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IMMUNOHAEMATOLOGICAL AND CYTOGENETICAL STUDIES ON HUMAN POPULATION EXPOSED TO SULFUR MUSTARD

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Abstract

The patients evaluated in this study were classified into three groups of severe, moderate, and mild according to the severity of exposure to sulfur mustard. We evaluated the Immunological and Cytogenetical status of the patients after eight years of their exposure to sulfur mustard. The investigations revealed that the number of neutrophils, lymphocytes and monocytes in the patients with severe exposure were significantly different than in the normal population, while moderate and mild groups showed no significant differences. We also evaluated the DNA indices (hypo diploid, diploid & hyperploid) in the severe, moderate and mild groups comparing to normal population. The results indicate that in patients with severe exposure 22 out of 26 patients had aneuploidy, while moderate and mild groups showed no significant differences compared to the normal population. Regarding the numerical aberration rate and chromosomal breakage, our results indicated that there were no significant differences among moderate exposure, mild exposure and normal population.

Keywords: Chromosome breakage; DNA index; Sulfur mustard

Introduction

Although international regulations strictly prohibit the use of chemical warfare, sulfur mustard has been employed in various regions of the world during the past decade and excessive resources have been allocated to this weapon [1]. Desperately, sulfur mustard is still a potential threat to the world so many years after the end

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of World- War I and effective therapeutic measures remain to be established for the victims of this markedly incapacitating agent.

The treatment and cure of patients exposed to sulfur mustard is a remaining challenge despite ongoing researches in this field. Severe suppression of the immune system still is the major cause of opportunistic infections, septicemia and death in these patients. Several reports concerning the clinical situation of patients exposed to sulfur mustard during eight years of Iraq-Iran war have been published in Iran [2-4]. The main pathological findings were depigmentation and wet lesions of the skin [5,6]; ophthalmologic manifestations including conjunctivitis and corneal abrasion [7,8], gastrointestinal symptoms, such as dysphagia, diarrhea & vomiting [9], hematological complications, such as leukopenia and bone marrow depletion [7-9], liver and kidney failure [7], deposit of sulfur mustard in tissue organs [10], and septicemia [8].

Some chronic pathological situations were also observed: the increased incidence of acute lymphatic leukemia, acute myeloid leukemia [11] and recurrent infections [12].

The most threatening effect of sulfur mustard exposure today has been determined by many investigators to be a severe suppression of the immune system [8], which, in the case of lesions and blisters, can lead to opportunistic infections, *i.e.* infections resistant to conventional antibiotics, septicemia, and subsequently death.

The presence of unmetabolized sulfur mustard in postmortem tissue samples from Iranian patients has been reported [10]. The concentration of sulfur mustard in the fat rich tissues such as thigh, abdominal skin and skin with subcutaneous fat were higher than other tissues [13]. Lung, spleen, liver, blood and urine also showed low concentrations of unmetabolized sulfur mustard [10,14]. The aim of this research was to look at the immune status including lymphocyte, granulocyte and monocyte and cytogenetic status including chromosome breakage and DNA index, which can help us in understanding the basic and clinical complications of our patients.

Materials and Methods

Patients

Peripheral blood samples from twenty healthy male subjects and seventy-five male patients after eight years of exposure to sulfur mustard were collected using standard procedures. Patients were classified into three groups of severe, moderate and mild (25 patients in each group) according to different levels of contamination, spirometry and clinical manifestations (Table1). Patients attending the hospital for routine check up or facing some complications were chosen according to the standard method, and their blood samples were collected before the patients received medical treatment. The patients and the control persons were age matched.

Measurement of DNA Index

DNA index was measured according to the method mentioned by Schafhauser *et al*. 1999 [15]. Five ml of Blood was collected from each patient and added to 3 ml of Ficoll Hypaqu and the lymphocyte layer was isolated and washed with buffer saline. Two hundred micrograms of lymphocyte was transformed to another test tube and mixed with 2 ml of 70% cold ethanol and incubated for 30 min at 4°C. The cells were then centrifuged and resuspended in 800 µl of buffer saline and 100 µl of 1 mg/ml Rnase and 100 µl of 400 mg/ml Propodium Iodide added and incubated for 30 min at 37°C. Samples were analyzed by flowcytometer wavelength 488 nm through FLAS and SS gates (Fig. 1a). Then the results were measured according to the following formula:

