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Volume 2

Section 4

Section 4 The effect of particles on the immune system

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Asbestos-Activated Peritoneal Macrophages Release a Factor(s) Which Inhibits Lymphocyte Mitogenesis

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Intraperitoneal asbestos injection in mice has previously been reported to elicit an activated macrophage population. In the present study supernatants from such macrophages were tested for their effect on thymocyte mitogenesis in response to concanavalin A; control supernatants were obtained from saline- and latex-elicited macrophages. Supernatants from asbestos-elicited macrophages were significantly inhibitory to thymocyte mitogenesis while saline- and latex-elicited macrophages did not release significant amounts of such activity. Asbestos-activated macrophage supernatants were inhibitory in a dose-dependent way and the activity was not secreted by macrophages from mice which had received asbestos in the long term. The inhibitory activity was partially dialysable. Supernatants prepared by treating macrophages *in vitro* with a lethal dose of asbestos were not inhibitory suggesting that the inhibitory activity in the supernatants of asbestos-activated macrophages did not leak from dead or dying cells. The asbestos macrophage supernatant was also significantly inhibitory to mature T-cell-enriched spleen cells but had no effect on fibroblasts, suggesting that the inhibitory effect could be lymphoid cell specific.

INTRODUCTION

Asbestos inhalation is associated with the lung diseases interstitial fibrosis, carcinoma, and mesothelioma as revealed by both epidemiological evidence and experimental pathology (Becklake, 1976; Selikoff and Lee, 1978). Additionally, epidemiological studies on the immune status of asbestotics has revealed alterations in both humoral and cellular immune profiles; in particular there has been persistent evidence of suppression of cell-mediated immunity and T-cell function (Kang *et al.*, 1974; Kagan *et al.*, 1977; Haslam *et al.*, 1978; Lange *et al.*, 1978; Gaumer *et al.*, 1981).

The alveolar macrophage has been implicated in the development of the asbestos-related diseases due to is central role in the handling of inhaled particles and the multiple regulatory roles of macrophages in immune response (Unanue, 1980), fibroblast growth (Leibovich and Ross, 1976; Martin *et al.*, 1981), and tumor development (Hibbs *et al.*, 1978; Currie, 1981).

Until recently the potentially lethal effects of asbestos on macrophages have been emphasized and macrophage damage and death have been implicated in fibrogenesis (Harington, 1974). Within the last few years, however, evidence for the importance of macrophage activation in host response to other pathogenic agents has prompted studies into the macrophage-activating potential of asbestos.

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These studies have revealed that asbestos can activate peritoneal macrophages *in vitro* (Davies *et al.*, 1974; Hamilton *et al.*, 1976) and *in vivo* by ip injection (Hamilton *et al.*, 1976; Miller and Kagan, 1976; Donaldson *et al.*, 1982) and alveolar macrophages by inhalation (Miller, 1979). Since activated macrophages have altered secretions and altered capacity to regulate target cell populations, macrophage activation may have a role in asbestos-related pulmonary pathology.

Using the mouse peritoneal injection model we have reported that asbestos injection elicits marcophages which are activated as judged by several criteria but are not tumoricidal (Donaldson *et al.*, 1982) and have altered membrane function (Donaldson *et al.*, 1983a). In the present study the supernatants from asbestos-activated peritoneal macrophages were tested for their effect on thymocyte mitogenesis, a commonly used indicator system for studies of lymphomodulatory factors (Nelson *et al.*, 1976).

MATERIALS AND METHODS

Animals and treatment. CBA/Ca mice, inbred in the Department of Surgery, University of Edinburgh, and 12–16 weeks old at the start of experiments, were used. Each mouse received either 2.5 mg chrysotile asbestos (UICC sample) in 0.5 ml Dulbecco A (Dul. A), 1% latex beads in Dul. A (0.81 μm diam Bacto-latex Difco), or 0.5 ml Dul. A intraperitoneally.

Harvesting of peritoneal exudate cells. Peritoneal exudate cells (PEC) were obtained, 3 days after injection of the above solutions, by washing out the peritoneal cavity with 3 × 2 ml of Dul. A containing 10 U/ml heparin (Pularin).

Preparation of macrophage supernatants. PEC were washed, counted, and 15 × 10^6 added to plastic tissue culture flasks (Corning 75 ml) and incubated for 1 hr at 37°C in complete RPMI (cRPMI) [RPMI 1640 (Gibco) containing 10% fetal calf serum (Gibco), 1 mM L-glutamine, penicillin, and streptomycin]. Nonadherent PEC were removed by washing and the macrophages incubated for 24 hr in cRPMI. Supernatants were collected, centrifuged, and frozen at −20°C until use within 4 weeks.

To obtain supernatants from macrophages treated with asbestos long term, PEC were obtained from mice injected with chrysotile or saline 280 days previously and which had been kept for studies on the development of pathology.

A supernatant from macrophages treated with asbestos *in vitro* was prepared by incubating control macrophages (10 × 10^6 in a 75-ml flask) with 500 μg chrysotile/ml cRPMI for 48 hr. At the end of this time there was 70% killing of the macrophages as measured by dye exclusion. The supernatant was centrifuged and stored frozen at −20°C before use.

Thymocyte mitogenesis assay. Thymuses were obtained from 3- to 6-week-old mice and gently disaggregated in a glass homogenizer. The thymocytes were spun-washed three times and adjusted to 5 × 10^6/ml in cRPMI + 2 × 10^{-5} M 2-mercaptoethanol (cRPMI + 2ME) containing concanavalin A (Con A) (Pharmacia). One hundred microliters of cell suspension were delivered to triplicate wells of microtiter plates (Sterilin) and to this was added 100 μl triplicate samples of various supernatants yielding final concentrations of 0, 1, or 2 μg/ml Con A and 10^{-5} M 2ME in a final volume of 200 μl. Plates were incubated at 37°C in

TABLE 1
EFFECT OF MACROPHAGE SUPERNATANTS ON THYMOCYTE MITOGENESIS

Supernatant	Con A (μg/ml)	cpm ($\bar{x}$ ± SD)	Mitogenic index ($\bar{x}$ ± SD)
None	1	5,078 ± 2336	—
	2	18,634 ± 8705	—
Saline[a]	1	5,155 ± 1808	1.10 ± 0.30
	2	19,054 ± 7638	1.07 ± 0.29
Latex[b]	1	3,620 ± 1966	0.76 ± 0.23
	2	18,609 ± 5759	0.89 ± 0.15
Chrysotile[a]	1	3,694 ± 835	0.59 ± 0.14
	2	12,804 ± 4112	0.65 ± 0.16

Note. No significant differences in tritiated thymidine uptake when expressed as cpm. When expressed as Mitogenic index, (Mitogenic index = (cpm in presence of supernatant + mitogen)/cpm in presence of mitogen only) significant suppressive effects produced by asbestos supernatants compared to saline macrophage supernatants. Significant differences when chrysotile is compared to saline—1 μg/ml Con A, $P < 0.02$; 2 μg/ml Con A, $P < 0.05$.

[a] Data for six separate experiments.

[b] Data for three separate experiments.

5% CO_2 for 48 hr, after which 0.25 μCi of [^{3}H]thymidine were added to each well in 10 μl: 24 hr later the wells were harvested using a cell harvester (Skatron) onto glass fibre paper. The papers were allowed to dry and 6 ml of scintillation cocktail (Wellcome) were added before determining the cell-bound [^{3}H]thymidine in a scintillation counter (Tri-Carb).

Mature lymphocytes. To test supernatants for their effect on mature lymphocytes, spleens were gently disaggregated and the splenocytes were passed through a nylon wool column. The eluted cells were 80% T lymphocyte as judged by immunofluorescence with anti-theta and 2% B cells as judged by surface immunoglobulin staining. These cells were used in Con. A mitogenesis studies as described above.

Nonlymphoid cells as indicator cells. To test the effect of the supernatant on proliferation of non-lymphoid cells, an early passage CBA/Ca fibroblast cell line, kindly provided by Dr. Jan Boyd, Department of Surgery, University of Edinburgh, was used. The assay involved inoculation of microtiter plate wells with 5×10^4 fibroblasts in 100 μl of cRPMI and 100 μl of supernatant. After 24 h, 0.25 μCi of [^{3}H]thymidine was added and the trypsin released cell-bound radioactivity determined 24 hr later using the cell harvester and liquid scintillometry as described above.

RESULTS

Thymocyte-Mitogenesis-Inhibiting Action of Supernatants from Asbestos-Activated Macrophages

Table 1 shows the summarized data from the experiments testing the effect of supernatants on thymocyte mitogenesis. When all experiments are considered there is no significant difference in [^{3}H]thymidine incorporation between any

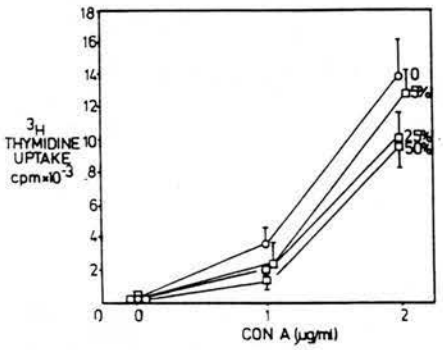


FIG. 1. Effect of chrysotile-induced macrophage supernatant, present in the incubation as 5, 25, or 50%, on Con. A mitogenesis of thymocytes. Significant reduction with 50% supernatant at 1 µg/ml ($P < 0.02$) and 2 µg/ml ($P < 0.05$) Con A. Symbols denote $\bar{x} \pm$ SD.

conditions due to variation between experiments. Such variations is well documented (Herberman, 1978) and can be taken into account by expressing the data as mitogenic indices for each condition within each experiment compared to controls. When the results are expressed in this way the supernatant from the asbestos-activated macrophages is seen to be significantly (P 0.05, see Table 1 for details) inhibitory to mitogenesis; neither saline- nor latex-induced macrophages were significantly suppressive to mitogenesis.

Dose relatedness of inhibition of mitogenesis. Figure 1 shows that inhibition of Con A mitogenesis by supernatant from asbestos macrophages is a dose-related phenomenon.

Use of Supernatants Prepared from Long-Term Asbestos-Injected Mice

Figure 2 represents the results of an experiment using supernatant prepared from macrophages harvested from the peritoneal cavity 280 days after injection of asbestos and saline. A 3-day saline and chrysotile macrophage supernatant is also included for comparison and it is evident that the 280-day chrysotile macrophage supernatant is not inhibitory compared to the 3-day chrysotile supernatant nor is it inhibitory compared to the 280-day saline macrophage supernatant. There is, in fact, significant stimulation of mitogenesis produced by both of the 280-day macrophage supernatants (see Fig. 2 for details).

Use of Supernatant Prepared by Treating Macrophages with Chrysotile in Vitro

To test whether the inhibitory factor in supernatants from asbestos-activated macrophages was actively secreted or passively released by dead or dying cells, control macrophages were treated with a lethal dose of chrysotile *in vitro*. Figure 3 shows that the centrifuged supernatants from this culture was not inhibitory to mitogenesis and that there was in fact some evidence of enhancement of thymidine uptake by this "*in vitro* supernatant."

Effect of supernatant on mature lymphocytes. Mitogenesis of nylon-wool-column-purified splenocytes (80% T cells, 2% B cells) was found to be signifi-

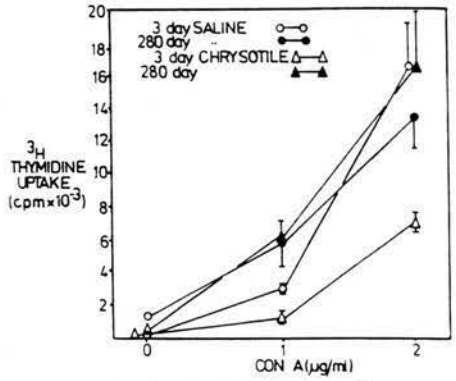


FIG. 2. Effect on Con A mitogenesis of 3-day and 280-day saline- and chrysotile-induced macrophage supernatants. Bars denote $\bar{x}$ + SD. Significant reductions compared to 3-day saline produced by 3-day chrysotile at 1 μg/ml ($P < 0.001$) and 2 μg/ml ($P < 0.01$) Con A; significant stimulation of mitogenesis produced by 280-day chrysotile compared to 3-day chrysotile at 1 μg/ml Con A ($P < 0.002$); significant stimulation of mitogenesis produced by 280-day saline compared to 3-day saline at 1 μg/ml Con A ($P < 0.05$).

cantly ($P < 0.05$, see Table 2 for details) inhibited by supernatants from asbestos-activated macrophages.

Effect of Dialysis on the Inhibitory Action of the Supernatant

Figure 4 shows the result of an experiment where supernatant from asbestos-activated macrophages was halved, one half dialysed against medium, then tested alongside the undialysed half in the mitogenesis assay. It is clear that dialysis reduced the inhibitory activity of the supernatant but significant (see Fig. 4 for details) inhibitory activity remained at 2 μg/ml Con. A even after dialysis.

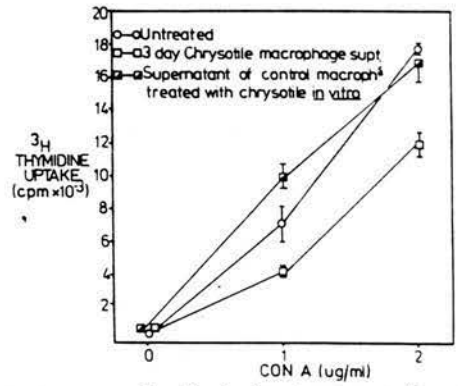


FIG. 3. Effect of supernatant, prepared by the *in vitro* treatment of control macrophages with chrysotile, on thymocyte mitogenesis by Con A. 3-day chrysotile (*in vivo*) macrophage supernatant included as positively suppressor supernatant. Symbols denote $\bar{x} \pm$ SD. Significant inhibition of mitogenesis produced by the *in vivo* chrysotile supernatant at 1 μg/ml ($P < 0.02$) and 2 μg/ml ($P < 0.001$) Con A. Significant enhancement of mitogenesis produced by *in vivo* chrysotile/macrophage supernatant at 1 μg/ml Con A ($P < 0.02$).

TABLE 2
EFFECT OF 3-DAY CHRYSOTILE OR SALINE MACROPHAGE SUPERNATANT ON NYLON-WOOL-COLUMN-PURIFIED SPLENOCYTE MITOGENESIS EXPRESSED AS MITOGENIC INDEX[a]

Supernatant	Con A (μg/ml)	
	1	2
Saline	0.88 ± 0.10[a]	0.98 ± 0.05
Chrysotile	0.70 ± 0.10	0.89 ± 0.02

Note. Significant differences, chrysotile vs saline: 1 μg/ml Con A, $P < 0.05$; 2 μg/ml Con A, $P < 0.05$.

[a] Mitogen index derived as in Table 1.

Effect of the supernatant on nonlymphoid cells. Supernatant from 3-day chrysotile-activated macrophages was tested in a fibroblast proliferation assay and, as shown in Table 3, no significant inhibition of fibroblast proliferation occurred.

DISCUSSION

The present study was undertaken to determine whether asbestos-activated peritoneal macrophages released activity which could modulate lymphocyte function; thymocyte mitogenesis with Con A was used as a model system. It was appropriate to study the asbestos-activated peritoneal macrophage in this regard since (i) alveolar macrophages from rats inhaling asbestos have previously been found to potentiate T lymphocyte proliferation (Miller and Kagan, 1981); (ii) epidemiological studies of asbestotics have revealed impairment of cell-mediated immunity and T-cell function which could, in view of the regulatory role of the macrophage in lymphocyte function, be macrophage mediated.

In the present study macrophages elicited in the mouse peritoneal cavity by asbestos injection and which contained asbestos as revealed by electron micros-

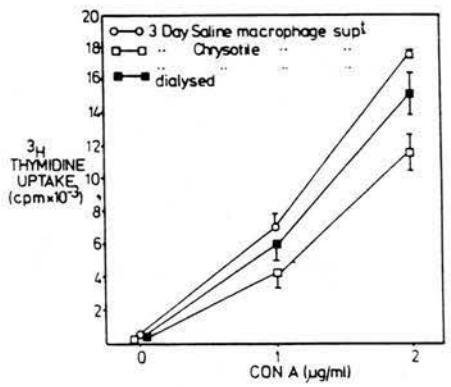


FIG. 4. Effect of dialysis on chrysotile-macrophage-supernatant-mediated suppression of mitogenesis. Significant reduction in mitogenesis caused by chrysotile supernatant compared to saline supernatant at both 1 μg/ml ($P < 0.05$) and 2 μg/ml ($P < 0.001$) Con A; significant reduction caused by dialysed supernatant at 2 μg/ml Con A only ($P < 0.01$). Symbols denote $\bar{x}$ + SD.

TABLE 3
EFFECT OF TREATMENT WITH VARIOUSLY INDUCED MACROPHAGE SUPERNATANTS ON UPTAKE OF TRITIATED THYMIDINE BY FIBROBLASTS

Supernatant	[^{3}H]Thymidine uptake (cpm ± SD)
None	1079 ± 164
Saline	922 ± 81
Chrysotile	1071 ± 171

copy, were found to release into the supernatant a factor(s) which significantly inhibited thymocyte mitogenesis by Con A. The inhibition was significant when variation between experiments was taken into account by expressing the results as "mitogenic indices" for each experiment. Expressed in the same way, supernatants from saline- and latex-elicited peritoneal macrophages were not inhibitory to thymocyte mitogenesis. The fact that latex injection did not elicit macrophages which released inhibitory activity is in agreement with our previous finding that such macrophages, in contrast to asbestos elicited macrophages, are not activiated (Donaldson *et al.*, 1982). The chrysotile-asbestos-activated macrophages were also inhibitory to mitogenesis of mature T-lymphocyte-enriched splenocytes.

The present study has revealed that the inhibition of mitogenesis was dose dependent and partially, but not wholly, dialysable.

The possibility that thymidine released by asbestos-activated macrophages into the supernatant was flooding the [^{3}H]thymidine label and causing "pseudo-inhibition" was considered. However, when the same supernatant was tested on [^{3}H]thymidine uptake by fibroblasts at a lesser incorporation rate in the present study, and on tumor cells at similar incorporation rates in a previous study (Donaldson *et al.*, 1983b), no inhibition of [^{3}H]thymidine uptake was observed. It is unlikely therefore that "cold" thymidine "pseudo-inhibition" was occurring in the experiments described here.

The inhibitory activity was not released by peritoneal macrophages derived from mice which had been injected with asbestos a long time previously, thereby suggesting that the inhibitory activity was an "acute" mediator.

Experiments also revealed that supernatants from control macrophages treated with a dose of asbestos *in vitro*, which caused considerable macrophage death, were not inhibitory to thymocyte mitogenesis. This suggests that the inhibitory factor(s) is not a cell component leaking passively from dead or damaged macrophages but represents an actively secreted component of activated macrophages.

The supernatant from asbestos-activated macrophages was not suppressive to fibroblast proliferation. When this result is taken together with our previous report that the same supernatants did not inhibit proliferation of a syngeneic fibrosarcoma cell line *in vitro* (Donaldson *et al.*, 1983b) it suggests that the inhibitory activity is lymphoid cell specific.

The release, by activated macrophages, of activities both stimulatory and in-

hibitory to lymphocyte function is well documented. The best characterized of the stimulatory molecules is Interleukin I (IL1) first described by Gery and Waksman (1972) and then termed lymphocyte activating factor (LAF). IL1 has been extensively purified and characterized (Mizel, 1979; Economou and Shin, 1980). IL1-type activity has been found to be released by activated macrophages from a variety of sources: alveolar macrophages treated with endotoxin *in vitro* (Murphy *et al.*, 1980), thioglycollate-elicited peritoneal macrophages (Tenu *et al.*, 1980), monocytes activated with endotoxin *in vitro* (Blyden and Handschumacker, 1977), and a macrophage cell line treated with phorbol myristate acetate (Mizel, 1979).

Other studies have, however, reported that activity inhibitory to lymphocyte proliferation is released by macrophages activated with a range of stimuli including peptone (Keller, 1976), *Toxoplasma gondii* infection (Wing and Remington, 1980), *Corynebacterium parvum* (Wing and Remington, 1977), and endotoxin (Nelson *et al.*, 1976). In addition activated macrophages have been shown to inhibit a number of proliferation-independent functions such as MIF release (Varesio and Holden, 1980), MAF release (Varesio *et al.*, 1981), and cytotoxic T-cell generation (Klimpel and Henney, 1978), suggesting that inhibiton of mitogenesis may be indicative of a more relevant and generalized inhibition of lymphocyte function.

Notwithstanding the fact that macrophages secrete at least eight substances which could inhibit lymphocyte function (Allison, 1978), inhibitory factors derived from activated macrophages have not been well characterized. Nelson *et al.* (1976) reported that macrophages from endotoxin-treated mice produced suppressor activity which was abolished by dialysis to reveal potentiating activity. Essentially the same result was reported by Calderon *et al.* (1974) who reported the suppressor factor to be 14,000 MW. With regard to the inhibitory activity from asbestos-activated macrophages the activity was only partially dialysable and there was no evidence of the appearance of potentiating activity. A role for prostaglandin in suppression by cells of the mononuclear phagocyte line has been suggested by Schechter *et al.* (1980) and a prostaglandin-secreting suppressor cell has been described (Goodwin *et al.*, 1977). In view of the reports that asbestos can induce prostaglandin release from macrophages (Humes *et al.*, 1977; Sirois *et al.*, 1980) it is possible that prostaglandin is an important factor in the inhibitory action of supernatants from asbestos-activated peritoneal macrophages described here. However, any of a number of macophage products (Allison, 1978) could be responsible for the observed inhibitory effect.

Several epidemiological studies of the immunological status of asbestotics have revealed impairment of cell-mediated immunity and peripheral T-cell mitogenesis (Kang *et al.*, 1974; Kagan *et al.*, 1977; Haslam *et al.*, 1978; Lange *et al.*, 1978; Gaumer *et al.*, 1981). It seems possible that macrophage activation in individuals inhaling asbestos could lead to the production of suppressor factors and to chronic impairment of cell-mediated immunity. In turn such a suppression of cell-mediated immunity could be a factor in the development of asbestos-related disease.

The present findings are in contrast to a study which reported that macrophages

from the lungs of rats inhaling asbestos, and which were activated as judged by several criteria (Miller and Kagan, 1976), were stimulatory to lymphocyte proliferation in the absence of mitogen (Miller and Kagan, 1981). This apparent contradiction could be due to a number of factors which have been reported to affect the expression of lymphomodulatory activity of macrophages including time in culture (Wing and Remington, 1980), cultural characteristics (Brune, 1980), and effector-to-target ratio (Wing and Remington, 1977). In addition the present study has utilized the peritoneal macrophage as a model whereas the alveolar macrophage represents the ideal cell for studies on the biological effects of asbestos.

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IMMUNOMODULATORY EFFECTS OF MINERAL DUST. I. EFFECTS OF INTRAPERITONEAL DUST INOCULATION ON SPLENIC LYMPHOCYTE FUNCTION AND HUMORAL IMMUNE RESPONSES *IN VIVO*

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SUMMARY Syngeneic PVG rats and C57BL6 mice were inoculated with liquid suspensions of UICC chrysotile A asbestos. DQ_{12} quartz or the inert particulate TiO_2. Rats received 10 mg and mice received 2·5 mg dust intraperitoneally on day 0. Injection of the pathogenic dusts asbestos and quartz was associated with a significant reduction in mitogenic response to PHA and Con A detected in splenocytes removed from animals 14 days after inoculation. Injection of TiO_2 had no significant effect on splenocyte mitogenesis. Pretreatment of C57BL6 mice with asbestos intraperitoneally also impaired subsequent production of antibody to the protein antigen KLH. These data show that intraperitoneal injection of chrysotile A asbestos and DQ_{12} quartz had a marked effect on generalized T lymphocyte function. An additional immunomodulatory effect of asbestos injection was shown in C57BL6 mice where *in vivo* humoral immune responses were suppressed.

Key words: Mineral dust, immunomodulation, splenic lymphocytes, humoral immune responses

INTRODUCTION

THE association between chronic inhalation of mineral dusts and lung disease is well known. In some cases, exposure to airborne pollutants including dusts has been shown to compromise the capacity of exposed individuals to resist infectious agents (1–3). Despite many epidemiological studies on general immune competence in dust-exposed workers (4–13), the role of the immune system in the development of mineral related diseases is still unclear.

In addition to the epidemiological studies there have been several experimental studies concerning the effect of *in vivo* mineral dust exposure on immune responses (14–19). Experimental studies on the cellular basis of the dust-related diseases have utilized several different methods of dust administration to animals to provide *in vivo* models of exposure. Thus, intraperitoneal (IP) injection, intracheal instillation (IT) and inhalation exposure have all been used and have provided valuable information on the biological effects of mineral dusts. Clearly, the inhalation route provides the most realistic method of dust administration relevant to occupational exposures. However, the difficulties and expense of generating stable, defined, respirable dust clouds have precluded the widespread use of inhalation techniques. Consequently IT has been used by many research groups an an alternative method of pulmonary dust deposition. The IP injection route has also proved useful particularly where recovery of representative, viable cell populations is required following dust exposure.

The pathogenesis of mineral dust-associated diseases is undoubtedly multifactorial and likely to be affected by a combination of events including macrophage/lymphocyte activation and production of soluble factors such as lymphokines. In order to assess the relative contribution of these factors in the generation of mineral dust induced diseases we have set out to study the immunomodulatory effects using defined routes of exposure. In the present paper we report some of the immunological consequences following intraperitoneal injection of asbestos, quartz and TiO_2 as assessed by splenocyte mitogenic responses to standard mitogens. We also present evidence for the immunomodulatory effects of asbestos on the generation

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of antibody to protein antigens. The results of studies on the immunomodulatory effects of exposure to mineral dusts by the inhalation route will be described separately.

Materials and Methods

Animals

Specific pathogen free inbred PVG rats were maintained by single-line brother-sister mating and supplied by Edinburgh University Centre for Laboratory Animals. Inbred C57BL6 mice were similarly maintained and used at approximately 10 weeks of age.

Mineral Dusts

Three dust preparations were used. Titanium dioxide (TiO_2—"Rutile") was obtained from TIOXIDE LTD., Cleveland, UK. The quartz dust was a Dorentrup sample, DQ_{12} fraction described as "less than 5 μm" particle size. Asbestos was a standard reference sample of chrysotile A, originally prepared under the auspices of the Union International Centre Cancer. All samples contained a substantial proportion of particles in the respirable size range.

In vivo *Manipulations*

All experimental animals were injected with suspensions of mineral dusts in saline or saline alone on day 0. Rats received a 10 mg dust suspension in a volume of 1 ml and mice received 2·5 mg dust suspension in 0·5 ml saline. Mice were immunized with 100 μg Keyhole Limpet Haemocyanin (KLH, CALBIOCHEM) emulsified in Freund's complete adjuvant (DIFCO) on day 7. All animals were killed on day 14 by exsanguination under ether anaesthesia and the spleens removed aseptically for mitogenesis.

Splenocyte Mitogenesis

Splenocytes were prepared from rats or mice by gentle disruption of whole spleens in a glass tissue homogenizer. Single cell suspensions were prepared after removal of course debris by sedimentation under unit gravity. Splenocytes were washed in complete medium (cRPMI) defined as follows: medium RPMI 1640 (GIBCO BIO CULT) supplemented with 2×10^{-3} M glutamine, 10^{-5} M 2-mercaptoethanol (SIGMA), 10% heat-inactivated foetal calf serum (GIBCO) and 0·06% gentacin (WINTHROP). Washed splenocytes were adjusted to 2×10^6 viable cells ml^{-1} and aliquots of 2×10^5 cells delivered into sterile 96-well microtitre plates (FALCON). Some experimentation was necessary to establish a routine method for rat splenocyte mitogenesis following reports of a requirement for special culture conditions (20) and these are summarized in Figure 1. Routine mitogenesis assays were carried out with mouse splenocytes by culturing cells in the presence of mitogen for 48 hr before addition of 0·25 μCi ^3H thymidine (AMERSHAM INTERNATIONAL) to each aliquot of 2×10^5 cells. Uptake of radiolabelled nucleic acid precursor was assessed after a further 20 hr by harvesting cells onto glass-fibre discs (Ilacon Sheet Processor) which were placed in plastic scintillation vials (KARTELL) and air dried before addition of 5 ml scintillation cocktail 1883 (VICKERS LABORATORIES). Radioactivity was measured by liquid scintillometry (LKB, RACKBETA).

Serum Antibody Assays

Serum antibody to KLH was measured by a solid-phase binding assay. Individual plastic microwells (Removawells, DYNATECH) were coated with antigen (KLH or control antigen, human serum albumin, HSA) at 100 μg ml^{-1} in 0·1 M Na_2CO_3, pH 9·6 at 4°C overnight. Wells were washed ×4 with assay diluent (phosphate buffered saline containing 0·1% (w/v) bovine serum albumin and 0·05% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20, SIGMA)) and 200 μl aliquots of test serum diluted 1/10 in assay diluent were added to each well. Following incubation in a humidified atmosphere at 37°C for 1 hr, the wells were washed ×4 in assay diluent and filled with 200 μl ^{125}I-labelled *Staphylococcus aureus* protein A (^{125}I-protein A). ^{125}I-protein A was prepared as described (21) except that L-cysteine (SIGMA) was used as the reducing agent instead of sodium metabisulphite. Approximately 10^5 counts min^{-1} were added to each well and after incubation at room temperature for 1 hr, the wells were washed ×4 in assay diluent and the remaining radioactivity was detected by gamma spectroscopy (LKB Rackgamma II) using a data reduction programme set to calculate the mean counts min^{-1} of triplicate samples with automatic background subtraction and calculation of standard curve.

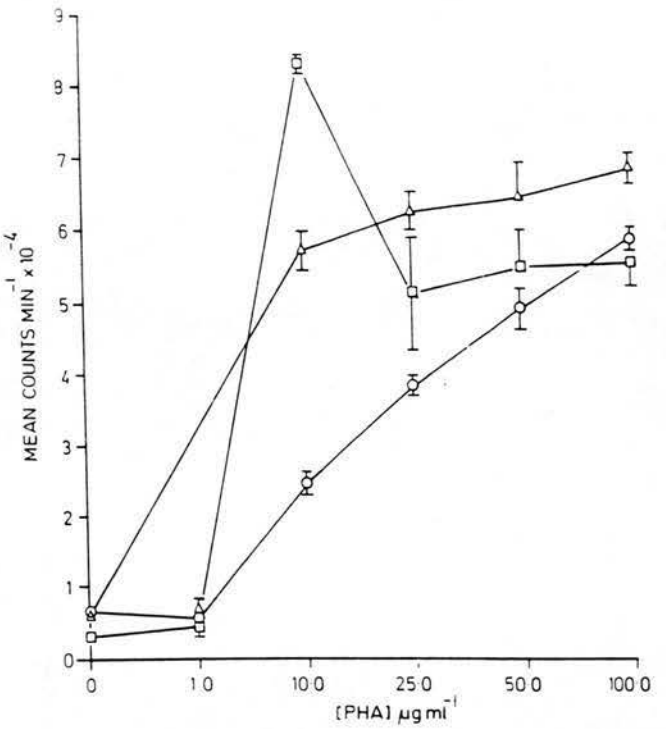


FIG. 1. Effect of culture conditions on PHA-induced mitogenesis of rat splenocytes. Washed splenocytes were cultured from day 0 in the presence of different concentrations of PHA as shown. Cells were labelled with 0·25 μCi ^3H thymidine and harvested 24 hr later as follows: ○ = label day 1, harvest day 2; △ = label day 2, harvest day 3; □ = label day 3, harvest day 4. Vertical bars = standard deviation of mean (n = 3).

