Indian J Med Res 90, December 1989, pp 407-414

Bronchoalveolar lavage study in victims of toxic gas leak at Bhopal

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Accepted September 4, 1989

Bronchoalveolar lavage using flexible fibreoptic bronchoscope was carried out in 50 patients 1-2½ yr after exposure to the 'toxic gas' at Bhopal. Thirty six patients in the analysis were categorised into 3 groups *(viz.*, mild, moderate and severe), depending upon the severity of exposure. There was an increase in cellularity in the lower respiratory tract (alveolitis) of the severely exposed patients (in both smokers and non-smokers), compared to normals ($P < 0.05$). The increase in cellularity in severely exposed non-smokers was due to abnormal accumulation of macrophages ($P<0.01$), and in severely exposed smokers, to macrophages ($P<0.01$) and neutrophils ($P<0.05$). Mild and moderately exposed patients did not show significant change in cellularity in lower respiratory tract, compared to normal individuals ($P>0.2$). There was a trend towards increasing cellularity, as the severity increased ($P < 0.0001$) and higher numbers of total cells were seen in severely exposed smokers, suggesting that smoking is a risk factor. It appears, therefore, that subjects severely exposed to the toxic gas at Bhopal may have a subclinical alveolitis characterised by accumulation and possibly activation of macrophages in the lower respiratory tract. Smokers, who were exposed to the gas had in addition, accumulation of neutrophils.

It may be recalled that leakage of over 40 tonnes of 'toxic' gas from an underground storage tank of a pesticide plant at Bhopal on 3rd December, 1984, resulted in the death of a large number of people due to acute pulmonary oedema and respiratory failure. The survivors had persisting respiratory symptoms and it was suspected that a proportion of these patients might develop interstitial lung disease. The suspicion was due to the fact that some of the patients had symptoms and radiological findings suggestive of interstitial lung disease, though physiological investigations did not show clear-cut evidence of interstitial disease. However, previous studies[1] had shown that normal results on tests of pulmonary function did not rule out alveolitis. It is widely accepted that alveolitis is the essential feature of the pathogenesis of chronic interstitial lung diseases and the alveolitis is responsible for the injury and fibrosis of the alveolar walls[2-5]. The technique of bronchoalveolar lavage[6] (BAL) had made it possible to understand the character and function of the effector cells in alveolitis. A study was therefore planned to evaluate the trachea-bronchial tree and to obtain samples of inflammatory and immune effector

cells from the lower respiratory tract, using the technique of bronchoalveolar lavage.

Material & Methods

Ail studies were carried out under protocols approved by the Indian Council of Medical Research, New Delhi. The procedures to be performed were explained in the local language and the consent was obtained in each case. This study was done 1-2½ yr after exposure to the toxic gas.

Pre-lavage assessment and investigations : Pre-lavage assessment and investigations of each patient included detailed history, physical examination, a full-plate PA chest X-ray examination, a 12 lead electrocardiogram, total and differential leucocyte count in the peripheral blood and pulmonary function tests (using Transfer Test Model C, P. K. Morgan Pvt. Ltd., Chatham, UK). Patients with pre-existing lung diseases were excluded from the study.

Bronchoalveolar lavage : Bronchoscopy and bronchoalveolar lavages were carried out as an out-patient procedure at Hamidia Hospital, Bhopal, in 50 patients. Patients were premeditated intravenously with valium (5-10 mg) and intramuscularly with atropine (0.8 mg). Four per cent xylocaine spray was used to anaesthetise the oropharynx and 5 per cent dextrose was given intravenously during the procedure. The flexible fibreoptic bronchoscope with an inner diameter of 2.6 mm (Olympus BF type IT 10, S. no. 2511484) was used for bronchoscopy and bronchoalveolar lavage. Usually the transnasal passage was used, but occasionally the bronchoscope was passed transorally. The lavages were usually done from three subsegments *viz.*, right middle lobe; lingula and left lower lobe. The tip of the bronchoscope was wedged in a subsegment BAL was performed with 300 ml sterile 0.9 per cent saline at room temperature. One hundred millilitres of sterile saline in five 20 ml aliquots were infused through the fibreoptic bronchoscope into each of the three lobes in the lower respiratory tract. After each aliquot was infused, the cells and lavage fluids were recovered by gentle suction using 50-100 mm water negative pressure with an usual clinical suction apparatus and collected in specimen traps (Chesebrough-Ponds Ltd., Ireland). The fluid obtained by lavage was pooled in a sterile plastic cup (Falcon-Plastics, Oxnard, CA). The bronchoscopy and bronchoalveolar lavage procedures were done under continuous cardiac monitoring. Supplemental oxygen was administered during and 1-2 h following lavage. All individuals were observed for 3-4 h after bronchoscopy. There were no complications during bronchoscopy and lavage.