DNA index = measurement of Sample / measurement of Diploid

Table 1. Grouping of patients exposed to sulfur mustard gas

Groups	Spirometry*	% Severity**
Normal State	>80	0
Mild	65 -80	5-20
Moderate	50-65	25-45
Severe	40-50	50-70

* The value presented in this table represent the lung efficiency based on FVC (mean Forced Vital Capacity), FEVI (mean Forced Expiratory Volume in the first second)

** % of severity was assessed according to spirometry and clinical manifestations

Karyotyping

Karyotype was determined according to the method mentioned by Berger *et al*. (1980) [16]. Some five milliliter of blood sample was collected from patients, and 0.5 ml of it was mixed with Roseuyl Park Memorial Institute (RPMI) containing 10% fetal calf serum, 15 µl

and 150 µl of Phytohemagglutinin (PHA) (1 mg/ml) and incubated for 72 h in CO2 incubator at 37°C. At the end of the third day, 5 µg of Colchicine was added to the sample and centrifuged for 5 min at 200 g. 10 ml of 0.075 M potassium Chloride was added to the cells and incubated in a water bath for 10 minutes at 37°C. The samples were then centrifuged at 200 g for 5 min and the supernatant was discarded and fixed with Carnoy's fixative. Cells re-centrifuged and stained with 5% Gimsa stain.

Statistics

One-way analysis of variance (ANOVA) and Kruskal-Wallis nonparametric test using SPSS software were used for evaluating the statistical significance.

Results

Long-term Effects of Sulfur Mustard on Immunological Parameters

In order to evaluate the percentages of lymphocyte, monocyte and neutrophil in all groups of the patients, 75 patients (25 in each group) were evaluated. The results indicated a significant increase ($p<0.05$) in the total number of WBC in the severe and moderate groups as compared with the controls, while no significant difference was noticed when comparing the mild group with the control (Table 2).

Long-term Effects of Sulfur Mustard on DNA Analysis

Ploidy studies showed 22 patients out of 26 studied patients in severe group had aneuploid population (hyper and hypo diploid in their peripheral blood) (Fig. 1b), while no significant differences were noticed in the moderate and mild groups comparing to the control groups (Table 3).

Long-term Effects of Sulfur Mustard on Chromosomal Breakage

No significant differences of the chromosomal breakages were noticed in moderate and mild groups comparing to control group (Table 4). Figure 2a shows the metaphase stage of the lymphocyte of the patients mildly exposed to sulfur mustard and Figure 2b shows the metaphase stage of the lymphocyte of the moderately exposed patients and Figure 2c shows that of severely exposed patients.

Discussion

Today, after ten years of their exposure to sulfur mustard, our patients are facing two main problems: high incidence of tumors [11] and recurrent infections [12]. In addition, there is a possible shift in the severity of illnesses from mild to either moderate or severe form.

Previous reports suggested that human exposure to sulfur mustard results in impaired immune endpoints, especially B and T helper lymphocyte numbers [17]. Low dose exposure to alkylating agents was shown to impair cell-mediated immunity, whereas high dose exposure could influence both cellular and humoral immune response [18,19]. So far, our study showed no significant differences between the lymphocytes of the patients with mild and moderate exposure comparing to the control population. While still moderate group and to some extent mild group show a significant increase in the incidence of tumors [11]. In case of sever patients, these results agreed with the previous studies in which lymphocytes showed a significant difference with the control population and still severe group having the highest incidence of the leukemia and lymphoma [11] and significant number of them suffering from recurrent infection [12].

In general, the appearance of clinical manifestations of patients with tumor indicates the presence of 1×10^{10} tumor cells [20] (in case of solid tumors) and less than that in leukemia and lymphoma. So correlation between the impairment of immune cells and tumor incidence is not so much significant comparing to the DNA indices. Malignant cells are frequently aneuploid and it is a prognostic factor in many human tumors, so that measurement of DNA content is of increasing importance in diagnostic pathology. Previous studies on the people treated with nitrogen mustard showed an increase of hypo- and hyperploids, which are indicative of a secondary tumor [21]

In this study, we aimed to understand the status of ploidy of the patients and the farther future of them. In the case of severely exposed patients, ploidy studies showed that 22 out of 26 studied patients had an aneuploid cell population (hyper or hypoploid) in their peripheral blood lymphocytes. Chi-square test for comparing aneuploidy state in patients and control population was significant [22,23]. In moderate and mild groups there was no difference in the ploidy comparing to the control group. These results correlate the high incidence of tumor in the severe group.