Statistical Methods

Because of the wide range of measurements between background and mitogen-stimulated cell proliferations, data were log-transformed before analysis of variance tests were applied. A plot of the residuals against the fitted values for the analysis of variance model showed that the residuals were randomly scattered, indicating that the analysis was appropriate. All significance tests were performed using the above method although data are presented in terms of untransformed counts min^{-1} in the figures and tables.

Results

Optimisation of Rat Splenocyte Mitogenesis

Optimum conditions were determined as summarized in Figure 1. The dose-response curves indicate that 48 hr culture in the presence of about 10 μg ml^{-1} PHA gave a reproducible proliferation response as measured by uptake of ^3H thymidine. Increasing the PHA concentration

Table 1 Effect of intraperitoneal asbestos inoculation on splenocyte mitogenesis in C57BL6 mice. Mean and standard deviation counts/min for 3 mice in each group. NS = not statistically significant

	Asbestos treated		*Saline treated*
Background	673 ± 106	$p < 0.001$	1,333 ± 34
PHA 10 μg ml^{-1}	9,206 ± 2,597	$p < 0.02$	14,657 ± 1,075
Con A 1 μg ml^{-1}	11,785 ± 9,878	NS	27,495 ± 476
Con A 10 μg ml^{-1}	42,069 ± 5,215	$p < 0.02$	59,043 ± 5,185

10-fold resulted in approximately 25% increase in uptake of radiolabel and incubation with mitogen for more than 48 hr caused some distortion of the dose response curve. Unlike Lyndsay and Allardyce (20) we found no beneficial effect of heat-inactivated normal human serum as a medium supplement compared with foetal calf serum (FCS) in rat splenocyte mitogenesis assays. Therefore FCS was used routinely in the present studies.

Effect of Intraperitoneal Dust on Splenocyte Mitogenesis

The effect of a single intraperitoneal injection of 2·5 mg chrysotile A asbestos on splenocyte mitogenesis in C57BL6 mice is summarized in Table 1. In the absence of mitogen, the background *in vitro* uptake of ^{3}H thymidine by 2×10^5 splenocytes was reduced in asbestos-treated mice compared with saline-treated mice ($p < 0.001$). There was also a significant reduction of proliferation in response to PHA and Con A by splenocytes from asbestos-treated mice ($p < 0.02$).

These studies were extended by examining the effects of intraperitoneal dust inoculation in rats, where we had the opportunity to study the effects of quartz and TiO_2 in addition to asbestos.

Following a single intraperitoneal injection of asbestos in PVG rats (fig. 2), proliferative responses to PHA and 2 concentrations of Con A were depressed compared with saline treated rats. Responses in rats treated with "inert" particulate TiO_2 were not significantly different from those in saline-treated rats.

In quartz-treated animals, the response to PHA could not be distinguished from saline or TiO_2 treated rats. However, the response to the lower concentration of Con A was depressed in quartz-treated rats compared with the saline-treated group and the response to the higher concentration of Con A was depressed compared with the TiO_2-treated group. Therefore PHA and Con A mitogenesis results in rats indicated that intraperitoneal injection of TiO_2 had no significant effect on splenocyte mitogenesis, whereas asbestos and quartz had a suppressive effect. The large standard deviations of the mean values in dust-treated rats appeared to be a feature of dust-

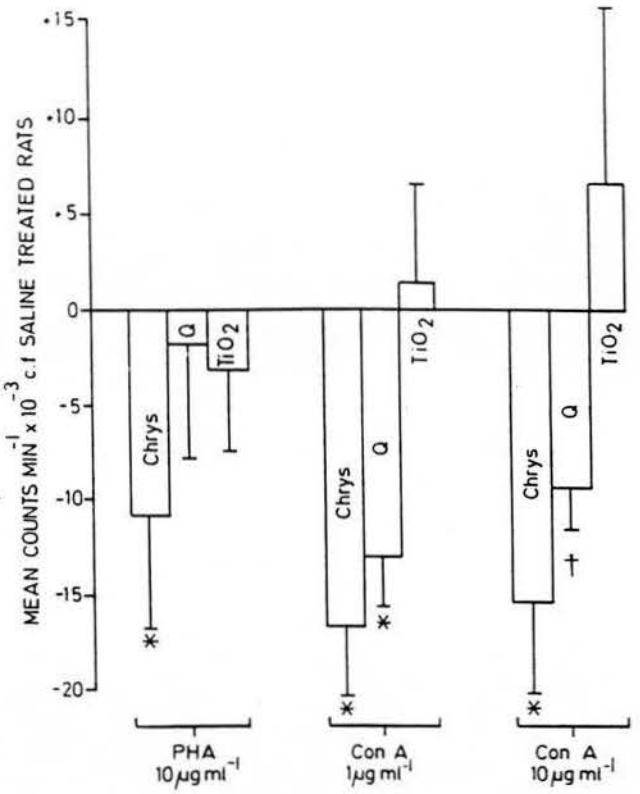


FIG. 2. Effect of intraperitoneal dust inoculation on splenocyte mitogenesis in PVG rats. Results are summarized as mean counts min^{-1} for 3 rats compared with saline-treated controls. Significances assessed by analysis of variance, * = $p < 0.05$. † = Suppression due to quartz inoculation is also significantly different from the effect of TiO_2, $p < 0.05$. Chrys = chrysotile A asbestos. Q = DQ_{12} quartz. Results are presented from 1 of 3 repeat experiments, each of which confirmed the results. Vertical bars = standard deviation of the mean.

inoculation rather than *in vitro* manipulation because standard deviations of the mean in saline treated rats were less variable (about 10% of mean counts min^{-1}—data not shown).

Antibody Production in Asbestos-treated Mice

Specific immunomodulation by asbestos was demonstrated by examining the production of antibody to a T dependent antigen (KLH) in mice following a single intraperitoneal injection of 2·5 mg chrysotile A asbestos. Mice which had been pretreated with asbestos showed a reduced antibody response ($p < 0.01$) to KLH compared with saline-pretreated mice (table 2). Non-specific antibody binding to KLH in non-immunized mice was not affected by asbestos pretreatment. Moreover, asbestos pretreatment combined with immunization with KLH had no effect on antibody binding to an irrelevant antigen (HSA).

Discussion

Previously published studies have revealed the immunomodulatory potential of dusts delivered by different routes.

Table 2 Effect of intraperitoneal asbestos inoculation on production of antibody to KLH in C57BL6 mice. Mean and standard deviations of counts/min for 6 mice in each group. Serum diluted 1/10 in assay diluent

Immunization with KLH	*Test antigen*	^{125}I-*protein A binding*		
		Asbestos treated		*Saline treated*
+	KLH	4,223 ± 995	$p < 0{\cdot}01$	5,948 ± 706
+	HSA	63 ± 4	NS	61 ± 7
−	KLH	71 ± 9	NS	63 ± 5

Results are mean counts min^{-1} ± SD for 6 mice.

For example, Miller and Zarkower (22) found that T lymphocyte activity in mice inhaling quartz was decreased in mediastinal lymph nodes and elevated in the spleens. Levy and Wheelock (23) found that antibody production to sheep erythrocytes and markers of cell-mediated immunity in quartz-treated animals were either variable or inhibited. Zarkower *et al.* (24) described changes in alveolar macrophage-mediated activity such as phagocytosis and mitogenesis accessory cell function in mice after fly-ash inhalation.

Epidemiological studies have provided information primarly concerning the inhalation route of exposure. An influence of quartz can be inferred from studies of coal miners who were exposed to multicomponent coal dusts containing quartz, where alterations in the humoral immunity of miners have been demonstrated (10–12). There have been several studies on aspects of immunocompetence of asbestos-exposed workers. Thus, impaired peripheral blood lymphocyte mitogenic responses and evidence of elevated antibody titres have been described (4, 6, 8, 9). However, it is important to note the difficulties of interpretation inherent in such epidemiological studies since it is not easy to distinguish between primary changes connected with dust exposure and second-order manifestations of chronic disease status.

We have demonstrated in the present investigations that IP injection of pathogenic dusts (quartz and chrysotile asbestos) is associated with a significant reduction in splenocyte mitogenesis. It has also been shown that this level of immunomodulation detectable by *in vitro* assay is associated with a demonstrable reduction in the capacity to mount an *in vivo* humoral immune response to the antigen KLH. Specific antibody production to the T-dependent antigen KLH was significantly reduced, whilst non-specific antibody was not affected and the total serum immunoglobulin also remained unchanged (data not presented).

It does not seem likely that the observed immunomodulatory effects are a consequence of direct movement of dust particles to the spleen. Widespread vascular dissemination of dust is not a recognized feature of IP injection and there was no histological evidence of dust in the spleens of dusted animals, or in the cell preparations used for mitogenesis assays.

In general, previous studies have shown that dust inhalation or parenteral exposure of animals induced prolonged changes in the size and composition of elicited cell populations (18, 25–28). Macrophage activation has been a consistent finding of our own studies. For example, we showed that intraperitoneal injection of asbestos elicited an activated macrophage population (29) which secreted increased reactive oxygen intermediates (30) and a factor(s) which was inhibitory to thymocyte mitogenesis (31). It is possible that IP injection of mineral dusts elicits populations of activated macrophages which secrete mitogenesis inhibitory factors having systemic effects. Therefore, splenocytes from dust-treated animals may be affected by inhibitory factors *in vivo* before being isolated for *in vitro* mitogenesis assays. Alternatively, activated macrophages may exist in the unfractionated splenocytes used for the mitogenesis assays and these macrophages may be able to confer their inhibitory effects by secretion of factors *in vitro*.

In addition to effects on splenocyte function, the present work has shown a significant reduction in antibody response to a T-dependent antigen. The relative contributions of macrophage-derived and lymphocyte-derived factors that influence these processes remains to be determined, but a consistent feature of the inhibitory effect is that it functions in the presence of cells taken from sites different from that of dust exposure. Clearly, modulation of immune responses to T-dependent antigens could also occur at several stages in the complex interactions between soluble factors, macrophages and lymphocytes during the *in vivo* generation of specific antibody. A major role for the T lymphocyte in the observed immunomodulatory effects should be anticipated either as a consequence of macrophage activation or as a result of direct interactions between lymphocyte subpopulations.

In analysing the complex cellular interactions which could result in the immunomodulatory effects we have reported in this study, we believe the macrophage could

be of particular importance. A common feature of chronic inhalation and IT exposure to mineral dusts is the recovery of increased numbers of alveolar macrophages (AM) compared with controls (32, 33) which are in an activated state (34). Similarly, IP injection of asbestos has been shown to induce increased numbers of peritoneal exudate cells comprising up to 70% macrophages (PM) (29). The well known dependence of lymphocyte functions on macrophage "help" increases the likely importance of AM and/or PM activation states following dust deposition in the lung or peritoneal cavity, particularly with respect to generalized lymphocyte responses to mitogens. Therefore, studies are in progress to consider the possible effects of dust-activated PM in the immunological perturbances we have demonstrated following IP exposure to mineral dusts.

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Antibody producing cells in the spleens of mice treated with pathogenic mineral dust

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ABSTRACT Experiments were carried out to assess the effect of intraperitoneal injection of the mineral dusts, titanium dioxide, quartz, or asbestos, on splenic lymphocyte antibody forming cells in immunised mice. Titanium dioxide and quartz caused similar, about one third, reductions in plaque forming cells; asbestos caused substantial reduction to about a quarter of the number found in control spleens. The inhibition of antibody forming cells in the spleen found with chrysotile was dose dependent and both chrysotile and crocidolite asbestos were similar in activity. Systemic immunomodulation after local deposition of mineral dust may be important to the development of disease.

Asbestos and quartz (silica) are two important mineral dusts, exposure to which is associated with the development of pulmonary disease in man.[1] In the case of quartz a nodular fibrosis (silicosis) develops, whereas with asbestos, diffuse interstitial fibrosis, tumours, and various forms of pleural disease arise. Leukocytes are considered to play an important part in these pathological processes[2,3] and the immune system is modulated in man[4–6] and animals[7,8] exposed to pathogenic dusts. We have ourselves studied the role of dust elicited leukocytes in inflammatory and immune responses after experimental treatment with quartz and asbestos dusts. These studies have shown inflammatory cell recruitment to sites of dust deposition,[9,10] production of inflammatory mediators,[11–13] and immunomodulatory factors[14] by these cells; we have also reported modulation of splenic lymphocyte T cell responses and circulating levels of specific Ig.[15] This latter parameter is an indirect measure of B cell function and in the present paper we set out to directly measure plaque forming cells (PFC) in the spleens of mice treated with the pathogenic mineral dusts, quartz and asbestos. A control dust titanium dioxide that is low in activity in biological systems[9,10] was also used.

Materials and methods

ANIMALS

Syngeneic, CBA, and C57B16 mice were used throughout the study.

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DUSTS

The following dusts were used: titanium dioxide (TiO_2, rutile form; Tioxide Limited, Stockton on Tees), UICC chrysotile asbestos standard sample 'A', and DQ_{12} quartz.

TREATMENT

Groups of three to eight mice were injected intraperitoneally with 0·5 ml of sterile phosphate buffered saline containing 2·5 ml of dusts; occasionally lower doses of dust were used. Control mice were injected with 0·5 ml of sterile saline alone.

ASSESSMENT OF SPLENIC PFCS

Plaques formed by antibody producing cells in spleens were determined according to the method of Mishell and Dutton.[16] Groups of mice were immunised by intraperitoneal injection with 0·2 ml of 10% sheep red blood cells (SRBCs). Spleens were removed from immunised mice four days later and 0·1 ml (10^5 cells) of splenic cell suspension were mixed with 0·1 ml guinea pig serum (preabsorbed with SRBCs) as a source of complement. This was added to the following mixture and kept at 45° C: 0·5 ml 10× concentrated Hanks balanced salt solution, 0·05 ml FCS, 0·4 ml 0·5% agarose, and 0·05 ml 20–30% SRBCs. The mixture was poured on to slides, allowed to gel, and then incubated at 37° C for two hours to allow plaques to form which were counted visually. Mice were treated with 2·5 mg of mineral dust three days before immunisation.

Table 1 *Inflammatory responses in the peritoneal cavity four days after injection of saline or dust (2·5 mg). Data given as $\bar{x}$(SEM) of total numbers of leukocytes (× 10^6) and $\bar{x}$(SEM) of percentages. (Three mice in each group)*

	Percentage			
Dust	*Macrophage*	*Neutrophil*	*Lymphocyte*	*Total No*
Saline	93·6 (3·0)	0·5 (0·3)	5·5 (0·6)	4·1 (1·0)
TiO_2	97·0 (2·6)	1·7 (2·1)	1·3 (0·6)	7·0 (2·5)
Quartz	70·3 (10·9)	23·5 (9·4)	5·3 (0·5)	11·4 (2·0)
Chrysotile	63·0 (8·5)	34·5 (9·2)	2·5 (0·7)	14·2 (1·2)
Crocidolite	64·3 (9·0)	33·3 (10·8)	2·1 (2·0)	27·9 (3·3)

Significant increase ($p < 0·1$–$0·001$) in total numbers of cells and percentage of neutrophils, with quartz, chrysotile, and crocidolite.

INFLAMMATORY RESPONSE IN THE PERITONEAL CAVITY

Inflammatory response in the mouse peritoneal cavity was assessed on cells lavaged using 3 × 2 ml volumes of phosphate buffered saline containing 10 units/ml of heparin, four days after injection; a volume of > 5 ml was invariably obtained. The total number of cells obtained from individual mice was counted in a Neubauer chamber and differential counts obtained from Diff-Quik (Merz-Dade, Dudingen, Switzerland) stained cytocentrifuge smears.

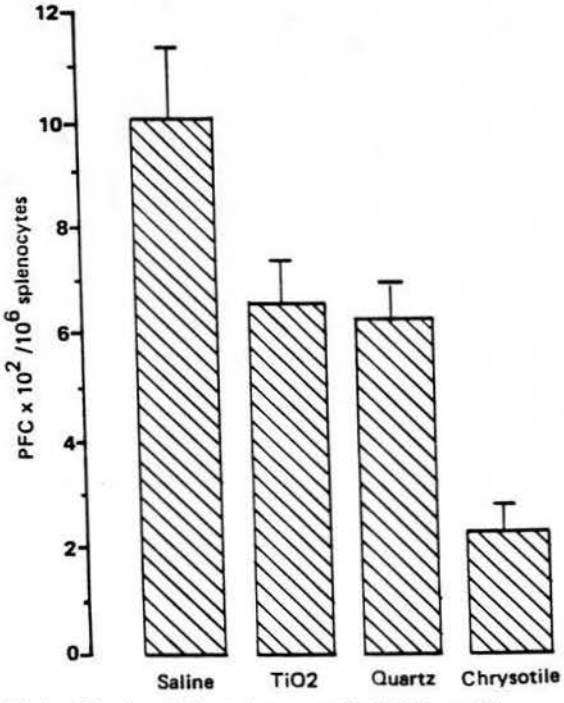


Fig 1 *Number of plaque forming cells (PFC) per 10^6 splenocytes in mice injected with 2·5 mg of indicated dusts three days previously. Results represent mean + SEM of five experiments, four mice in each experimental group. Significantly less PFC with quartz, TIO_2, and chrysotile compared with saline ($p < 0·01$–$0·001$); significantly less PFC with chrysotile compared with TiO_2 ($p < 0·001$); NSD between quartz and TiO_2.*

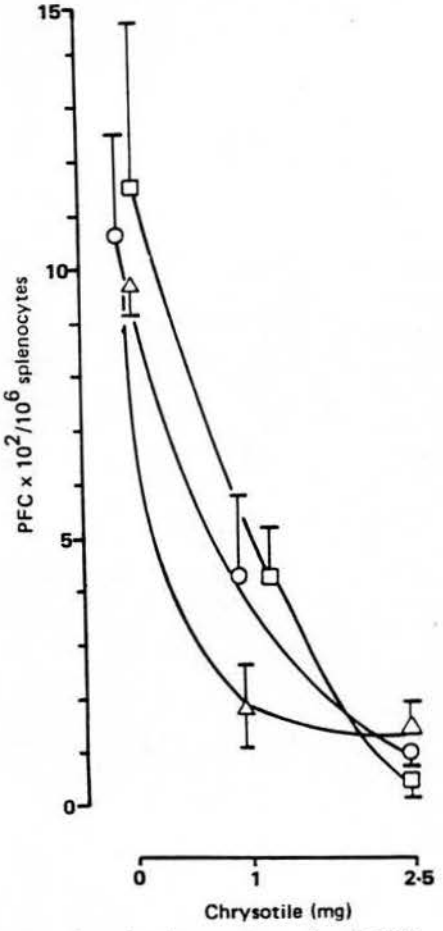


Fig 2 *Dose dependent decrease in number of PFC in spleens of mice treated with chrysotile asbestos. Results represent mean ± SEM of five mice in each treatment group shown for three separate experiments.*

Results

INFLAMMATORY RESPONSE TO MINERAL DUSTS

Table 1 shows the inflammatory response in the peritoneal cavity to dusts injected at the doses used for studying PFCs in spleen. These showed that TiO_2 had no inflammatory effect other than slight increase in macrophage numbers. Quartz and the two types of asbestos produced substantial amounts of inflamma-

Table 2 *Effect of intraperitoneal crocidolite or chrysotile on antibody producing cells in the spleen three days after injection*

	Treatment*	
Saline	Chrysotile	Crocidolite
1327 (229)†	539 (266)	589 (191)

*0·5 ml of saline or saline containing 2·5 mg of crocidolite or chrysotile asbestos.
†x̄(SEM) of plaque forming cells per 10^6 splenocytes. Significant ($p < 0·01$) reductions with asbestos compared with saline alone.

tion at day 4, as assessed by increased macrophages and neutrophils; crocidolite asbestos produced more inflammation than quartz or chrysotile.

PFCS IN THE SPLEENS OF RATS TREATED WITH MINERAL DUST

In fig 1 the results from five different experiments show that a single intraperitoneal injection of TiO_2 or quartz both produced about one third reductions in the numbers of splenic PFCs. By contrast, chrysotile caused a far greater reduction than both TiO_2 and quartz, down to about a quarter of the number found in control immunised spleens.

DOSE RESPONSE OF INHIBITION OF PFCS CAUSED BY CHRYSOTILE

Figure 2 shows that, in three different experiments, there was a pronounced dose response in the inhibition of splenic PFCs caused by injection of chrysotile asbestos.

COMPARISON OF CHRYSOTILE AND CROCIDOLITE ASBESTOS IN CAUSING REDUCTION IN SPLENIC PFCS

Table 2 shows the results of a single experiment where equal doses of crocidolite or chrysotile asbestos were used and shows similar reductions with both asbestos types in the number of PFCs present in the spleens of the injected mice.

Discussion

These experiments have shown that after exposure to mineral dust by single intraperitoneal injection the number of antibody producing cells in the spleens of immune mice is reduced. This reduction was caused by both TiO_2, a non-pathogenic dust, a fibrogenic dust quartz, and asbestos which is both fibrinogenic and carcinogenic. The decrease, however, was much greater with both chrysotile and crocidolite asbestos than with quartz or TiO_2. The ability of the dusts to cause this type of immunosuppression was not simply related to their ability to cause inflammation at the site of deposition, since quartz and chrysotile asbestos caused much more inflammation than TiO_2, yet chrysotile was much more immunosuppressive than quartz. Additionally, the immunosuppression caused by chrysotile and crocidolite was similar although crocidolite caused more inflammation at the time studied.

The inhibition of PFCs found with the inert dust TiO_2 is in keeping with several previous studies showing that inhalation exposure to a particulate load of relatively inert dust, such as fly-ash[17] or olivine,[18] cause modulation of immune function. Previous work from our own laboratories suggest that intraperitoneal TiO_2 has little inflammatory effect[9] or effect on splenic lymphocyte responses to T cell mitogens.[15] The present study has shown inhibition of specific humoral immune response, as assessed by PFCs in spleens, on injection of TiO_2. This indicates that a complex systemic immunomodulatory effect is present in the spleen of mice exposed to TiO_2 which may involve induction of suppressive T cells in the complex environment of the spleen.

With quartz our previous studies have shown that intraperitoneal exposure causes inhibition of mitogenic T cell responses.[15] In inhalation and intraperitoneal studies quartz has been reported to cause suppression of humoral and cellular immune responses both locally and systemically (in the spleen).[7] Splenic PFC responses in mice inhaling quartz and the non-toxic dust olivine have also shown reductions in immune responsivity with both quartz and the non-toxic dust; however, the suppression was greatest with quartz.[18]

With chrysotile asbestos our previous studies have shown reduction in serum antibody response after both intraperitoneal and inhalation exposure.[15] By contrast, epidemiological studies on workers exposed to asbestos have tended to show suppression of T cell immunity with a slight adjuvant effect on humoral immunity.[5 19 20] These findings in exposed workers are in contradiction to the inhibition of antibody forming cells in the spleens of mice treated with asbestos by intraperitoneal injection. The present study, however, was carried out three days after exposure to a large dose of the mineral and experiments carried out using eight day exposures showed a substantial decrease in the inhibitory activity of the asbestos on splenic PFC number (data not included). The responses shown here may therefore represent an acute response to asbestos deposition whereas chronic exposure may well result in a different pattern of change in the systemic immune system.

The experimental model used here could be criticised on the basis that the immunogen was deposited in the peritoneal cavity where dust induced inflammation had already been caused. This, however, mimics the situation in the lung where deposited mineral dusts such as asbestos and quartz cause alveolitis with

leukocyte recruitment.[10] The aim of the present study was to examine the effect of just such an inflammation on the response to any immunogen depositing in the lung. The model, therefore, is an appropriate one for studying immune responses imposed on mineral dust induced inflammation in the peritoneal cavity.

The immune response modifier which is responsible for affecting splenic lymphocyte responses may be assumed to pass from the peritoneal cavity, where the dust is deposited, to the spleen itself. Although this could be dust itself, which in the case of amosite can directly inhibit Ig production by B cells in vitro,[21] there is no convincing evidence that dust is transferred to the spleen in large amounts. Evidence that the signal for immunomodulation may be produced in the spleen itself has come from studies where we injected a lysate of washed splenocytes from quartz exposed rats into naive recipients and assessed their splenic mitogen responses. This showed that splenocytes from dust exposed rats, but not control rats, released factors causing profound immunosuppression.[22] Leukocyte traffic from the peritoneal cavity could be responsible and many products, now known to be released by dust exposed leukocytes, could be important in causing immunomodulation. Macrophages from rats exposed to silica[8] and asbestos[23] are reported to produce increased amounts of Interleukin-1; this would, however, be expected to produce enhanced B cell responses[8] rather than the inhibition evident in the present study. Other potentially important leukocyte products with ability to affect lymphocytes and known to be released by dust treated macrophages are reactive oxygen intermediates,[11] proteases,[13] and prostaglandins.[24] A low molecular weight inhibitor of splenocyte mitogenesis released from dust exposed peritoneal macrophages[13] may be of particular importance.

Other potentially important substances which could have systemic effects are the acute phase proteins. We have shown that these are found in greatly raised amounts in the serum of mice exposed to asbestos, compared with those exposed to TiO_2.[25] Furthermore, the levels produced by quartz were similar to those produced by TiO_2 (data not published) mimicking the pattern of response found here for immunosuppression in the spleen.

The results have shown no difference between chrysotile and crocidolite asbestos in causing immunosuppression, although crocidolite caused more inflammation, at the time studied, than chrysotile.

The implications of the present study are, of course, that inhaled dust causes important immunomodulation systemically which could be important for development of dust related disease or responses to other substances—for instance, infectious agents. In particular, the immunosuppression associated with the carcinogenic asbestos fibres could be important.

The peritoneal model differs from inhalation with regard to (*a*) site of deposition and (*b*) single hit nature of the stimulus in the peritoneal model compared with the multi-hit nature of daily inhalation exposure. Further work is clearly warranted to determine the importance of the immunomodulatory effects described here and of the peritoneal cavity as a site to study the effect of dusts on the immune system.

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Immunomodulation in mineral dust-exposed lungs: stimulatory effect and interleukin-1 release by neutrophils from quartz-elicited alveolitis

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SUMMARY

Quartz deposition in the rat lung causes an intense and persistent neutrophil alveolitis leading to parenchymal fibrosis. Bronchoalveolar leucocytes (BAL) from quartz-exposed rat lungs were studied for their effects on splenic lymphocyte proliferation; titanium dioxide (TiO_2) was used as a control, non-fibrogenic dust. Seven days after the intratracheal instillation of 1 mg of quartz or TiO_2 suspended in phosphate-buffered saline (PBS), BAL were recovered by lavage; the effect of PBS alone was also studied. TiO_2-elicited BAL (macrophages >98%) inhibited splenocytes responding to suboptimal phytohaemagglutinin (PHA) more than PBS-elicited BAL (macrophages >98%); the effect was dependent on the BAL:splenic lymphocyte ratio. Quartz-elicited whole BAL (macrophages 49%, neutrophils 51%), and an alveolar macrophage-enriched population with purity of 87% separated from it, were less inhibitory to splenocyte mitogenesis than PBS-elicited BAL. A neutrophil-enriched population, with a purity of 80%, markedly enhanced splenocyte response to PHA. In addition, whole quartz BAL and the macrophage-enriched population obtained from it enhanced the mitogenesis of T cell-enriched lymphocytes at a much lower BAL:lymphocyte ratio. The neutrophil-enriched quartz BAL enhanced mitogenesis substantially more than the whole or macrophage-enriched population from quartz-exposed lung. Supernatants from normal macrophages, PBS BAL, TiO_2 BAL, quartz BAL and both alveolar macrophage and neutrophil-enriched quartz populations were assessed for interleukin-1 (IL-1) activity. Quartz-BAL, quartz macrophages and quartz neutrophils all produced significantly higher IL-1 levels than PBS BAL; the supernatants from quartz neutrophils, however, showed the highest IL-1 activity. These findings suggest that quartz-elicited bronchoalveolar leukocytes, especially neutrophils, enhance lymphocyte proliferation and that increased IL-1 secretion by these cells is likely to be the effector molecule involved. These findings have important implications for immune response in mineral dust-stimulated lung and for inflammatory lung disease in general.

Keywords immunomodulation lungs quartz macrophages neutrophils

INTRODUCTION

Mineral dusts such as asbestos, quartz and coalmine dust, cause pulmonary fibrosis (pneumoconiosis) (Morgan & Seaton, 1984). The products of dead (Harington, 1974) and activated macrophages (Schmidt *et al.*, 1984; Brown, Monick & Hunninghake, 1988) following phagocytosis of dust are generally considered to be important mediators of this process. Neutrophils are also found in increased numbers in the bronchoalveolar lavage from dust-exposed workers and experimentally exposed animals (Gellert *et al.*, 1985; Begin *et al.*, 1986; Donaldson *et al.*, 1988).

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Alveolar macrophages activated by inhaled fibrogenic dusts have been reported to have altered immunomodulatory properties (Miller & Kagan, 1981; Hartmann, Georgian & Kagan, 1984a; Hartmann *et al.*, 1984b) and altered immune responses in mineral dust-exposed worker populations have been described (Soutar, Turner-Warwick & Parkes, 1974; Kagan *et al.*, 1977; Robertson *et al.*, 1983). This immunomodulation could be caused by alveolar macrophages which, following phagocytosis of dust, have altered immunological status and migrate to lymphoid tissue (Harmsen *et al.*, 1985) where they are capable of affecting lymphocyte responses. Indeed it has been shown that *in vivo* or *in vitro* exposure of rat alveolar macrophages to quartz leads to their enhanced production of the important inflammatory and immunomodulatory cytokine interleukin-1 (IL-1) (Oghiso & Kubota, 1986b, 1987). Neutrophils also migrate to lymph nodes (Harmsen *et al.*, 1985) and have been reported to show immunomodulatory activity (Goto *et al.*, 1984).

The present study was aimed at investigating effects of inflammatory bronchoalveolar leucocytes (BAL), recruited to quartz-exposed lung, on lymphocyte proliferation. We report here the enhancement of lymphocyte proliferation by quartz-elicited bronchoalveolar macrophages and neutrophils.

MATERIALS AND METHODS

Animals

Inbred, female PVG rats aged 12–15 weeks and weighing 250–300 g were supplied by the Breeding Unit of the Institute of Occupational Medicine. Rats were maintained in specific pathogen-free conditions.

Dust

Two kinds of dust were used in the experiments: titanium dioxide (TiO_2) (rutile; Tioxide, Stockton on Tees, UK); and quartz dust (DQ12 standard).

Preparation of purified T lymphocytes

Rats were killed by i.p. injection of an overdose of nembutal. Spleens were aseptically removed and disaggregated with a glass homogenizer in RPMI 1640 (HEPES-buffered; GIBCO, Paisley, UK). After haemolysis of erythrocytes with hypotonic buffer, splenocytes were washed with RPMI 1640 and suspended in RPMI 1640 supplemented with 5% fetal calf serum (FCS), 2 mM glutamine and 100 mg/l kanamycin. Two millilitres of splenocyte suspension at a concentration up to 50×10^6 cells/ml were loaded onto a 0·6-g packed nylon wool column (Fenwal, Deerfield, IL) which was equilibrated with the same medium. Columns were incubated at 37°C in 5% CO_2 for 45 min. Non-adherent cells were eluted with 15 ml of warmed medium at a flow rate of 1 ml/min. Recovered cells were then passed through a second column. Nylon wool-separated splenocytes were resuspended in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 50 mM 2-mercaptoethanol and 100 mg/l kanamycin (cRPMI) and co-cultured with bronchoalveolar leucocytes from rats as described below.

