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Fibrogenic cytokine levels in bronchoalveolar lavage aspirates 15 years after exposure to sulfur mustard

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Aghanouri, Reza, Mostafa Ghanei, Jafar Aslani, Hossein Keivani-Amine, Ferdos Rastegar, and Ali Karkhane. Fibrogenic cytokine levels in bronchoalveolar lavage aspirates 15 years after exposure to sulfur mustard. *Am J Physiol Lung Cell Mol Physiol* 287: L1160–L1164, 2004. First published July 30, 2004; doi:10.1152/ajplung.00169.2003.—Over 100,000 Iranian war veterans suffer from chronic effects of mustard gas exposure. Sulfur mustard was used by Iraq during the Iraqi-imposed war on Iran (between 1980 and 1988). The major complaints of these patients are mild interstitial fibrosis and bronchiolitis. We aimed to determine the state of fibrosis progression and assessed transforming growth factor (TGF)-β1 levels in pulmonary samples and in bronchoalveolar lavage (BAL) aspirates. A total of 126 war veterans confirmed for lung disease were assessed and compared with three control groups: *1*) 64 veterans not exposed to chemical agents, *2*) 12 idiopathic pulmonary fibrosis civilian patients, and *3*) 33 normal persons. BAL was performed via a flexible fiberoptic bronchoscope and the standard manual method. Total protein was measured by Bradford assay, and samples were corrected with regard to coefficients. Samples were concentrated 15-fold by lyophilization and resolubilization. Samples were double-checked using an ELISA test kit. The Mann-Whitney test was used for the data analysis using commercial software. We detected that significant differences between TGF-β1 levels between the case group and control *group 1* ($P = 0.001$) and control *group 3* ($P = 0.003$). No significant differences were found between the case group and control *group 2* ($P = 0.57$). Inflammation and fibrotic processes in lung tissue of patients exposed to sulfur mustard may be progressive so IFN-γ may be a useful drug to these patients' treatment.

transforming growth factor-β; bronchiolitis; mustard gas; idiopathic pulmonary fibrosis

DURING THE LATE STAGES of the Iraq-Iran war (1984–1988), Iraq attacked Iranian combatants with various chemical warfare agents. Some of these agents such as nerve gas resulted in almost instant death, whereas others such as sulfur mustard had gradual and in many cases more long-term effects. Both war veterans as well as civilians were victims of these attacks and now suffer from residual effects of biochemical exposure. Exact information concerning the actual number of people exposed to these agents and consequent specific organ disorders is lacking. However, estimates indicate that >100,000 patients in Iran suffer from symptoms related to mustard gas exposure.

Mustard agents were the most frequently used chemical agents. The basic structure of this gas is $C_4H_8Cl_2S$. It is very irritating and lethal gas (18, 38). Lung injury is common after inhalation of this gas and leads to chronic bronchitis and interstitial lung diseases. A large number of the victims present with signs of chronic lung disease long after their exposure to mustard gas. The laboratory pathology in these patients resembles a mild fibrosis in their respiratory parenchyma. Bronchiolitis and dyspnea are common clinical findings. Exaggerated fibroblast proliferation and increased collagen synthesis represent two critical events in the pathogenesis of this type of pulmonary fibrosis (6, 33).

A substantial number of growth factors, most of which promote fibroblast replication and collagen accumulation, have been identified (1, 30, 33). Transforming growth factor (TGF)-β1 is the most abundant isoform found in the normal lung parenchyma. Expression of this gene is altered during pulmonary fibrosis (10, 11). Previous research has shown overexpression of TGF-β1 in macrophages, mesenchymal, and mesoendothelial cells in pulmonary fibrosis and suggests that fibrogenic factors are activated during inflammatory and progressive processes leading to bronchiolitis and other chronic lung diseases (3, 12, 17, 27, 29, 34). Previous other studies have shown that TGF-β1 suppresses IFN-γ inhibition of class 2 major histocompatibility complex (MHC-II) gene expression by inhibiting expression of class 2 transactivator mRNA (16). For these reasons IFN-γ is being used under new drug procedures for treatment of idiopathic pulmonary fibrosis (IPF) patients (31, 39).