Immediately after lavage, the fluid was filtered through three layers of sterile surgical gauze and the volume was measured accurately. Cells were evenly resuspended by repeated aspirations with a 10 ml pipette. An aliquot was removed for determinations of cell number and preparations of filters for determinations of differential cell count. Rest of the fluid was centrifuged and preserved for biochemical and immunological investigations. The cells recovered by lavage were counted on a haemocytometer, using the unconcentrated lavage fluid[7] and expressed as cells per 100 ml of recovered fluid.

The lavage cell differentials were determined using filtration method[7,8]. Nitrocellulose filters 25 mm in diameter with 5.0 µm pores (SMWP-025-00, Millipore Corporation, Bedford, MA) were pre-soaked in absolute alcohol for 5 sec and mounted above a paper pad (AP-10-025-00, Millipore Corp.),

in a 15 ml graduated funnel with a fritted glass base (XX-100-25-14, Millipore Corporation). The filter was then washed with 15 ml of 0.9 per cent sodium chloride. 2 x 10^5 lavage cells were added to the funnel and the filter was washed with 15 ml of absolute alcohol. The filters were then removed from the funnel apparatus, mounted onto 25 x 75 mm microscope slides using bell clips (Bell Products Co.).

Cells collected on filters were stained with haematoxylin and eosin. The filters mounted on glass slides were washed in tap water (5 min), and distilled water (1 min) and stained in Harris haematoxylin (30 sec). Filters were 'blued' by washing in lukewarm tap water for at least 20 min. After 'bluing', filters were dipped in 50 per cent ethanol (1 min), and 80 per cent ethanol (1 min), and counterstained in eosin (2.5 min). The filters were then dipped in 3 changes of absolute alcohol (1 min each), one change of 2-propanol (2 min) and three changes of xylene (1-2 min each), until they are transparent and mounted on 25 x 75 mm glass microscope slides under glass coverslips using permount. Using oil immersion of a microscope, alveolar macrophages, lymphocytes, neutrophils and eosinophils were identified and 400 cells were counted from each preparation for deriving the differentials. Bronchial epithelial cells counted were always less than 5 per cent.

Normal controls : Ideally, lavages should have been done in normal individuals not exposed to 'toxic' gas at Bhopal. However, lavages could not be done in normal people because of the difficulty in obtaining consent from them. In the present study, therefore, the results of lavage performed on 12 non-smoking individuals from Madras (Vijayan, V.K., unpublished data) were used for analysis. None of these subjects had respiratory symptoms or abnormal physical findings, and all had normal chest X-rays and normal pulmonary function tests. None of the subjects were on any medication. Our results in normal subjects in Madras are comparable to those reported from Delhi[9] and Western countries[10].

Classification of severity of exposure : As the degree of exposure to the gas may not be the same in all individuals, the patients were categorised into three groups depending upon the severity of exposure. The three categories were as follows :–

(i) Severe exposure–If one of the members of the family died due to the toxic gas exposure or the patient had severe ophthalmic and respiratory symptoms, requiring immediate medical help with assistance from others, the patient was classified as having severe exposure.

(ii) Moderate exposure–After exposure to the gas, if the patient developed respiratory symptoms and required immediate medical relief, the patient was classified as having moderate exposure.

(iii) Mild exposure–After exposure, if the patient developed respiratory symptoms, but did not seek immediate medical relief because of mild symptoms, the patient was classified as having mild exposure.

Analysis : Fourteen patients were excluded from analysis due to the following reasons viz., poor recovery of BAL fluid (6); acute respiratory infections (3); interstitial fibrosis (2) ; prior bronchogram (1) ; old pulmonary tuberculosis (1); and normal smoker (I). The two patients with interstitial fibrosis were excluded because one (20 yr female) admitted that she had respiratory disease

prior to exposure to the gas. The second patient (a 65 yr old female) was excluded because she attributed all her symptoms to gas exposure (unconvincing history) and showed pus in the tracho-bronchial tree during lavage procedure.