Nylon wool-separated splenocytes comprised 80% T cells as judged by immunofluorescence with a monoclonal mouse anti-rat T cell (Serotec, Oxford, UK) and 2% B cells as judged by surface immunoglobulin staining as reported previously (Donaldson, Davis & James, 1984). Thus, nylon wool-separated splenocytes were used as a source of T cells for the following experiments.

Splenocyte or T cell-enriched lymphocyte mitogenesis

Splenocytes or T cell-enriched lymphocytes (2×10^5) with or without BAL were cultured in cRPMI in 96-well microtitre plates in the presence or absence of a suboptimal dose (10 μg/ml) of phytohaemagglutinin (PHA) (Sigma, Poole, UK) for 2 days at 37°C in 5% CO_2. The cultures were then pulsed with 9·25 kBq ^{3}H-thymidine (Amersham International, Amersham, UK), incubated overnight, and the uptake of ^{3}H-thymidine was determined by liquid scintillation counting. T cell-enriched lymphocytes gave about half of the ^{3}H-thymidine uptake shown by whole splenocytes.

Effects of bronchoalveolar cells from dust-exposed rats o splenocyte mitogenesis

Groups of three rats were anaesthetized with ether, the intratracheally instilled with 1 mg of quartz dust or TiO suspended in 0·5 ml phosphate-buffered saline (PBS) through small incision in the trachea; the skin was then closed with meta clips. PBS alone was injected into rats as control. BAL wer obtained by lavage 7 days later at time-points where th inflammatory response had stabilized. Lungs were remove from rats killed by nembutal overdose and then lavaged wit 4×8-ml volumes of warm (37°C) saline; BAL were obtained b centrifugation, washed with RPMI 1640 and suspended i cRPMI; and cells from separate animals were pooled. BAL fron quartz-treated rats were separated into the alveolar macro phage-enriched population (QMAC) and the neutrophil enriched population (QPMN) by density gradient centrifuga tion through Sepra-cell medium (Sepratech, USA). Total an differential counting was done on Diff-Quick (Merz-Dade Dudingen, Switzerland) stained cytospin preparations an viability was assessed by trypan blue exclusion. Viable whole c separated BAL were added to splenocytes or lymphocytes a final ratios of 1:64 to 1:8. The cultures were incubated an assessed for mitogenesis as above. It was possible that the BA themselves could take up ^{3}H-thymidine, so this was tested. A the highest number of BAL (1:8) the uptake was negligible whe BAL alone were incubated with ^{3}H-thymidine (ranges: norma BAL, 9–28 ct/min; whole quartz, 25–34 ct/min; QMAC, 8–44 ct min; QPM, 15–28 ct/min).

Preparation of supernatants from BAL

Normal BAL, TiO_2 BAL, PBS BAL, quartz whole BAL QMAC and QPMN were adjusted to 1×10^6 viable cells/m These were incubated for 24 h at 37°C in 5% CO_2. Supernatant were collected, spun, filtered through 0·2-μm filters and froze until use in the IL-1 assay.

IL-1 activity

Two-fold dilutions of supernatants from cultures of BAL wer incubated with C3H mice thymocytes in cRPMI at 6×10^5/we in microtitre plates. PHA was added to a final concentration c 4 μg/ml and the plates were cultured for 72 h. Thymocyt proliferation was determined by the incorporation of ^{3}H thymidine added during the final 16 h of culture. Supernatan from unfractionated spleen cells of mice cultured with 10 μg/m concanavalin A (Con A) served as a positive control; Con A activity was neutralized with methylmannoside.

IL-2 assay

Supernatants from normal, PBS-elicited whole, quartz BAL QMAC and QPMN were tested for IL-2 activity. In brie supernatants were prepared as described above. Serial dilution of supernatant were added to 5×10^3 cells of an IL-2-dependen cell line (CTLL-2; kindly supplied by Dr Julian Symonds suspended in cRPMI without HEPES in each well of 96-we microtitre plates. A Con A-induced, IL-2-rich supernatant fror rat splenocytes was used as a positive control (Gearing Johnstone & Thorpe, 1985). The cells were incubated in 5% CO at 37°C for 24 h, pulsed with ^{3}H-thymidine and incubate further for 6 h and harvested. Uptake of ^{3}H-thymidine wa determined by liquid scintillation counting.

istical analysis

the effects of BAL on splenocyte or lymphocyte mitogene-as variation between experiments was large, the data were ·essed as mitogenic indices (MI) for each condition; the MI ɛ obtained by dividing the suboptimal PHA-driven mitoge-s with BAL by the mitogenesis without treatments. Typical *r*-data' from an experiment on the effect of PBS BAL and rtz BAL on T cell-enriched splenocytes are given as an mple (all data given as mean ± s.d. of ct/min at ratio of 1:8) nocytes alone 7713 ± 756, PBS BAL 1488 ± 511 (MI 0·19), rtz BAL 1035 ± 266 (MI 0·13), QMAC 640 ± 344 (MI 0·08), QPMN 20 518 ± 1142 (MI 2·66). The differences in mean values of MI between treated and untreated were tested by Student's *t*-test. The mean MI from the cultures with TiO_2 BAL or quartz BAL, QMAC or QPMN were compared with those in the cultures with PBS BAL at every ratio. In the IL-1 assay, the ^{3}H-thymidine uptake by the cultures with various supernatants were compared with that by cultures with supernatants from normal BAL. The differences in the mean mitogenic indices or mean ^{3}H-thymidine uptake were evaluated by Student's *t*-test. The differences were considered significant if P was $< 0{\cdot}05$.

le 1. Total and differential counts of normal BAL, PBS-BAL, TiO_2-L, quartz-BAL and both macrophage-enriched (QMAC) and neu-hil-enriched populations (QPMN) separated from the quartz BAL

	Total (10^6 per rat)	Differential (%)		
		Macrophage	Neutrophil	Lymphocyte
rmal ($n=3$)	4·16 ± 0·33	> 98	0	< 2
S ($n=3$)	3·56 ± 1·53	> 97	< 1	< 2
t_2 ($n=3$)	4·61 ± 0·95	> 97	< 1	< 2
artz ($n=6$)	20·69 ± 5·40	49 ± 7	51 ± 7	< 2
IAC	NA	87 ± 3	13 ± 3	< 2
MNC	NA	20 ± 3	80 ± 3	< 2

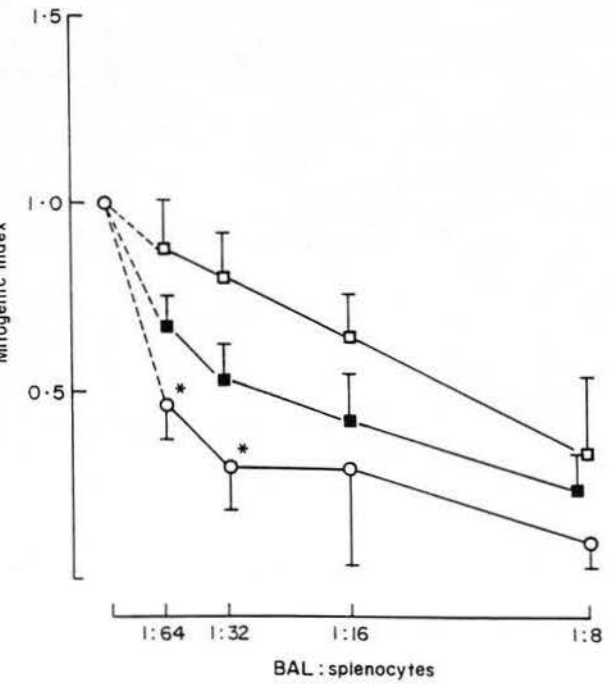
Lungs lavaged 7 days after treatment.
Results are mean ± s.d. NA, not applicable.

RESULTS

The 1-mg dose of quartz used in the present study has been shown by us to cause recruitment of inflammatory macrophages and PMN into the BAL population in the long-term and to lead to fibrosis and alveolar lipoproteinosis (Brown *et al.*, 1989).

Effects of BAL on splenocyte mitogenesis

Table 1 summarizes total numbers and differential count of the normal BAL, PBS-BAL, TiO_2-BAL, quartz-BAL, QMAC and QPMN. The recovery efficiency after the separation was 68% (8 s.d.) ($n=6$). Exposure to quartz clearly provoked inflammation



g. 1. Bronchoalveolar leucocytes (BAL) from normal (□), PBS-tilled (■) and TiO_2-instilled (○) rats inhibit splenocyte mitogenesis in lose-dependent manner. TiO_2 BAL were more inhibitory than PBS AL at ratios of BAL:splenocytes of 1:64 and 1:32. Mitogenic index rived as the ratio of mitogenesis with BAL: without BAL. Data (mean d s.d.) from three separate experiments in triplicate samples. * Signifi-nt difference from PBS alone.

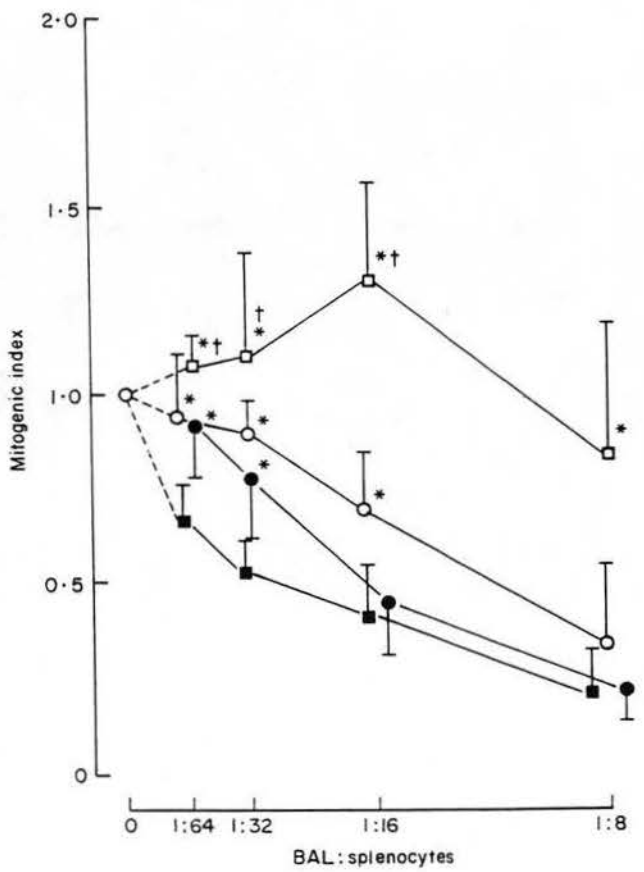


Fig. 2. Quartz-elicted bronchoalveolar leucocytes (BAL) and an alveolar macrophage-enriched population (●) separated from the whole cells (○) were less inhibitory to splenocyte mitogenesis than PBS-elicited (■) BAL at 1:64, 1:32. A neutrophil-enriched population (□) enhanced mitogenesis at every ratio. Data from three separate experiments in triplicate samples. * Significant difference from PBS BAL; † significant difference from splenocytes alone.

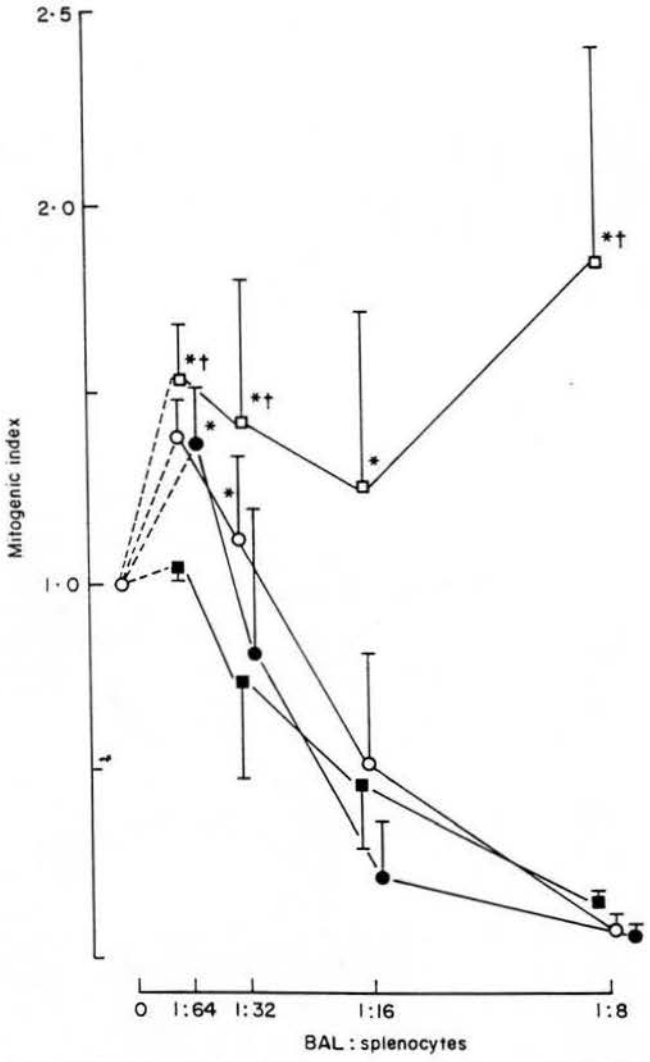


Fig. 3. Whole bronchoalveolar leucocytes (BAL) from quartz-instilled rats and its macrophage-enriched population (QMAC) enhanced T cell-enriched lymphocyte mitogenesis at 1:64. A neutrophil-enriched population (QPMN) enhanced substantially more. ■, PBS; ○, whole; ●, QMAC; and □, QPMN. Data from three separate experiments in triplicate samples are shown as mean and s.d. * Significant difference from PBS; † significant difference from T cell-enriched splenocytes.

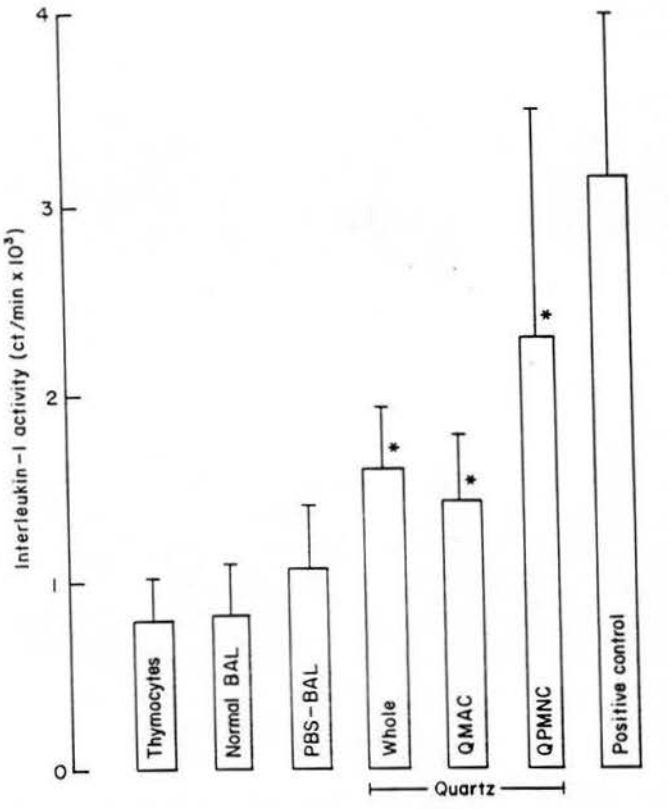


Fig. 4. Interleukin-1 activity in supernatants from quartz-elicited whole bronchoalveolar leucocytes (BAL), the alveolar macrophage-enriched population (QMAC) or the neutrophil-enriched population (QPMN). Data (mean and s.d.) from three different supernatants for each population in triplicate samples. * Significant difference from normal BAL.

as shown by the pulmonary neutrophil influx. QMAC or QPMN had purity of ⩾80%. Normal BAL, PBS BAL and TiO_2 BAL showed an inhibitory effect on splenocyte mitogenesis which was BAL:splenocyte ratio-dependent. TiO_2 BAL were more inhibitory to mitogenesis than PBS BAL at 1:64 and 1:32 (Fig. 1). BAL from quartz-treated rats was significantly less inhibitory to splenocyte proliferation at ratios of 1:64, 1:32 and 1:16 than PBS BAL (Fig. 2). QMAC and whole quartz BAL showed less inhibition than PBS BAL at ratios of 1:64 and 1:32 and, in comparison to TiO_2 BAL, the quartz BAL were substantially less inhibitory. In contrast to the inhibitory effect of quartz BAL or QMAC, QPMN did not inhibit mitogenesis at 1:64 and 1:32 and in fact significantly enhanced it at 1:16, 1:32 and 1:64.

Effect of quartz BAL on T lymphocyte mitogenesis

PBS-BAL inhibited T lymphocyte mitogenesis in a BAL:lym-

Table 2. Absence of interleukin-2 activity in supernatants from normal bronchoalveolar leukocytes (BAL)

Supernatants		^{3}H-thymidine uptake (mean ± s.d. ct/min)
Positive control		3596 ± 78
CTLL cells only		135 ± 18
Normal BAL		106 ± 8
PBS-elicited BAL		108 ± 23
Quartz-elicited whole BAL		118 ± 21
	QMAC	119 ± 19
	QPMN	107 ± 32

PBS-BAL, quartz-BAL, a macrophage-enriched population (QMAC) and a neutrophil-enriched population (QPMN), both obtained from the quartz BAL. Data show results from three separate supernatants diluted 1:4 in triplicate samples. Positive control rat interleukin-2 was present at 5%.

yte ratio-dependent fashion. Quartz-elicited whole BAL QMAC were inhibitory to mitogenesis at 1:16 and 1:8 but of these populations enhanced it at 1:64. QPMN enhanced nphocyte response at every ratio and nearly reconstituted plenic lymphocyte proliferation at 1:8 (Fig. 3).

activity

re 4 shows the IL-1 activity in the supernatants from the al, PBS BAL, quartz BAL, QMAC and QPMN. Two-fold ons (1:2 to 1:64) of supernatants were tested and it was d that ^{3}H-thymidine uptake was greatest with the 1:4 and dilutions (data not shown). Quartz-elicited whole BAL, AC and QPMN had significantly elevated IL-1 activity pared to the normal BAL; PBS BAL did not. QPMN ed the highest IL-1 levels although not significantly greater whole quartz BAL.

activity

ernatants from various BAL populations at various dilu- failed to show detectable IL-2 activity. The positive rol caused IL-2-dependent CTLL cells to proliferate in a ion-dependent manner. Table 2 summarizes the results of L-2 assay with supernatants at a dilution of 1:4.

DISCUSSION

eolar macrophages are situated at the air-tissue interface, egically located for initial contact and phagocytosis of led particulates. They also play a crucial role in pulmonary une responses. Normal rat alveolar macrophages have been d to suppress the mitogenesis of lymphocytes (Holt, 1978, 9), although subfractions of alveolar macrophages have vn heterogeneity in suppression/enhancement of lympho- proliferation (Shellito & Kaltreider, 1984; Oghiso, 1987). results coincide with these findings.

In our rat model system, normal BAL (nearly all alveolar rophages) were inhibitory. However, quartz-elicited macro- ges were less inhibitory to whole splenocyte mitogenesis and fact caused enhancement of T cell-enriched lymphocyte ogenesis at low ratios. Enhancement of T cell immunity ld be particularly important in view of the role that T cell phokines could have in modulating further macrophage mulation, activation and inflammation in the lung of rtz-exposed individuals.

The diminished inhibition of lymphocyte function by quartz L compared with PBS BAL may be associated with IL-1 eted by QMAC. *In vitro*, rat or rabbit alveolar macrophages ete IL-1 on exposure to quartz (Oghiso & Kubota, 1986a, 7; Kampschmidt, Worthington & Mesecher, 1986) and the ernatant from human alveolar macrophages dusted with a *in vitro* cause fibroblast proliferation (Schmidt *et al.*, 1984; wn *et al.*, 1988). Inhalation exposure to silica also causes etion of IL-1 by alveolar macrophages and causes an ease in Ia-positive lung cells (Oghiso & Kubota, 1986b). A liminary study also showed that alveolar macrophages from rtz-dusted rats secrete IL-1 in a dose-dependent manner en cultured with quartz *in vitro* (data not shown).

In vitro, silica-dusted alveolar macrophages have orted to enhance T lymphocyte mitogenesis (Oghiso & bota, 1987). In our study, *in vitro*, alveolar macrophages from quartz-dusted lungs inhibited lymphocyte proliferations at high ratios. Products such as prostaglandins secreted from alveolar macrophages may be involved in the inhibition of lymphocyte mitogenesis (Demenkoff *et al.*, 1980). Inhalation exposure to quartz provokes more severe inflammation than TiO_2 or asbestos and prolongs inflammation even after exposure (Donaldson *et al.*, 1988). Accordingly, quartz could be expected to cause enhanced lymphocyte proliferation during continuous inhalation exposure if QMAC and lymph node lymphocytes interacted at crucial ratios. We are aware, however, of the possible differences between the intratracheal, single-hit model used here and the lung exposed to quartz by inhalation.

QPMN showed an augmentary effect on both whole splenocytes and T cell-enriched lymphocyte proliferation stimulated with mitogen. The IL-1 activity in the supernatants from QPMN may explain these findings along with the fact that macrophages are not present to exert other inhibitory effects.

Our QPMN could be contaminated with up to 20% alveolar macrophages and it is clear that alveolar macrophages activated by fibrogenic dusts secrete IL-1. Accordingly, two possible explanations for the IL-1 activity released by the QPMN population are that neutrophils do not secrete IL-1 and that the contaminating macrophages are responsible; and/or that neutrophils secrete IL-1. If we take the former, we must explain further why whole BAL with a macrophage population of 50% and QMAC with purity of 80% secrete levels of IL-1 similar to QPMN which had macrophage contamination of only 20%. Thus we adopt the latter explanation, and suggest that QPMN secrete levels of IL-1 similar to those produced by alveolar macrophages. Further evidence that neutrophils can enhance lymphocyte response was found when, in a companion study to the present one, using coalmine dusts, we showed a strong positive correlation between percentage PMN in any inflammatory BAL population and enhanced lymphocyte response (manuscript in preparation). It is, however, impossible to preclude the possibility that a sub-population of macrophages from inflamed lung, high in IL-1 producing activity, separates along with the neutrophils and contributes to the apparent production of IL-1 by the neutrophils. Other investigators have reported that neutrophils, which share the same bone marrow stem cell as macrophages, secrete IL-1: thus normal human peripheral blood PMN, when stimulated with endotoxin (Tiku, Tiku & Skosey, 1986); bovine peripheral blood PMN (Canning & Neill, 1989); and inflammatory PMN recruited into the mouse or rabbit peritoneal cavity all secrete IL-1 (Goto *et al.*, 1984, 1988). We believe the present report to be the first demonstration of IL-1 release by pulmonary PMN in the rat. This IL-1-like activity from lung neutrophils appears to have a molecular weight of < 10 kD in preliminary studies (data not shown). Such a low molecular weight might suggest the presence of an active IL-1 fragment following proteolytic cleavage (Dinarello *et al.*, 1984). In this regard, increased proteolytic activity of the quartz population has been demonstrated previously and this could be important in generating the active cleavage product (Brown & Donaldson, 1988). Purification and characterization of pulmonary neutrophil IL-1 is under way in our laboratory.

Although lymphocytes were present in BAL at only a very low level, we demonstrated that IL-2 was not involved in the enhancement of splenocyte and lymphocyte proliferation caused by BAL supernatants.

This study suggests that, in the lungs of individuals inhaling

quartz, the alveolar macrophages may be affected by phagocytosed dust to release a range of mediators which could modulate lymphocyte responses in the local environment of the lung and lung-associated lymph nodes. Additionally, neutrophils which are recruited into dust-exposed lung could enhance immune responses leading to the presence of persistent localized T cell activation. Any subsequent 'adjuvant-type' effect on the immune system could contribute directly to heightened responses within the lung both to quartz dust itself as we have previously described (Hannant, Donaldson & Bolton, 1988) and to infectious agents, both of which could contribute to the tissue injury and fibrosis found in pneumoconiosis.

Neutrophil recruitment to the lung occurs in several diseases where immune responses are apparently disordered (e.g. cryptogenic fibrosing alveolitis), and we suggest that a PMNC-mediated adjuvant effect, via IL-1, may also be important in these diseases.

ACKNOWLEDGMENTS

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EXPERIMENTAL STUDIES ON THE EFFECT ON THE IMMUNE SYSTEM OF EXPOSURE TO COALMINE DUST AND QUARTZ.

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INTRODUCTION

Immune effects of mineral dusts may influence the development and progression of pneumoconiosis. In coalworkers' pneumoconiosis, progressive massive fibrosis and Kaplan's Syndrome are said to be related to immunologic abnormalities.[1] In addition, experimental studies have revealed that fibrogenic mineral dust alter immune responses.[2,3]

As part of a programme to examine the effect of silica and coalmine dust on the immune system, two approaches were taken: firstly immunocompetent cells from the rat spleen were exposed to dust *in vitro* and their mitogenic responses were assessed; secondly, dusts were intratracheally administered to rats and the effects of elicited bronchoalveolar leukocytes on splenocyte mitogenesis were studied.

MATERIALS AND METHODS

Animals

Twelve to fifteen-week old, female, SPF-maintained, inbred PVG rats were supplied by the Institute of Occupational Medicine breeding unit.

Dusts

Four kinds of dusts were used in the experiments: two were coalmine dusts collected from the air of British collieries mining 1) anthracite coalmine dust (A) and 2) low rank coalmine dust (L), 3) titanium dioxide (TiO_2; rutile, Tioxide Ltd. Stockton on Tees), a dust of low biological activity, 4) quartz dust (DQ12 standard).

Splenocyte Mitogenesis

Rats were killed by intraperitoneal injection with Nembutal and spleens were aseptically removed and disaggregated with a glass homogenizer. After lysing the erythrocytes, splenocytes were suspended in Hepes-buffered RPMI 1640 medium supplemented with 50mM 2-Mercaptoethanol, 2mM glutamine, 100mg/l kanamycin and 10% fetal calf serum (cRPM1). Finally 2×10^5 splenocytes, in medium were delivered to each well of 96-well microtiter plates.

The splenocytes, with or without dust suspensions, supernatant or bronchoalveolar cells, were cultured in the presence or absence of a suboptimal dose of phytohemagglutinin (PHA, 10 µg/ml) for three days at 37°C in 5% CO_2. The cultures were then pulsed with 0.25uCi tritiated thymidine, incubated overnight, and the uptake of ^3H Thymidine was determined by liquid scintillation counting.

Effect of Dusts on Splenocyte Mitogenesis

The four kinds of dusts were autoclaved and suspended in cRPMI. Each aliquot was added to splenocytes to obtain a final concentration in the well of 10, 50 or 100 µg/ml. They were then co-cultured at 37°C in 5% CO_2 for 24 hours and stimulated with suboptimal PHA for a further three days in culture. A preliminary study showed that 24 hours of co-culture of the splenocytes with dusts led to the optimal response to PHA. The cultures were assessed for mitogenesis as described above.

Effect of Supernatants on Mitogenesis

Splenocytes were adjusted to 1×10^6 cells/ml and aliquots of 5ml were delivered to plastic flasks. The splenocytes were allowed to adhere for six hours (adherence efficiency 27 ± 10%, $\bar{x}$ ± sd) and non-adherent cells were removed by washing. The adherent splenocytes were cultured with dusts at a final concentration of 100 µg/ml for 24 hours and supernatants were collected which were spun, filtered and frozen until use. The supernatants, at various dilutions were delivered to wells containing 2×10^5 splenocytes and these were cultured and harvested as described above.

Interleukin-1 Activity in Spleen Cell Supernatants

Three-fold dilutions of supernatants from cultures of adherent spleen cells exposed to dust at 100 µg/ml, were incubated with C3H mouse thymocytes at 6×10^5 per well in microtiter plates. PHA was added at a final concentration of 4 µg/ml and the plates cultured for 72 hours. Thymocyte proliferation was determined by the incorporation of tritiated thymidine added during the final 16 hours of culture. Supernatant from unfractionated spleen cells cultured with 10 µg/ml Concanavalin A (Con A) served as a positive control. Con A activity was neutralized with methylmannoside before use in the thymocyte assay.

Effect of Bronchoalveolar Leukocytes from Dust-Exposed Rats on Splenocyte Mitogenesis

Rats were intratracheally instilled with 1mg of the four different kinds of dusts suspended in 0.5ml Phosphate Buffered Saline (PBS). PBS alone was injected into rats as a control. Bronchoalveolar cells (BAC) were obtained by lavage seven days later. BAC were washed with RPMI164O and sus-

ıded in cRPMI. BACs from quartz-treated rat were ıarated into a macrophage and neutrophil-enriched popula-ns by density gradient centrifugation through Sepra-cell dium. Total and differential counting was done on Diff-ck stained cytospin preparations and viability was assessed trypan blue exclusion. Total or separated BACs were add-to splenocytes at final ratios of from 1:4 to 1:128. The tures were incubated and assessed for mitogenesis as ɔve.

atistical Analysis

ıce variation between experiments was large, the data were ɔressed as mitogenic indices for each condition: the togenic indices were obtained by dividing the suboptimal A-driven splenocyte mitogenesis with dust, supernatant bronchoalveolar leukocytes, by the mitogenesis without se treatments. The differences in mean values of mitogenic lices between treated and untreated were tested by paired st. The differences were considered as significant if values re less than 0.05. In the IL-1 assay, the ^3H uptake by the tures with various supernatants were compared to those th control supernatant (no dust treatment).

RESULTS

Effect of Dusts on Splenocyte Mitogenesis *In Vitro*

Both quartz at 10, 50 or 100 µg/ml and low rank coalmine dust L at 50 and 100 µg/ml significantly enhanced mitogenesis. Quartz especially augmented splenocyte proliferation even without mitogen (data not shown). On the contrary, both TiO_2 and coalmine dust A suppressed splenocyte proliferation in a dose-dependent manner (Figure 1).

Effect of Supernatant from Dust-Exposed Adherent Splenocytes on Mitogenesis

The supernatant, tested at various dilutions did not cause enhancement of mitogenesis and, in fact, supernatant from splenocytes treated with quartz at a high dose were inhibitory to mitogenesis (typical results of 1:16 dilution shown in Figure 2).

Interleukin-1 Activity in Supernatants

Despite the lack of enhancement in the spleen cell mitogenesis assay, the thymocyte assay did show interleukin 1-like activity in the quartz supernatant diluted at 1:7.5 (Figure 3).