We attempted to determine the amounts of TGF-β1 in war veterans exposed to mustard agents to determine the progressive nature of respiratory tissue injuries and whether IFN-γ treatment can be of value in the treatment protocol of these patients.

MATERIALS AND METHODS

Case group. Inclusion criteria for the case group were confirmed exposure to mustard gas 15–16 yr ago during the Iran-Iraq war (clinical signs such as blisters or ocular injuries documented in patient charts) and mild fibrosis and bronchiolitis confirmed from high-resolution computed tomography (HRCT) scan and biopsy samples taken during bronchoscopy. Patients with other chronic lung diseases, autoimmune disease, chronic infectious disease, cancer, and acquired autoimmunodeficiency syndrome and smokers, addicts, and patients treated with corticosteroids were excluded. We selected 126 patients for further tests. All cases were male with a mean age of 41.5 ± 5.6 yr.

Control groups. Three control groups were chosen as follows: *1*) 64 male veterans who had been in combat zones but not exposed

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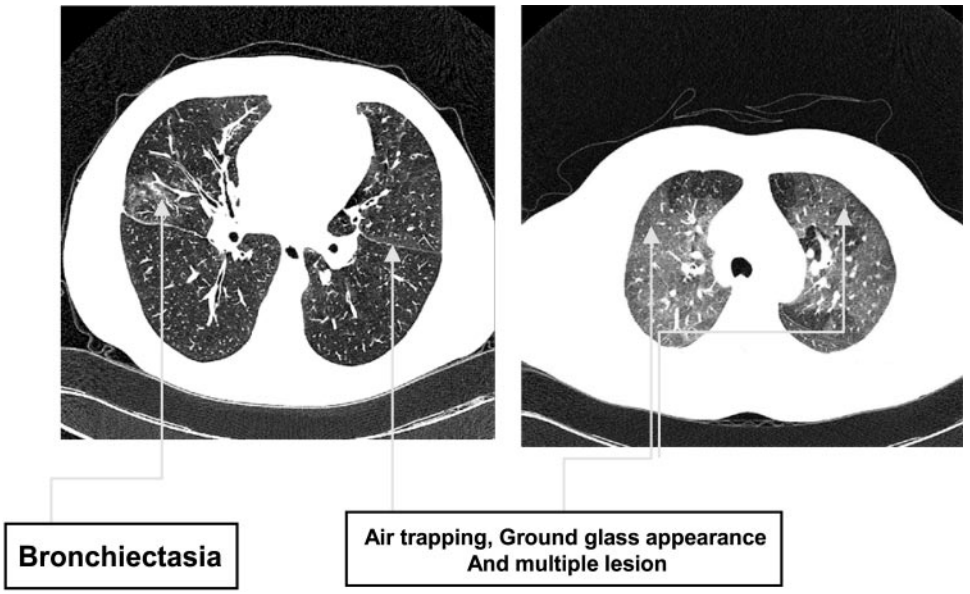

Fig. 1. Cases: high-resolution computed tomography view.

to chemical agents with a mean age of 42.25 ± 6.3 yr; *2*) 12 civilian patients (nine females with a mean age of 53.2 ± 2.4 yr and three males with a mean age of 63.6 ± 5.3 yr) who were not exposed to mustard agents but had IPF; and *3*) normal persons who had no symptoms of pulmonary disorder or significant disease in body organs (n = 33: 21 male with mean age of 40.2 ± 4.6 yr and 12 female with mean age of 32.1 ± 8.3 yr). All exclusion criteria were observed for case and control groups and for final tests; patients with exclusion criteria were omitted, and other case or controls were substituted.

