Long-term hematological and immunological complications of sulfur mustard poisoning in Iranian veterans

Mahmoud Mahmoudi¹,*, Mehrdad Hefazi², Maryam Rastin¹, Mahdi Balali-Mood²

¹Immunology Research Center, Bu-Ali Research Institute, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran
²Medical Toxicology Center, Imam Reza Hospital, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran

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Abstract

Background: Sulfur mustard (SM) is an alkylating chemical warfare agent that was widely used during World War I and by Iraqi forces against Iranian soldiers. We aimed to study late toxic effects of SM on the blood and the immune system of severely intoxicated Iranian veterans.

Methods: All SM-poisoned veterans in the province of Khorasan, Iran who had severe clinical complications were studied. Blood cell count, flow cytometric analysis of immune cells, serum protein electrophoresis, and determination of serum immunoglobulins and complement components levels were performed for the patients and 35 healthy age-matched controls. Hematological and immunological parameters were compared between the patients and the control group, using Mann–Whitney U test.

Results: Forty male subjects (aged 43.8 ± 9.8 years) who had confirmed SM poisoning 16 to 20 years prior to this study were investigated. Total counts for WBC and RBC, as well as Hct percentage were significantly (p ≤ 0.042) higher in the patients than in the controls. The percentages of monocytes and CD3⁺ T-lymphocytes were significantly higher (p = 0.013 and p = 0.037, respectively) and the percentage of CD16⁺ 56 positive cells was significantly lower in patients (p = 0.006). IgM and C3 levels were significantly higher in patients (p ≤ 0.030). The absolute level of α₂ globulin, as well as absolute levels and the percentages of α₁ and β globulins were significantly higher in patients compared to the control group (p ≤ 0.024).

Conclusions: SM can cause long-term effects on the immune system in patients with severe intoxication. The impaired immunity is probably responsible for the increased risk of infections in these patients.© 2005 Elsevier B.V. All rights reserved.

Keywords: Sulfur mustard; Poisoning; Long-term; Immunological; Hematological; Natural killer cells

1. Introduction

Sulfur mustard (SM) was the most widely used chemical warfare agent (CWA) in the Iran–Iraq War, resulting over 100,000 chemical casualties between
1983 and 1988 [1]. It acts as an alkylating agent with long-term toxic effects on several body organs, mainly the skin, eyes, and respiratory system [2–4]. The extent of tissue injury depends on the duration and intensity of exposure [5].

When absorbed in large amounts, SM can damage rapidly proliferating cells of bone marrow and may cause severe suppression of the immune system [4]. Early investigations on SM casualties during the Iran–Iraq War showed decreased immunoresponsiveness, expressed as leukopenia, lymphopenia, and neutropenia, as well as hypoplasia and atrophy of the bone marrow [4,6,7].

Chronic exposure to SM has been associated with the impairment of natural killer cells among workers of poison gas factories in Japan [8]. Although depression of both cell-mediated and humoral immunity have been observed in Iranian veterans several years after their exposure to SM [9–12], there is still paucity of information regarding the long-term immunosuppressive properties of SM in the setting of battlefield exposure to this agent.

We conducted this study to determine long-term toxic effects of SM on the serum immunoglobulins, complement factors, and serum proteins, as well as on the cell blood counts and populations of immune system cells in severely intoxicated Iranian veterans.

2. Materials and methods

2.1. Patients and study design

According to the medical expert committee of the Khorasan Veteran Foundation in June 2001, 47 veterans had severe SM complications at least in one of the target organs of respiratory system, skin or eyes. Of these, 40 patients volunteered to participate in this study and signed the written informed consent. In addition a total of 35 healthy male volunteers, who had no previous history of exposure to sulfur mustard, were recruited as a control group. After approval by the medical ethics committee of the university, blood samples were taken from each patient, as well as from the control participants, using the standard procedures.

2.2. Hematological studies

Complete blood cell count was performed with a hematometry autoanalyzer (Techincon H1; Bayer Medical Systems, NY). The main parameters evaluated were white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb) level, hematocrit (Hct) percentage, and platelet (Plt) count.

2.3. Immunofluorescence staining and flow cytometry

For the lymphocyte subsets, 3 ml of blood samples was collected into EDTA tubes and kept at room temperature. Blood was processed within 3 h of collection. Briefly, 100 μl of each blood samples was immunostained with 20 μl of monoclonal antibodies. The following two-color reagents were used for phenotypic analysis: anti-CD3/CD4 (Cat # 342405, BD Biosciences), anti-CD3/CD8 (Cat # 342406, BD Biosciences), anti-CD3/CD19 (Cat # 342404, BD Biosciences) and anti-CD3/CD16+CD56 (Cat # 342403, BD Biosciences).

