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Original article

Level of Eosinophil Cationic Protein in Sputum of Chemical Warfare Victims

[1]Majid Mirsadraee, [2]Hassan Ghobadi-Marallu, [3]Mohammad Reza Khakzad , [4]Shahrzad M Lari, *[4]Davood Attaran , [4]Mohammad Towhidi, [5]Mohammad Khajedalouee, [6]Amir Hosein Jafarian

Abstract

Objective(s)

Considering fair response to inhaled corticosteroids and reports of severe air way hyper responsiveness in chemical warfare victims (CWV), a role for eosinophilic inflammation (i.e. asthma) was postulated. The objective of this study was to determine the presence of eosinophilic inflammation in CWV by evaluation of Sputum cellularity and eosinophil cationic protein (ECP).

Materials and Methods

Forty CWV and 15 control subjects entered this cross sectional study. Demographic data, dyspnea severity scale, spirometry results and 6 min walk test were determined. Sputum was collected with inducing by nebulizing hypertonic saline and analyzed for total inflammatory cell count, the cellular differential count and ECP level. Control group was normal volunteers with PC_{20} more than 8 mg/ml.

Results

Mean±SD of eosinophil percentage (11.7±11.1%) and ECP level in sputum of CWV (46.1±19.5 ng/ml) were significantly more than control group. Regression analysis showed significant correlation between ECP level and percentage of eosinophils in sputum (r= +0.43, $P< 0.01$). ECP level of CWV subjects with obstructive pattern did not show any significant difference from CWV with normal spirometry. ECP level in CWV subjects who revealed more than 12% improvement in forced expiratory volume in one second (FEV1) was significantly higher than CWV who had improvement less than 12% ($P= 0.01$). BO and asthma as final clinical diagnosis of CWV did not show any significant difference of sputum ECP.

Conclusion

Bronchial inflammation in different types of pulmonary complication of CWV is eosinophil dependent. ECP level of sputum in CWV could guide physician to select CWV who would respond to corticosteroids.

Keywords: Chemical warfare agents, Eosinophil, Eosinophil cationic protein, Mustard gas, Sputum

1-Department of Pulmonary Medicine, Islamic Azad University- Mashhad Branch, Mashhad, Iran
2- Department of Pulmonary Medicine, Ardabil University of Medical Science, Ardabil, Iran
3- Department of Immunology, Islamic Azad University-Mashhad Branch, Mashhad, Iran
4- Department of Pulmonary Medicine, Lung Diseases and TB Research Center Mashhad University of Medical Science, Mashhad, Iran
*Corresponding authors: Tel: +98-511-8440303; Fax: +98-511-8817385; email: attarand@mums.ac.ir
5-Department of Epidemiology, Mashhad University of Medical Science, Mashhad, Iran
6- Department of Pathology, Mashhad University of Medical Science, Mashhad, Iran

Introduction

Sulfur mustard (SM) is a vesicant chemical weapon which was used widely by Iraq between 1983 and 1988 during Iran-Iraq war. This chemical warfare causes delayed complications in organs such as lung, eyes, skin, and peripheral nerves (1,2). Lung lesions are the most prevalent (42%) of these complications (3). The most frequent pulmonary complications of chemical warfare agent occur in air ways. Early report during and after the Iran and Iraq war corroborated chronic bronchitis and bronchiectasis (2). But after precise investigation, Ghaneie and co workers proved that predominant pathologic and imaging findings in chemical warfare victims (CWV) was bronchiolitis obliterans (4, 5). Natural course of this disease is typically progressive and usually does not respond to treatment. But CWV usually managed with high dose inhaled corticosteroids (ICS) and long acting beta 2 agonists. In another study, Ghanei *et al* treated CWV with systemic corticosteroids (6). These results raised the question that: Does pulmonary lesions of chemical warfare cause eosinophil dependent lung lesion such as asthma? Previous reports showed that airway hyper responsiveness was found to be present in 75% of CWV (7). In fact in our experience there is a group of CWV who were asymptomatic for a period of time after their exposure and then presented with asthma symptoms that partially responded to ICS therapy.

The aim of this study was to determine the presence of eosinophilic inflammation by evaluating the cellularity of sputum and eosinophil cationic protein (ECP) to prove the presence of eosinophilic inflammation in CWV.