Bronchoscopy and bronchoalveolar lavage sampling. Bronchoalveolar lavage (BAL) was performed in all subjects via a flexible fiber-optic bronchoscope (Olympus BF1T, Tokyo, Japan). The upper respiratory tract was anesthetized with 2% lidocaine. Atropine (0.75 mg intramuscularly) was administered before the procedure. Supplemental oxygen was given throughout the procedure, and the oxygen saturation was monitored by continuous pulse oxymeter. The bronchoscope was wedged for lavage in the middle lobe segmental bronchus, and four 60-ml aliquots of sterile physiological saline solution warmed to 37°C were infused. The fluid was immediately recovered by gentle suction after each instillation. The first aliquot, consisting of a bronchial sample, was sent for cell counting, whereas the others were pooled for study in two major Tehran hospitals. Bronchoscopy was advised for all patients for confirmation and follow-up of their diagnosis. The patients were appropriately informed of their situation and the reasons for the recommendations, and patient consent was obtained. All subjects were studied under protocols approved by institutional review board at Tehran University of Medical Sciences.

Transbronchial lung biopsy and pathological examination. Transbronchial lung biopsy was done via bronchoscope by using small pinchers attached to a long cable threaded through the bronchoscope. The operation sites were chosen at the discretion of the clinician. Biopsy forceps (18-mm cup; Bard Interventional Products, Billerica, MA) were employed, and two specimens were obtained from each patient. Pathologists examining the specimens were blinded as to the patient's clinical presentation. In IPF patients for whom the biopsy procedure produced no histological diagnosis, open lung biopsy was offered only to confirm diagnosis. Lung biopsies were not offered for other control groups. Paraffin-embedded lung samples were cut and microscopically screened for the presence of pathologic lesions using hematoxylin-eosin stain for general morphology and Verhoeff stain for exact identification of the margins of subepithelial fibrosis. In the latter sections, the extent of airway obstruction by bronchiolitis obliterans (BO) lesions was measured under an Olympus BX50 microscope (Olympus, Hamburg, Germany) and expressed as percentage of the airway surface.

HRCT scan. Chest HRCT examinations were obtained on one scanner (High Speed Advantage; General Electric Medical Systems, Milwaukee, WI). Each HRCT examination consisted of five 1.0-mm collimation images obtained during both deep inspiration and full

Table 1. *Descriptive pattern of study groups*

Groups	n	Male (mean age, yr)	Female (mean age, yr)	TGF-β1
Case*	126	126 (41.5±5.6)		43.63±26.5
Control group 1†	64	24 (42/25±6.3)		13.34±7.78
Control group 2‡	12	3 (63.6±5.3)	9 (53.2±2.4)	57.5±6.36
Control group 3§	33	21 (40.2±4.6)	12 (32.1±8.3)	9.7±4.54

Values are means ± SE. TGF, transforming growth factor. *Cases are frequent because of large patient number with pulmonary disease symptoms; †real control groups of documented war veterans without exposure; ‡patients with idiopathic pulmonary fibrosis (prevalence of this disease is 1/100,000 in normal population of Iran); §Normal persons without any signs of pulmonary disease or inclusion criteria for bronchoscopy and no confirmed pulmonary disease after bronchoscopy.

Table 2. *HRCT pathological patterns*

Groups	LUL	LLL	RUL	RML	RLL	
Reticular pattern	12.4	51.2	12	7.3	38.8	68
Honeycombing	5.2	7.2	0	2	4.2	11
Air brochogram	6.2	16.4	1	0	2.2	18.5
Ground-glass pattern	7.9	37	6.2	3.2	27.9	34
Bronchiectasia	11.8	8.5	9.5	3.8	7.3	32
Pleural thickening	8.2	9.3	3	4.8	3	16

Results, given as percentages, of high-resolution computed tomography (HRCT) diagnosed by a pulmonologist and a radiologist. The radiologist was blinded to the study groups (single blind). Intraobserver (P = 0.86, P = 0.91) and interobserver (0.74) differences for ground-glass appearance as an index were not significant. Conditions were supine and prone. LUL, left upper lobe; LLL, left lower lobe; RUL, right upper lobe; RML, right middle lobe; RLL, right lower lobe.