The mixture of cells and antibodies was incubated for 20 min at room temperature in dark. After labeling, cells were fixed in 1% paraformaldehyde. Red blood cells were lysed with 100 μl of FACS lysing solution for 10 min. White blood cells were then centrifuged at 300 × g for 5 min, washed twice by adding 2 ml of phosphate-buffered saline (PBS) (pH=7.2) solution to each tube and centrifuged again at 300 × g for 5 min. Next, the supernatant was discarded and sample was stored on ice in dark for < 6 h. Before flow cytometry analysis, each sample was resuspended in 500 μl PBS.

Two-color immunofluorescence analysis was performed on FACScalibur flow cytometer (Becton-Dickinson, San Jose, CA), equipped with the Cellquest software. 10,000 events were analyzed per tube. Isotypic controls (γ1-FITC/γ2a-PE) were used for each assay to determine non-specific staining. The blood samples were gated on forward scatter versus side scatter to exclude debris and cell aggregates. We used a combination of anti-CD45 and anti-CD14 antibodies (Cat # 342408, BD Biosciences), to establish precisely the lymphocyte gate and to measure the proportion of leukocytes in each sample. Phenotypes were expressed as the percentages of cells stained with specific antibodies.
2.4. Measurement of immunoglobulins and complement in serum

Serum immunoglobulin levels of IgA, IgG, IgM, and complement factors C3 and C4 were determined using radial immunodiffusion (SRID). Briefly, 5 μl of serum was placed in the well of the SRID plate using a capillary micropipette. High calibrator was placed in a well on each plate as a control. As instructed, the plates were closed and left to stand at room temperature until the diffusion was complete (48 h for IgA, IgG, C3, and C4 and 72 h for IgM). Precipitation ring diameters were measured, using a specific calibrated scale. The immunoglobulin concentrations related to the measured diameters were read directly from the table of reference values. The SRID plates, control serum, calibrated scale and table of reference values were purchased from Biogen; Mashhad, Iran.

Total serum IgE levels were measured with enzyme-linked immunosorbent assay (Radims; Rome, Italy) according to the manufacturer’s instructions, and were expressed in international units per milliliter (IU/ml).

2.5. Serum protein electrophoresis

Cellulose acetate membrane electrophoresis was carried out on an automated system (Cosmo FED-2; Cosmo, Japan) according to the manufacturer’s instructions. The membranes were pre-equilibrated in barbital buffer, at pH 8.6. We applied 0.4 ml of each sample and carried out the electrophoresis at 10 mA for 20 min in the barbital buffer. Proteins were fixed with mixture of sulfosalicylic acid and trichloroacetic acid, and visualized with a Ponceau 3R stain. Peak area quantitation was conducted on a scanner with COSMO software.

2.6. Statistical analysis

All data were expressed as mean (± S.D.) unless otherwise indicated. The data distribution was assessed by Kolmogrov–Smirnov Z test. Intergroup comparisons were analyzed by Mann–Whitney U test. SPSS version 11.5 (SPSS Inc, Chicago, USA) was used throughout with the minimum level of significance set at p = 0.05 for all the comparisons.

3. Results

The age range of the patients was 32 to 76 (43.8 ± 9.8) years and that of the control group was 26 to 55 (42.1 ± 8.8) years (p = 0.479). The patients were studied 16 to 20 (18.0 ± 1.5) years after their initial exposure. Revision of the patients’ medical records revealed no respiratory impairment [ Forced Vital Capacity (FVC) ≥ 80 and Forced Expiratory Volume in the First Second (FEV1) ≥ 80] in 2 (5%), mild respiratory impairment (80%>FVC≥60% or 80%>FEV1≥60%) in 11 (27.5%), moderate respiratory impairment (60%>FVC≥50% or 60%>FEV1≥40%) in 14 (35%) and severe respiratory impairment (50%>FVC or 40%>FEV1) in 13 (32.5%) patients.

3.1. Hematological findings

The hematological comparison of 40 patients and 35 control subjects is summarized in Table 1. Total counts for WBC and RBC, as well as HCT percentage were significantly (p ≤ 0.042) higher in patients than in the control group. There was no significant difference in Hb level and Plt counts between the two groups.

3.2. Flow cytometric findings

Flow cytometric parameters of patients and controls are summarized in Table 2. The percentages of monocytes and CD3+ lymphocytes were significantly higher (p = 0.013 and p = 0.037, respectively), and the percentage of CD16+56 positive cells was significantly lower (p = 0.006) in patients than in the control group.