Materials and Methods

Subjects

Forty CWV entered this study. All had proved history of exposure to chemical weapons such as sulfur mustard or nerve agents and experienced skin symptoms after exposure for a period of time. These subjects suffered from obstructive lung disease such as asthma or bronchiolitis obliterance and for this reason they were treated with ICS (beclomethasone dipropionate or fluticasone dipropionate) 300-1000 μg/day for 3-15 years. Additional drugs like short or long acting inhaled beta 2 agonists such as salbutamol or salmetrol were also used.

Control group was 15 normal subjects who didn't have any complaint of respiratory symptoms and in methacholine challenge test their PC20 (Provocation concentration producing a 20% fall in forced expiratory volume in one second (FEV1)) was more than 8 mg/ml. The body mass index (BMI), age, and sex were matched with case group. All the case and control subjects were male, nonsmokers who didn't consume any systemic steroids. Subjects with peripheral blood eosinophilia or other known eosiniphilic disease were also excluded.

The experiments were approved by the Ethical Committee of Mashhad University of Medical Sciences, Mashhad, Iran and each subject gave an informed consent.

Technique and protocol

The study was designed as prospective, case control study, and performed in pulmonary function laboratory of the Ghaem hospital, Mashhad, Iran (2007-2008). A questionnaire regarding the respiratory symptoms was designed and completed. Severity of dyspnea was evaluated by Modified Medical Research Council score (MMRC) questionnaire.

Spirometry and imaging

At the beginning of each collection of specimen, standard spirometry was performed using a standard spirometer (Chest Inc, Tokyo Japan). Before spirometery, the operator demonstrated the required maneuver, and subjects were encouraged and supervised throughout test performance. Main variables measured consisted of forced vital capacity (FVC), FEV1 and FEV1/FVC. Measurements of these variables were performed using the acceptability standards outlined by the American Thoracic Society (ATS) (8), in a sitting position inside the box and wearing nose clips. Values were expressed as percentage of the predicted values. Response to bronchodilator was assessed by prescribing 400 μg of salbutamol inhaler via spacer and the FVC maneuver was repeated. Severities of

obstructive lung disease were classified according to American Thoracic Society recommendations to mild (FEV1 60-80% predicted), moderate (40-59% predicted) and severe (less than 40% predicted) disease (9). Type and dose of ICS were recorded and considered for evaluation in case group. Daily steroid dosage was classified according to Global Initiative for Asthma guideline (10). Exercise tolerance was evaluated by 6 min walk test (6MWT). High resolution computed tomograpghy (HRCT) of lung in mediastinal and paranchymal windows in deep inspiration and expiration were used to classify specific disease. Final diagnosis was made by using clinical findings, spirometry and HRCT.

Collecting sputum specimen

Before procedure, 400 μg salbutamol was delivered to CWV via a large spacer to prevent bronchospasm. Inducing sputum was performed by nebulizing hypertonic saline (nebulizer used was Omron NE -U17). The concentration of used saline was 3%, 4% and 5% and subjects had 5 min rest between each step. In each step subjects could expectorate enough sputum. By the end of procedure the specimens were sent to laboratory in less than two hours.

Sputum processing

The saliva contamination of sputum was winnowed by sampler, and the volume of the samples was measured by graduated cup. For homogenization, the specimen was transferred to a petri dish and was treated by adding four times volumes of 0.1% dithiotheritol (DTT) (sputolysin 10%; Calbiochem corp.; San Diego, CA). The petri dish was placed on a rotator mixer for 15 min to ensure complete homogenization. To remove cell debris and mucus, the suspensions were filtered through 48 μm nylon gauze (B&SH Thompson, Scarbough, Ontario, Canada); for preserving bronchial cells. The resulting suspension was centrifuged at 1400 g for 10 min at 4 °C. The supernatant was aspirated and were frozen at -24 °C for subsequent measurement of ECP.