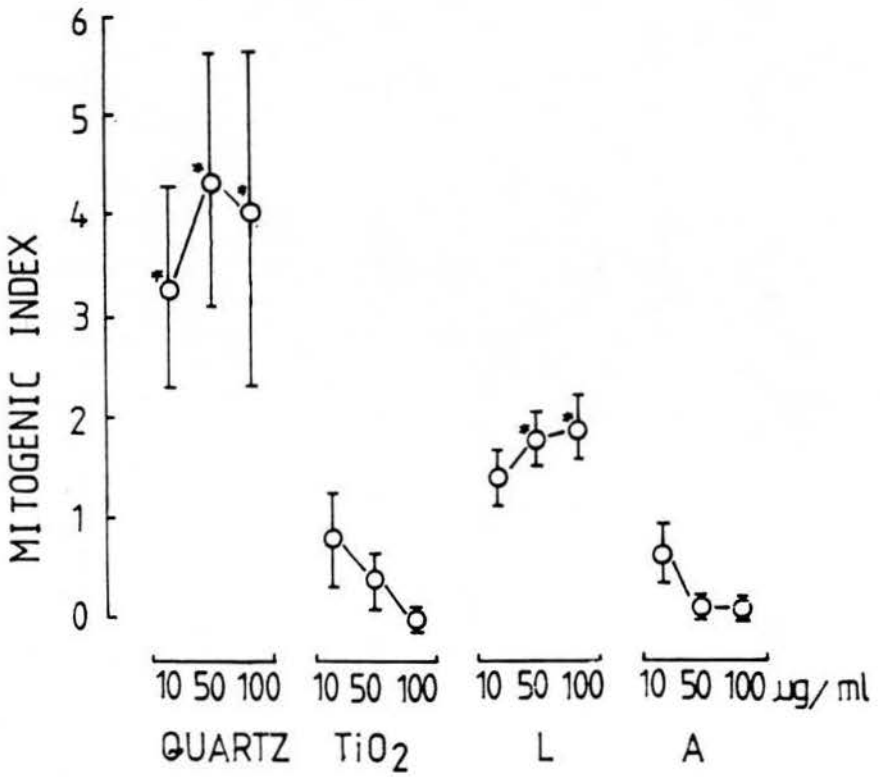


Figure 1. Mitogenic indices (means with standard errors) of splenocytes cultured with four kinds of dusts. Mitogenic index derived as the ratio of mitogenesis with dust:mitogenesis without dust. An asterisk denotes a significant ($p<0.05$) difference from the control.

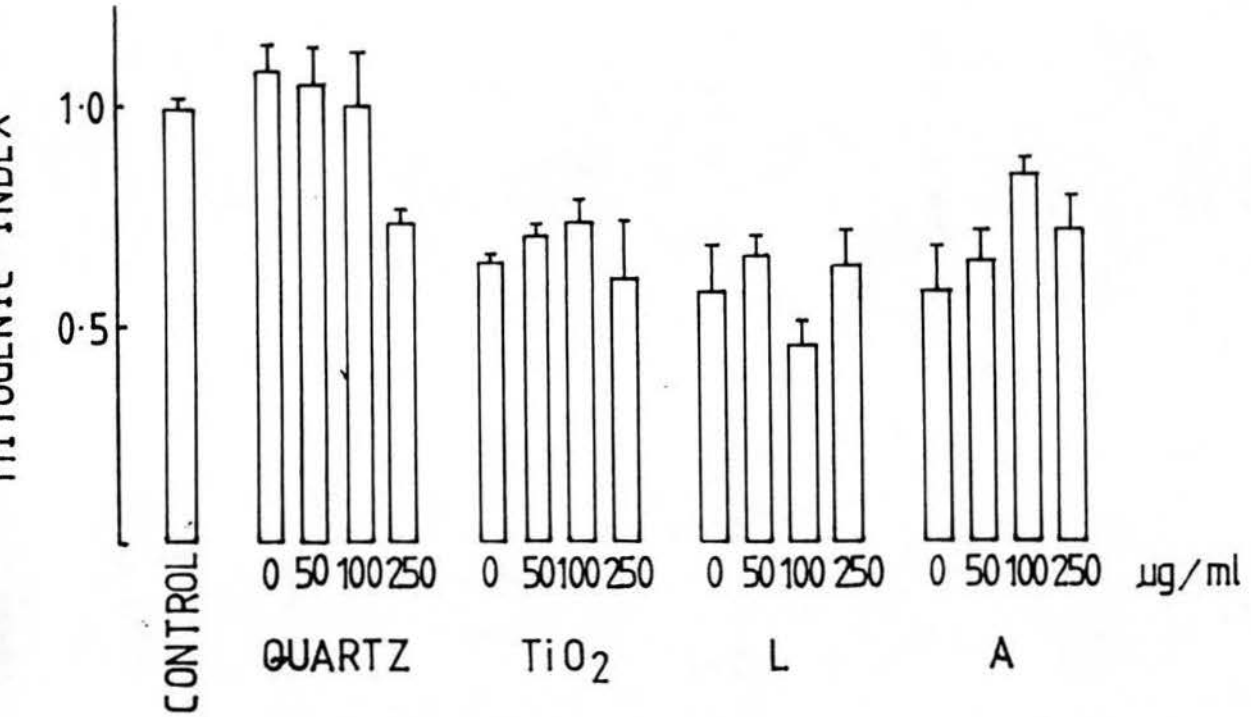


Figure 2. Effects of supernatants from dust-exposed adherent splenocytes on mitogenesis. Data are shown as mitogenic indices (means with standard deviations). Mitogenic index derived as the ratio of mitogenesis with supernatant from dusted or not-dusted adherent splenocytes:mitogenesis without supernatant.

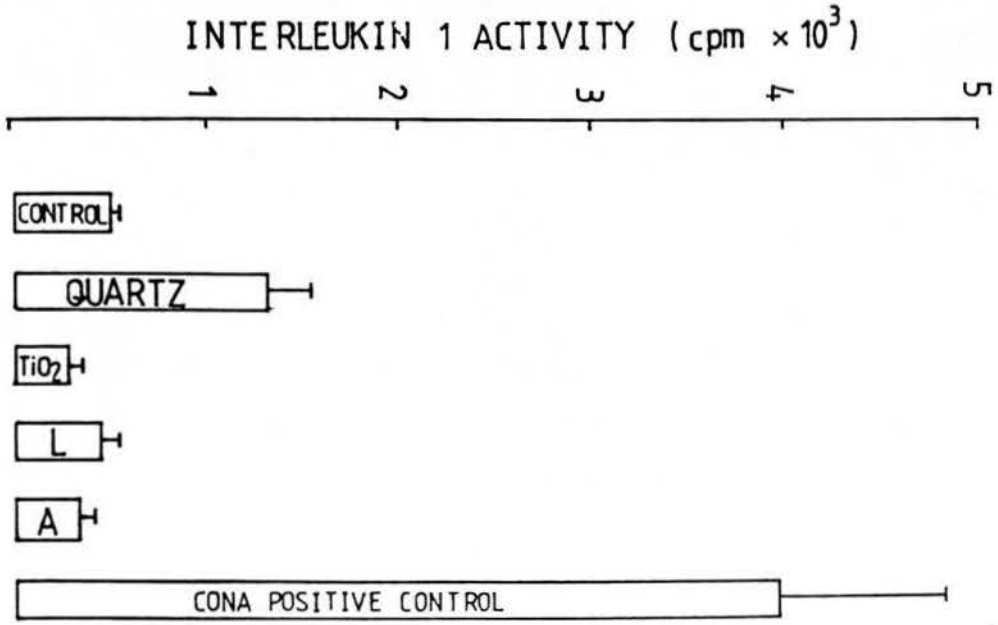


Figure 3. Interleukin 1 activity in supernatants from dust-exposed adherent splenocytes. An asterisk denotes a significant ($p < 0.05$) difference from the control supernatant.

gher concentrations of supernatant were inhibitory and ernatant from spleen adherent cells treated with other dust l no detectable Il-1 activity.

ect of Bronchoalveolar Leukocytes from Dust-posed Lungs on Mitogenesis

ntrol, PBS-elicited bronchoalveolar leukocytes (total 4.16 0.33×10^6 ($\bar{x} \pm$ sd) cells per rat, macrophages >98%, bility >95%) showed an inhibitory effect on splenocyte :ogenesis which was effector:indicator cell ratio-)endent. The bronchoalveolar leukocytes from coal dust total $3.08 \pm 0.76 \times 10^6$ cells per rat, macrophages 96 2%, neutrophils 3 ± 2%, viability >91%), coalmine dust) (total $2.58 \pm 0.33 \times 10^6$ cells per rat, macrophages 99%, viability >92%) or TiO_2 (total $4.61 \pm 0.33 \times 10^6$ crophages >99%, viability >93%) did not affect togenesis. Figure 4 shows the results for BAC from almine dust A.

e BAC from quartz-treated rats (total $16.83 \pm 4.64 \times$ ^{5} cells per rat, macrophages 42 ± 4%, neutrophils 57 ± ;) was significantly less inhibitory to splenocyte proliferan, at ratios of 1:64, 1:32 and 1:16, than the control. After e separation into macrophage- and neutrophil-enriched fracns (recovery rate 60 ± 1%), the macrophage-enriched pulation (macrophages 89 ± 5%) also showed less inhibin at ratios of 1:64 and 1:32. In contrast to the inhibitory effect of the total leukocytes or separated macrophages, the neutrophil-enriched population (neutrophils 82 ± 2%) markedly enhanced mitogenesis compared to control BAC (Figure 5).

DISCUSSION

In our rat model system, we have examined the effects of exposure to mineral dusts on the immune system. The splenic lymphocytes were taken as indicator cells for the direct effect of dust on the immunomodulatory role of leukocytes within the lung.

Both quartz and coalmine dust with a high (>5%) quartz component, enhanced splenocyte mitogenesis *in vitro*. Supernatant from adherent splenocytes, presumed to be mostly macrophages, treated with quartz showed increased IL-1 activity, whilst supernatant from coalmine dust or TiO_2-treated macrophages had no such activity. None of these supernatants caused enhanced mitogenesis. These apparently conflicting findings may be explained as follows. Adherent macrophages secrete, in addition to IL-1, a variety of substances including prostaglandins and hydrogen peroxide, which are inhibitory to lymphocyte proliferation.[4,5] Subsequently the ability of any supernatant to influence mitogenesis is likely to be the product of both the inhibitory and enhancing activity present in it. Evidence that inhibitory factors were present, and could be diluted out was

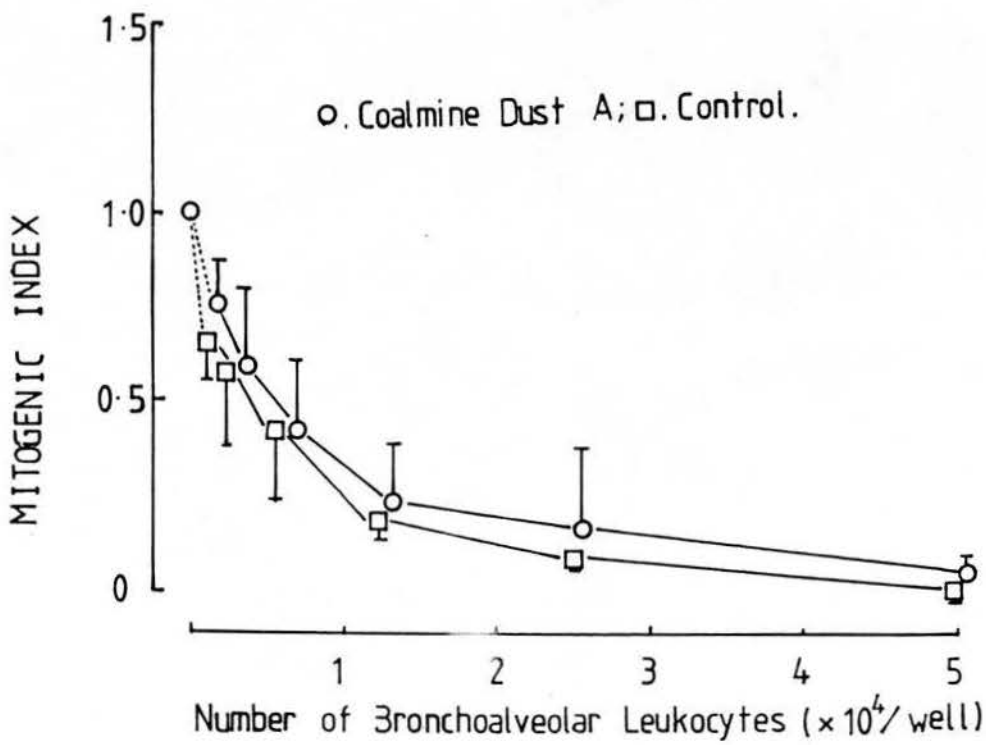


Figure 4. Bronchoalveolar leukocytes from coalmine dust L—instilled rats inhibited splenocyte mitogenesis in a dose-dependent manner. No significant differences from control leukocytes were present. Mitogenic index derived as the ratio of mitogenesis with bronchoalveolar leukocytes:without leukocytes.

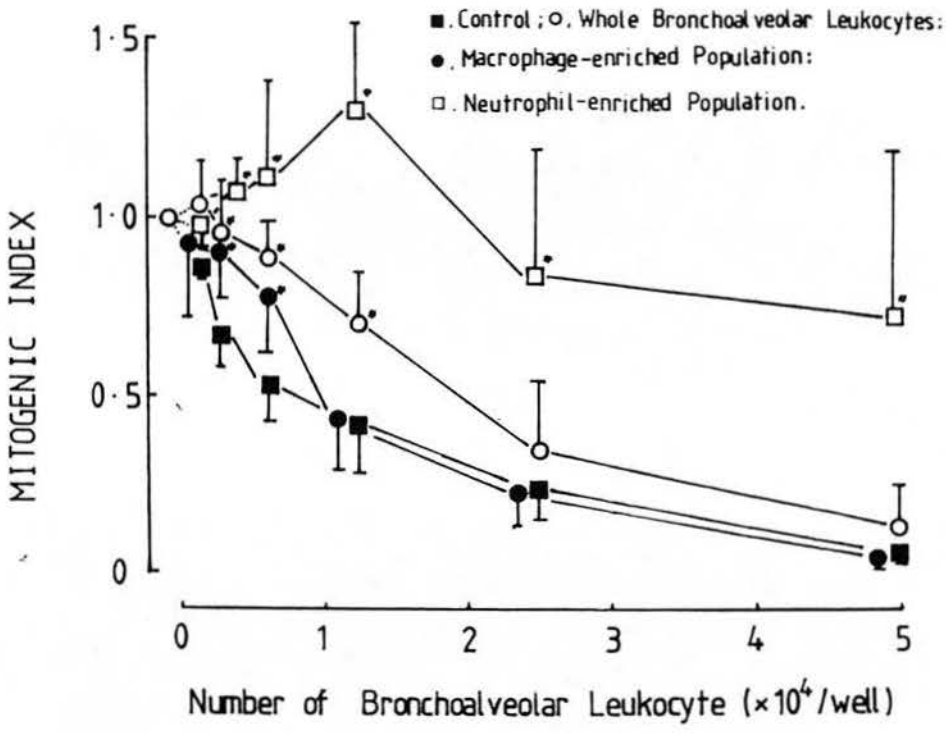


Figure 5. Effects of bronchoalveolar leukocytes from quartz-injected rats on splenocyte mitogenesis. Data are shown as mitogenic indices (means with standard deviations). An asterisk denotes a significant difference ($p<0.05$) from controls treated with PBS.

vn by the fact that IL-1 activity in the supernatant from tz-exposed splenocytes was expressed only at higher ions. Further studies are needed to elucidate the hanism of enhanced mitogenesis by quartz and low rank mine dust *in vitro* including further characterization of ecreted product present in supernatant form dust-treated rophages.

olar macrophages are situated at the air-tissue interface, egically located for initial contact with inhaled par- ates. They also play a crucial role in pulmonary immune onses. Alveolar macrophages in some species including are said to be poor accessory cells for mitogen or antigen- ved lymphocyte proliferation.[6] In our study normal nchoalveolar macrophages inhibited splenocyte genesis in a dose-dependent manner. TiO_2 and two s of coalmine dusts did not affect this down-regulatory tion of alveolar macrophages. However the whole BAC the alveolar macrophage-enriched population from tz-treated rats inhibited lymphocyte response to a lesser nt than control BAC although this may be due to con- nating neutrophils as described below. Further studies eeded to confirm whether alveolar macrophages elicited xposure to quartz have altered immunomodulatory prop- es, as suggested by this study.

neutrophils separated from quartz BAC strikingly anced mitogenesis and this could be mediated through ease[7] or an Il-1 analogue which has been described in etions from peritoneal neutrophils.[8]

Inhalation exposure to asbestos fiber, another type of fibrogenic dust, causes recruitment of Ia-positive alveolar macrophages and secretion of Il-1 by alveolar macrophages.[9,10] Additionally, alveolar macrohpages from asbestos-exposed rat enhanced T lymphocyte proliferation *in vivo*.[11] *In vitro* fibrogenic dust such as asbestos and silica stimulated alveolar macrophages to secrete IL-1.[12] Inhalation exposure to silica also causes secretion of IL-1 by alveolar macrophages when stimulated with endotoxin.[13] These studies suggest that fibrogenic dusts have immunostimulatory effects on alveolar macrophages and our results partially support these findings. However the complex effect of recruitment of newly exudated, monocyte-derived populations with altered cytokine production and the role of neutrophils, which are also found in dust exposed lung,[14] remain to be resolved.

This study suggests that, in the lungs of individuals inhaling quartz or quartz-containing coalmine dusts, the alveolar macrophages may be affected by phagocytosed dust to release a range of mediators which could modulate lymphocyte responses in the local environment of the lung. Additionally, neutrophils which are recruited into dust-exposed lung could also enhance immune responses leading to localized immunodulation. Any "adjuvant-type" effect on the immune system could contribute directly to heightened responses within the lung both to dust itself and to infectious agents, both of which could contribute to the tissue injury and fibrosis found in pneumoconiosis.

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Alveolitis Caused by Exposure to Coal Mine Dusts: Production of Interleukin-1 and Immunomodulation by Bronchoalveolar Leukocytes

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Two kinds of coal mine dust, low rank with high quartz (bituminous) and high rank with low quartz (anthracite), were assayed for ability to induce alveolitis and to stimulate interleukin-1 release from normal alveolar macrophages *in vitro*. Dust-elicited bronchoalveolar leukocytes were also assessed for their effects on macrophage-depleted splenocyte mitogenesis and their ability to produce interleukin-1. Quartz and titanium dioxide were used for comparison as toxic and inert dusts, respectively. Both coal mine dusts caused substantial release of interleukin-1 from normal alveolar macrophages *in vitro* and the levels were higher than those caused by quartz. Bituminous coal mine dust provoked acute but rapidly subsiding macrophage/neutrophil alveolitis which was greater than that provoked by titanium dioxide; anthracite caused less alveolitis than titanium dioxide and quartz caused large scale, persistent alveolitis. Whole bronchoalveolar leukocytes, including polymorphonuclear neutrophils, elicited by exposure to dust, were less inhibitory to lymphocyte mitogenesis than normal alveolar macrophages and bituminous coal mine dust-induced neutrophils were augmentary to lymphocyte mitogenesis; macrophages from inflamed lungs were, on the whole, inhibitory to lymphocyte mitogenesis. Alveolar macrophages from bituminous coal mine dust- or titanium dioxide-exposed lungs showed increased ability to release interleukin-1 on stimulation *in vitro*. These findings suggest that bronchoalveolar leukocytes elicited by coal mine dust could modulate immunity by means of interleukin-1 release and enhancement of lymphocyte proliferation. © 1990 Academic Press, Inc.

INTRODUCTION

Occupational exposure to coal mine dust is associated with the respiratory disease coal workers' pneumoconiosis (CWP), in which nodular fibrosis of the lung parenchyma is found to varying degrees (Morgan and Seaton, 1984). Although the higher prevalence and rapid progression of CWP are partly attributed to differences in the quality of coal mine dusts, including quartz content (Seaton *et al.*, 1981), little is known about the effect of coal mine dust of different rank and quartz content on the early part of the pulmonary response to the deposition of dust. Silica is known to cause persistent pulmonary inflammation and pulmonary fibrosis (Donaldson *et al.*, 1988a).

Interleukin-1 (IL-1), a cytokine crucial to immunity and inflammation (Oppenheim *et al.*, 1986; Martin and Resch, 1986), is secreted from macrophages/

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monocytes exposed to silica (Gery *et al.*, 1981; Lepe-Zuniga and Gery, 1984), and IL-1 from silica-exposed macrophages and its release by alveolar macrophages have been implicated in silicotic inflammation and fibrosis (Schmidt *et al.*, 1984; Kampschmidt *et al.*, 1986; Oghiso and Kubota, 1986a; Brown *et al.*, 1988).

Alveolar macrophages are situated at the air–tissue interface and play a crucial role in pulmonary immune responses. Resident alveolar macrophages are said to be poor accessory cells for mitogen-driven or antigen-driven lymphocyte proliferation (Holt, 1979), and in some species they inhibit lymphocyte proliferation (Holt, 1979). In silicotic humans and animal models of silica exposure, lymphocytes are found to infiltrate the lungs (Morgan and Seaton, 1984; Donaldson *et al.*, 1988a, b; Struhar *et al.*, 1989); alveolar macrophages exposed to silica *in vitro* cause significant lymphocyte proliferation (Oghiso and Kubota, 1986a). We have previously reported that bronchoalveolar cells (BAC) from quartz-inflamed rat lung are less suppressive to lymphocyte mitogenesis than resident alveolar macrophages and that purified neutrophils from these populations enhance lymphocyte mitogenesis, probably by means of IL-1 production (Kusaka *et al.*, 1989).

Previous studies have identified altered immunomodulatory activities in alveolar leukocytes from rats exposed to quartz and asbestos (Oghiso and Kubota, 1986b). The present study was aimed at determining whether another fibrogenic dust—coal mine dust—had the ability to induce alveolitis and cause IL-1 secretion by alveolar leukocytes *in vitro*. We also sought to assess the effects of whole BAC, and enriched populations of alveolar macrophages and neutrophils from coal mine dust-exposed rat lungs, on *in vitro* lymphocyte proliferation. Two other kinds of dust were used: (i) pure quartz with high toxicity, and (ii) TiO_2 with low biological activity; these were chosen to determine the scale of response within which the effects of the coal mine dusts were gauged.

MATERIALS AND METHODS

Animals

Twelve- to fifteen-week-old, female, SPF-maintained, in-bred PVG rats were supplied by the Institute of Occupational Medicine breeding unit.

Dust

Four kinds of dust were used in the experiment:

(1) quartz dust (DQ_{12} standard); density, 2.65 g/cm^3; median volume diameter, 1.85 μm;

(2) the rutile form of titanium dioxide (TiO_2, Tioxide Limited, Stockton-on-Tees); density, 4.25 g/cm^3; median volume diameter, 2.4 μm;

(3) a low-rank bituminous coal (coal mine dust L);

(4) a high-rank anthracite dust (coal mine dust A).

Coal mine dusts were collected from the air of selected British collieries by drawing air by fan through Bondina socks placed in the return roadway of a single face. Analysis of dust showed that dust L consisted of 53% ash, 18% kaolin, 0% mica, and 7% quartz; dust A consisted of 11% ash, 1% kaolin, 4% mica, and 1%

quartz. Details of sampling and analysis of coal mine dust are described elsewhere (Donaldson *et al.*, 1988a).

Exposure of Rats to Dusts

Rats were anesthetized with ether, their tracheas were exposed by dissection, and 0.5 ml sterile saline containing 1 mg of dust was instilled into the lungs through a small incision in the trachea. The skin was closed with metal clips and the animals recovered rapidly with no ill effects.

Bronchoalveolar Lavage

To obtain bronchoalveolar cells, rats were killed by Nembutal overdose and the lungs were lavaged with 4 × 6 ml vol of phosphate-buffered saline (37°C). Cells were concentrated by centrifugation and counted, and the proportion of different leukocyte types was assessed in Giemsa-stained cytospin preparation.

Preparation of Supernatants from Normal Alveolar Macrophages Treated with Dust in Vitro

In normal rats the BAC are predominantly macrophages and these were recovered from rats by bronchoalveolar lavage as described elsewhere (Donaldson *et al.*, 1988b). They were washed with RPMI 1640 (Hepes-buffered; GIBCO, Paisley) and resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS; GIBCO, Paisley), 2 m*M* L-glutamine, 50 m*M* 2-mercaptoethanol, and 100 mg/liter kanamycin (cRPMI). Total and differential cell counts were carried out on Giemsa-stained cytospin preparations and viability was assessed by trypan blue exclusion. Bronchoalveolar leukocytes from untreated rats comprised more than 98% macrophages, showed more than 96% viability, and are referred to hereafter as alveolar macrophages.

Alveolar macrophages were adjusted to 5×10^5 viable cells/ml and incubated with or without the four kinds of dust for 24 hr at 37°C in 5% CO_2. The concentrations of each dust were 50, 100, 250, 500, or 1000 μg/ml. The supernatants were collected, spun, and frozen at −70°C before use in IL-1 and interleukin-2 (IL-2) assays.

Preparation of Lymphocytes

Rats were killed by intraperitoneal injection of an overdose of Nembutal. Spleens were aseptically removed and disaggregated with a glass homogenizer in RPMI 1640. After hemolysis of red cells with hypotonic buffer, splenocytes were washed in RPMI 1640 and finally suspended in RPMI 1640 supplemented with 5% fetal calf serum, 2 m*M* L-glutamine, and 100 mg/liter kanamycin. Splenocytes were depleted of macrophages by passage through Sephadex G-10 (Pharmacia, USA). Sephadex G-10 was presoaked in three times the bed volume of distilled water for 24 hr, autoclaved, washed three times in PBS, and stored at 4°C suspended in 1 vol of PBS. A small amount of nylon wool (Fenwall) was packed into 10-ml syringe columns and 7 ml of prepared Sephadex G-10 was added. Columns were washed with warmed RPMI 1640 supplemented with 5% FCS and preincubated at 37°C in 5% CO_2 for 1 hr. Two milliliters of splenocyte suspension at a

concentration of up to 50×10^6 cells/ml was loaded onto a column and incubated for 45 min at 37°C in 5% CO_2. Nonadherent cells were eluted with 15 ml of warmed medium at a flow rate of 1 ml/min and the recovered cells were then passed through a second column. Recovery efficiency was 50% on average. Sephadex G-10-separated splenocytes were resuspended in cRPMI and cocultured with bronchoalveolar leukocytes from rats as described below. Sephadex G-10-separated splenocytes comprised 23% B cells and 71% T cells on average, as judged by surface immunoglobulins. The depleted splenocytes contained less than 3% nonspecific esterase-positive cells and less than 4% latex bead-phagocytosing cells. Hence Sephadex G-10-separated splenocytes were used as a source of purified lymphocytes and are referred to as lymphocytes in the following experiments.

Splenocyte and Lymphocyte Mitogenesis

Splenocytes or lymphocytes (2×10^5) with or without BAC were cultured in cRPMI in 96-well microtiter plates in the presence or absence of a suboptimal dose (10 μg/ml) of phytohemagglutinin (PHA, type P, Sigma, Poole) for 2 days at 37°C in 5% CO_2. The cultures were then pulsed with 9.25 kBq of tritiated thymidine (Amersham International, Amersham) and incubated overnight, and the uptake of [^{3}H]thymidine was determined by liquid scintillation counting. Lymphocytes gave about 30% of the tritiated thymidine uptake shown by whole splenocytes.

Effects of Bronchoalveolar Leukocytes from Dust-Exposed Rat Lungs on Lymphocyte Mitogenesis

BAC were obtained from dust-treated rats by lavage 1, 3, or 7 days later. Cells were washed in RPMI 1640 and suspended in cRPMI. Of the two coal mine dusts, dust L gave the greatest increase in total leukocytes and polymorphonuclear neutrophils and thus the BAC from dust L-treated rats were separated into macrophage (MAC)- and neutrophil (PMN)-enriched populations by density gradient centrifugation through Sepracell medium (Sepratech). Recovery efficiency following separation was 67 ± 17% (mean ± SD). Total and differential counts were carried out on Giemsa-stained cytospin samples and viability was assessed by trypan blue exclusion. Viable whole or separated BAC were added to lymphocytes at final ratios of BAC:lymphocytes from 1:64 to 1:8. The cultures were then incubated and assessed for mitogenesis as above.

Preparation of Supernatants from BAC

Normal BAC, dust L BAC (MAC or PMN), dust A BAC, or TiO_2 BAC were adjusted to 1×10^6 viable cells/ml in cRPMI. They were incubated in the presence or absence of an optimum dose (100 ng/ml) of lipopolysaccharide (LPS 0.127:B8; Sigma, Poole), for 24 hr at 37°C in 5% CO_2. Supernatants were collected, spun, and frozen until use in the IL-1 and IL-2 assays.

IL-1 Assay

Twofold dilutions of supernatant from cultures of BAC were incubated with

C3H mouse thymocyte suspensions in cRPMI at 6×10^5 cells/well in microtiter plate wells. PHA was added at a suboptimal concentration of 5 μg/ml and the plates were cultured for 3 days. Thymocyte proliferation was determined by the incorporation of tritiated thymidine added 1 day before harvesting. Supernatants from mouse peritoneal macrophages, cultured with 10 μg/ml lipopolysaccharide (LPS 0111:B4; Sigma, Poole), served as a positive control.

IL-2 Assay

Supernatants from cultures of BAC were also tested for IL-2 activity. In brief, supernatants were prepared as described above and serial dilutions were added to 5×10^3 cells of an IL-2-dependent cell line (CTLL-2) suspended in cRPMI without Hepes in each well of 96-well microtiter plates. A concanavalin A-stimulated, IL-2-rich supernatant from rat splenocytes was used as a positive control (Gearing *et al.*, 1985). The cells were incubated in 5% CO_2 at 37°C for 24 hr, pulsed with [^{3}H]thymidine, incubated a further 6 hr, and then harvested. Uptake of tritiated thymidine was determined by liquid scintillation counting.

Statistical Analysis

As variation between experiments on the effects of BAC on lymphocyte mitogenesis was large, the data were expressed as mitogenic indices for each condition: the mitogenic indices were obtained by dividing the suboptimal PHA-driven mitogenesis with BAC by the mitogenesis without BAC. The mean mitogenic indices from the cultures with dust L BAC on MAC, dust A BAC, or TiO_2 BAC were compared to those in the cultures with normal BAC at every ratio. The differences in mean values of mitogenic indices between dust BAC and normal BAC were tested by *t* test and considered significant if the *P* value was less than 0.05.

With regard to PMN, since PMN were augmentary rather than inhibitory to lymphocyte mitogenesis, the mean mitogenic indices from the cultures with PMN at every ratio were compared with the index of one from untreated cultures. The differences in mean values of mitogenic indices between cultures with PMN and cultures without PMN were tested by paired *t* test. The relationship between mitogenic indices and proportion of PMN in various BAC was evaluated using positive linear regression. The correlation coefficient was tested by Student's *t* test. In the IL-1 assay the ^{3}H uptake by the cultures treated with various supernatants from dust-elicited BAC or dusted normal BAC was compared to that by cultures with supernatants from normal BAC. The differences in the mean mitogenic indices or mean ^{3}H uptake were considered significant if the *P* value was less than 0.05

RESULTS

IL-1 Activity in Supernatants from Normal Macrophages Treated with Dust in Vitro

During the 24 hr culture period, the viability of alveolar macrophages was not changed and Fig. 1 shows IL-1 activity of various supernatants. It was found that

tritiated thymidine uptake in the IL-1 assay was greatest with a 1:4 dilution of supernatant from alveolar macrophages and with a 1:8 dilution of the positive control (data not shown). All dust caused release of IL-1 significantly higher than that of the normal untreated BAC. Quartz caused greater IL-1 production at lower doses than the other three dusts. At higher doses, however, quartz was less active than the coal mine dusts.

