Production of interleukin 1 by rat pleural leucocytes in culture after intratracheal instillation of crocidolite asbestos

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Abstract

This study was undertaken to investigate the production of interleukin 1 (IL-1) by pleural leucocytes in culture and to evaluate the influence of intratracheal instillation of crocidolite asbestos on this production. Normal pleural leucocytes spontaneously released IL-1 in culture and stimulation with lipopolysaccharide (LPS) dramatically increased production. Intratracheal instillation with crocidolite asbestos induced recruitment of pleural leucocytes in the longer term and changed IL-1 production by the leucocytes. Reduced production of IL-1 was found by one day after instillation of asbestos and this was correlated with the dose of asbestos. With increasing time after instillation, however, release of IL-1 by pleural leucocytes gradually recovered to normal until, one month after asbestos injection, the leucocytes produced augmented IL-1 in culture compared with control pleural leucocytes. Our data show that pleural leucocytes possess the potential to produce IL-1 in vitro and this capability is altered by intratracheal instillation of crocidolite asbestos. This may be relevant to development of pleural diseases associated with inhalation of asbestos.

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Interleukin 1 (IL-1) belongs to a family of proteins, the interleukins, which are usually described as performing important regulatory functions between

leucocytes.¹ Mononuclear phagocytes seem to be the major producers of IL-1, although other cells, including reticulum cells, endothelial cells, and neutrophils also produce IL-2.^{2,3} Although small amounts may be released spontaneously, most normal macrophages produce IL-1 only in response to exogenous stimulants such as microbial toxins, inflammatory agents, complement, and clotting components.⁴ Interleukin 1 possesses a wide spectrum of immunological and non-immunological activities including tissue remodelling, repair, and inflammation, by helping to coordinate the activities of many cell types.^{5,6}

Asbestos has been in widespread industrial use for a long time⁷ and a large body of evidence has confirmed that inhaled asbestos is associated with many lung disorders such as parenchymal fibrosis (asbestosis) and bronchial carcinoma.⁸ The roles of IL-1 in asbestos related lung disorders have been investigated and studies have shown that exposure to asbestos results in abnormal release of IL-1 by macrophages.^{9,10}

Pleural pathological processes, such as effusion, pleural plaques, pleural fibrosis, and mesothelioma,¹¹ are seen in a proportion of asbestos exposed persons. The effects of asbestos on the activity of pleural leucocytes have not, however, received much attention. Very little is known about the role of cytokines in pleural pathology caused by fibrous dusts such as asbestos.

The objective of this investigation was to study the ability of pleural leucocytes to produce IL-1 in vitro and to assess the influence of crocidolite asbestos, given intratracheally, on this production.

Materials and methods

ANIMALS

Syngeneic PVG rats more than 12 weeks old were obtained from the Institute of Occupational Medicine animal unit.

INTRATRACHEAL INSTILLATION AND PLEURAL LAVAGE
Rats were injected intratracheally with 0.5 ml of phosphate buffered saline (PBS) containing from 1 mg to 10 mg UICC crocidolite asbestos. At different periods after infection, rats were killed by

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injection of Nembutal (Ceva, Watford) and the chest wall was exposed. After insertion of a blunt needle into the pleural cavity between two lower ribs, four sequential 5 ml volumes of PBS were injected into the cavity and the chest wall was gently massaged before withdrawal. The cell suspension from the lavage was pooled in plastic containers and kept on ice.

PREPARATIONS OF CELLS AND SUPERNATANTS

The pleural leucocytes were centrifuged and resuspended in F10 medium (Gibco, Paisley) plus 2% bovine serum albumin (BSA, Sigma, Poole). The total cell number from each rat was assessed in a Neubauer chamber. The proportion of each cell type was obtained by staining cytocentrifuge smears with Diff-Quik (Merz-dade, Duding, Switzerland).

Pleural leucocyte supernatants were obtained by resuspending the cells at 1×10^6 cell/ml in F10 medium plus 2% BSA. The cells were incubated at 37°C in 5% CO₂ for 24 hours. Supernatant fluids were collected and spun at 3000 rpm to clarity, then aliquoted and stored at -70°C before assay. When required lipopolysaccharide (LPS, Sigma, Poole) was added into cell cultures at 100 ng/ml.