The cell suspension was aspirated and adjusted to one milliliter by PBS buffer. Homogenized sputum was used to determine the total cell count with a standard hemocytometer. For cell type determination, two smears of the unfixed sputum were prepared (air-dried) and stained by the hematoxylin & eosin. Three hundred non-squamous cells were counted in each slide. The mean of results of two slides were recoded. The samples with more than 20% squamous cells were considered as 70% contaminated with saliva and samples with more than 42% squamous cells were considered as more than 80% contaminated (11). Sputum with more than 42% squamous cells was excluded from study. As a control, comparison of CWV and control groups by chi square method showed that frequency of sputum contamination was not significant different in two groups (χ^2= 0.32, *P*= 0.84). The concentration of ECP was determined using an ELISA kit (MBL Co.LTD; Naka-ka Nagoya, Japan. Code No.7618E). The lowest detection limit was 2 μg/l.

Statistical analysis

Sample size was calculated according to 5% alpha error, 80% power and 2/1 ratio of control to case group (40 CWV and 15 control subjects). Kolmogerov- Smirnof test was done for evaluating the homogeneity of samples and nonparametric tests was used when the data showed no homogenous pattern. Mean values for age, spirometric data and ECP level were quoted as arithmetic mean and standard deviation (SD). For comparing values of spirometric data and ECP between normal and chemical war victims unpaired "t" test was used. Analysis of variance was used to test differences between the CWV severity groups. Statistical significance was accepted at $P< 0.05$.

Results

General data

Forty CWV with average age of 47.80±10.3 years (range 35-78) were enrolled in this study. Control group were 15 subjects with average age of 40±13.7 years (range 18-62) (Table 1).

The most frequent clinical findings were dyspnea (in all CWV) followed by cough in 38 subjects (95%). Hemoptysis was presented in 3 (7.5%) CWV. Physical exam showed

wheezing in 13 CWV (33.3%) and rales in 5 CWV (12.8%).

Spirometry results

The most frequent spirometry pattern in CWV group was obstructive in 27 subjects, however normal spirometry and restrictive pattern were also detected in 6 and 3 cases respectively. Spirometry data in obstructive group of CWV showed low level of FVC percentage of predicted (62±14%) and FEV1 percentage of predicted (52±15%) (Table 1). Significant improvement in FEV1 after bronchodilator was seen in only 6 cases of CWV group.

Cellularity of sputum

Five specimen of CWV and one specimen of control group were excluded from study for the reason that mucoid part of sputum was absent. Mean±SD of total inflammatory cells in CWV was 1465±2354 cells per ml that did not show significant difference from control group (Table 1). Mean±SD of eosinophilwas 11.7±11.1 % that was significantly more than control group (*P*= 0.02), in comparison lymphocyte in control group was significantly more than CWV group (Table 2). Neutrophile, macrophage and epithelial cells were not significantly different between groups. Approximately half of the cases in both groups showed epithelial cells less than 21% and less than 27% showed epithelial cells more than 42%.

ECP level in sputum

ECP level in sputum of CWV was 46.1±19.5 ng/ml that was significantly more than control group (Table 1). Eighty five percent of CWV subjects showed ECP level more than mean ECP level of control group. Twenty five CWVsubjects with obstructive pattern in spirometry revealed ECP level more than 45 ng/ml and in 5 subjects ECP was less than this level. Regression analysis showed significant correlation between ECP level and percentage of eosinophilin sputum (r= +0.43, *P*< 0.01) (Figure 1). On the contrary regression analysis did not show any significant correlation between FEV1 and ECP level (r= 0.12, P= NS). ECP level of CWV subjects who showed obstructive pattern was 46±2.4 ng/ml that did show any significant difference from CWV with normal spirometry (47.2±19.5 ng/ml, t= 0.2, *P*= 0.98).

According to response to bronchodilator and improvement in FEV1, CWV subjects were categorized into two groups: 1- responder (asthma like) 2- non responder (COPD or bronchiolitis like). Comparison of sputum differential cell count did not show any significant difference between eosinophil and neutrophil percentage in these two groups. However, ECP level in bronchodilator responder group was significantly higher than non-responder group (*P*= 0.01).

Final diagnosis of pulmonary complication of CWV was established according to spirometry and HRCT findings. Although higher levels of eosinophlie count seem to be presented in asthma groups but statistical analysis showed not significant difference between groups (Table 3).