Time Course of Dust-Induced Inflammation

Table 1 summarizes the total and differential counts of leukocytes lavaged from the lungs of rats treated with dust L, dust A, and TiO_2; the effect of quartz is included for comparison. Figure 2 shows the time course of inflammation as indicated by total lavageable leukocyte numbers recovered per rat. Coal mine dust L caused more severe inflammation than TiO_2, and dust A was the least active, being even less than TiO_2. By 7 days, except in quartz-treated rats, inflammation had subsided, although many macrophages were seen to contain dust particles.

Effects of BAC on Lymphocyte Mitogenesis

Normal BAC and dust A-elicited BAC at 1 day inhibited lymphocyte mitogenesis in a dose-dependent manner (Fig. 3). However, both dust L-elicited BAC and TiO_2-elicited BAC at 1 day were substantially less suppressive than normal BAC at 1:64 and 1:32. Both dust L- and TiO_2-elicited BAC contained 50% or more neutrophils on average, while dust A-elicited BAC comprised 31% neutrophils on average. Accordingly, to test the hypothesis that inhibition of lymphocyte mitogenesis was related to neutrophil numbers, we plotted the mitogenic indices at 1:64 for different types of BAC, against the percentage neutrophil content. Figure 4 shows a significantly positive linear relationship between neutrophil content and mitogenic index ($r = 0.704$, $n = 12$). This finding suggests that neutrophil content

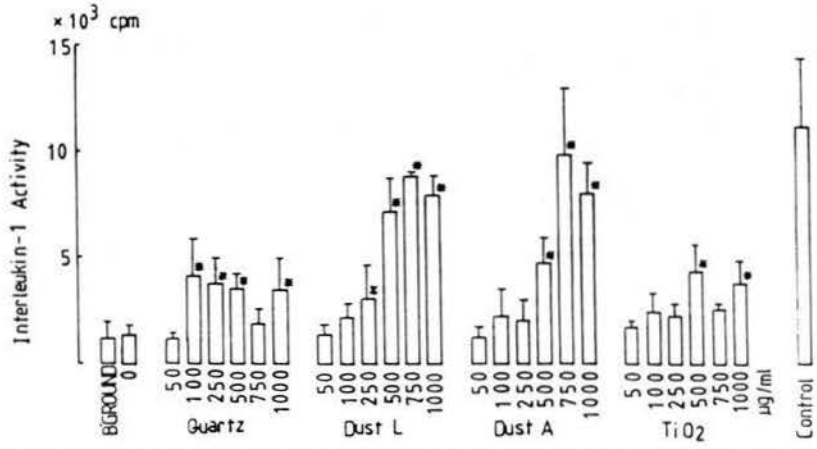


FIG. 1. Interleukin-1 secretion by normal alveolar macrophages exposed *in vitro* to quartz; low-rank, high-quartz bituminous coal mine dust (Dust L); high-rank anthracite coal mine dust (Dust A); and TiO_2. Data are presented as means plus SD from more than three separate experiments in triplicate samples. An asterisk denotes a significant ($P < 0.05$) difference from normal macrophages.

TABLE 1
TIME COURSE OF PULMONARY INFLAMMATION INDUCED BY DUSTS

Treatment	Day bronchoalveolar leukocytes recovered								
	1			3			7		
Normal									
T[a]		3.3 (1.2)			4.3 (1.3)			3.6 (0.8)	
	MAC	PMN	LYM	MAC	PMN	LYM	MAC	PMN	LYM
D[b]	>98	<1	<1	>98	<1	<1	>98	<1	<1
Quartz									
T		17.3 (0.9)			14.9 (2.7)			20.7 (5.4)	
	MAC	PMN	LYM	MAC	PMN	LYM	MAC	PMN	LYM
Mean	36	64	<1	67	32	<1	49	51	<2
(SD)	(3)	(2)		(4)	(2)		(7)	(7)	
Dust L									
T		10.0 (3.6)			5.7 (1.0)			4.5 (1.1)	
	MAC	PMN	LYM	MAC	PMN	LYM	MAC	PMN	LYM
D	44	50	<1	92	7	<2	97	<2	<1
	(12)	(7)		(3)	(1)				
Dust A									
T		4.9 (1.4)			3.5 (0.6)			3.5 (0.4)	
	MAC	PMN	LYM	MAC	PMN	LYM	MAC	PMN	LYM
D	58	31	<1	92	7	<2	>97	<2	<1
	(21)	(21)		(6)	(5)				
TiO_2									
T		7.6 (2.8)			3.8 (0.3)			2.7 (0.5)	
	MAC	PMN	LYM	MAC	PMN	LYM	MAC	PMN	LYM
D	47	52	<1	90	9	<2	>98	<1	<1
	(22)	(22)		(1)	(1)				

Note. Data represent mean and the standard deviation from three (dust-exposed) or five (control) rats. MAC, macrophages; PMN, polymorphonuclear neutrophils; LYM, lymphocytes.

[a] The total number of cells/rat recovered by bronchial lavage as mean (SD).

[b] The differential counts of bronchoalveolar leukocytes as mean (SD) percentage.

is related to loss of suppression of lymphocyte mitogenesis in BAC at this ratio of BAC:lymphocytes. The experiment using purified neutrophils at 1 day clearly emphasizes this (Fig. 5). Thus the polymorphonuclear neutrophil-enriched population for the dust L-exposed lungs (neutrophil, 82 ± 7%, alveolar macrophages, 18 ± 7%; lymphocytes, less than 1%) was significantly augmentary to lymphocyte mitogenesis at 1:64, 1:32, and 1:16. The macrophage-enriched population from the dust L-exposed lung (macrophage, 89 ± 2%, neutrophil, 10 ± 2%; lymphocytes, less than 2%) was less inhibitory than control macrophages at 1:64 and 1:32, as was the whole BAC. At 3 and 7 days all dust BAC inhibited lymphocyte mitogenesis to the same extent as normal BAC (data not shown).

IL-1 Activity in Supernatant of BAC

All supernatants caused the greatest IL-1 activity when diluted at 1/4 and 1/8 and all data are expressed at the optimum uptake produced by each supernatant (Fig. 6). At 1 day all dust-elicited BAC, including dust L PMN, secreted IL-1, but

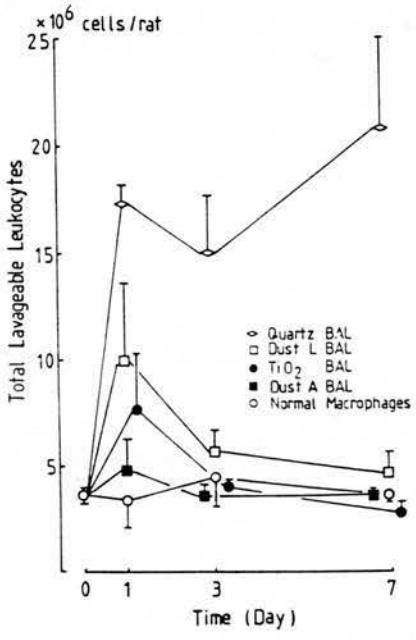


FIG. 2. Time course of pulmonary inflammation indicated by total bronchoalveolar lavage cells (BAC) from dust-exposed rat lungs. Data represent mean and SD from more than three experiments.

the levels were not significantly increased compared to those of normal BAC. At 7 days, TiO_2 BAC showed significantly higher IL-1 without LPS and supernatants from both dust L and TiO_2 BAC stimulated with LPS showed significantly higher levels of IL-1 activity.

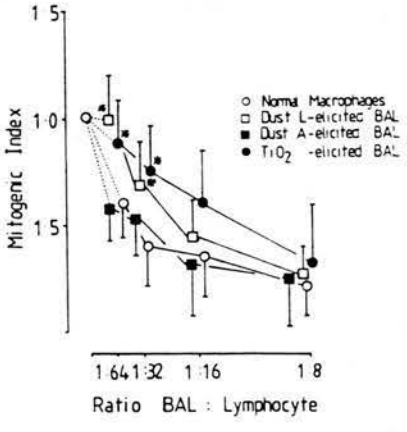


FIG. 3. Effect of bronchoalveolar leukocytes (BAC) from coal mine dusts L- and A- and TiO_2-exposed rat lungs on lymphocyte mitogenesis at BAC:lymphocyte ratios of 1:64 to 1:8. Data (mean and SD) are from more than three experiments. An asterisk denotes a significant ($P < 0.05$) difference from normal macrophages at any ratio.

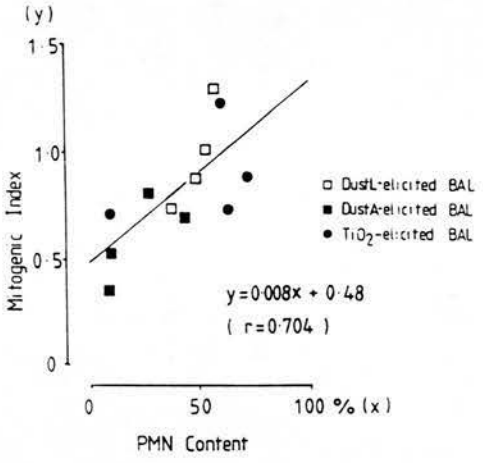


FIG. 4. Mitogenic index of lymphocyte mitogenesis in the presence of dust-elicited BAC plotted against polymorphonuclear neutrophil content (%). Data are from four separate experiments. Regression line is shown.

IL-2 Activity in Supernatant of BAC

No IL-2 activity was detected in any of the supernatants in normal BAC, normal BAC dusted *in vitro*, or dust-elicited BAC (data not shown).

DISCUSSION

The aim of the present study was to determine whether BAC from rats exposed

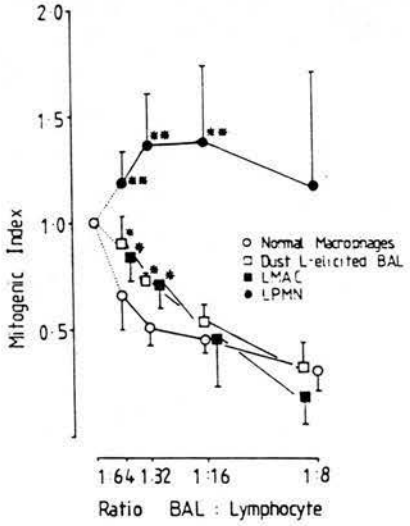


FIG. 5. Effect of whole dust L-induced BAC and macrophage-enriched population (MAC) and PMN-enriched population (PMN) on lymphocyte mitogenesis at ratios of 1:64 to 1:8. Data (mean and SD) from three separate experiments. A single asterisk denotes a significant ($P < 0.05$) difference from normal macrophages at each ratio. Double asterisks denote a significant ($P < 0.05$) difference from the mitogenic index of one.

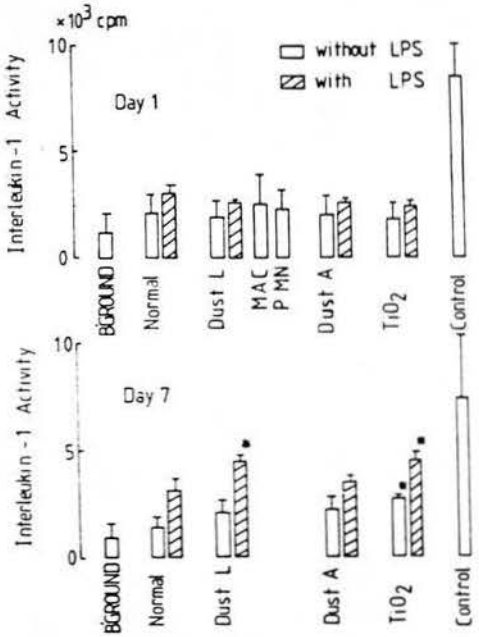


FIG. 6. Interleukin-1 activity in supernatants of dust-elicited BAC recovered at 1 day and 7 days; spontaneous release and LPS-stimulated release are shown. An asterisk denotes a significant ($P < 0.05$) difference from normal macrophages.

to coal mine dust were able to modulate lymphocytes and so might contribute to altered pulmonary immune and inflammatory responses in coal workers. Previous studies have identified altered immunological activities in BAC from animals exposed to other fibrogenic dusts, including quartz and asbestos (Oghiso and Kubota, 1986a). When we found that coal mine dust-elicited BAC were less suppressive to lymphocyte responses than normal BAC, we assessed the ability of normal BAC (alveolar macrophages) to produce IL-1 in response to dust *in vitro*. The study clearly showed that, as we and others have described previously for silica and asbestos, coal mine dust particles are stimulants of IL-1 secretion by alveolar macrophages. However, TiO_2 was capable of promoting IL-1 secretion by alveolar macrophages, contrary to the finding by Oghiso and Kubota (1986b). This conflict may be due to differences in strains of rat used, physical characteristics of TiO_2 (rutile versus anatase), or particle size of the TiO_2 sample.

It is notable that workers are unlikely to be exposed to levels of TiO_2 or coal mine dust as high as those found to stimulate high IL-1 production *in vitro*. Of particular importance, however, is the high level of IL-1 secretion from macrophages in response to doses of quartz compared to the other mineral dusts. Quartz was a more effective inducer of IL-1 production at lower doses *in vitro* than any other dust, but this effect was not sustained at the higher dust doses. This was probably due to the toxicity of quartz at the higher concentrations (Donaldson and Brown, 1988). The likelihood remains that, at the low level of dust depositing in the alveolar region, quartz is likely to generate more IL-1 than any of the other

dusts tested here and so could contribute to the increased pathological change and immunomodulation found in quartz-exposed individuals.

With regard to acute inflammogenic potential in the lung the two kinds of coal mine dust differed, with the low rank, high quartz bituminous sample provoking more severe macrophage and neutrophil alveolitis than the anthracite: anthracite was less toxic than even TiO_2 in this regard. The low rank bituminous coal contains 7% quartz and this level of quartz may contribute to the acute pulmonary toxicity. Our previous inhalation study showed that the same anthracite coal mine dust as that used in the present study, as well as low rank coal, caused substantially more severe alveolitis than TiO_2 when delivered by inhalation. TiO_2 was virtually inactive in terms of its ability to cause inflammation (Donaldson *et al.*, 1988a). The chronic inhalation study better reflects the chronic effects of dusts on the lung and highlights the drawbacks of the instillation exposure route.

Rat alveolar macrophages are reported to be inhibitory to lymphocyte proliferation (Holt, 1986), and our findings are in concordance with this. Whole inflammatory cells from coal mine dust-exposed lung in the present study, like quartz-elicited leukocytes (Kusaka *et al.*, 1989), were similar in their lymphocyte inhibitory effects to the control alveolar macrophages. The exception to this was the finding that dust L BAC, dust L MAC, or TiO_2 BAC at 1 day was less inhibitory to lymphocyte mitogenesis at low ratios of BAC:lymphocytes than normal macrophages. This effect was probably due to the presence of neutrophils as shown by the fact that, as described below and in a previous study, neutrophils were found to enhance mitogenic responses of splenocytes (Kusaka *et al.*, 1990).

During the inflammatory process IL-1 attracts neutrophils and causes prostaglandin synthesis in macrophages (Oppenheim *et al.*, 1986; Martin and Resch, 1986). The inflammation induced by dusts, except for dust A in the present study, increased the ability of macrophages to produce IL-1 in response to a second stimulus, in this case lipopolysaccharide (LPS). In a BCG-treated rat model (Fuchs *et al.*, 1988) and a silica-exposed rat model (Oghiso and Kubota, 1986b) increased IL-1 secretion was not seen in lavaged macrophages until these were stimulated with LPS *in vitro*. In a previous study we found that, in quartz alveolitis, macrophages recovered 7 days after instillation secreted higher IL-1 than normal macrophages without any second stimulus (Kusaka *et al.*, 1990). The exposure to anthracite (dust A) coal mine dust in the present study caused only minimal inflammation and macrophages from anthracite-exposed lung did not have the ability to produce increased IL-1 in response to LPS. By contrast, both bituminous coal mine dust and TiO_2 caused inflammation and the inflammatory leukocytes were able to be stimulated by LPS to release more IL-1. It seems likely that the ability of these leukocytes to be further stimulated by LPS to produce IL-1 may be related to leukocyte-priming cytokines present in the inflammatory milieu.

We obtained neutrophil-enriched and macrophage-enriched populations from low-rank coal mine dust BAC because only this coal mine dust caused a sufficiently increased total BAC count to allow separation. However, we believe that dust A and TiO_2 neutrophils will have characteristics similar to those of dust L neutrophils in view of the finding that the neutrophil component of dust A BAC or

TiO_2 BAC contributed to a significantly positive linear regression relating numbers of neutrophils to mitogenic index (Fig. 4). Since neutrophil-enriched populations were contaminated with alveolar macrophages, greater augmentation of lymphocyte mitogenesis by neutrophils at a high ratio might be masked by the presence of significant numbers of inhibitory alveolar macrophages. In contrast to the suppressive activity of alveolar macrophages, dust-elicited pulmonary neutrophils are devoid of lymphosuppressive activity and are frequently augmentary to it.

Inflammatory neutrophils from the mouse peritoneal cavity (Goto *et al.*, 1984) and supernatants from neutrophils (Vischer *et al.*, 1976) have also been found to augment lymphocyte mitogenesis. Protease (Panush, 1986) has been demonstrated to enhance lymphocyte proliferation. In this regard it is important to note that we have described increased proteolytic activity of leukocytes from quartz-inflamed lung (Brown and Donaldson, 1988) and of bronchoalveolar leukocytes from rats inhaling coal mine dust (Brown and Donaldson, 1989) and that both neutrophils and macrophages are the source of this protease production (Brown *et al.*, 1989a).

Production of elevated levels of interleukin-1 by neutrophils is not a new finding and both human blood neutrophils and bovine peripheral neutrophils have also been found to release IL-1 when stimulated with endotoxin (Tiku *et al.*, 1986; Canning and Neil, 1989). We have reported that pulmonary inflammatory neutrophils recruited by exposure to quartz augment the mitogenesis of lymphocytes (Kusaka and Donaldson, 1990) and that both quartz-elicited pulmonary neutrophils and neutrophils elicited by the killed bacterial preparation of *Corynebacterium parvum* secrete high levels of IL-1. IL-1β from inflammatory mouse peritoneal neutrophils has also been proved to be involved in augmented lymphocyte proliferation (Goto *et al.*, 1988). Although PMN in the present study did not secrete IL-1 at levels significantly higher than that of normal macrophages, a small amount of IL-1 from neutrophils, combined with an inability to suppress lymphocyte mitogenesis (unlike macrophages), may lead to the observed enhancement of lymphocyte mitogenesis. Our rat inhalation study with coal mine dust (Donaldson *et al.*, 1988b) and bronchoalveolar lavage studies in coal miners (Voisin *et al.*, 1983; Begin *et al.*, 1986) have revealed that exposure to coal mine dust causes persistent macrophage and neutrophil alveolitis. Alveolar macrophages activated by inflammatory stimuli may have increased ability to produce IL-1 and hence the potential to secrete high levels of IL-1 in response to a second stimulus; they may therefore modulate lymphocyte responses in the local environment of the lung and lung-associated lymph nodes.

Inflammatory neutrophils could counteract the normally suppressive influence of alveolar macrophages and so allow lymphocyte expansion to proceed. This neutrophil enhancement of immunity may be particularly important during the initial stages of an inflammation when neutrophils are recruited. The precise role of lymphocytes in the pathogenesis of lung diseases associated with both mineral dust and organic dust inhalation is still controversial (Pernis and Vigliani, 1982; Hunninghake *et al.*, 1984), but inflammatory neutrophils, through their production of IL-1, could be important in any dust-related lymphomodulation.

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INFLAMMATION AND IMMUNOMODULATION CAUSED BY SHORT AND LONG AMOSITE ASBESTOS SAMPLES

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ABSTRACT

In order to try and understand the processes underlying carcinogenesis in the peritoneal cavity following deposition of fibrous dusts, we instilled long and short fibre samples of amosite asbestos into the peritoneal cavity of mice and assessed some aspects of the inflammatory and immune response. In previous studies we have demonstrated that the long fibre sample causes mesotheliomas following intraperitoneal instillation while the short fibre sample is virtually inactive in this respect. There was a dramatic inflammatory response to a very low dose of long fibres and no effect with the same dose of short fibres. In animals immunised with Sheep Red Blood Cells, treatment with the long fibres caused a suppression of systemic humoral immunity, as assessed by the numbers of antibody-producing cells in the spleen; splenic T-cell responses were also depressed. The short fibre sample caused much smaller effects in inhibiting these immune responses. Long fibre amosite also caused a greater release of the cytokines Interleukin-1 and TNF from alveolar macrophages than did the short fibre amosite sample. Inflammation and immunosuppression may play a role in the pathogenic processes following peritoneal cavity deposition of fibres that lead to mesothelioma; cytokine release by macrophages may also play a role in these processes.

Mechanisms in Fibre Carcinogenisis
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INTRODUCTION

Fibre length is a major descriptor of the pathogenicity of fibrous dusts (Davis, 1989) although surface chemistry of fibres also has a role to play. We have used two samples of asbestos differing in fibre length and demonstrated that, when delivered by inhalation, the long fibre sample has the ability to cause more lung fibrosis and more lung tumours. The long fibre sample also produces more mesotheliomas when injected into the peritoneal cavity than does the short fibre sample (Davis *et al.*, 1986).

Great interest has been shown in the role of leukocytes in pathological change and in the responses of the mesothelial surfaces (both pleural and peritoneal) to asbestos. We therefore investigated the effects of the long and short fibres following intraperitoneal exposure on the two key leukocyte-mediated defence systems - the immune and inflammatory responses. We injected long and short fibre samples of amosite asbestos into the peritoneal cavity of mice and assessed general measures of immune response as well as the local inflammatory response. We also assessed the ability of the two different fibre types to stimulate the release of cytokine from macrophages *in vitro*.

MATERIALS AND METHODS

Dust samples

The dust samples used in the present study were those used previously in our laboratory (Davis *et al.*, 1986; Donaldson *et al.*, 1989). These samples were obtained from the same batch of South African amosite. The two samples did not differ elementally or crystallographically. The dimensions of the two samples are shown in Figures 1 and 2. From these figures it is evident that the samples do not differ substantially in their diameters but are quite different in the length with very few fibres longer than 10 μm in the short fibre sample. The UICC standard sample of chrysotile asbestos was also used.

Animals

Syngeneic C57Bl6 mice of 12-16 weeks were used throughout. Rats of the HAN/Wistar strain were used as a source of alveolar macrophages.

Inflammation Assay

Inflammatory potential of the two fibre samples was assessed by injecting 5 μg of dust, in a 0.5 ml suspension, into the peritoneal cavity of mice. Two days later the peritoneal cavity was lavaged with 3 x 2 ml volumes of phosphate buffered saline to retrieve the free cell population; the total number of cells was estimated and a differential cell count was made in Giemsa-stained preparations.

Assessment of the systemic humoral immunity

The ability of splenic lymphocytes to mount a humoral immune response was measured as the number of lymphocytes able to produce specific antibody following immunisation with Sheep Red Blood Cells (SRBC). On day 0 mice were exposed to fibre as described above, by intraperitoneal injection. On day 3 mice were immunised with an intraperitoneal injection of 0.2 ml of a 10% solution of SRBC. On day 7 the mice were killed, their spleens removed aseptically, and the proportion of splenocytes producing antibody to SRBC was determined using a haemolytic plaque assay as described in detail

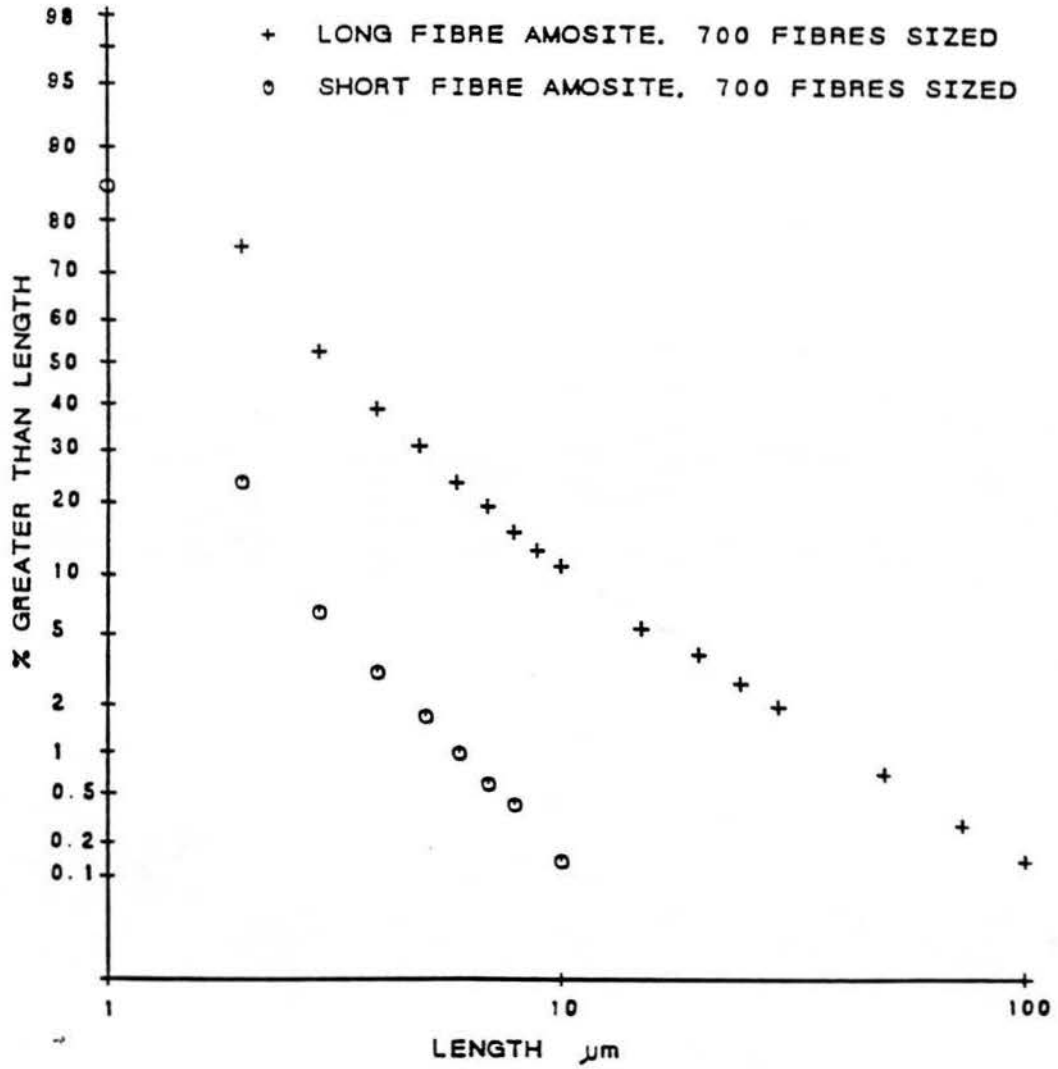


Figure 1. Length characteristics of the long and short fibre amosite samples. Results from 700 sized fibres.

in Szymaniec and co-workers (1990). Plaque-forming cells (PFC) were expressed as either PFC per spleen or PFC per 10^6 spleen cells.

Mitogenic responses of splenic T cells

The status of T cells in fibre-exposed mice was ascertained by exposing to fibre and, 3 days later, measuring the splenic T lymphocyte responses to the T cell mitogen phytohaemagglutinin (PHA). Spleens were removed aseptically and mechanically dispersed before bringing into microtitre plate wells and assessing the mitogenic response to PHA, added at 2.5 and 5 μg/ml. Plates were then incubated for 2 days and pulse labelled with tritiated thymidine. Proliferation was measured by liquid scintillation counting 16 hours later. The method is described in detail in Hannant and co-workers (1985).

Production of Interleukin 1 and Tumour Necrosis Factor

Rat alveolar macrophages were obtained by bronchoalveolar lavage from normal rats and brought into culture. Long or short fibre amosite asbestos at 25 μg/ml was added to the cultures and these were incubated for 24 hours. The supernatants were collected, centrifuged to clarity and stored at -20°C. Interleukin 1 (IL-1) was assayed using

TABLE 1. The peritoneal inflammatory response to 5 μg of long or short fibre amosite instilled 2 days previously. Significant increase in the numbers of macrophages and neutrophils with long amosite treatment ($P < 0.001$)

AMOSITE	MACROPHAGES	NEUTROPHILS
SHORT	*4.65(0.56)	0.20(0.02)
LONG	23.25(3.56)	13.28(3.71)

*CELLS $x10^6$ (mean($\pm$s.d.))

enhancement of the sub-optimal PHA mitogenesis of C57BL6 mouse thymocytes as described in detail in Kusaka and co-workers (1990). Tumour Necrosis Factor (TNF) was assayed using the TNF-sensitive cell line L929. By using standard cytokine preparations in the same assay systems we are able to determine the absolute amounts of IL-1 and TNF in the different samples of supernatant.

Statistical Analysis

Data from repeat experiments was analysed by analysis of variance and significance of differences between treatment groups was tested using a T test.

RESULTS

Inflammatory potential of the long and short amosite samples

As shown in Table 1 the long fibre sample caused substantially more inflammation in the mouse peritoneal cavity than the short fibre sample.

Immunomodulatory activity of the long and short fibre samples

The short fibre sample caused a reduction in the splenic antibody response of the same order of magnitude as that caused by exposure to the UICC chrysotile sample, when they were injected at equal mass (Figure 3). In contrast, the short fibre amosite sample suppressed splenocyte responses much less, although it did cause a significant reduction in PFC compared to the saline control.

Dose response of the humoral immunosuppression caused by long fibre amosite

When the PFC response was measured in mice exposed to lower levels of the long amosite it became clear in 3 separate experiments that, at very low doses (5 μg) of dust, there was still profound immunosuppression which appeared greater at the lower doses (Figure 4).

T-cell responses in the spleens of mice exposed to long and short fibre

As shown in Figure 5, intraperitoneal instillation of both the short and long fibres caused a decrease in T cell responses to PHA with the short fibre sample. The long fibre

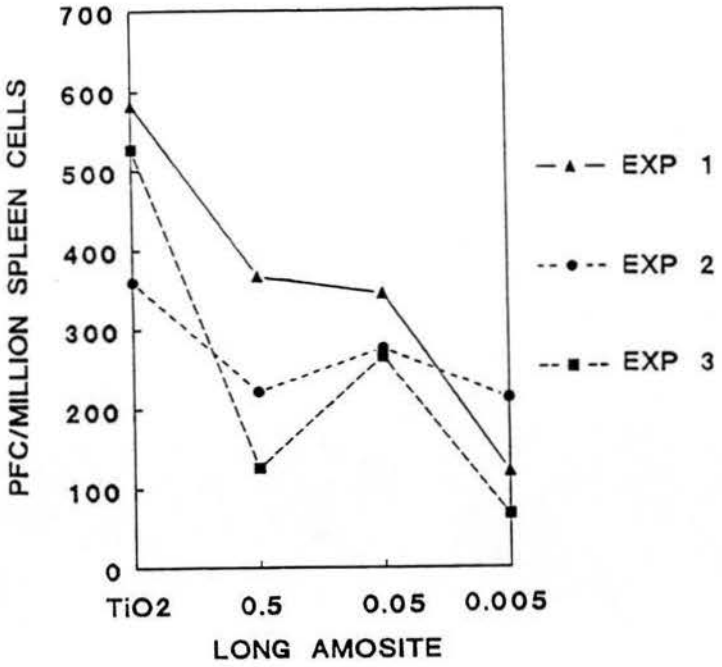


Figure 4. Numbers of anti-SRBC PFC in the spleens of mice injected intraperitoneally with 0.5 mg of TiO_2, 0.5, 0.05 or 0.005 mg of long amosite. Results are the means from 3 separate experiments. Significant reductions with all treatments compared to TiO_2 ($P<0.01$).