<table>
<thead>
<tr>
<th>Patients (n = 40)</th>
<th>Control group (n = 35)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (1000/ml)</td>
<td>7.24 ± 1.90</td>
<td>5.79 ± 1.16</td>
</tr>
<tr>
<td>RBC (million/ml)</td>
<td>5.46 ± 0.45</td>
<td>5.19 ± 0.28</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>48.3 ± 3.5</td>
<td>45.5 ± 1.9</td>
</tr>
<tr>
<td>Hb (mg/dl)</td>
<td>15.9 ± 0.7</td>
<td>15.6 ± 0.7</td>
</tr>
<tr>
<td>PLT (1000/ml)</td>
<td>255 ± 99</td>
<td>238 ± 101</td>
</tr>
</tbody>
</table>


* p-value is determined with Mann–Whitney U test.
Table 2
Flow cytometric parameters in 40 Iranian veterans with late complications of sulfur mustard poisoning and in 35 normal controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=40)</th>
<th>Control group (n=35)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Lymph</td>
<td>31.5 ± 8.4</td>
<td>30.5 ± 8.0</td>
<td>0.651</td>
</tr>
<tr>
<td>%Mono</td>
<td>4.8 ± 1.6</td>
<td>3.9 ± 1.1</td>
<td>0.013</td>
</tr>
<tr>
<td>%Poly</td>
<td>63.8 ± 8.7</td>
<td>65.4 ± 8.7</td>
<td>0.372</td>
</tr>
<tr>
<td>%CD3</td>
<td>71.1 ± 8.6</td>
<td>65.6 ± 10.7</td>
<td>0.037</td>
</tr>
<tr>
<td>%CD4</td>
<td>57.7 ± 8.3</td>
<td>57.8 ± 8.1</td>
<td>0.983</td>
</tr>
<tr>
<td>%CD8</td>
<td>37.1 ± 8.3</td>
<td>34.1 ± 7.8</td>
<td>0.099</td>
</tr>
<tr>
<td>%CD19</td>
<td>11.9 ± 5.9</td>
<td>13.6 ± 6.2</td>
<td>0.187</td>
</tr>
</tbody>
</table>

Table 3
Serum immunoglobulins and complement levels in 40 Iranian veterans with late complications of sulfur mustard poisoning and in 35 normal controls

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=40)</th>
<th>Control group (n=35)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 (mg/dl)</td>
<td>109.8 ± 30.1</td>
<td>90.9 ± 14.8</td>
<td>0.030</td>
</tr>
<tr>
<td>C4 (mg/dl)</td>
<td>31.1 ± 11.6</td>
<td>35.5 ± 15.4</td>
<td>0.542</td>
</tr>
<tr>
<td>IgA (mg/dl)</td>
<td>302.6 ± 142.1</td>
<td>233.1 ± 59.9</td>
<td>0.154</td>
</tr>
<tr>
<td>IgG (mg/dl)</td>
<td>1438.6 ± 486.1</td>
<td>1140.0 ± 244.2</td>
<td>0.065</td>
</tr>
<tr>
<td>IgM (mg/dl)</td>
<td>235.3 ± 84.4</td>
<td>136.8 ± 58.3</td>
<td>0.000</td>
</tr>
<tr>
<td>IgE (IU)</td>
<td>92.4 ± 112.1</td>
<td>86.5 ± 146.3</td>
<td>0.161</td>
</tr>
</tbody>
</table>
* p-value is determined with Mann–Whitney U test.

3.3. Serum immunoglobulins and complement

The comparison of serum immunoglobulins and complement for the 40 patients and 35 control subjects is summarized in Table 3. While serum IgA, IgE, and C4 did not show any significant difference between the two groups, IgM (p = 0.000) and C3 (p = 0.030) levels were significantly higher in patients compared to the control group. Serum IgG was slightly higher in patients than in the control group but the difference was not significant (p = 0.065).

3.4. Plasma protein electrophoresis

The comparison of plasma protein levels between patients and control subjects is summarized in Table 4. α2 globulins (both G/L and %) were significantly higher in the patients than in the controls (p < 0.0001). Absolute level (G/L) of α1 globulin, and the absolute levels (G/L) as well as the percentage of β globulins were significantly (p ≤ 0.024) higher in the patients than in the control group. The percentage of albumin and the total proteins (G/L) were significantly (p < 0.017) lower in the patients than in the control group. There was no significant difference between the two groups, in the percentages (%) of albumin and α1 globulin or the percentage (%) and the absolute level (G/L) of γ globulins.

4. Discussion

The search for chemical agents with antitumor activities has provided a clue that sulfur and nitrogen mustards are immunotoxic. As alkylating agents, they form covalent linkages with biologically important molecules, resulting in disruption of cell function, especially cell division [13,14]. As a result, these agents are particularly toxic to rapidly proliferating cells including neoplastic, lymphoid, and bone marrow cells.