ASSAY FOR IL-1 ACTIVITY

The IL-1 activity of supernatant fluids was assessed by using the standard mouse thymocyte proliferation assay as described by Mizel.¹² Thymocytes obtained from three to six week old C3H mice at 0.6×10^6 cells per well were incubated in RPMI-1640 medium (Gibco, Paisley) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley) and 20 µM 2-mecaptoethanol (Sigma, Poole) or in test supernatants diluted with the medium at 37°C, under 5% CO₂ for 72 hours. Proliferation was assessed in the presence of a suboptimal dose (5 µg/ml) of phytohaemagglutinin (PHA, Sigma, Poole). Sixteen hours before the end of the incubation period thymocytes were labelled with 9.25 KBq ³H-methylthymidine (Amersham International, Aylesbury Buckinghamshire) and harvested on glass fibre filters. The filters were dried and the cellular thymidine incorporation was measured by liquid scintillometry in a β counter. The activity of test samples was calculated by comparison with the standard curve of a series of dilutions of IL-1β standard (a gift from Dr D Burnett, Lung Immunobiochemical Research Laboratory, General Hospital, Birmingham).

ASSAY FOR IL-2 ACTIVITY

The IL-2 was assayed using the IL-2 dependent CTLL-2 cell line. The CTLL-2 cells were maintained in RPMI 1640 medium with 10% FCS as well as 5% of IL-2 enriched rat splenocyte supernatant. When required the cells were washed thoroughly and then suspended in RPMI 1640 medium supplemen-

ted with 10% FCS. Fifty µl of cell suspension containing 5×10^3 cells and 50 µl of test supernatants were dispensed into each well of the microtitre plate and then incubated for 24 hours at 37°C under 5% CO₂. Proliferation of CTLL-2 cells was assayed by the uptake of tritiated thymidine as described and compared with the IL-2 standard.

ENDOTOXIN MEASUREMENT

To determine whether the background release of IL-1 by normal pleural leucocytes in vitro was caused by low concentrations of endotoxin in the media or serum used in the assay, a kit (COATEST, KABI Diagnostica, KabiVitram Ltd, Uxbridge, Middx) was used to detect the endotoxin concentrations in all media used in the study.

STATISTICS

Multiple repeat experiments were carried out and results were subjected to analysis of variance. When there was a significant F value for the effect of treatment, individual means were compared for significance using *t* tests.¹³

Results

PLEURAL LEUCOCYTE POPULATIONS

Normal pleural leucocytes were composed mainly of four types of cells: macrophages, mast cells, eosinophils, and lymphocytes, most cells being macrophages (table 1).

Table 2 shows that with increasing time after the intratracheal injection of 5 mg crocidolite asbestos, the total cell numbers of pleural leucocytes increased significantly compared with those of leucocytes from normal pleura. Considering the change in the proportion of different cell types, the percentage of macrophages declined after injection of asbestos whereas the proportion of eosinophils increased significantly. Other cell types remained at the same level; neutrophils were rarely seen in any of the populations of pleural leucocytes.

The most significant difference arose in that a small number of neutrophils appeared in the pleural cell population when the doses of intratracheally injected crocidolite increased up to 10 mg; however, there were no obvious differences among total cell numbers and the percentages of other components of pleural leucocytes (table 3).

Table 1 Proportions of normal pleural leucocytes

Total number ($\times 10^6$)	Percentage			
	Macrophages	Lymphocytes	Mast cells	Eosinophils
4.51 (0.29)	82 (1.55)	1 (0.34)	11 (1.50)	6 (0.77)

Results are mean (SEM); n = 14 rats.

Table 2 Cell populations of pleural leucocytes after intratracheal instillation of 5 mg crocidolite asbestos

Time (day)	Total no ($\times 10^6$)	Percentage				
		Macrophages	Neutrophils	Lymphocytes	Mast cells	Eosinophils
1	4.54 (0.30)	78 (1.1)*	0	1 (0.3)	12 (1.5)	9 (0.9)*
3	6.30 (0.73)*	76 (1.5)**	0	2 (0.8)	9 (1.4)	13 (1.5)***
14	6.92 (0.60)**	72 (3.0)**	0	1 (0.5)	9 (2.1)	18 (1.4)***
30	6.96 (2.08)**	75 (1.8)**	0	1 (0.4)	9 (1.5)	15 (0.7)***

Results are mean (SEM); n = 5-7 rats.

*p < 0.05; **p < 0.01; ***p < 0.001 compared with controls.