Table 3. *Transbronchial lung biopsy pathological pattern*

Definition	Percentage of Cases
No interstitial fibrosis	17
Variegated mild interstitial fibrosis	56
Variegated moderate interstitial fibrosis	20
Variegated severe interstitial fibrosis	6
Diffuse mild interstitial fibrosis	4
Usual interstitial pneumonias with bronchiolitis	99
Collagen deposition (mild)	96
Collagen deposition (none)	7
Hyaline deposition (none)	95
Total mild hyaline deposition	5

Needle biopsies from cases (%) were taken during bronchoscopy from pathological sites shown in HRCT. Pathological patterns were diagnosed by 2 pathologists who were blinded to the chemical exposure of the subjects. All samples were screened by 2 pathologists. Interobserver variation was not meaningful ($P = 0.9$). Total mild hyaline deposition was found in patients with severe inflammation.

expiration, respectively, with the patient lying in a supine position. Images were obtained at the levels of the aortic arch, midway between the aortic arch and tracheal carina, tracheal carina, midway between the tracheal carina and the right hemi diaphragm, and 1 cm above the right hemidiaphragm. All images were reconstructed using high-spatial-resolution algorithm and displayed at standard (level −700, width 1,500) and narrow (level −700, width 1,000) lung window settings.

Inspiratory images were read before expiratory ones, and images were displayed at standard windows before narrow window settings. The inspiratory images were assessed for the presence of bronchiectasis according to previously established computed tomography criteria. The mosaic parenchymal pattern was defined as areas of heterogeneous lung attenuation in a lobular or multilobular distribution in expiratory phase. The expiratory images were also assessed for the presence and lobar distribution of air trapping. The criteria used to diagnose the presence of air trapping were alteration of normal anterior posterior lobar attenuation gradients and/or lack of homogeneous increase in lung attenuation resulting in persistent areas of decreased attenuation. We quantified and classified the extent of air trapping using the same system as defined for hyperlucent regions on inspiratory images, considering that limited air trapping has been reported in normal individuals. Presence of air trapping was considered indicative of BO only if it exceeded 25% of the cross-sectional area of an affected lung on at least one scan level. Expiratory images were displayed at standard and narrow window settings. These were directly compared to determine differences in the conspicuity of air trapping (samples are seen in Fig. 1).

Determination of TGF-β1. In many measurements, no coefficient correction for BAL is applied to the samples collected. In our procedures, we measured the amount of total protein by Bradford assay and determined whether when correcting for equal amounts of protein TGF-β1 was overexpressed or not. We added a cocktail protease inhibitor to the aspirate before centrifuging at 800 *g* at 4°C for 10 min. After centrifugation, we filtered all samples through a 100-μm filter pore and finally determined the amounts of TGF-β1 in the processed BAL samples by an ELISA technique described previously (8). To perform this assay, 3 ml of the samples were lyophilized and resolubilized in 200 μl of double-distilled water, yielding a 15-fold increase in concentration.

Table 4. *Inflammation of pathological biopsies*

Definition	Percentage of Cases
Variegated mild inflammation	37
Variegated moderate inflammation	14
Variegated severe inflammation	8
Diffuse mild inflammation	4

Table 5. *Inflammatory cell pattern in biopsies*

Definition	Percentage of Cases
Neutrophil	58
Plasma cell	44
Lymphocyte	100
Macrophage	8
Eosinophil	0

These data were computed from the mean of 5 microscopic observations in biopsies. Percentage values in Tables 2, 3, 4, and 5 are the frequency of pathological patterns or cell types found in total case samples.

ELISA. ELISA was performed for all samples according to the method recommended by the manufacturer (IBL) for its kit, and some samples were double-checked in dual time to determine test accuracy. This kit measured total (active and latent) TGF-β1 level by acidification-neutralization before ELISA procedure. The assay was reliable for the 15× concentrates. Data analysis was performed by the Mann-Whitney test and commercial computer software.