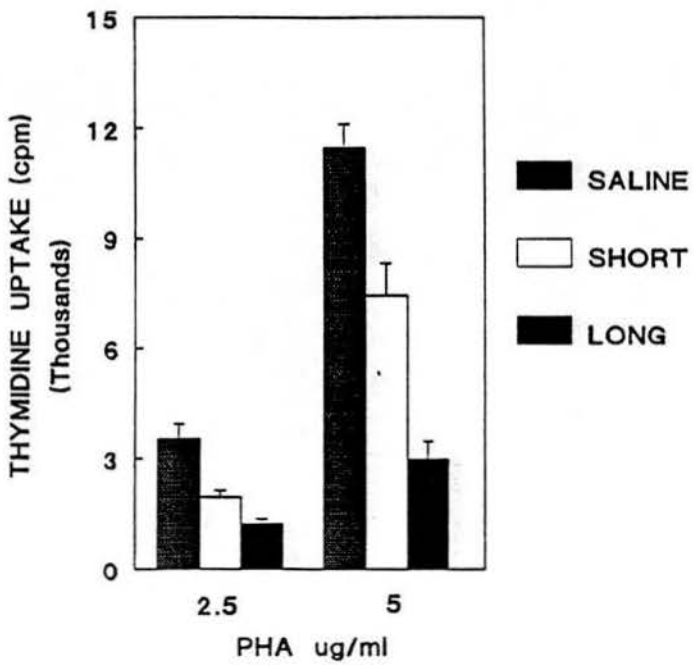


Figure 5. PHA-stimulated proliferation of splenocytes from mice injected intraperitoneally with the indicated dusts or saline. Results are the mean and standard deviation from 3 mice in each group in a single experiment. Significantly reduced proliferation with short and long amosite compared to saline and long compared to short amosite ($P<0.05$).

sample caused substantially more suppression of T cell response than did the short fibre sample.

Release of cytokine by macrophages exposed to long and short fibre *in vitro*

Figures 6 and 7 reveal that the long fibre sample had more ability than the short fibre sample to stimulate the release of cytokine when alveolar macrophages were the indicator cells.

DISCUSSION

We set out to determine whether there was any difference in the ability of a long and a short fibre amosite asbestos sample to affect the two main body defence systems of immunity and inflammation. The two samples have greatly different abilities to cause lung fibrosis and cancer when inhaled by rats with the long fibre sample being considerably more carcinogenic and fibrogenic than the short fibre sample (Davis *et al.*, 1986).

We used the reaction of the mouse peritoneal cavity to assess these effects because it represents a more reliable site than the lung to deposit a known quantity of a fibrous dusts by instillation and it has been used as a site to study fibre carcinogenesis (*e.g.* Bolton *et al.*, 1982). It is, however, very different to the lung, having no clearance mechanism and a macrophage population which differs in several important respects. It is questionable, therefore, whether studies on the peritoneal cavity reveal information applicable to an understanding of events inside the lung itself. They should, however, aid understanding of the mesothelioma response found after intraperitoneal instillation.

Fibrous dusts deposited in the peritoneal cavity give rise to a fibrogenic (Donaldson, 1982) and carcinogenic (Bolton *et al.*, 1982) response and the ability of fibrous dusts to cause mesotheliomas in the peritoneal cavity following instillation has been used as a bio-assay for fibre carcinogenesis and to investigate some of the determinants of fibre carcinogenesis. When the long and short fibre amosite samples were injected into the rat peritoneal cavity in a previous study, there was a high proportion of mesothelioma in the long fibre-treated rats but little response in those animals treated with the short fibre (Davis *et al.*, 1986). We sought therefore to further understand the effect of instillation of these dusts into the peritoneal cavity on the immune and inflammatory responses.

The present study revealed that the long fibre sample was very active in causing inflammation in the peritoneal cavity and that in long fibre-treated rats there was an inhibition of a humoral immune response and impaired T cell function in the spleen. The short fibre sample was able to cause a small amount of inflammation but was many times less active than the long fibre sample. The short fibre sample also reduced both the B- and T-cell responses but this was modest in comparison to the long fibre sample.

We have reported previously that the non-carcinogenic dusts quartz and TiO_2 caused inflammation in the mouse peritoneal cavity but that this was substantially less than that caused by fibrous dusts (Donaldson *et al.*, 1988). We have also shown that TiO_2 and quartz can reduce the PFC response in the spleen in experiments similar to those shown here (Szymaniec *et al.*, 1990). However, the ability of these dusts to cause this immunosuppressive effect was markedly less than that of UICC chrysotile asbestos.

The peritoneal cavity has its own macrophage population and is lined by mesothelial cells. Macrophages are an important cell type in the initiation and execution of both inflammatory and immune responses and we have shown macrophage activation in the peritoneal cavity following injection of asbestos (Donaldson *et al.*, 1982). It is assumed

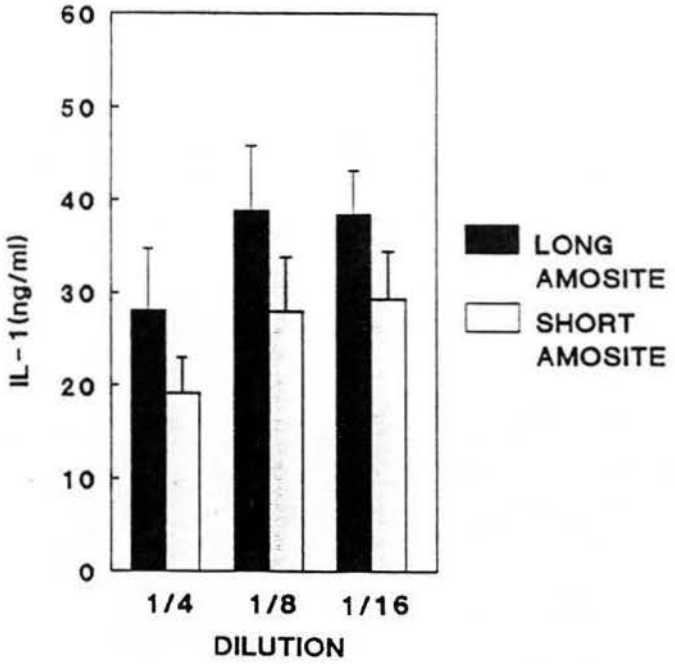


Figure 6. Production of Interleukin-1 (IL-1) by alveolar macrophages exposed *in vitro* to long or short amosite asbestos. Results are mean and standard deviation obtained from 3 separate experiments. Significantly increased IL-1 at all dilutions ($P < 0.05$).

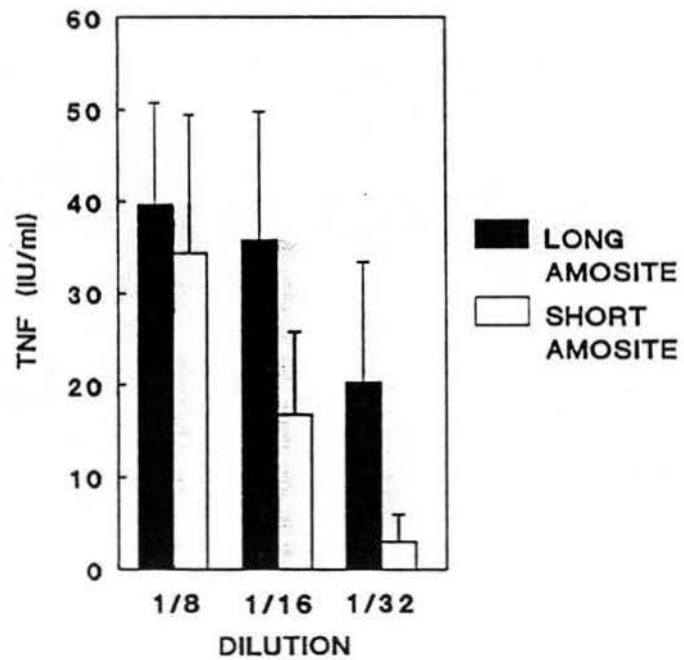


Figure 7. Production of Tumour Necrosis Factor (TNF) by alveolar macrophages exposed to long or short amosite asbestos *in vitro*. Results are mean and standard deviation from triplicate wells in 2 separate experiments. Significantly increased production with long amosite compared to short amosite at 1/16 and 1/32 dilutions ($P < 0.05$).

that, following instillation of asbestos, the macrophages phagocytose the fibres and this is an important event in leading to inflammation, immunomodulation and, ultimately, pathological change. The other cell type which could be directly affected are the mesothelial cells. One potential factor in inflammation and immunomodulation following fibre deposition and phagocytosis is the production of macrophage cytokines. These have powerful pro-inflammatory activity and also the ability to stimulate lymphocytes. The increased production of cytokine by alveolar macrophages treated with the long fibre *in vitro* demonstrated here, suggests one route whereby inflammation could be caused by the long fibres. It was notable that TNF showed a greater difference in response between the two fibre types than was shown by IL-1. This suggests that TNF could be more important than IL-1 in the development of fibre-associated pathology. The production of these particular cytokines is unlikely to be a primary cause of the immunosuppression that is seen with the long fibre sample. It does, however demonstrate that the long fibre sample can stimulate secretion; the production of other substances such as prostaglandin, with well-identified immunosuppressive activity, is therefore possible.

In general terms the layout of the experiment on plaque-forming cells would be expected to enhance immune responses since the antigen is deposited into the peritoneal cavity where inflammation is proceeding. In inflammatory sites the up-regulation of Ia on the surface of the macrophages should favour the immune response and the increased recruitment of macrophages should also aid the transfer of antigen to the lymph nodes for induction of the immune response. It is all the more surprising therefore that with long amosite, where the inflammation is greatest, there is profound suppression of immune response. We have, however previously reported that macrophages from the peritoneal cavity, following instillation of asbestos, release a factor(s) that inhibits lymphocyte response (Donaldson *et al.*, 1984). This factor is likely to be released locally but may have some function in the systemic immunosuppression described here for the long amosite fibres.

Inflammation and immunity are likely to be important in the fibrogenic and carcinogenic process. Leukocytes play an important role in fibrosis through the release of growth factors which aid in the accumulation, proliferation and synthetic activities of fibroblasts. In the case of carcinogenesis, growth factors may be important and many tumour promoters are inflammogens.The immune response, through surveillance for transformed cells, is considered to be important in preventing neoplasia; cancer arises in immunosuppressed individuals.

We would suggest that the activities that we have demonstrated here in the highly carcinogenic, long fibre sample *i.e.* the ability to cause inflammation in combination with an ability to suppress the immune response, could be important in the carcinogenic and fibrogenic properties of this preparation in the peritoneal cavity. Following deposition of a bolus of dust containing long fibres, it can be assumed that there will be an intense inflammatory response with the release of macrophage cytokines; there is also likely to be immunosuppression. These events may play a key role in the subsequent pathological response.

These changes to the inflammatory and immune response may also be of general relevance to fibre carcinogenesis following inhalation although direct extrapolation from the peritoneal cavity to the lung is perilous. However inflammation (Begin *et al.*, 1986; Donaldson *et al.*, 1988) and immunosuppression (Kagan *et al.*, 1977; Hannant *et al.*, 1985) have been reported in workers and animals exposed to asbestos. The processes that underlie carcinogenesis in the lung are likely to be fundamentally the same as those that occur during carcinogenesis in the pleural and peritoneal cavities.

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A NOVEL MODEL FOR HUMAN INTERSTITIAL LUNG DISEASE: HAPTEN-DRIVEN LUNG FIBROSIS IN RODENTS

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SUMMARY

A novel model is described of chronic pulmonary fibrosis in rodents. The condition is induced by a single intratracheal instillation of a well-characterized fluorescent haptenic antigen, fluorescein isothiocyanate (FITC), into non-immune animals. This results in an acute inflammatory response involving a granulocytic infiltrate, which disappears over a week and is replaced by a chronic mononuclear infiltrate in which T lymphocytes predominate. Over several months, a chronic patchy fibrosis is accompanied by a sustained mononuclear interstitial infiltrate localized at sites of persistent FITC deposition. Where no FITC is present, the lung tissues are apparently normal. An immune response is mounted, as measured by the appearance of specific anti-FITC serum antibodies. This model has relevance to the pathogenesis of some forms of human interstitial lung disease.

KEY WORDS—interstitial lung disease; fluorescein isothiocyanate

INTRODUCTION

Interstitial lung disease (ILD) represents a clinically important, heterogeneous group of disorders accounting for a significant number of patients seen by respiratory physicians. ILD includes a number of clinically disparate disorders, characterized histologically by interstitial fibrosis associated with a variable inflammatory cell population. Despite intensive investigation, the aetiology and pathogenesis of most forms of ILD, including the most common type cryptogenic fibrosing alveolitis (interstitial pulmonary fibrosis, idiopathic pulmonary fibrosis), remain poorly understood. Because of the persistence and the nature of the inflammatory infiltrate seen in these conditions, chronic immune system stimulation has been postulated to underlie much of the damage seen in ILD. However, specific antigenic triggers have only been identified in a minority of cases (reviewed in refs 1–3).

In order to understand the cellular mechanisms and interactions which could be responsible for various forms of ILD and to aid in the design of new therapeutic approaches, it is necessary to be able to study the condition in detail from its initiation. This is impossible in patients who present with established and often end-stage disease. To this end, various animal models have been devised. Much work has been done on models of ILD induced by drugs such as bleomycin,[4,5] metal salts,[6] reactive chemicals,[7] or dusts to which industrial workers are exposed.[8,9] However, although the immune system has been proposed to be involved in all of these systems, none of them allows easy measurement of specific immune responses to the initiating agent *per se* and the inducing agents are not readily tracked in the tissues. The pattern of the inflammatory process seen in the tissues in these models is also not

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typical of that seen in human interstitial lung disease, more closely resembling the diffuse alveolar damage with scarring that is seen in 'shock lung' syndromes.

We report here the development of a novel rodent model for interstitial lung disease, in which patchy fibrosis is induced following intratracheal instillation of the fluorescent hapten fluorescein isothiocyanate (FITC). This molecule has been used previously as a skin sensitizing agent and is known to induce specific immune responses.[10] The induced fibrosis is accompanied by a mononuclear infiltrate in the interstitium in which T lymphocytes predominate. Critically, in this model the foci of lung damage are confined to areas of long-term FITC deposition.

MATERIALS AND METHODS

Antibodies and reagents

Biotinylated anti-mouse immunoglobulin (catalogue No. E354), ABC complex horseradish peroxidase (HRP) (catalogue No. K355), soluble complex of HRP and rabbit anti-HRP (catalogue No. Z113), swine anti-rabbit immunoglobulin (catalogue No. Z196), rabbit anti-FITC antibodies (catalogue No. V403), monoclonal anti-proliferating cell nuclear antigen (PC10) (catalogue No. M879), and biotinylated affinity isolated swine anti-rabbit immunoglobulin (catalogue No. E353) were purchased from DAKO U.K. Ltd. HRP-conjugated rabbit anti-rat immunoglobulin (catalogue No. AAR10P for ELISA and STAR51 for immunohistochemistry), monoclonal anti-rat-CD4, -CD8, and α/β T-cell receptor (TCR) antibodies were all purchased from Serotec Ltd. Fluorscein isothiocyanate (FITC), ubiquitin, FITC–ubiquitin, bovine serum albumin (BSA), carbonate/bicarbonate buffer capsules, *o*-phenylenediamine tablets, and monoclonal anti-FITC were all purchased from Sigma U.K. Ltd. Normal swine serum was obtained from SERA-LAB U.K. Ltd. Normal rabbit serum was obtained from the Scottish Antibody Production Unit, Law Hospital Carluke.

Animals

Normal female BALB/c mice were bred and maintained in the Department of Medical Microbiology animal house, University of Edinburgh. The mice were 8–12 weeks old and a minimum weight of 20 g prior to use. Adult male HAN rats were maintained in the animal facility of the Regional Virus Laboratory, City Hospital. All were 12 weeks old when first used.

Intratracheal instillation of FITC

Mice were anaesthetized by inhalation of the volatile anaesthetic halothane. An incision was made to expose the trachea and FITC (0·007 mg per g body weight) in sterile 0·1 ml Dulbecco's phosphate-buffered saline, pH 7·2 (PBS) or PBS alone was instilled using a 1 ml syringe and a 25 G needle. The incision was closed using surgical clips. Mice regained consciousness within 5 min of the procedure. Rats were anaesthetized by injection of valium intraperitoneally (IP) and Hypnorm intramuscularly (IM). An incision was made to expose the trachea and 0·5 ml of 1 mg/ml FITC in PBS was instilled via a blunted needle.

Bronchoalveolar lavage (BAL)

Mice were killed by diethyl ether overdose, the thoracic cavity was opened, and the lungs were cannulated via the trachea. The lungs were lavaged gently *in situ* with three sequential aliquots of PBS at room temperature. The volume depended on the size of the mouse. Rats were killed by Nembutal overdose, the thoracic cavity was opened, and the lungs were cannulated and removed. Lungs were lavaged with four sequential 6 ml aliquots of normal saline at 37°C and massaged gently between each wash. The recovered BAL fluid was placed on ice. The cells were centrifuged at 1000 rpm for 10 min, resuspended in PBS, and counted. Cytocentrifuge preparations were made with approximately 10^5 cells per slide, fixed in methanol, and stained with Giemsa/May-Grunwald for differential counting. A minimum of 400 cells were counted at × 1000 magnification.

Tissue processing and immunohistochemistry

Fixed tissues—Lungs were inflated with 10 per cent formol saline to standard pressure. Fixed tissues were routinely paraffin-embedded, sectioned, and processed for staining. Sections from each block were stained with haematoxylin and eosin (H & E). Serial sections were stained with a polyclonal rabbit anti-FITC using a peroxidase/anti-peroxidase method. For direct immunofluorescence to localize FITC on the confocal

microscope, thick (20 μm) sections were dewaxed and mounted in glycerol saline. Sections were also stained with the monoclonal antibody PC10, which stains the proliferating cell nuclear antigen of all mammalian species and is associated with dividing cells in non-malignant populations.[11]

Frozen tissues and BAL cytospins—Lungs were inflated with OCT freezing medium (Miles, U.K. Ltd.)/PBS (1:1, v/v) solution, immersed in undiluted OCT, snap-frozen in liquid nitrogen, and stored at -70°C until processed. Cryostat sections or cytospins were fixed in acetone at room temperature for 10 min, stained with H & E, or immunochemically stained with murine monoclonal antibodies against rat CD4, CD8 or α/β T-cell receptors, using an indirect immunoperoxidase method.

Bronchoalveolar epithelial permeability measurement

Control and FITC-instilled rats were anaesthetized as above. A butterfly G25 attached to a 1 ml syringe containing 100 units/ml heparin in PBS solution was placed in the femoral artery. An incision was made in the trachea and 0·1 ml of PBS containing ^{125}I-labelled BSA (Amersham, U.K.) was instilled, followed by 1 ml of air. Blood samples (0·1 ml) were drawn through the butterfly at 6, 7, 8, 9, and 11 min after the start of tracheal instillation. Blood samples were counted for radioactivity in a gamma counter. The percentage of leaked protein was calculated according to the formula:

$$\text{percentage} = \frac{\text{cpm blood} \times 160^* \times 100}{\text{total cpm injected}}$$

Bronchoalveolar lavage protein measurement

Total protein present in BAL fluid of control rats and animals instilled with FITC 6 h or 1, 3, or 7 days previously was measured by the conventional Coomassie blue method, obtained in kit form from Bio-Rad, Hemel Hempstead, Hertfordshire, U.K.

ELISA for serum anti-FITC antibodies

Ubiquitin or FITC-conjugated ubiquitin (0·05 μg/well) was coated onto ELISA microtitre plates (Costar, U.K. Ltd.) overnight at 4°C in carbonate/bicarbonate buffer (pH 9·6). Plates were washed and blocked with 1 per cent BSA in PBS (pH 7·4) containing 0·05 per cent Tween 20 (BDH, U.K.) for 1 hour at room temperature. Doubling dilutions of 100 μl of rat serum (pooled from at least three animals) from 1:25 to 1:3200 in PBS containing 1 per cent BSA, 5 per cent NRS, and 0·05 per cent Tween 20 were added to triplicate wells and incubated overnight at 4°C. Plates were washed and incubated for 2 h at room temperature with 100 μl of a 1:400 dilution of HRP-conjugated rabbit anti-rat serum added to each well. Plates were washed and incubated with 100 μl of 0·4 mg/ml *o*-phenylenediamine dihydrochloride per well. The reaction was stopped by adding 100 μl of 4 M H_2SO_4 to each well. The plates were read at 490 nm on a Dynatech MR5000 ELISA plate reader.

*The blood volume of an average rat is assumed to be 16 ml; 0·1 ml samples were measured.

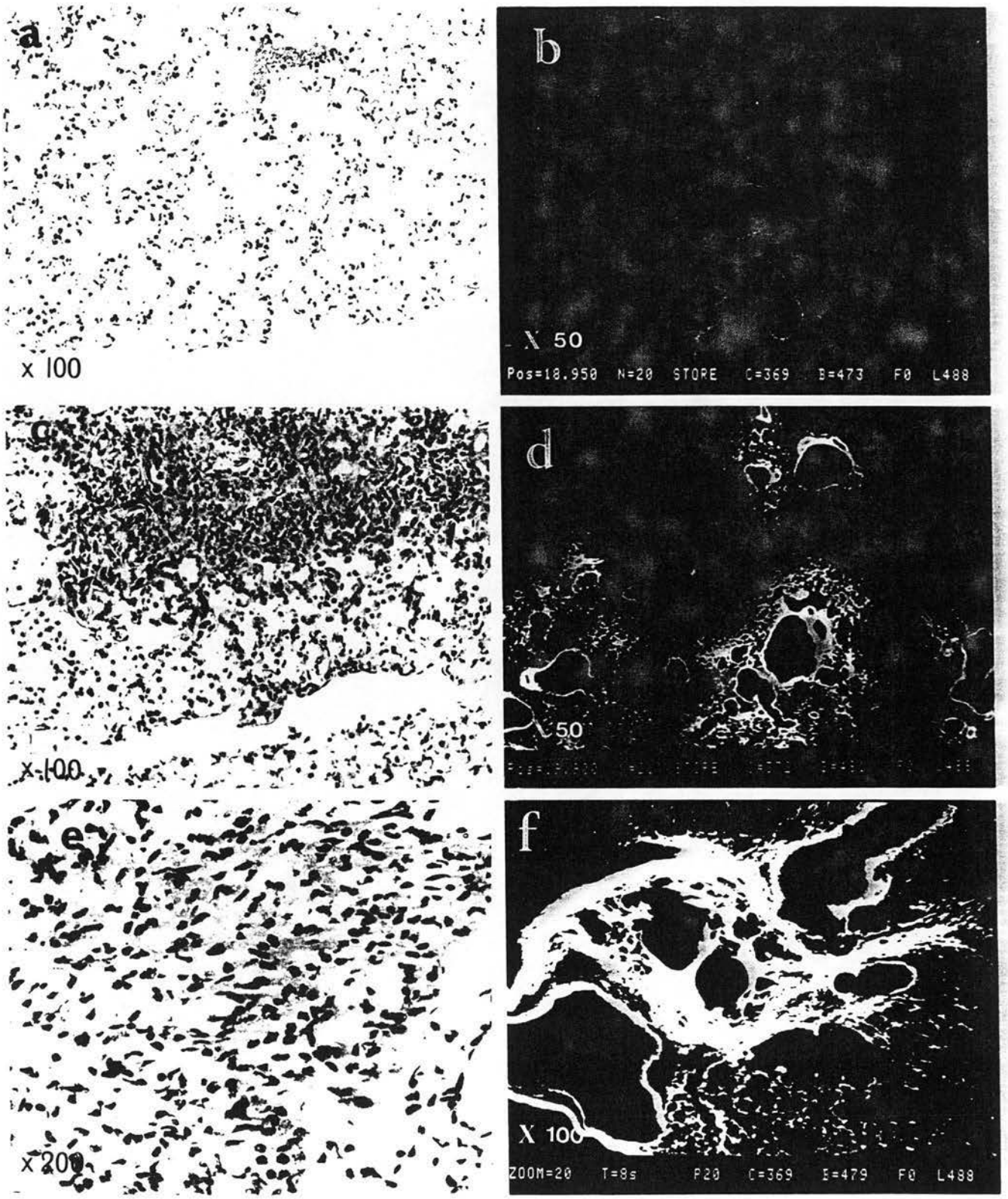
RESULTS

Tissue histology and FITC localization

The typical histology seen in untreated mice and at 3 days and 5 months after intratracheal instillation of FITC is shown in Figs 1a, 1c, and 1e and the distribution of FITC in the lungs detected by confocal microscopy at the same time points is shown in Figs 1b, 1d, and 1f. Similar appearances were also observed in rats.

At 3 days, sections revealed a focal abnormality in the pulmonary parenchyma. The lung architecture was preserved, but there was a marked infiltrate of mononuclear cells and neutrophils within the lung interstitium, extending into the alveolar spaces. The areas of abnormality appeared to be centred principally around the respiratory bronchioles and focal evidence of alveolar oedema was noted. There was marked bronchial epithelial cell hyperplasia, which was confirmed immunohistochemically in the rat, using the monoclonal antibody PC10. Figure 2 illustrates PC10 staining at various times after instillation of FITC IT. The appearances 3 days after instillation of FITC were suggestive of acute lung injury without evidence of interstitial fibrosis (Fig. 1c) and the areas of abnormality appeared confined to the areas of FITC deposition as assessed immunohistochemically and by confocal microscopy (Fig. 1d).

At 5 months, there was patchy, focal destruction of the normal lung architecture with focal interstitial fibrosis. The inflammatory infiltrate was


a
x 100
b
X 50
Pos=18.950 N=20 STORE C=369 B=473 F0 L488
c
x 100
d
50
e
x 200
f
X 100
ZOOM=20 T=8s P20 C=369 B=479 F0 L488

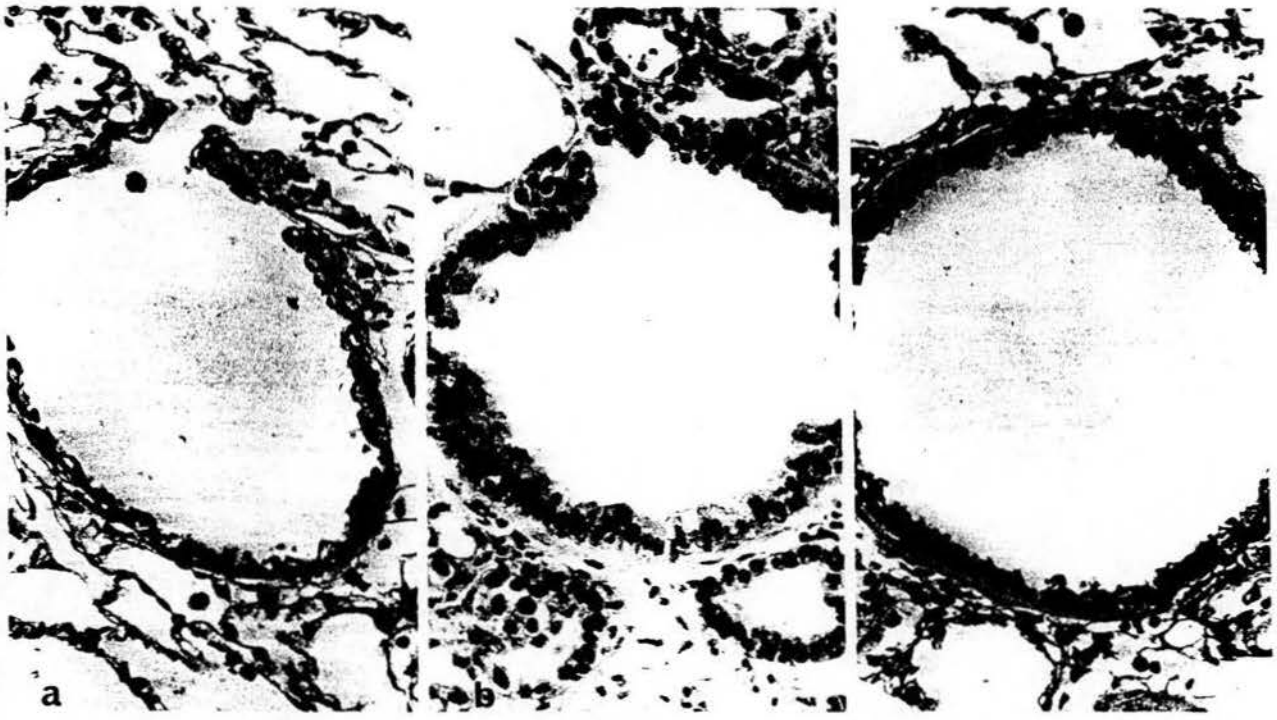


Fig. 2—Paraffin sections of lungs of control and FITC-treated rats stained with the antibody PC10 (immunoperoxidase technique). No significant staining of epithelial cells within the airway or adjacent lung is identified in the control (a). At 3 days, the majority of the epithelial cells show nuclear positivity suggesting proliferative activity (b). By 7 days, the number of positive cells has decreased (c)

predominantly mononuclear, although occasional neutrophils were still evident. Both the infiltrate and the scarring were confined to peribronchial areas of FITC deposition, as demonstrated by immunohistochemistry, with other areas of the lung showing no histological abnormality (Fig. 1e).

Figure 1f shows the appearance of the lung at 5 months using the confocal laser microscope. The FITC can be seen in association with parenchymal components, which from their distribution and appearance are thought to be elastin fibres. The lung architecture can be seen to be severely disrupted, with the formation of fine honeycomb spaces.

Frozen sections proved to be of poor morphological quality, but indicated that the interstitial infiltrate comprised monocyte/macrophage lineage cells and lymphocytes. Rat lungs 7 days after instillation with FITC were stained with monoclonal anti-CD4 and anti-CD8 and are illustrated in figs 3a and 3b. Of the lymphocytes, more than 95 per cent were $\alpha\beta$ TCR-positive, with both CD4- and CD8-positive subsets being present in keeping with the results obtained with lavage.