Concerning the numerical aberration rate and chromosomal breakage in the previous studies, a significant aberration rate (specially chromosome 5&7)

Table 2. The percentage of the cell type constituents in patients and control groups

Patients	No. of cases	WBC count Mean±SD	Neutrophil % Mean±SD	Lymphocyte % Mean±SD	Monocyte % Mean±SD
Severe					
Patient	25	9269±2457	71±8.1[b]	22±6.9[a]	2.35±1.4[a]
Control	10	7607±1244	54.7±7.8	33.6±6.5	9.8±11.8
Moderate					
Patient	25	8752±1730	63.23±5.8	30±6.05	4.8±2.5
Control	10	7480±1508	55±4.2	34±2.8	4.8±1.4
Mild					
Patients	25	8122±1767	57.66±3.3	35.63±2.8	5.2±0.96
Control	10	7110±1507	56.9±7	35.5±3.7	5.13±0.82

[a] $P<0.05$; [b] $P<0.1$ indicates a significant difference in comparison with control group as shown by Kruskal-Wallis test

WBC: White Blood Cell; SD: Standard Deviation

Table 3. The ratio of ploidy in metaphase of the peripheral blood of severe, moderate, mild and control groups

Subject	No. of cases	Hypoploid Mean±SD	Diploid Mean±SD	Hyperdiploid Mean±SD
Severe				
Patient	25	0.89±0.109	1.00±0.04	1.08± 0.08
Control	10	0.89±0.045	1.00±0.02	1.08±0.01
Moderate				
Patient	25	0.05±0.064	0.9±0.09	0.06±0.036
Control	10	0.155±0.065	0.93±0.07	0.06±0.01
Mild				
Patient	25	0.89±0.03	0.90±0.13	0.99±0.79
Control	10	0.92±0.03	0.99±0.056	1.26±0.1

Table 4. The percentage of chromosomal breakage in the peripheral blood of patients and control groups

Severity	No. of cases	Mean±SD
Moderate		
Patient	25	4.83±5.85
Control	10	3.15±5.62
Mild		
Patient	25	2.67±4.30
Control	10	2.5±4.20

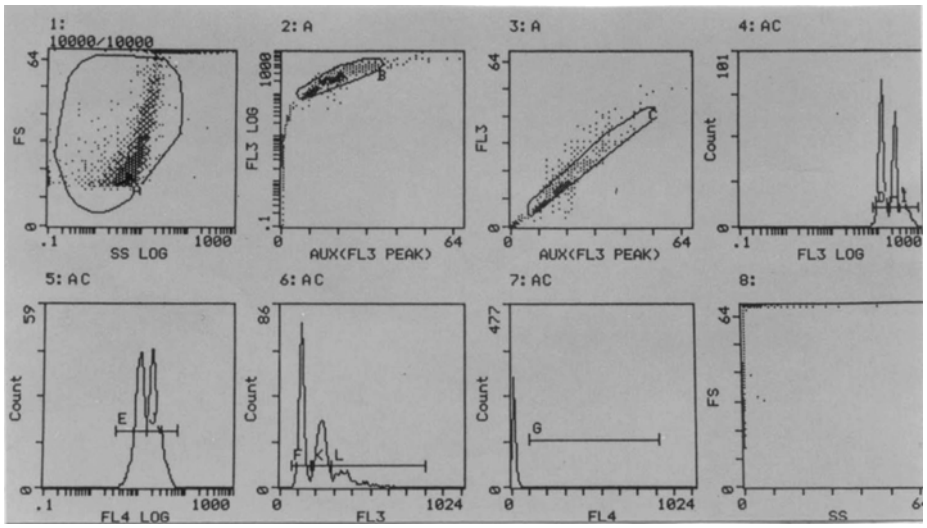

Figure 1a. Histogram of the flowcytometer.