Table 3 Pleural leucocyte populations one day after intratracheal instillation of different doses of crocidolite asbestos

Dose (mg)	Total no ($\times 10^6$)	Percentage				
		Macrophages	Neutrophils	Lymphocytes	Mast cells	Eosinophils
1	4.83 (0.22)	79 (1.2)	0	2 (1.0)	10 (1.5)	8 (0.8)*
5	4.54 (0.30)	78 (1.1)	0	1 (0.3)	12 (1.5)	9 (0.9)*
10	4.78 (0.21)	78 (0.9)	2 (0.9)	1 (0.4)	10 (1.2)	9 (0.7)**

Results are mean (SEM); n = 5-7 rats.

*p < 0.05; **p < 0.01 compared with controls.

PRODUCTION OF IL-1 BY NORMAL PLEURAL LEUCOCYTES WITH AND WITHOUT LPS STIMULATION

Figure 1 shows that leucocytes from normal pleura produced IL-1 activity in culture, even in the absence of stimulation by endotoxin. The activity of IL-1 in supernatants of normal leucocytes without stimulation was about 167 U/ml compared with IL-1 β standard. After stimulation with 100 ng/ml of LPS, the activity of IL-1 reached about 268 U/ml, roughly double the normal values. At the highest concentration of LPS stimulated supernatant (1:4) the abnormal reduction of IL-1 activity might be due to too high a concentration of IL-1 in the supernatant, which inhibits the mitogenesis of C3H mouse thymocytes.

Figure 1 Production of IL-1 by pleural leucocytes with or without stimulation with LPS. Results are presented as mean (SEM) of 10-14 separate experiments performed in triplicate. Asterisks denote a significant difference from corresponding dilution of controls: **p < 0.01; ***p < 0.001.

The measurement of endotoxin in media used in the study indicated a small contamination of endotoxin: F10 + 2% BSA contained 106 pg/ml and PBS 16 pg/ml of endotoxin. There was no detectable endotoxin in RPMI-1640 + 10% FCS and saline.

RELEASE OF IL-1 BY CULTURED PLEURAL LEUCOCYTES FROM RATS INTRATRACHEALLY INSTILLED WITH CROCIDOLITE ASBESTOS

Intratracheal instillation of 5 mg crocidolite asbestos resulted in a decrease in activity of IL-1 in the supernatants (fig 2). One day after injection of asbestos, the IL-1 activity decreased significantly.

Figure 2 Production of IL-1 by pleural leucocytes from rats at various times after intratracheal instillation of 5 mg crocidolite asbestos. Each column represents mean (SEM) of triplicate wells in 9-15 separate experiments. Dilution of pleural leucocyte supernatant is 1:8. Asterisks denote a significant difference from controls: *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 3 Production of IL-1 by pleural leucocytes from rats one day after intratracheal instillation of different doses of crocidolite asbestos. Each column represents mean (SEM) of triplicate wells in 9–16 separate experiments. Dilution of pleural leucocyte supernatant is 1:8. Asterisks denote a significant difference from controls: *** $p < 0.001$.

With increasing time after instillation, the activity of IL-1 recovered towards normal. Moreover, IL-1 activity in the supernatants was significantly higher than control values by 30 days after instillation.

Figure 3 shows the dose related effect of instilled asbestos on production of IL-1 by pleural leucocytes in culture. The decrease in activity of IL-1 was correlated with the increase in the dose of injected asbestos.

MEASUREMENT OF IL-2 ACTIVITY

To rule out IL-2 as a factor in the thymocyte assay,¹⁴ IL-2 activity was measured with the IL-2 dependent CTLL-2 cell line. Assays of pleural leucocyte supernatants maximally stimulating C3H thymocytes failed to show detectable amounts of IL-2 when tested for their capacity to support CTLL-2 cell growth (data not shown).

Discussion

Although a large body of work has been carried out on production of IL-1 by monocytes and macrophages,¹ few studies have been carried out on pleural leucocytes in this regard. In view of the important role of the pleura in basic lung function and the pleural pathology caused by fibrous dusts, we were interested in the functions of pleural leucocytes after exposure of the airspace to asbestos.

The present study indicates that, without added stimulation, pleural leucocytes from normal rats produced IL-1 in culture. When stimulated with LPS this production increased considerably. Production of IL-1 was decreased when rats received intratracheal administration of crocidolite asbestos and this effect was related to the dose of asbestos in the lungs. With increasing time after instillation of asbestos, IL-1 production by the leucocytes returned

to normal and was increased over the control values by one month after the instillation.