Mean of 6MWT in CWV was 351±99 m. Statistical analysis showed correlation of 6MWT and sputum eosinophil and ECP levels were not significant. Dyspnea scale revealed that stage 2 of MMRC had the highest frequency (45%) followed by stage 3 (25%). Stage 1 and 4 consisted only 21% and 9% of CWV (none for stage 5). Mean of eosinophilcount and sputum ECP were not significantly different in MMRC different stages. The correlation of MMRC and sputum ECP and eosinophilcount was evaluated and did not show any significant correlation (Table 4).

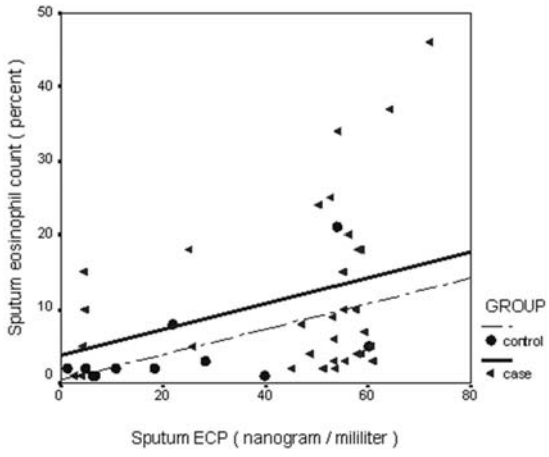

Figure 1. Correlations between sputum ECP and sputum eosinophilia in mustard gas lung injured patients and healthy control groups.

Table 1. Comparison of major demographic, spirometric data, cellularity and eosinophilic cationic protein (ECP) between chemical warfare victims (CWV) and normal control groups.

	Age Yrs	Sex M/F	FEV1 % pred	FEV1/FVC %	Sputum ECP µg/ml	Sputum eosinophile cells/mm^3
CWV (N=40)	47.8±10.3 (35-78)	40/0	59.2±18 (27-105)	64.1±13.4 (33-91)	46.1±19.5 (2-72)	229±490 (2-2304)
Normal (N=15)	40±13.7 (18-62)	8/7	90.5±30.1 (63-118)	101.0±18.6 (84-118)	23.6±19 (1.2-60)	64±73.6 (1-250)
P value	0.19	<0.0001	<0.0001	0.001	0.001	0.02

Table 2. Comparison of sputum volume, cellurity and eosinophilic cationic protein (ECP) between chemical warfare victims (CWV) and normal control groups.

	CWV	Control	*P* value
Volume (µl)	974±897	1000±781	0.92
Total cells (count per ml)	1965±2354	1360±1325	0.62
Eosinophil(%)	11.7±11.1	4.2±5.9	0.04
Neutrophil(%)	16.5±10.8	16.7±16.9	0.97
Lymphocyte (%)	6.6±6.5	11.1±6.8	<0.01
Macropage (%)	67.1±19.8	68.8±19.1	0.8
Epithelial cell (%)	25.2±17.5	27±8.5	0.75
ECP (ng/ml)	46.1±19.5	23.6±19.5	0.001

Table 3. Comparison of sputum eosinophil(percentage and total) and eosinophilic cationic protein (ECP) in specific lung disease of chemical warfare victims (CWV) groups.

	Eosinophilpercentage	Eosinophilcount	ECP (ng/ml)	High ECP (>45 ng/ml)
BO	12±13	207±418	47±19	80%
Asthma	14±10	444±752	42±22	70%
Brronchiectasis	4.5±3.5	45±55	52±10	100%
Interstitial Fibrosis	11±10	42±51	53±6.7	100%

Table 4. Correlation of sputum eosinophilic cationic protein (ECP) and eosinophilwith 6MWT and MMRC.