Krumbhaar observed that among the first changes in the circulating blood of patients exposed to SM was an exhaustion of leukocyte forming centers [15,16]. Leukocytosis is common within the first few days after exposure. WBC counts then begin to drop on the third and fourth days after exposure and reach...
their minimum level around the ninth day [4]. Lymphocytes are the first to disappear and granulocytes are also severely affected but lag behind the lymphocytes in their rate of decrease [17]. Bone marrow biopsies have shown hypo-cellular marrow and atrophy involving all elements [6,7]. In a study by Willems, severe leukopenia was indicative of fatal exposure and all Iranian victims with WBC counts of 200 cells/ml or fewer died during their initial admissions [4].

While leukopenia, thrombocytopenia, and anemia are known to be main acute hematological effects following SM poisoning [4,18], long-term follow-up of our patients revealed a significant increase in the total counts of WBC and RBC, as well as the Hct percentage. Differential counts of lymphocytes, monocytes, and granulocytes revealed no significant difference between the patients and the control group. The significant increase in RBC count and Hct percentage is probably due to the hypoxemic status of the patients as a result of their chronic respiratory problems. Significantly increased WBC count is probably related to the high frequency of acute respiratory infections in these patients rather than direct toxic effects of SM on the bone marrow. This was supported by the revision of the patients’ medical records, which revealed moderate to severe respiratory impairment in 67.5% of the patients. A mean of 0.66 ± 1.37 (range, 0–6) hospitalization due to the respiratory infections was also found for each patient during the last year prior to the study.

Along with the appearance of clinical disorders, both C3 and C4 titers begin to increase [19]. Ghannadpour indicated that the percentage of patients with increased levels of C3 and C4 was higher than in the healthy controls during the first week and up to the sixth month. The percentage remained higher 3 years post-exposure especially in the severely intoxicated group [20]. Razavi and colleagues studied CH50 in 120 Iranian combatants in a period of 51 days after exposure to SM. The mean level of CH50 increased in the first 2 weeks, but began to decrease after the second week, reaching a level not significantly different from the controls [21].

In our study, neither C3 nor C4 complement components showed out of range fluctuations. Sixteen to twenty years after exposure to SM, C3 level was significantly higher in the patients, while there was no significant difference on C4 level between the patients and the control group. Complement changes are probably related to the acute phase response following frequent infections, and possibly indicate the efficiency of the classic pathway of the complement system.

Studies have revealed that SM has short-term and long-term influences on antibody production in animals and humans [22–24]. Dayhimi et al. studied 100 CWA victims through 1 year after their exposure. None of the immunoglobulin classes showed out of range fluctuations, although an early increase followed by a slow return in IgG and IgA levels was noticed [19]. Hassan and Ebtekar demonstrated that the majority of SM exposed patients had increased levels of IgG and IgM during the first weeks and up to the 6th month after exposure. Even 8 years after exposure, the percentage of patients with increased IgM, IgG, and IgE were still significantly higher compared to the controls [10]. Our study indicated that the IgM level is significantly higher 16 to 20 years after exposure, compared to the control group. IgG level was also noticeably ($p=0.065$) higher in patients than in the controls, but the difference was not significant.

Previous reports suggested that exposure to SM could result in the impairment of human immune function, especially in the number of B and T lymphocytes [25]. In one of the few studies on long-term effects of SM, Zandieh and colleagues noted depression of the cell-mediated immunity 1, 2, and 3 years after exposure. Helper T cells were significantly decreased in patients, whereas T suppressors were increased [11].

Occupational exposure to SM has been reported to cause impairment of natural killer cells in Japanese SM gas workers [8]. Ghotbi and Hassan also demonstrated that the percentage of natural killer cells in severely intoxicated Iranian veterans was significantly lower than that of the normal controls, 10 years after exposure to SM [12]. Similar long-term effects were found in our patients. Sixteen to twenty years after exposure to SM, the percentage of CD16+56+ cells was still significantly lower in the patients than in the control group. Natural killer cells are lymphocytes that can non-specifically destroy certain virally infected or tumor cells. Their impairment can therefore be responsible for the increased risk of tumors
and recurrent infections in patients with severe exposure to SM.

The percentages of monocytes and CD3+ lymphocytes were significantly higher in the patients than in the controls. However, this may just represent chance fluctuations since the absolute difference is fairly small. The percentages of CD19+ B-lymphocytes, CD4+ T-helper, and CD8+ T-suppressor lymphocytes revealed no significant difference between the two groups.

No previous report on the serum protein levels of SM casualties has been published to date. Our results indicated a significant increase in the absolute levels of α1, α2, and β globulins of the patients compared to the control group, while albumin and γ globulin revealed no significant difference between the two groups. Percentage of albumin and the albumin/globulin ratio were lower in patients than in the controls, whereas total protein level was significantly higher in the patients.

The limitations of this study were its small sample size and a lack of clinical data on confounding variables such as medications taken by the patients and their potential immunosuppressive effects. Further studies are needed to assess the long-term toxic effects of mustard gas on the blood and the immune system.

Acknowledgements

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