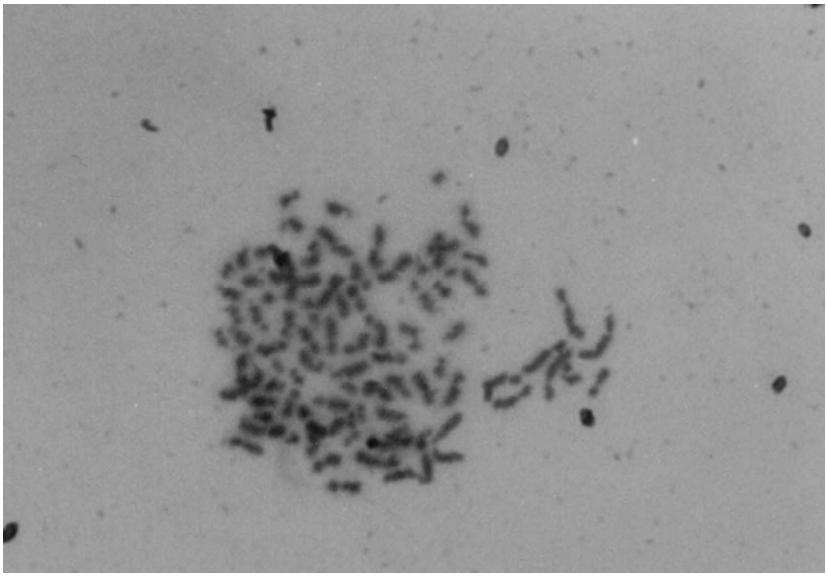

Figure 1b. Metaphase stage of the lymphocyte of the patients exposed to sulfur mustard (hyperdiplody).

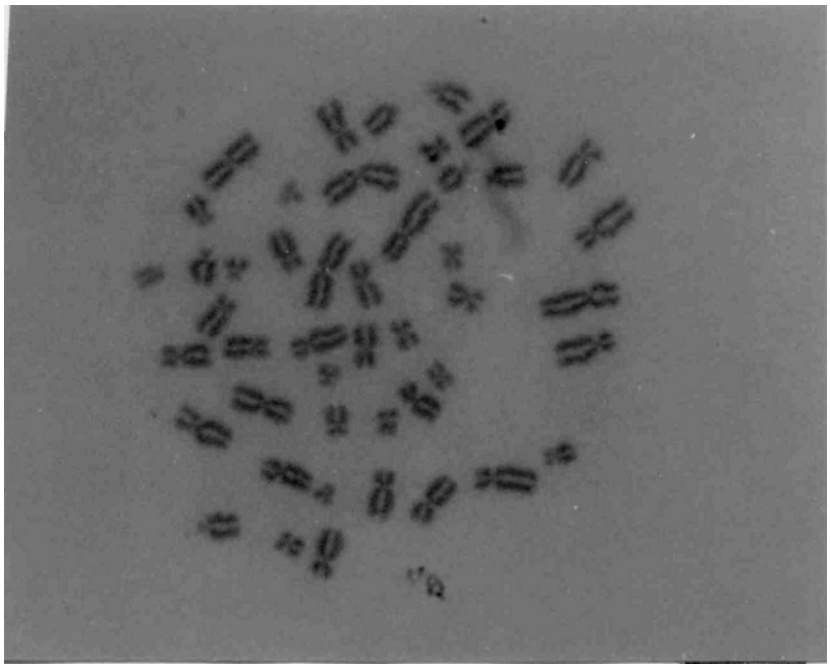

Figure 2a. Metaphase stage of the lymphocyte of the patients mildly exposed to sulfur mustard (normal picture).

and chromosomal breakage (specially chromosome 5, 7&9) were shown following seven years after exposure with nitrogen mustard [24,25]. In our study, we could not find any significant difference in the numerical aberration rate and chromosomal breakage among mild, moderate and control groups.