All data between groups were compared using the two tailed Students' 't'-test and also with Mann-Whitney U test, and the results werefound to be similar. The trend Chi square test was applied to see whether the level of exposure had any trend effect OR the number of cells.

Results

All patients (n=36) included in the analysis were males. Female patients were not willing to undergo the procedure. The mean age was 35.7±10.6 yr (range 18-60 yr). There were 6 patients with mild exposure, 5 with moderate exposure and 25 with severe exposure. Eight patients were smokers and all of them had severe exposure. All mildly exposed patients had normal chest X-rays and pulmonary function. Three of five moderately exposed patients had radiographic abnormality of 1/0 (ILO, 1980 classification") and two had obstructive ventilatory defect. Radiographic abnormalities of 1/0 or 1/1 were observed in 18 of 25 severely exposed patients. Obstructive and restrictive ventilatory defects were observed in 8 patients each in severely exposed group. The total and differential leucocyte counts and electrocardiograms were within normal limits in all subjects studied.

The mean values of total and differential cell counts in mildly and moderately exposed patients were not significantly different (P> 0.2) from Madras normals (Table I). Severely exposed patients had a significantly elevated

Table I. Total and differential cell counts in lower respiratory tract

(Data are mean ± SD)

Group	Total cells (x 10^6/dl)	M %	L %	N %	E %
Normal (Madras) (n=12)	14.4 ±6.5	83.6 ±7.1	14.3 ±7.0	0.9 ±0.9	1.3 ±1.2
Mild exposure (n = 6)	10.7 ±3.2	86.8 ±5.4	12.3 ±4.9	0.8 ±0.98	0
Moderate exposure (n = 5)	20.6 ±4.3	92.6 ±3.7	6.6 ±3.4	0.6 ±0.4	0
Severe exposure (n=25)	39.2* ±24.2	87.0 ±9.4	10.7 ±8.7	1.7 ±2.4	0.5 ±1.6

M, macrophages; L, lymphocytes; N, neutrophils; E, eosinophils
*P<0.05 as compared to the normal group

(P<0.05) total cell count in the lower respiratory tract compared to Madras normals. This was true whether all individuals or only severely exposed non-smokers were considered. However, the proportion of different types of cells recovered was similar to that of normals in all groups (P>0.2, normal compared to all others).

The range of total cells (x 10^6/dl) recovered from the lower respiratory tract was 5.5 to 13.8 in mildly exposed, 13.8 to 25.3 in moderately exposed, 15.3 to 71 in severely exposed (non-smokers) and 21 to 136 in severely exposed (smokers) patients. Eleven of 17 (64.7%) severely exposed (non-smokers) and 7 of 8 (87.5%) severely exposed (smokers) had more than Z-fold increase in cells in the lower respiratory tract, whereas only one of 11 (9 %) mildly and moderately

exposed patients had more than 2-fold increase. With increasing severity of exposure, there. was a tendency for a higher proportion of patients to have increasing cellularity in the lower respiratory tract and this trend was statistically significant (P<0.0001).

The absolute numbers of different types of cells (total cells times differential percentages) are given in Table II. Among the inflammatory and immune effector cells recovered from the lower respiratory tract, macrophages showed a significant rise in severely exposed patients, compared to normals (P<0.01). This was true whether all individuals or only non-smokers were considered. There was also a significant rise in neutrophils in severely exposed patients who continued to smoke tobacco (P<0.05).

Table II. Mean absolute values of inflammatory and immune effector cells

	cells ($x 10^6$/dl)			
	M	L	N	E
Normal (n = 12)	12.10 ±5.54	2.10 ±1.39	0.1 ±0.17	0.2 ±0.14
Mild exposure (n = 6)	9.38 ±3.12	1.33 ±0.47	0.07 ±0.08	0
Moderate exposure (n = 5)	19.08 ±4.09	1.38 ±0.89	0.16 ±0.11	0
Severe exposure (non-smokers) (n= 17)	29.69* ±14.47	3.93 ±3.20	0.56 ±0.82	0.19 ±0.47
Severe exposure (smokers) (n = 8)	46.58* ±37.48	2.49 ±2.17	0.50** ±0.38	0.04 ±0.11