Cellular composition of bronchoalveolar lavage

The absolute numbers of cells obtained at 3 h, 6 h, 24 h (1 day), 72 h (3 days), and 148 h (7 days) after intratracheal instillation of FITC, and from untreated controls are shown in Fig. 4a. Comparisons were made between FITC-treated mice and untreated and PBS-treated controls. The time

Fig. 1—Paraffin sections of control and FITC-treated lungs examined by light microscopy and a laser scanning microscope. The control, untreated appearance is shown in (a) and (b). The lung architecture is normal with no apparent inflammatory process. The confocal image reveals only autofluorescence due to collagen and elastin in airway and vessel walls. In contrast, at 3 days (c), a focal inflammatory infiltrate is identified within the lung, composed of neutrophils and macrophages. The corresponding confocal image shows focal deposition of FITC in a peribronchial distribution (d). At 5 months (e), a predominantly mononuclear inflammatory infiltrate is identified associated with focal scar formation in a peribronchial distribution corresponding to foci of FITC retention in the tissues (f)

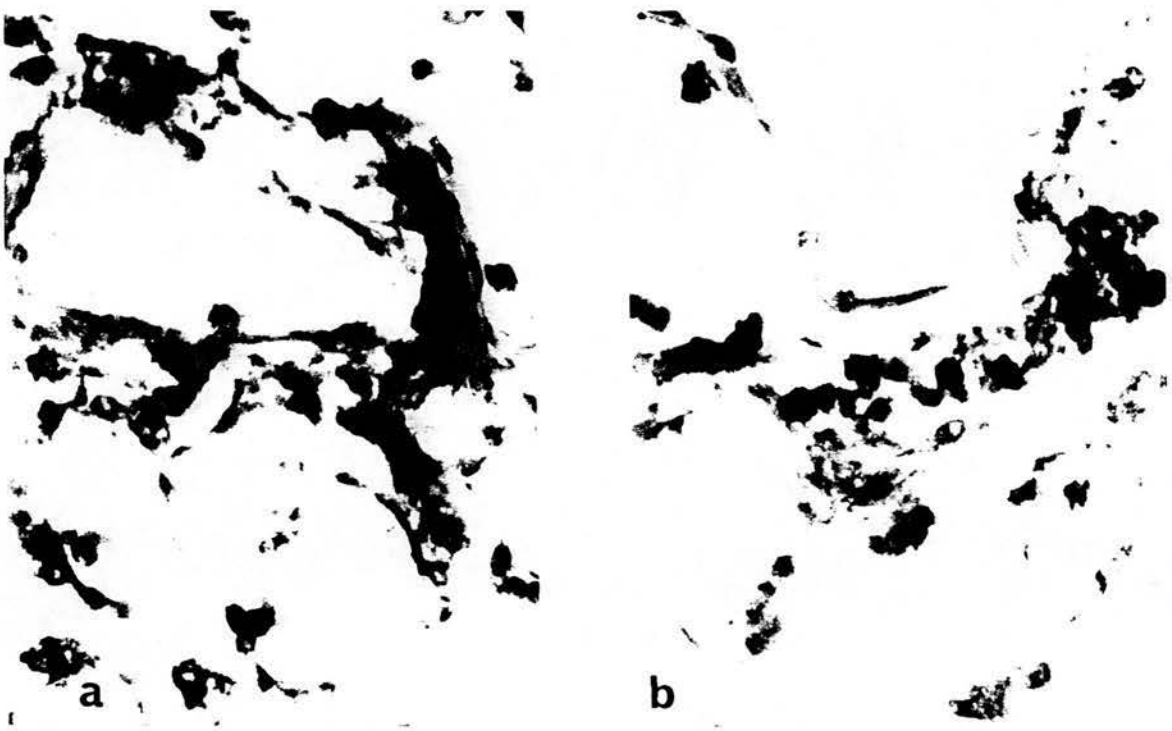


Fig. 3—Cryostat sections of rat lung 7 days after instillation of FITC showing the presence of (a) CD4- and (b) CD8-positive cells (immunoperoxidase technique)

point 3 h appears to represent a transitional phase where a response to the administered antigen is not yet evident in all individuals, hence the large standard deviation. By 6 h, the acute response is in progress in all individuals, with a much elevated number of cells in the lavage relative to both untreated and PBS-administered controls. There is a fall in the number of cells recovered at 1 day, which is possibly due to the nature of the inflammatory process making it difficult to lavage the lungs properly at this time. A greater number of cells were recovered at 3 days and this was maintained up to 7 days after antigen administration. Experiments in rats demonstrated a similar rapid increase and gradual decrease in the number of cells recovered by BAL after administration of FITC (data not shown).

Differential cell counts were performed on cytospins of BAL obtained from mice at the same time points as mentioned above. Figure 4b demonstrates the percentage changes occurring in the cellular composition of lavage fluid. In BAL fluid recovered from untreated control mice (time 0), macrophages/monocytes compose almost the entire population of cells, with only a very low percentage of granulocytes and lymphocytes. However, after FITC instillation there is a rapid influx of granulocytes, mainly neutrophils, which by day 1 make up over 60 per cent of the total number of recovered cells. After this time, the numbers of granulocytes decrease. Lymphocytes begin to appear by day 7.

Immunocytochemistry of rat BAL cytospins indicated that the lymphocytes seen on day 7 were predominantly (>95 per cent) α/β TCR-positive T lymphocytes, with both CD4- and CD8-positive cells being present in approximately equal numbers (data now shown).

Protein accumulation in the alveolar space

The permeability of the rat endothelial and epithelial barriers to blood-bone ^{125}I-labelled BSA is shown in Fig. 5. It is clear that 6 h after FITC instillation there is marked leakage of protein from the blood to the alveolar space. However, by day 1 this has returned to control values. The obvious increased levels of protein apparent in the BAL fluid even at 7 days post-instillation (Fig. 6) are unlikely, therefore, to be a consequence of plasma exudation. As described below, this pattern is more likely to be due to shedding from the epithelial surface, evident in histological sections.

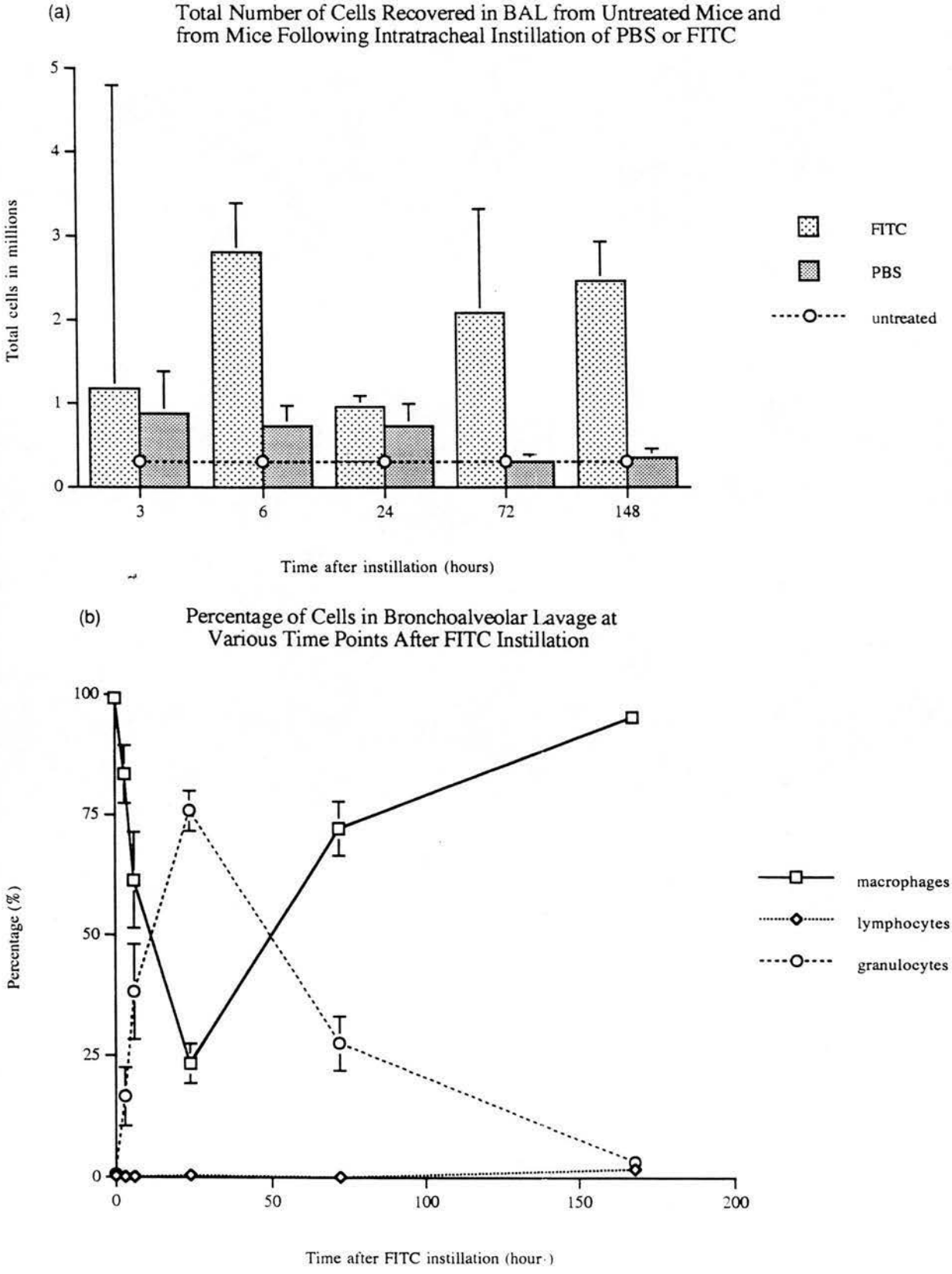


Fig. 4—(a) Graph illustrating the changes in total cell number recovered by BAL in mice at various time points following instillation of FITC or PBS alone. Data expressed as mean and SEM from 4–7 mice at each time point. (b) Graph illustrating the differential cell count of the BAL recovered cells at each time point. Data as geometric mean and SEM from 4–7 mice at each point

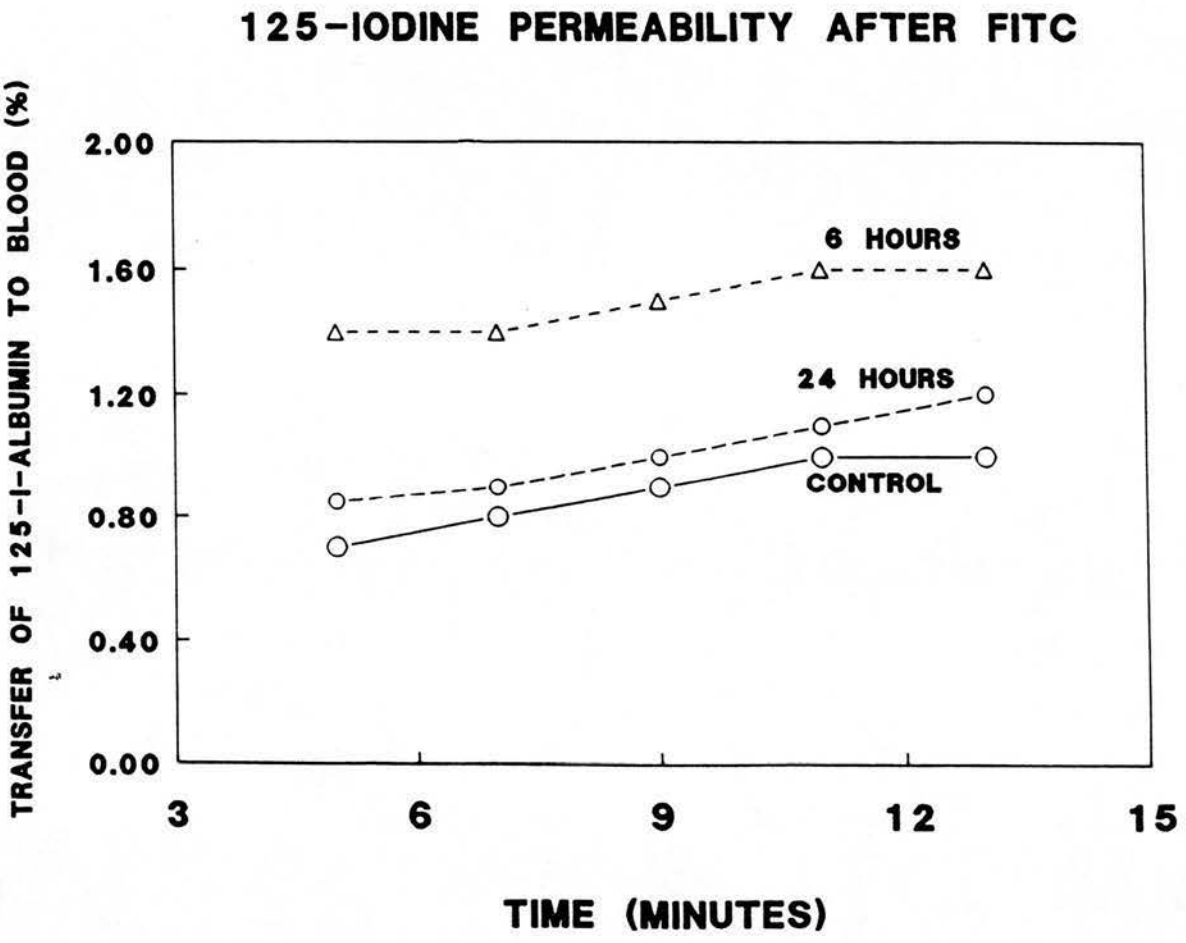


Fig. 5—Leakage of ^{125}I-labelled BSA from the alveolar space to blood at various times after FITC instillation. See the Materials and Methods section for details. The presented data were from pooled samples of at least three animals at each time point

ELISA measurement of anti-FITC antibodies

Table I illustrates the level of anti-FITC antibodies measured in rats at different time points after FITC instillation. Antibodies were detectable in serum 7 days after intratracheal FITC and were still detectable 6 weeks later. FITC given by the intraperitoneal route induced similar levels of anti-FITC antibodies.

DISCUSSION

In this paper we have described a new model to study the involvement of the immune system in the development of pulmonary fibrosis. We have used the fluorescent hapten FITC, a reagent commonly used to label proteins for use in immunological techniques. When FITC was instilled into the lungs of rodents, it remained in the interstitium, associated with connective tissue fibres, for up to 5 months. This is associated with a chronic mononuclear interstitial infiltrate localized to the areas of FITC deposition. Serum antibodies against FITC have been demonstrated.

If the chronic inflammation were a consequence of on-going cell injury caused by the presence of FITC, as seen with non-immunological cytotoxic stimuli such as quartz,[9] then a persistent protein leak and large-scale neutrophil influx would be anticipated. In fact, BAL analysis revealed that by 7 days the number of neotrophils was at almost normal levels, whilst the protein leak present at 6 h had resolved by 24 h. From this we assume that the driving force for the inflammation beyond the first week is an immunological process.

From the position of the FITC in the interstitium and its appearance, we contend that FITC/host protein conjugates are the source of the

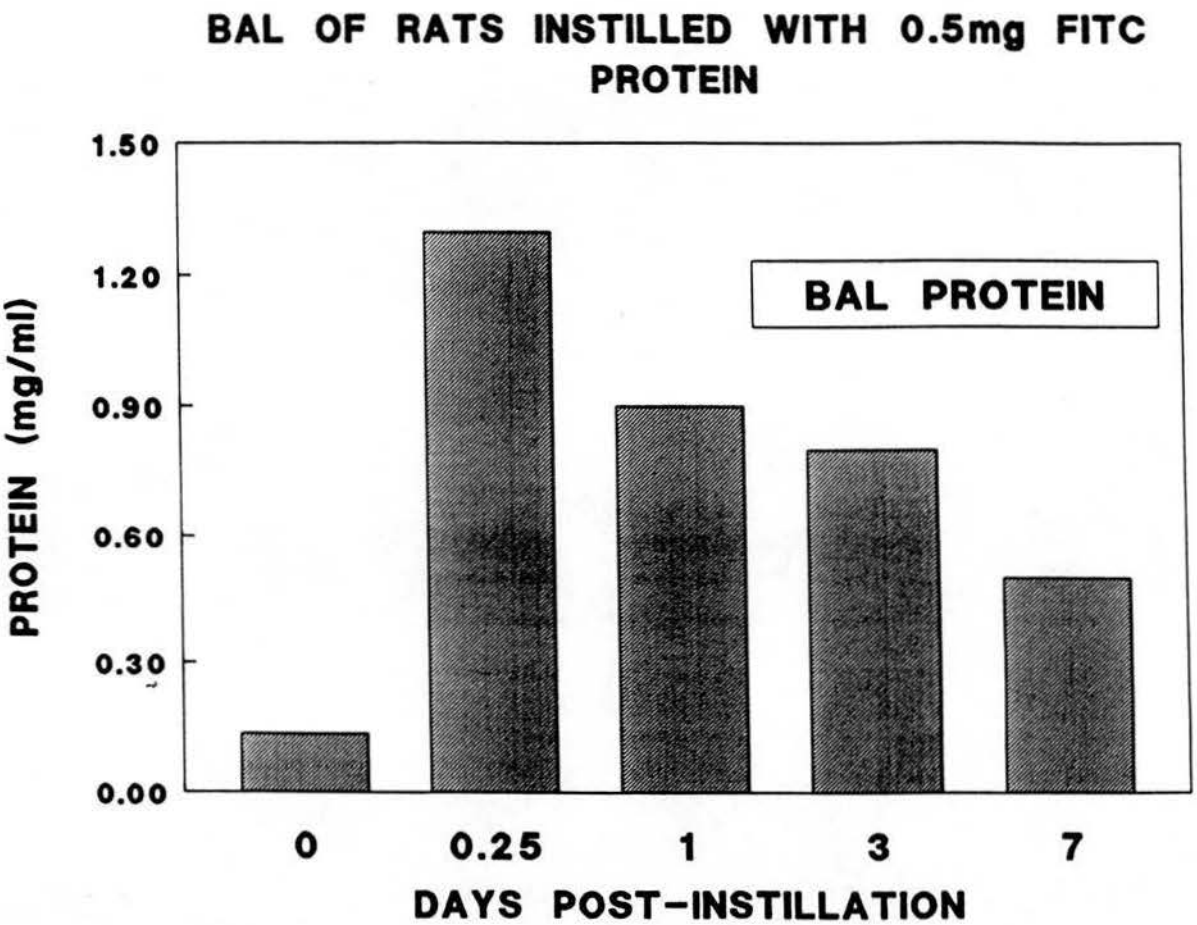


Fig. 6—Protein measurement (mg/ml) in BAL fluid at various times after FITC instillation. The presented data were from pooled samples of at least three animals at each time point

ng-term immune stimulation. We propose, as a quence of events, that the instilled FITC, con- ned to a small area of the lung at high concentra- on, is transiently cytotoxic to the epithelial lining lls. This was demonstrated by the histological ppearance of damaged and shed epithelial cells nd associated proliferation in the lung 3 days ter instillation. The FITC then gains access to e interstitium at the regions of breached epi- elium and forms conjugates with any proteins at it then contacts. Due to the rapid turnover of e majority of proteins, most of these conjugates ill be cleared from the interstitium within a few ays. However, histological evidence supports e notion that relatively stable proteins such as astin[12] may form conjugates with FITC, which ersist in the medium and long term, stimulating a hronic immune response.

The FITC model has some similarities with ther animal models of ILD. Bleomycin has been used to cause fibrosis in rats and mice, with a report that the fibrosis was markedly diminished in athymic mice compared with their heterozygous littermates.[13] The contention that T cells are important in bleomycin fibrosis is further supported by the finding that in non-responder mice, a cyclophosphamide-sensitive T-cell population is important in maintaining the non-responder status.[14] Further, cell-mediated immunity to normal collagen is reported in bleomycin-treated rats,[15] although there is no evidence that bleomycin is a hapten. However, work with a classical immunological hapten, trinitrophenol (TNP), has shown that the lung can be sensitized. Hamsters were treated on their skin with TNP and a subsequent exposure in the lung was found to cause pulmonary fibrosis.[7]

The particular benefits of the FITC model are that the hapten/protein complex can be seen easily by fluorescence, so that its association with

Table I—ELISA estimation of anti-FITC serum antibodies

Treatment		Titre
Nil	(n=3)	<1:50
IT FITC 3 days	(n=3)	<1:50
IT FITC 7 days	(n=3)	1:400
IT FITC 4 weeks	(n=9)	1:400
IT FITC 6 weeks	(n=9)	1:800
IP FITC 4 weeks	(n=9)	1:400

Pooled sera from rats given intratracheal (IT) FITC or intraperitoneal (IP) FITC. All animals were given 0·5 mg of FITC in PBS. Titres given are the highest doubling dilution which gave a value greater than 3 standard deviations above the mean value for normal rat serum: see the Materials and Methods section for details.

on-going response can be gauged, and that FITC is a well-characterized immunological reagent against which specific B- and T-lymphocyte responses can be measured. The persistence of the FITC/protein conjugate on a long-lived interstitial component such as elastin obviates the need for the prior sensitization needed in the TNP model. The FITC system allows close observation of the kinetics associated with the development of pulmonary fibrosis after a single exposure to a sensitizing agent. We are currently using the model to investigate in detail the pathogenesis and immunobiology associated with pulmonary fibrosis, which may be of relevance to those forms of human ILD which involve specific immune responses within the pulmonary interstitium. The haptogenic agent trimellitic anhydride, used in plastics manufacture, can generate new antigenic determinants on human serum albumin. These new antigens are recognized by IgE and total antibody in workers suffering from a range of syndromes including late respiratory systemic syndrome, which has been described as 'a variant of hypersensitivity pneumonitis'.[16,17] Epidemiological evidence linking the development of cryptogenic fibrosing alveolitis (CFA) with exposure to a variety of environmental agents including metal dust, household fire, and cattle[18] gives support to the idea that reactive chemicals could be a factor in the development of this important fibrotic lung disease, although there are differences in the nature of the infiltrate between CFA and our model, particularly in the presence of granulocytes seen in CFA. Nevertheless, this is an important model in understanding the development and consequences of chronic patchy lung fibrosis, in the apparent absence of persistent direct epithelial damage.

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Section 5

Section 5 Studies on asbestos and the pleural space

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Studies on the morphological patterns of asbestos induced mesotheliomas *in vivo* and *in vitro*

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Abstract

Cell lines were established *in vitro* from five peritoneal mesotheliomas produced in rats by the injection of crocidolite asbestos. These tumours exhibited the previously described diverse range of histological patterns normally associated with mesotheliomas. This diversity of appearance was also evident in culture but cell patterns *in vitro* did not necessarily correspond to the histology of the original tumour. With increased time in culture most cell lines tended towards a pattern of random orientation associated with the piling up of cells to form discrete masses. Despite this variation in cell pattern in culture, few ultrastructural differences were seen during electron microscope examination. Following varying periods in culture, some cell lines from these tumours were injected into either rats or athymic mice. Only one typical rat mesothelioma was produced but all cell lines produced tumours in athymic mice. The implications of these findings are discussed in relation to the aetiology of asbestos induced mesotheliomas.

Introduction

The existence of specific tumours arising from the mesothelial surfaces of the pleural and peritoneal cavity was disputed for many years (1,2) and even those pathologists who accepted the existence of mesotheliomas agreed that they were extremely rare. However, following the discovery of Wagner *et al.* in 1960 (3) that workers in the South African asbestos mining industry had an unexpectedly high incidence of this type of tumour, the importance of the mesothelioma in an industrial, asbestos-using, society was accepted by most workers. Reports of cases of mesothelioma in asbestos workers have now appeared from most industrial nations and there is concern that some cases appear to have developed after a relatively low exposure to this mineral (4). These findings have given rise to the suggestion that contamination of the urban environment with low levels of asbestos from industrial processes could cause mesotheliomas in the general population and even the contamination of water supplies with minute amounts of these minerals fibres has caused concern (5).

Animal experimental studies have confirmed that mesotheliomas are found occasionally following asbestos inhalation (6,7), but they occur in a high percentage of animals following intrapleural or intraperitoneal injection (8,9). There is, however, still much to be learned about the biology of this type of tumour in animals and man. In spite of cases having occurred in man after only a relatively low exposure to asbestos, the tumour is still rare even among heavily exposed asbestos workers although this may be due to increased mortality from other causes. In addition mesotheliomas appear to occur after an extremely long latent period and many cases do not develop until 30- 40 years after first exposure (10). Mesotheliomas are unusual tumours in that they show a variety of histological patterns and appear to contain elements of both epithelial and connective tissue type. Moreover, there are differences between tumour formation in the pleural and peritoneal cavities (11, 12, 13).

As mesotheliomas show such variable morphology *in vivo* it was felt that *in vitro* studies offered scope for the elucidation of the cell biology of these tumours. Peritoneal mesotheliomas were used in this study since they develop more quickly than pleural mesotheliomas in rats following asbestos injection and furthermore the presence of ascites provides a simple indication of tumor development in these animals.

Materials and Methods

Male random bred white SPF Wistar rats of the AF/HAN strain were given a single intraperitoneal injection of 25 mg of UICC crocidolite asbestos in 1 ml of phosphate buffered saline (PBS), at the age of three months. The first tumours were found nine months after injection.

For the culture of mesothelioma cells the peritoneal cavity of five rats with these tumours was opened aseptically and washed out with about 20 ml of Dulbecco's PBS. The resulting cell suspensions were centrifuged. Cells from the pellets were resuspended in medium and plated out in 25 ml tissue culture flasks at approximately 4 x 10^6/flask. Pieces of solid tumour were also removed and either plated out as explants using the technique described by Greene in 1973 (14) or dissociated with trypsin/EDTA (Gibco-Biocult Ltd.). This treatment involved washing the pieces of tumour with PBS followed by agitation for one hour at 37°C in the enzyme solu-

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tion. At intervals during this period the larger fragments of tissue were allowed to settle out and the supernatant, containing small clumps and single cells, removed and plated out at approximately 2 x 10^6 cells/25 ml tissue culture flask. The medium used for all cultures was Ham's nutrient mixture F_{10} containing 10% foetal calf serum and antibiotics. After the primary cultures reached confluence they were subcultured and thereafter maintained by three medium changes each week and passaged at a ratio of approximately 1:8 once each week. The morphological patterns of typical cultures were photographed prior to subculture.

After varying periods in culture, cell lines from the first four tumours were re-injected into the peritoneal or pleural cavities of three-month-old isologous rats. In addition cell lines from all five tumours were injected into the peritoneal cavities of six-week-old athymic nude mice. Two rats and three mice were injected with each cell line. Prior to injection, cell suspensions were prepared by incubation of monolayer cultures in trypsin/EDTA solution. The trypsin was neutralised by the addition of serum when the cells had become detached from the substrate. The cells were then centrifuged and suspended in PBS at a concentration of 4 x 10^6 cells/ml. One ml of this suspension was then injected intraperitoneally into the groups of rats or athymic mice.

For light microscopical examination, tumour material from the rats or mice used in this study was fixed in formol saline, embedded in paraffin wax and stained with either haematoxylin and eosin, Van Gieson's method for collagen, or Gordon and Sweet's stain for reticulin. For electron microscopy cell suspensions were fixed in buffered osmium tetroxide, embedded in araldite and stained with a combination of lead citrate and uranyl acetate.

Results

Morphology of the original tumours in rats and their characteristics in vitro

The five mesotheliomas cultured *in vitro* in the present study covered the full range of histological structures previously described for this type of tumour by Davis in 1974 (9). The histology of these tumours and their *in vitro* growth patterns are described below. The morphological growth patterns for any one tumour were identical regardless of whether the cultures originated from ascitic fluid or pieces of solid tumour.

Tumours 1 and 2 were of similar morphology and were typical of the most frequently occurring pattern for rat peritoneal mesotheliomas. They consisted mainly of nodules 0.2 and 5.0 mm in diameter attached to the body wall or abdominal viscera (Figure 1). Nodules less than 1 mm in diameter were frequently pedunculated and attached only by a thin strand of connective tissue but larger nodules were usually firmly adherent over a large portion of their surface area. There was, however, no evidence of tissue invasion in these two tumours. The smallest nodules consisted of loosely arranged and irregularly shaped connective tissue cells surrounded by a few peripheral layers of rounded cells of an epithelial type. In the central regions both collagen and reticulin were present and these were often arranged in a characteristically whorled pattern. The larger masses also had surface layers of epithelial-like cells, but their central regions often consisted of densely packed spindle cells with relatively few connective tissue fibres between them. Within these large tumour masses, however, it was still common to find small, acellular areas of whorled collagen.

As well as being morphologically similar, tumours 1 and 2 also behaved similarly *in vitro*. The cultures initially consisted of a mixture of apparently normal fibroblastic and mesothelial cells but over a period of

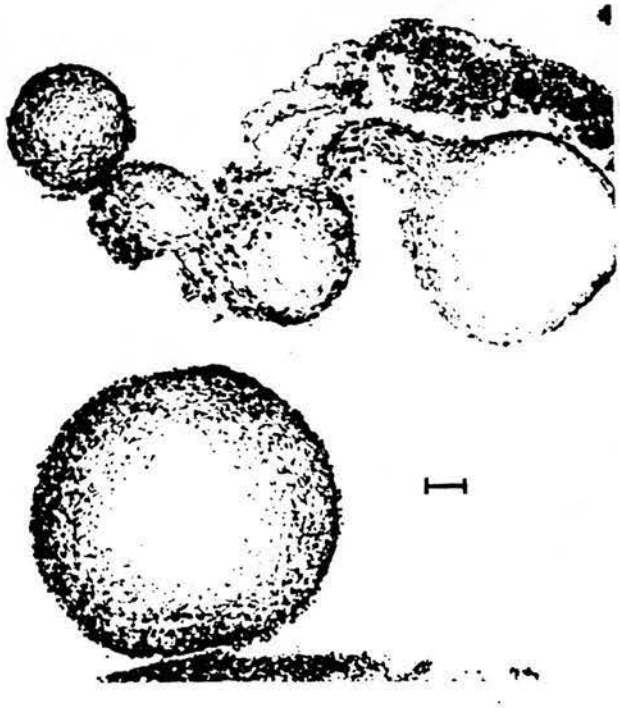


Fig. 1. Rat peritoneal mesothelioma produced by the injection of crocidolite asbestos (Tumour 1). This tumour consisted of multiple tumour nodules 0.2-5 mm in diameter, most of which were attached to the viscera or body wall by thin strands of connective tissue. Magnification × 60. Bar denotes 100μ.

approximately three months they changed to show two different patterns in culture. The first of these consisted of randomly orientated cells which crossed over each other and piled up (Figure 2). In older cultures, 5 to 7 days after subculture, this piling up became extensive and large clumps of cells appeared (Figure 3). Alternatively, the cells formed a flat grainy cell sheet (Figure 4). This latter cell growth pattern was never associated with the large cell clumps that were observed when the cells were randomly orientated. Initially the two types of growth pattern predominated in the subcultures apparently at random and could change from one form to the other from subculture to subculture. Single cell clones were established from both types of culture using the technique described by Martin (15). These clones behaved exactly like the parent cultures with both growth patterns occurring in them. With increasing time in culture, however, the randomly orientated form of cell sheet tended to predominate.

Tumour 3 showed an entirely different histological pattern to tumours 1 and 2. The smallest lesions of this tumour consisted of papillomatous outgrowth of the mesothelial surface consisting mainly of relatively rounded cells. Amongst these rounded cells, however, some large tissue spaces were present, lined

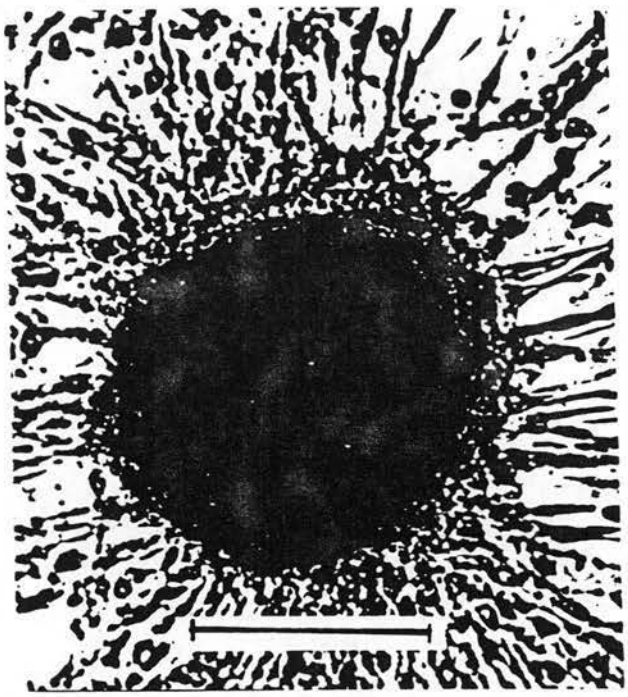


Fig. 3. One of the growth patterns of Tumour 1 after 8 months in culture. The cells have piled up to produce a large cell mass. Bright field. Magnification × 350. Bar denotes 100μ.