It has been reported that most normal cell types and some cell lines produce IL-1 only in response to various stimulants.¹⁵ Production of IL-1 by normal cells can probably be attributed to a continuing response to previous stimulation *in vivo* or alternatively, the presence of contaminating endotoxin.

Culture media, blood separation media, FCS, and organic buffer used in culturing IL-1 producing cells may contain endotoxin, depending on the source.¹⁶ In the present study, low concentrations of contaminating endotoxin were detected, which may contribute to spontaneous production of IL-1 in culture. Despite this background release of IL-1, however, the leucocytes could still be stimulated with LPS to release considerably more IL-1 in culture than controls, which indicates the extensive potential for production of IL-1 by the cells.

The present study indicates that the increased production of IL-1 is concurrent with the recruitment of pleural leucocytes into the pleural space with increasing time after instillation of asbestos. It has been reported that normal alveolar macrophages are at least 1000 times less sensitive to LPS in terms of IL-1 release than blood monocytes. Also, denser alveolar macrophages and blood monocytes released more IL-1 than the less dense components; this dense population is considered to be the less mature monocytic population.^{17,18} If young mononuclear phagocytes are the prime source of IL-1, IL-1 release in the pleural space may be regulated, at least in part, by factors that control the influx of young monocytes. In fact, our results show a persistent recruitment of pleural leucocytes with increasing time after exposure to asbestos. As these monocytic cells are continuously recruited into the pleural space, eventually the IL-1 activity released by those cells may exceed control values, as shown in the study.

The changed pattern of release of pleural leucocyte IL-1 at different stages after deposition of asbestos in the airspace cannot be fully explained by changes in cell populations. We presume, therefore, that the difference in IL-1 production is a result of alteration in cellular activity. Pleural leucocytes are sensitive to stimulation to produce cytokine during culture and, after intratracheal instillation of asbestos *in vivo* this potential may be modulated or impaired by asbestos fibre in the lungs.

Increasing dose of injected crocidolite resulted in slight changes to the pleural cell population with the appearance of a few neutrophils in the pleural space at the highest dose. A dose related decrease in IL-1 production by the leucocytes was, however, present. Because we failed to detect migration of asbestos fibres or other similar size particles into the pleural space after intratracheal instillation,¹⁹ we suggest that the inflammation caused by asbestos in the airspaces

may cause the transfer of as yet unidentified inflammatory mediators to the pleural cavity. These may down regulate the ability of pleural leucocytes to release IL-1 in culture.

Normally, there is increased IL-1 release by macrophages exposed to asbestos. Kagan *et al*¹⁰ showed that inhalation of either crocidolite or chrysotile asbestos in rats was associated with augmented release of IL-1 by alveolar macrophages. An in vitro study also showed that exposure of peritoneal macrophages to chrysotile asbestos resulted in increased intracellular IL-1 in those cells.⁹ Long amosite asbestos also stimulates increased release of the cytokine tumour necrosis factor compared to short amosite.²⁰

In the present study we have shown that asbestos in the airspace results in a decrease in the IL-1 secreting activity of pleural cells. Because deposition of asbestos in the airspace also suppressed production of tumour necrosis factor by pleural leucocytes in culture,¹⁹ this suppression may have some general selective value in the pleural space. Our data on the increased production of plasminogen activator inhibitor after the same type of asbestos treatment²¹ confirms that the effect is not a general inhibition but suggests that there is selective inhibition of cytokine production by the macrophages. The potency of prostaglandin E₂ in inhibiting secretion of IL-1²² suggests an autocrine role for this substance.

In the longer term, IL-1 production by pleural leucocytes was increased and this may partly explain the pleural pathology, such as pleural adhesion and pleural fibrosis, seen in persons exposed long term to asbestos.¹¹ Fibroblasts and their functions, such as raised rates of synthesis of collagen, prostaglandin production, and fibroblast migration, may be stimulated by IL-1.^{5,23} Thus the long term increase in production of IL-1 could lead to fibroplasia in the pleura. Increased production of plasminogen activator inhibitor by pleural leucocytes, which we have also shown in the same model,²¹ could produce a fibrous matrix for fibroblast growth that would encourage fibrous tissue growth.

In summary, the ability of pleural leucocytes to produce IL-1 in culture showed a dose dependent decrease in the acute period after exposure to asbestos; the pleural leucocytes obtained a longer time after exposure, however, showed augmented ability to produce IL-1. These complex changes in production of IL-1 by pleural leucocytes are not understood but may be related to the pleural pathology seen after exposure to asbestos.

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