CD4^+$ lymphocytes was significantly lower (P<0.001) in sulfur-mustard–exposed patients than it was in control subjects, and total white blood cell and polymorphonuclear cell counts were significantly higher in sulfur-mustard–exposed veterans than they were in control subjects (P=0.008 and P<0.001). The $CD8^+$ lymphocyte count and the CD4/CD8 ratio were not significantly different between the 2 groups.

Although international regulations strictly prohibit the use of chemical warfare, sulfur mustard has been used excessively in various regions of the world during the past decade, and vast global resources have been allocated to this weapon [8]. Hence, sulfur mustard remains a potential threat to the world; effective therapeutic measures must be established to counter the short- and long-term effects of sulfur mustard contamination. Studies to evaluate these effects will give us insight on how to prevent and treat sulfur mustard exposure complications.

A study that evaluated the consequences of sulfur mustard 6 years after exposure found that bronchitis, folliculitis, dermatitis, and pharyngitis were the most-frequent infections among veterans [9]. Unfortunately, neither that report nor ours reported the most-prevalent infections or the most-frequent causative organisms among sulfur-mustard–exposed veterans after 25 years' follow-up.

Previous reports have suggested that exposure to sulfur mustard can result in impairment of human immune function, especially in the number of B and T lymphocytes, resulting in impairment of humoral and cellular immune functions [10–12]. One study [13] found depression of cell-mediated immunity in Iranian veterans 1, 2, and 3 years after exposure to sulfur mustard. In that same study, helper T cells were significantly decreased in patients, whereas T-suppressor cells were increased. Sixteen to 20 years after exposure to sulfur mustard, the percentage of CD16+56+ cells still was significantly lower in the patients than in control subjects [11]. Long-term immunologic effects also were found in our patients 25 years after exposure to sulfur mustard. Total white blood cell and polymorphonuclear cell counts were still significantly higher in sulfur-mustard–exposed patients than in control subjects. Contrary to the results of Mahmoudi and associates [11], we found the percentage of $CD4^+$ cells to be significantly lower in veterans compared with control subjects. The percentage of $CD8^+$ lymphocytes was not significantly different between the 2 groups; this is consistent with the results of Mahmoudi and associates.

It is well known that sulfur mustard can produce toxicity via formation of reactive electrophonic intermediates, which in turn covalently modify nucleophilic groups in biomolecules such as DNA, RNA, and protein. A decrease in the number of lymphocyte cells in patients is probably due to the destructive effect of this alkylating agent on lymphocyte cell precursors in bone marrow. Injury resulting from systemic absorption of sulfur mustard may be transitory or it might lead to permanent damage of more-sensitive proliferating tissues, such as bone marrow, the reticuloendothelial system, and the intestinal tract. Intrastrand DNA cross-linking, which is probably responsible for the delayed toxic effects on rapidly proliferating tissues, such as bone marrow, produces a lethality at the lowest frequency of occurrence and at the lowest concentration of the agent. However, at a higher cellular

exposure, mechanisms such as NAD depletion and glutathione inactivation become important and produce rapid cell death in a dose-dependent manner [2].

CONCLUSIONS

Finally, regarding the proven immunologic consequences of exposure to sulfur mustard, it will be of interest to do a more comprehensive study to evaluate all the subtypes of immunologic and hematologic cells to achieve thorough information regarding the long-term immunohematologic complications of this chemical warfare agent.

Use of sulfur mustard as a blister agent in the past century has been documented as producing enduring toxic effects that include hematologic complications, initial leukocytosis followed by leukopenia, and bone marrow depletion with devastating consequences in cases of severe bone marrow suppression. Our experiences are meant to be informative in case of further use of the agent in military conflicts or terrorist attacks.

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