RESULTS

When comparing study and control groups, we detected the following differences: there were significant differences between TGF-β1 levels in control *group 1* (veterans not exposed to chemical agents) and the study group ($P = 0.001$). There were also significant differences between control *group 3* and the study group ($P = 0.003$). This latter group was found by bronchoscopy to be normal although the individuals had been referred for bronchoscopy because of apparent respiratory disorder. In contrast to the above results, no significant difference in TGF-β1 measurements between control *group 2* (patients with IPF disorders) and the study group ($P = 0.57$) was found (Table 1). Pathological patterns of case biopsies are shown in Tables 2, 3, 4, and 5. Pathological signs of BO such as chronic inflammation, chronically infiltrated inflammation, airway obstacle, and airway loss were more common findings in pathological samples that were positive for >46% of cases (Figs. 2 and 3).

DISCUSSION

Mustard agents, among the worst weapons of mass destruction, were used against Iranians during the Iraq-imposed war (7, 26, 35). Respiratory, ocular, and cutaneous injuries resulting from sulfur mustard are well-known findings after acute exposure. Chronic effects are also common in these organs and are much more complicated (9, 19, 23, 32). Respiratory disorders are the major cause of disability and mortality in mustard victims. There are several studies relevant to long-term respiratory disorders after mustard exposure in Iranian warfare victims (9, 19). Our study like others confirmed that fibrotic pathways were pursued following respiratory organ damage subsequent to sulfur mustard exposure (Fig. 3) (20, 21).

Other studies showed that mustard exposure may affect the immune system and lead to blood cancer (23, 25). In patients

with BO, the pulmonary lobules that have normal airways increase in density during the expiratory phase, whereas areas with diseased airways cannot empty and remain radiolucent secondary to the obstructive bronchiolar inflammatory and fibrotic changes (22, 28, 36). This is observed also in our cases.

Others documented the presence of air trapping had 87.5% sensitivity and specificity for the diagnosis of BO (5). In another, smaller study, an air-trapping score provided a sensitivity of 74% and a specificity of 67% for histopathologically proven BO (5). The pathological character of our findings in HRCT and needle or open lung biopsy documented in this paper confirmed mixed airway obliterative and mild parenchymal lung fibrosis. This may therefore create some mixed findings in the clinical and paraclinical symptoms of chemical casualties' chronic lung diseases (Tables 2–4). Some of these findings have also been reported in a 5-yr study of Kurdish Iraqis exposed to sulfur mustard (15).

The determination of fibrotic pathway activity and fibrogenic markers is vital because of its chief end organ damage and the need to design new therapeutic protocols for these patients.

TGF-β is one of the well-characterized growth factors that is one of the most important regulators of inflammation in connective tissue synthesis in vivo and in vitro. TGF-β1, an isoform of TGF-β, has been described to play an important role in the pathogenesis of progressive inflammatory and fibrotic diseases such as IPF and BO (4, 13). Previously, others found that TGF-β1 concentration in BAL fluid was significantly increased in patients with IPF compared with controls (8). In other studies, IFN-γ 1b and low-dose prednisolone significantly decreased TGF-β1 ($P = 0.004$) and increased IFN-γ gene expression at transcriptional levels and was useful in the treatment of IPF patients (29, 31, 39). One of these studies demonstrated a sixfold increase in the TGF-β1 transcriptional level in biopsy samples (39).

In this study, comparison of TGF-β1 levels in warfare victims with controls not exposed to chemical agents showed a significant difference of approximately sixfold. IFN-γ testing was not conducted because the suppression of IFN-γ target protein level in aspirated (BAL) fluid was such that many samples showed negative results (a pilot study showed six negative in 10 samples). IFN-γ converts macrophages from a resting to an active state and induces the synthesis of an array of receptors for binding to pathogens and endothelia, degradative enzymes, transcription factors, and cytokines involved in host defense (2). TGF-β1 has become probably the most important cytokine underlying progressive pulmonary fibrosis, owing to its strong activation of mesenchymal growth and its ability to modulate cellular immunity.