P values, * <0.01; l *<0.05 as compared to normals

Discussion

In severely exposed patients (both smokers and non-smokers), there was a significant increase in inflammatory and immune effector cells in the lower respiratory tract. Accumulation of inflammatory cells in the alveolar structures is referred to as alveolitis[12] and the observation of increased cells in severely exposed patients suggest that a proportion of these patients had alveolitis. The increase in cellularity in severely exposed non-smokers was due to abnormal accumulation of macrophages, whereas in severely exposed smokers it was due to macrophages and neutrophils. An increase in macrophages and neutrophils in the lower respiratory tract of smokers has been described by earlier workers as well [10].

Activated macrophages had been shown to produce various mediators which can cause injury to and fibrosis of the lung parenchyma[13]. The observation of increased number of macrophages in the lower respiratory tract in non-smoking severely exposed patients, even 1 to 2½ yr after exposure to the gas, suggests that alveolitis in this group of patients may have a deleterious effect on lung parenchyma. Usually, two types of cells are involved in alveolitis, such as macrophage-lymphocytic alveolitis in sarcoidosis[14] macrophage-neutrophilic alveolitis in idiopathic interstitial fibrosis[15] and macrophage-eosinophilic alveolitis in Tropical Eosinophilia[16]. However, a predominant increase in macrophages had been described in siIicosis[17] and respiratory bronchiolitis[l8]. Soon after exposure to the toxic gas, an increase in neutrophils varying from 72 to 98 per cent in BAL had been reported[19]. The occurrence of increased neutrophils in the lower respiratory tract Soon after exposure[19],

and the observation of increased macrophages 1-2½ yr after exposure in the present study may be similar to the observation in mouse lung exposed to short Crocidolite asbestos fibres in which there was a rapid elevation of polymorphonuclear leucocytes, followed by a decline in polymorphonuclear leucocytes and a significant increase in alveolar macrophages between 5 days and 8 weeks, though it produced only minimal lung injury and fibrosis[20]. Similar increase in macrophages in BAL were reported in other experimental studies[21,22].

It had been recently demonstrated in non-smokers with long-term occupational exposure to inorganic dusts and functional evidence of interstitial diseases (asbestosis, coal workers' pneumoconiosis and silicosis), that the inflammation in the lower respiratory tract was dominated by alveolar macrophages[23]. The single exposure of large amounts of the toxic gas in our subjects has also resulted in an exaggerated number of alveolar macrophages in the lower respiratory tract, especially in severely exposed patients. Though the occurrence of increased numbers of alveolar macrophages may be non-specific in character, the demonstration that activated alveolar macrophages in various occupational lung diseases are capable of spontaneously releasing exaggerated amounts of oxygen radicals (O_2^- and H_2O_2), fibronectin and alveolar macrophage derived growth factor[23] suggests that long-term follow up of toxic gas exposed patients is essential to know whether the expanded numbers of alveolar macrophages are 'activated' and if so, cause any injury to the lung parenchyma.

The increasing cellularity in the lower respiratory tract, as the severity of exposure increases and also the higher total cells in severely exposed smokers compared to non-smokers, suggest that smoking is a risk factor. It was also further observed that the severely exposed smokers had a significant increase of neutrophils in the lower respiratory tract. Recent studies[13,24] had shown that activated macrophages and neutrophils in the lower respiratory tract can release mediators which were toxic to the pulmonary tissue resulting in permanent destructive changes. In view of the possibility that a portion of the increased alveolar macrophages and/or neutrophils (and/or their mediators) may be in direct contact with small airway epithelium[25], and some of these patients had already obstructive ventilatory defect, these cells/mediators may further damage the epithelium, resulting in progressive chronic obstructive lung disease. Health education of the community to avoid any type of pollution such as smoking tobacco, domestic and environmental pollutions is essential to prevent the early development of chronic lung diseases in these patients. Similarly, the role of steroids in suppressing alveolitis in severely exposed patients has also to be critically evaluated.

Acknowledgment

The authors thank the Indian Council of Medical Research, New Delhi, for providing facilities to undertake this study. The support provided by Dr R. Prabhakar, Director, Tuberculosis Research Centre (TRC), Madras, is also gratefully acknowledged. Statistical analysis was done by Shri P. Venkatesan, TRC, Madras.

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