	6MWT		MMRC	
	R	*P* value	R	*P* value
ECP	+0.16	0.4	+0.26	0.17
Eosinophil	-0.12	0.3	+0.31	0.12

Discussion

This study was conducted on CWV who suffered from long lasting pulmonary disease. The main anticipated differential diagnoses were bronchiolitis obliteranse, asthma, bronchiectasis and chronic bronchitis (2, 4). Pulmonary function test mostly showed obstructive pattern that can be considered for all the differential diagnosis mentioned above. BO was observed in 44% of CWV 80% of whom revealed high eosinophilpercentage and ECP levels. Six subjects revealed normal spirometry who their ECP levels were high that can be classified as mild asthma. Five subjects revealed low sputum ECP levels and was classified as BO. Also, three subjects showed restrictive pattern, according to HRCT two subjects proved to suffer from IPF. Both of these subjects showed high level of

eosinophiland ECP. Due to normal HRCT, the remainder of the cases was classified as asthma that were not very cooperative during spirometry. Response to bronchodilator was helpful to diagnosis asthma in only 6 subjects. Totally we conclude that from 36 CWV subjects, 16 subjects should have suffered from BO and 15 subjects suffered from asthma. High Sputum ECP was found in fraction of subjects of all groups.

For differentiating between asthma and chronic airway disease such as bronchiolitis obliteranse and COPD, several devices were used. One of them is spirometry and especially improvement of FEV1 after using bronchodilator. In this regard every subject who showed more than 12% increase in FEV1 or FVC classified as asthma (9). But in severe longstanding asthma with remodeling of bronchial structure, reversibility of air way obstruction may decrease, the term that we called "Irreversible asthma" (12). Therefore asthma cannot be ruled out in subjects who do not improve post bronchodilator use. Bronchodilator response was evaluated in 22 subjects of this study. Eighteen cases (82%) of these subjects did not significantly improve with bronchodilator, although all of them showed high level of sputum ECP.

High resolution computed tomography (HRCT) of lung especially when comparing inspiration and deep expiration imaging is another helpful study for diagnosis of bronchiolitis obliteranse, COPD (emphysema) and bronchiectasis. Results of previous study on HRCT of CWV showed air trapping, bronchial thickening and bronchial dilatation (4). In this report micronodular pattern was not seen. These pictures were also described in severe resistant asthma with airway remodeling (13, 14). As a result, using HRCT for differentiating asthma from bronchiolitis obliteranse is not very helpful.

In this study we used sputum for defining the pattern of bronchial inflammation in CWV. Collecting sputum by inducing sputum was very effective and without complication. Increasing ECP level was also detected in obstructive lung disease other than asthma. In a study on COPD patients, ECP level was detected up to 22±6 µg/l (15). In another study on lung transplant recipients who suffered from BO, ECP in BALF was in the range of 0-200 µg/l (median 40). In this study the eosinophilic count in BALF was low or zero. Analysis of sputum obtained by induced sputum in IPF also showed 2.1% eosinophiland elevated ECP (median 1.1 mg/ml) (16). In this study the authors suggested that the eosinophiles were originated from airways and cough symptoms in subjects showed eosinophilia and/or elevated ECP in sputum may respond to steroids better than other IPF patients. In our series 2 subjects showed IPF and both revealed high level of ECP which could get benefit from steroids.

However this study showed that 85% of CWV demonstrate high level of sputum ECP in favor of eosiniphilic inflammation in air ways. This finding is not related to type of lung disease. We believe that eosinophilic inflammation is the result of chemical injury occurred in air ways. Fortunately inhaled corticosteroid and other steroids could suppress eosinophilic inflammation and may be useful for management of CWV. According to the results of this study 82% of CWV did not showed post bronchodilator response, so this test can not be used for differentiation between asthma and BO. Therefore sputum ECP can help physician to classified CWV to eosinophilic dependent CWV and non eosinophilic dependent CWV (regardless of type of pulmonary lesion). In CWV who suffer from eosinophilic inflammation, treatment with corticosteroids could be very effective. Other subjects with low ECP may benefit from long term treatment with Macrolids or immunosuppressive drug (17, 18).

Unfortunately ECP level did not reveal any correlation with MMRC, 6MWT or FEV1. Therefore pulmonary complication of CWV should be a little bit different from routine asthma and ECP level can not be used for evaluating the severity of pulmonary complication of CWV.

Conclusion

According to categorization through sputum

eosinophiland ECP, most of CWV suffer from eosinophilic inflammation in lung (including asthma). In addition to spirometry and HRCT, sputum ECP can be recommended for diagnosis, classification and directing corticosteroid therapy for pulmonary complication of chemical weapon.

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