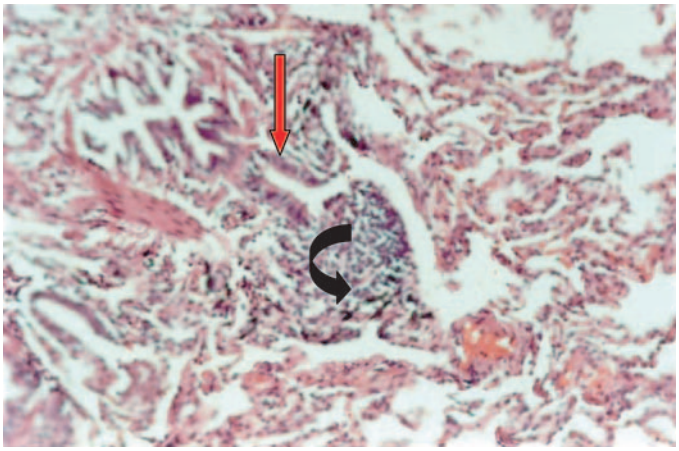

Fig. 2. Bronchiolitis obliterans in one of our cases. Chronic infiltrated inflammation and airway obstacle (black arrow) and airway loss (red arrow) are common findings.

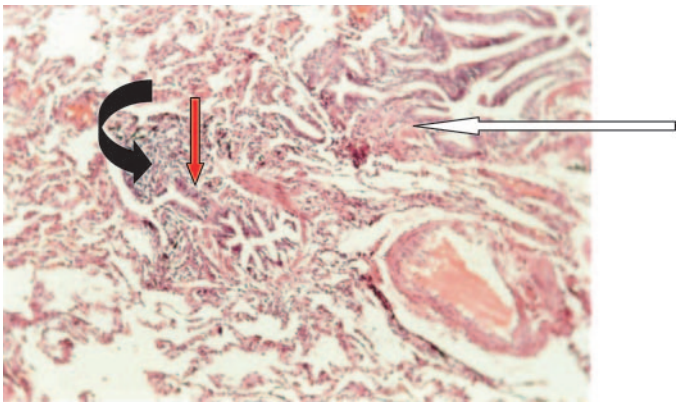

Fig. 3. Bronchiolitis obliterans in another of our cases. Chronic inflammation (fibrosis as white arrow), chronic infiltrated inflammation and airway obstacle (black arrow), and airway loss (red arrow) are common findings.

In animal models, the administration of IFN-γ causes reduced expression of TGF-β1 together with a reduction in the amount of fibrosis apparently at the transcriptional level; needle sample volume was not enough for gene expression tests such as Northern blot hybridization (24, 37).

One of theories regarding TGF-β1 overexpression is genetic mutation and genotype variation. It has been shown that the genotype variations in TGF-β1 genes are apparent in fibrotic lung diseases, perhaps resulting from overexpression of the TGF-β1 gene (4), and could thus increase synthesis of fibrillar collagen types I and II and also significantly decrease matrix metalloproteinase-1 gene expression (17, 29, 34). This pathway is associated with extracellular matrix deposition, explaining the fibrogenic potency of TGF-β1 in vitro and emphasizing the biological role of this agent in the pathogenesis of lung fibrosis. Also, TGF-β1 suppresses IFN-γ induction by inhibiting the class II transactivator, which affects class II MHC gene expression at the transcriptional level (16).

With regard to our data and the previous studies, we conclude that progression of inflammation and fibrotic processes in pulmonary tissue of chemical warfare victims exposed to mustard agent is not an exception. TGF-β1 target protein is substantially increased in BAL aspirates and target tissues in these cases. Thus use of IFN-γ may be beneficial for these patients. We are currently undertaking further studies to detect TGF-β1 expression by determining target proteins and mRNA levels in warfare victims with lung tissue injury to explain the condition of TGF-β1 receptors and to determine whether there are genotype variations in TGF-β1 genes in these patients.

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