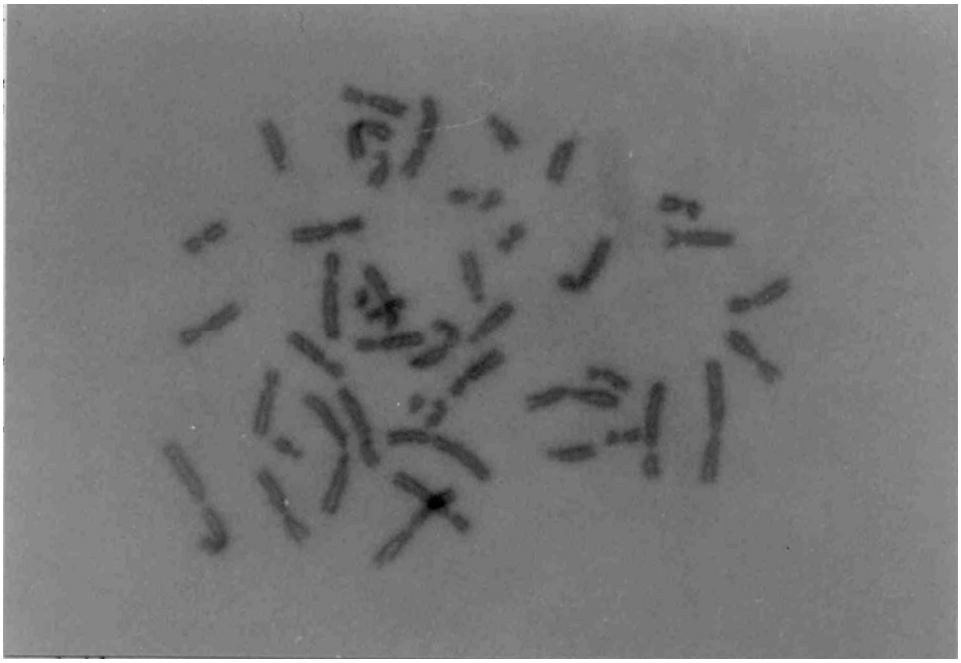

Figure 2b. Metaphase stage of the lymphocyte of the patients moderately exposed to sulfur mustard (45 chromosomes).

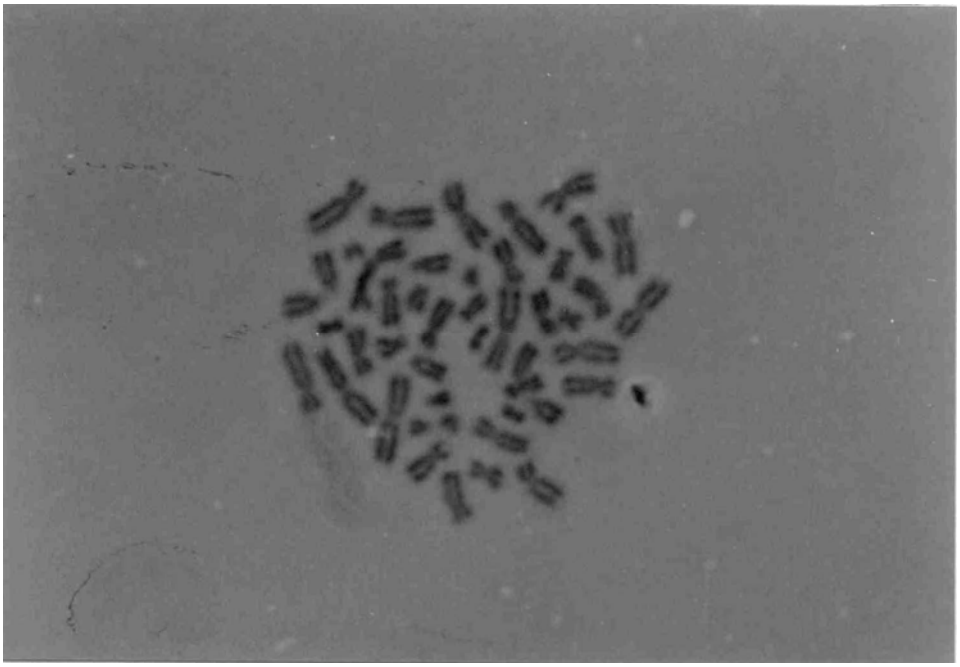

Figure 2c. Metaphase stage of the lymphocyte of the patients severely exposed to sulfur mustard (47 chromosomes).

Acknowledgements

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