Fig. 2. *In vitro* growth pattern of Tumour 2 after 8 months in culture. The cells show a random orientation and are beginning to pile up. Phase contrast. Magnification × 350. Bar denotes 100μ.

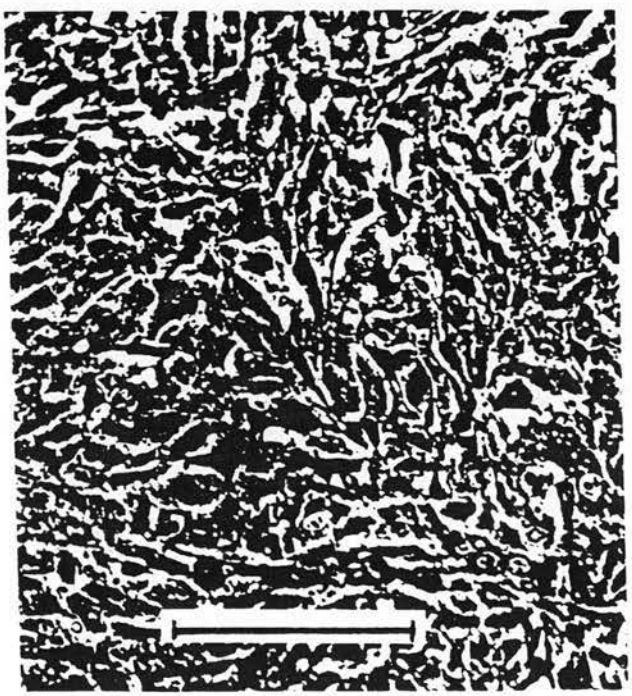


Fig. 4. Tumour 2 after 6 months in culture. The cells have formed a flat grainy layer with no evidence for cells piling up. Phase contrast. Magnification × 350. Bar denotes 100μ.

by extremely flattened and extended cells. Some areas of these outgrowths contained collagen and/or reticulin fibres. The larger tumour masses which were up to 5 mm in diameter consisted of areas of closely packed spindle shaped cells interspersed with groups of large tissue spaces which were up to 200 μm in diameter and lined with cells that were often extremely flattened and extended (Figure 5). Mesotheliomas with this structure are rarely found in the rat peritoneum although they frequently occur in the pleural cavity (9). As was found for tumours 1 and 2, large masses of tumour tissue were present in the peritoneal cavity, with no evidence of invasion of normal tissues.

Tumour 3 also differed from tumours 1 and 2 in its growth patterns *in vitro*. The initial cultures were composed of fibroblast-like cells although these appeared to be shorter and more densely packed than normal fibroblasts. After approximately two months in culture, many rounded cells appeared on the surface of confluent cell sheets and tended to float off into the medium (Figure 6). By three months all of these cultures had changed to a growth pattern showing randomly orientated cells. Some piling up of cells was observed in older cultures (5 to 10 days after subculture) but these cell clumps never reached the size observed with tumours 1 and 2 (Figure 7). Once the cultures had changed to cells showing a random growth pattern this type always persisted.

Tumour 4 differed from the previous three tumours in that, although macroscopically the tumours consisted of numerous nodules scattered over the peritoneal surface, there was much less structural variation. All the nodules exhibited a uniform fibrosarcomatous structure throughout. The cells were relatively rounded and there was only a thin network of collagen and reticulin between them (Figure 8). This tumour was malignant and had invaded the body wall and the muscular layers of the intestine. The cells growing out from this tumour initially had the appearance of normal fibroblasts but after approximately one month in culture the growth pattern changed and the cells became randomly orientated with cells piling up in clumps as has been described for tumours 1 and 2 (Figure 9). The flat grainy cell sheets described for tumours 1 and 2 were, however, never seen with tumour 4.

Tumour 5 was atypical in that it consisted of a single tumour mass instead of the more nodular form of mesothelioma associated with ascites that was



Fig. 5. An area from a crocidolite induced rat peritoneal mesothelioma (Tumour 3). This tumour consisted of closely packed spindle shaped cells interspersed with large tissue spaces lined with extremely flattened and extended cells. Magnification × 400. Bar denotes 100μ.

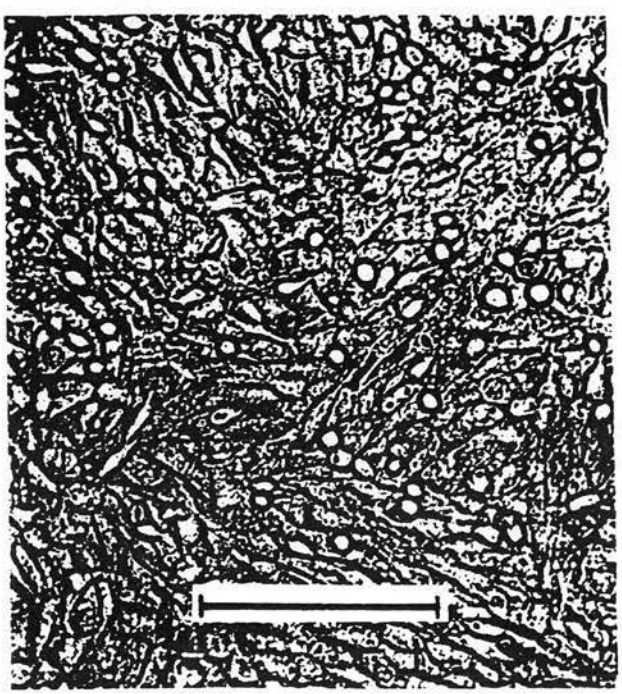


Fig. 6. Tumour 3 after 3 months in culture. The cells have formed a fibroblastic-like monolayer but rounded cells are appearing on the surface of the cell sheet. Bright field. Magnification × 350. Bar denotes 100μ.



Fig. 7. Typical growth pattern of Tumour 3 after 5 months in culture. The cells are growing in a randomly orientated manner and piling up to form cell masses but these have not attained the size seen in tumours 1 and 2. Bright field. Magnification × 350. Bar denotes 100μ.

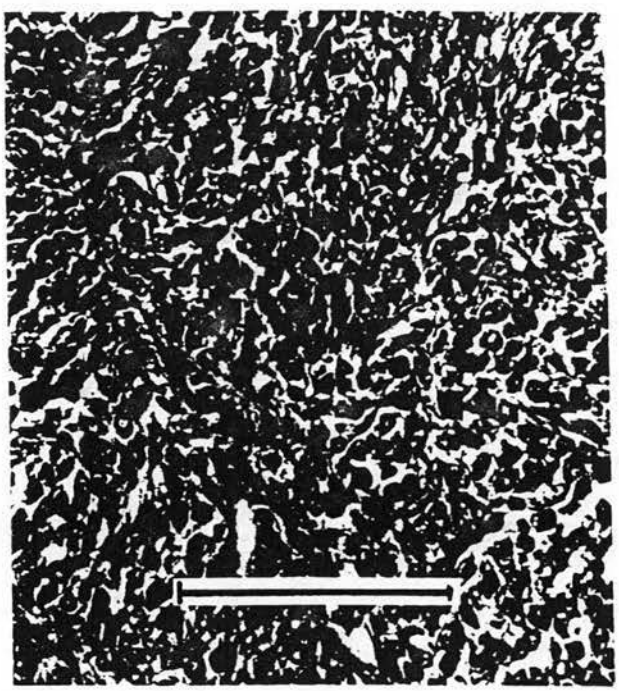


Fig. 8. An area from a rat peritoneal mesothelioma induced by crocidolite asbestos (Tumour 4). All the nodules of this tumour exhibited a uniformly fibrosarcomatous pattern. The tumour cells were, however, relatively rounded and there was only a thin network of collagen and reticulin between them. Magnification × 400. Bar denotes 100μ.

observed with the other four tumours. This tumour exhibited a uniform fibrosarcomatous structure with spindle shaped cells intermingled with a dense network of connective tissue fibres (Figure 10). Like tumour 4, this tumour was malignant and had invaded the body wall and the muscular integument of the intestines. Initially, the cultures obtained from this tumour consisted of flattened cells with many of the characteristics of normal mesothelial cells (Figure 11) but after a short period colonies composed of a different type of cell appeared in the cultures. These cells were smaller and often had many processes extending from them and rounded up cells appeared on the surface. This cell type soon predominated in the cultures and the proportion of rounded up, loosely adherent cells increased with time in culture (Figure 12). The piling up of cells seen with the other tumours was never observed in cultures from tumour 5.

Electron microscope studies of the mesothelioma cell lines in culture showed relatively few differences in cell ultrastructure regardless of the many variations seen in the patterns of cell growth. All the cells examined had the general ultrastructural pattern previously reported for rat peritoneal mesotheliomas (9). They usually had a well developed granular endoplasmic reticulum which was often seen in a greatly extended condition and a very active Golgi apparatus. Intracytoplasmic crypts lined by microvilli were found in all cell lines and most cells contained lipid droplets. Cells were removed from the surfaces of culture vessels and pelleted by centrifugation before fixation, and consequently they usually appeared spherical when examined (Figure 13); however, there were two exceptions to this. When cells of definite flattened mesothelial type were present in the cultures many of these cells remained flattened in the cell pellet and could be seen cut in transverse section as in Figure 14. Where cells had aggregated to form dense masses as in Figure 3, they were seen in the electron microscope to be elongated with nuclei of typical fibroblastic type (Figure 15). In addition, while the surface membranes of most cells examined were covered with numerous elongated microvilli or blebs, these elongated cells had relatively few surface processes and their flat cell membranes were in contact with those of nearby cells



Fig. 9. Tumour 4 after 6½ months in culture with the cells growing in a randomly orientated pattern. Bright field. Magnification × 350. Bar denotes 100μ.

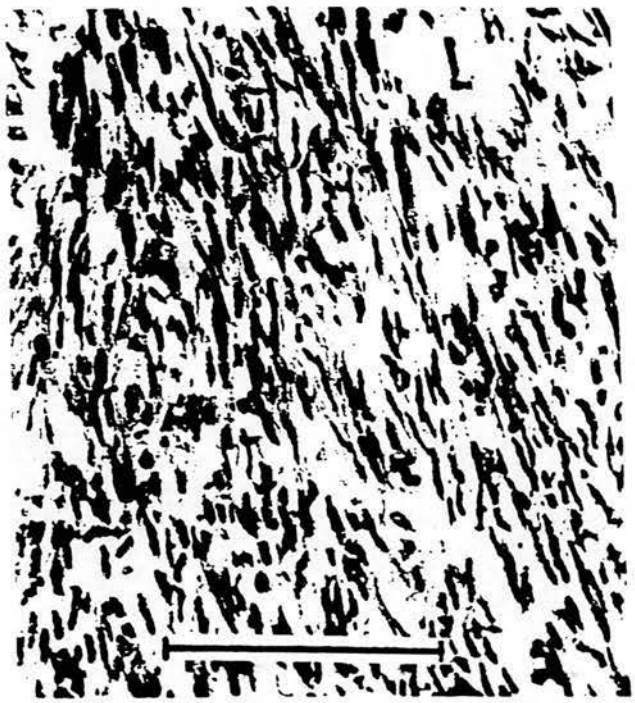


Fig. 10. An asbestos induced rat peritoneal mesothelioma showing a fibrosarcomatous histological pattern (Tumour 5). The cells are elongated and spindle shaped and intermingled with a dense network of connective tissue fibres. Magnification × 400. Bar denotes 100μ.

over quite large areas. Whereas the cells from most of the lines examined had a haphazard mixture of microvilli and globules on the surface membranes, those from tumours 3 and 4 contained many round cells where microvilli were absent and blebs were the predominant feature, each bleb containing elements of the rough endoplasmic reticulum (Figure 16).

Passage of the mesothelioma cell lines in rats and athymic nude mice

Following varying periods in culture a few of the mesothelioma cell lines from the first four tumours were reinjected into an isologous strain of the AF/HAN rats. One animal injected with a 'randomly orientated' cell line from tumour 4 (maintained in culture for six months) developed a typical rat peritoneal mesothelioma five and a half months after injection. This tumour had a histological pattern which was more closely related to the original appearance of tumours 1 and 2 than to tumour 4. A rat injected intraperitoneally with a randomly orientated cell line from tumour 1 developed a pleomorphic sarcoma after 26 months that grew as sheets covering and invading many of the abdominal viscera. This tumour consisted of large, irregularly shaped and loosely arranged cells which did not appear to be associated with the production of connective tissue fibres. Another rat injected intrapleurally with a different randomly orientated cell line from tumour 1 developed, after 15 months, a large fibromatous growth on the visceral pleural surface. This tumour which consisted of closely packed spindle shaped cells had compressed the lungs without invading them. There were, however, no adhesions between the tumour mass and the parietal pleural surfaces.

When only one tumour had been found after six months in rats injected with mesothelioma cell lines it was considered that this random-bred strain of animals might be unsuitable for passaging these tumours. Mesothelioma cell lines from all five tumours were therefore injected into athymic nude mice which have been shown to be particularly suitable for the passaging of tumours (16). Some lines were injected on more than one occasion after different numbers of passages in culture in order to determine whether or not growth patterns could change during long periods of culture.

All athymic mice injected with cell lines from tumours 1 and 2 had died within five weeks of injec-



Fig. 11. Tumour 5 after 3½ months in culture. Many flattened mesothelial-like cells are present and they often show the striations that are observed in normal mesothelial cells. Phase contrast. Magnification × 350. Bar denotes 100μ.

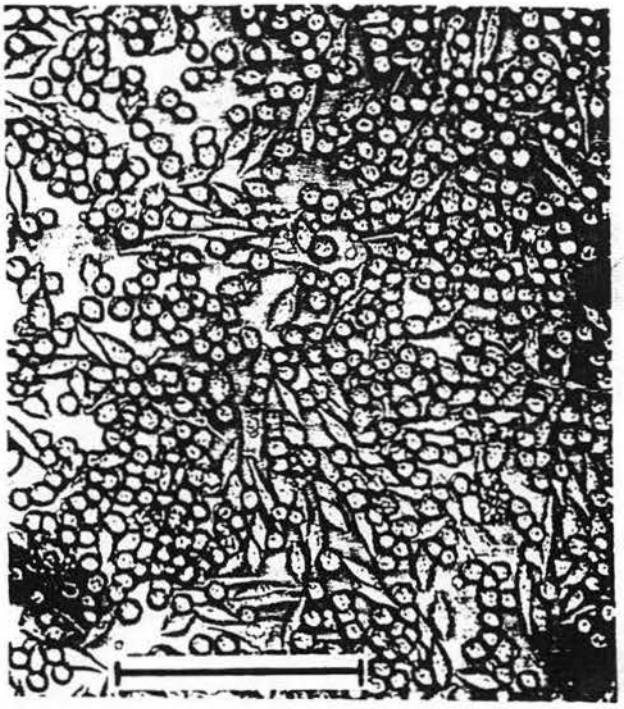


Fig. 12. Tumour 5 after 25 months in culture. No mesothelial-like cells can be seen. Many of the cells are rounded up and only loosely adherent to the culture vessel. The cells never showed evidence of piling up. Bright field. Magnification × 350. Bar denotes 100 μ.

tion. At this stage tumours were found to have grown as numerous small nodules between 1 and 2 mm in diameter, mostly unattached in the peritoneal cavity. No nodules attached to any of the peritoneal surfaces were found with lines from tumour 1 but a few attached nodules were found with tumour 2. Nodules from tumour 1 all consisted of a peripheral cellular region with a necrotic centre and there was no evidence of collagen production. Unattached nodules from tumour 2 showed the same basic pattern but with numerous small spherical areas of acellular collagen embedded in the central necrotic regions (Figure 17). Where nodules had become attached to the peritoneal surfaces, however, they had become vascularised and were cellular throughout. There was no central necrotic region with or without collagen production (Figure 18). Cell lines from tumour 3 showed some variation of growth patterns in athymic mice. Some animals died as early as four weeks after injection with the peritoneum distended with blood stained ascites containing very large numbers of tumour cells. In these animals small nodules 1 to 2 mm in diameter and sheets of tumour cells were present in many areas. However, none of these tumour masses, whether obviously attached to the peritoneal surface or not, showed any signs of central necrosis. They consisted of layers of rounded cells on the surface with spindle cells forming the deeper layers. In a few nodules small areas of the original vesiculated histological pattern had developed but this was comparatively rare. Among the larger spindle cell areas there was relatively little evidence of collagen or reticulin production but floating free among the ascites cells it was common to find very small islands of collagen only about 100 microns in diameter surrounded by single areas of flattened cells and containing an occasional spindle shaped cell (Figure 19).

With tumour 3 the production of blood stained ascites in athymic mice was particularly associated with one cell line first injected after three months in culture. When this line was reinjected after eight months, ascites did not develop in any of the animals used and the tumour developed as a series of solid nodules. However, when cells cultured for three months and then stored in liquid nitrogen were recovered and reinjected into athymic mice, ascites again developed in all animals within four to six weeks. Mice injected with any of the tumour 3 lines

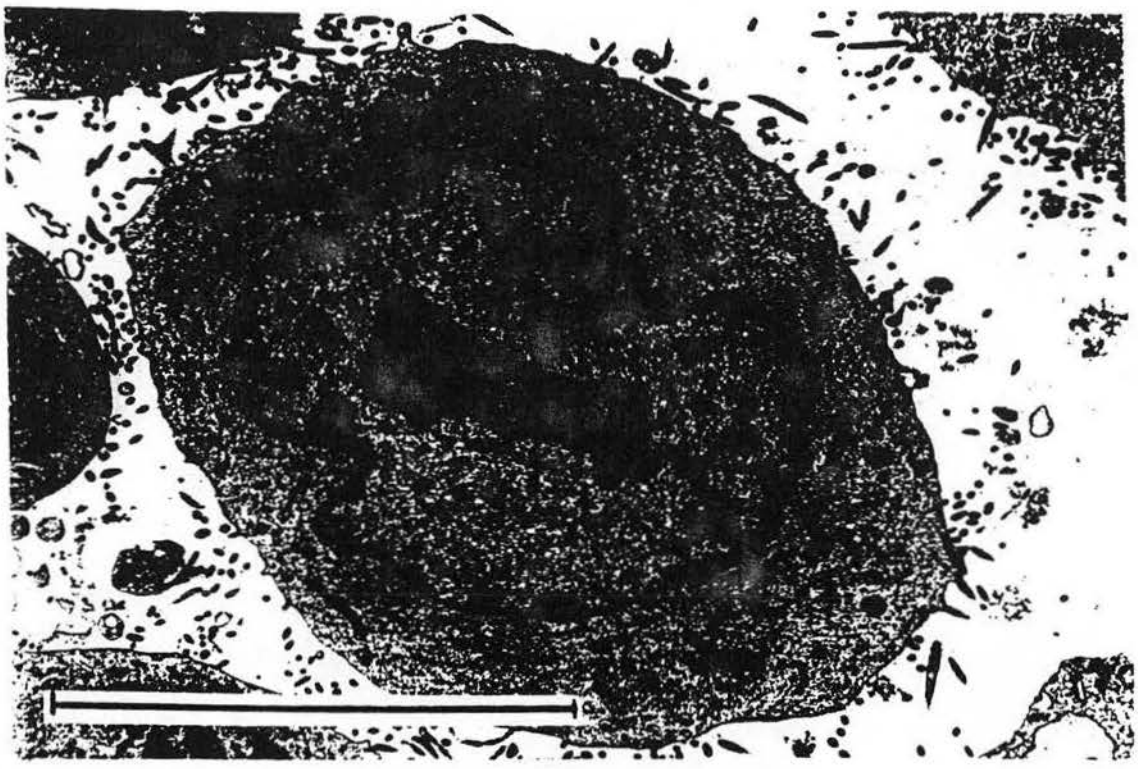


Fig. 13. Electron-micrograph of a rounded cell from a rat mesothelioma culture (Tumour 1). This cell has a well developed granular endoplasmic reticulum and its surface membrane is covered by elongated microvilli. Magnification × 7,500. Bar denotes 10μ.

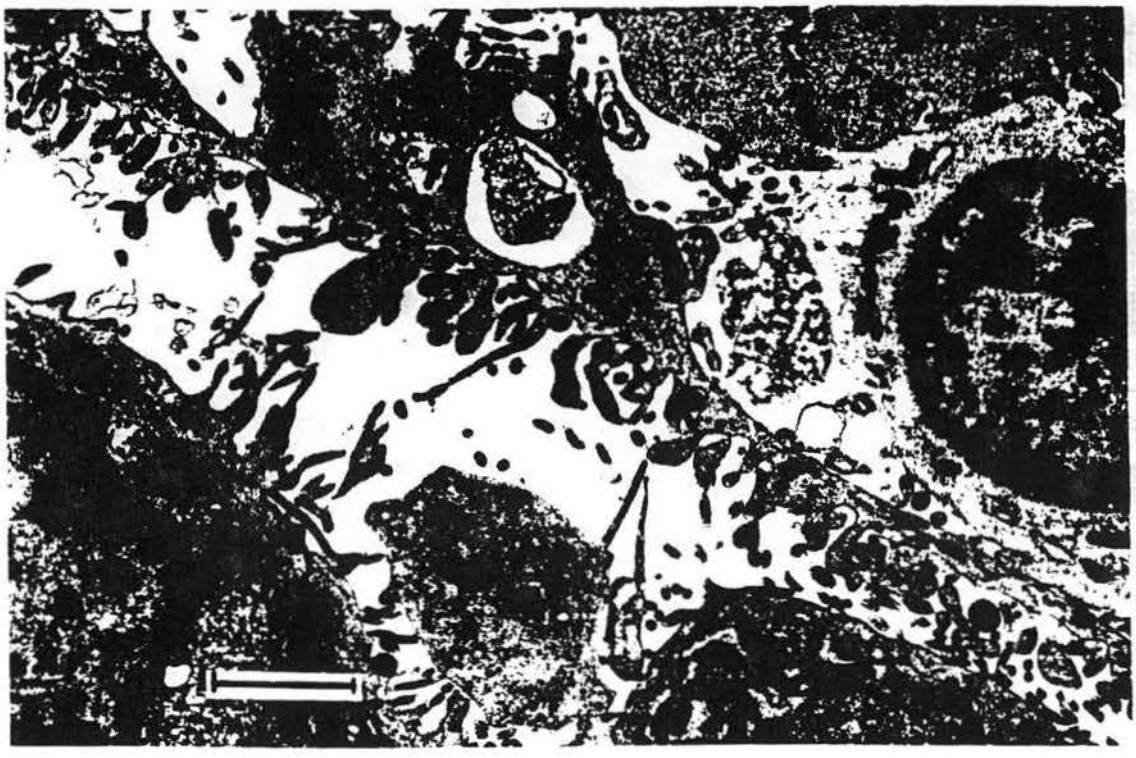


Fig. 14. Electron-micrograph of part of a cell pellet from a rat peritoneal mesothelioma maintained *in vitro* (Tumour 5). Most of the cells are rounded but in the centre of the photograph is a transverse section of a flattened and extended area of cytoplasm. The upper surface of this is covered by closely packed globular processes and microvilli. Magnification × 20,000. Bar denotes 1μ.

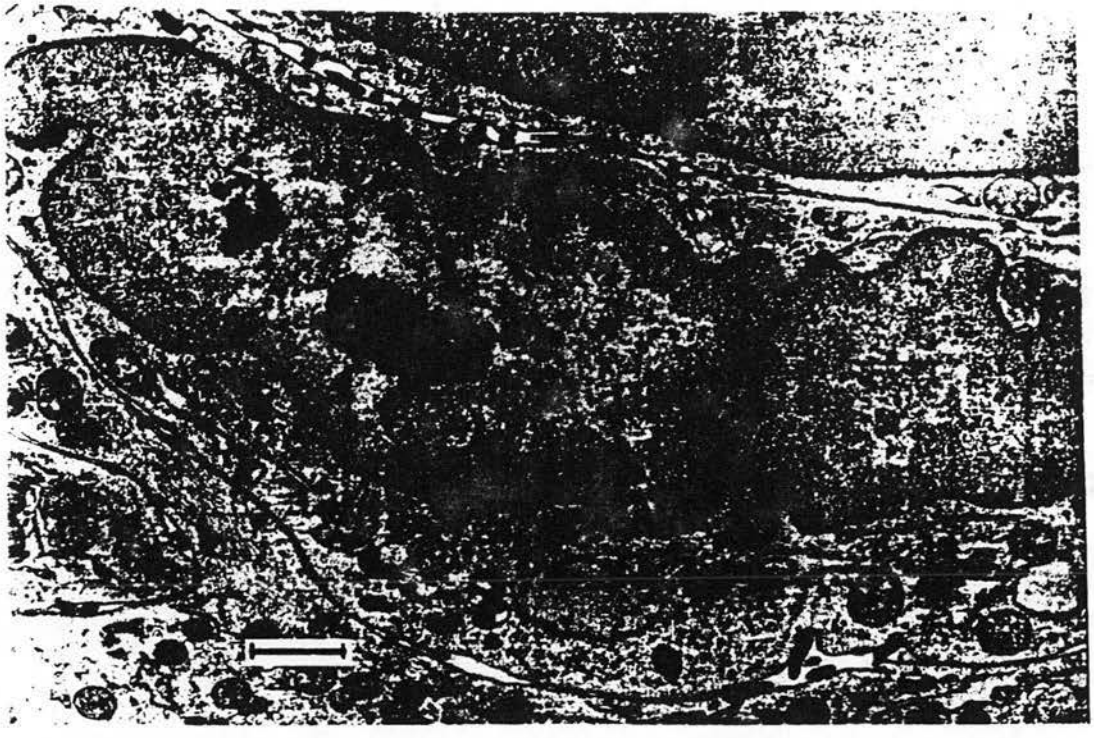


Fig. 15. Electron-micrograph of a group of cells from one of the dense cell masses from tumour 1 that were illustrated in Fig. 3. The cells are elongated and similar in appearance to fibroblasts, having a well developed endoplasmic reticulum frequently seen in an extended condition. The cell membranes are closely apposed to one another over large areas and only a few microvilli are present between the cells. Magnification × 14,000. Bar denotes 1μ.



Fig. 16. Electron-micrograph of part of a cell from a culture of rat peritoneal mesothelioma. The cell membrane has become extended to produce a series of large blebs which contain cytoplasmic organelles, particularly segments of endoplasmic reticulum. Magnification × 24,500. Bar denotes 1μ.

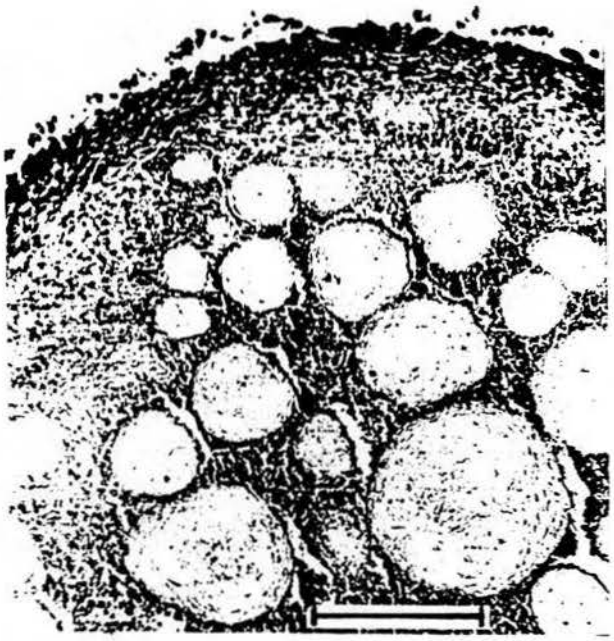


Fig. 17. Part of a tumour nodule resulting from the injection of cultured rat mesothelioma cells from tumour 2 into an athymic mouse. Several layers of cells at the periphery are still alive but the majority of the central area nas become necrotic. Within this area of necrosis, however, are large numbers of round nodules of acellular collagen. Magnification × 250. Bar denotes 100μ.



Fig. 18. A small tumour nodule resulting from the injection of cultured rat mesothelioma cells from tumour 2 into an athymic mouse. This nodule has become attached to the surface of the pancreas and is vascularised. The central regions of the nodule show no signs of necrosis but one small spherical area of collagen production has occurred. Magnification × 150. Bar denotes 100μ.

that survived more than six weeks usually showed no evidence of ascites and most had a typical pattern of cellular nodules and sheets of tumour with no evidence of invasion. However, two mice that had survived for more than eight weeks had developed large tumour masses with a uniform spindle cell structure that had invaded the body wall and the surface layers of some of the abdominal viscera. Some ascites fluid from nude mice injected with tumour 3 was recultured. The cell patterns seen in these cultures were very similar to those seen in the original cultures and illustrated in Figure 6.

Cell lines from tumour 4 when injected into athymic mice grew as sheets and nodules consisting mainly of spindle shaped cells but with some areas of large pleomorphic cells especially in those animals that survived for the longest time (Figure 20). Occasionally areas of large tissue spaces lined with flattened cells were also found. All cell lines from this tumour were found to be invasive in athymic mice.

Cell lines from tumour 5 showed similar masses of tumour growth in athymic mice to tumour 4 but areas of spindle cells were more evenly mixed with areas of round cells. Some small areas of large pleomorphic cells still occurred but no cell-lined tissue spaces were found within the tumour. As with tumour 4, all cell lines from tumour 5 were invasive when grown in athymic mice.

Discussion

Cell culture techniques have been used for many years in the study of tumour cells and their properties but only a few *in vitro* studies of the mesothelioma have been described previously and these, for the most part, tended to be short-term studies on tumours from individual patients (17,18). The extreme structural variability of these tumours in both man and animals has been emphasised in many previous publications (11, 12, 13, 8, 9), but no extensive study of their morphology in long-term culture *in vitro* has been reported. As the rat has proved to be a suitable experimental model for the study of mesotheliomata *in vivo*, a description of the establishment and morphology of cell lines from rat mesotheliomata is described in this report.

The five tumours available for culture in the present study covered all the known histological varieties of these tumours in rats and ranged from a well dif-



Fig. 19. Cells from a cultured rat peritoneal mesothelioma (tumour 3) growing as an ascites tumour in an athymic mouse. Most of the cells remain separate and rounded but some have become involved in the formation of small islands of collagen production (C). These islands contain an occasional spindle shaped cell and are surfaced by a single layer of flattened cells. Magnification × 470. Bar denotes 100μ.



Fig. 20. An area from a tumour nodule resulting from the injection of cultured rat mesothelioma cells from tumour 4 into an athymic mouse. The histological pattern is that of a pleomorphic sarcoma with many small irregularly shaped cells intermingled with very large cells, some elongated and others rounded. Magnification × 400 Bar denotes 100μ.

ferentiated fibromatous form to a vesiculated pattern where the cytoplasm of some cells was greatly extended to form a lining for large tissue spaces. The cell lines that were established in culture, however, showed little relationship to the *in vivo* histology. Thus tumour 5, the most fibrosarcomatous neoplasm of all, initially produced cultures containing many flattened cells resembling mesothelial cells but tumour 3 which had contained flattened cells in its vesicular histology began to grow in culture as cells similar to fibroblasts. The cell lines from tumours 1 and 2 appeared to alternate between a random orientation of cells and the production of a flat uniform sheet. In many cases the randomly orientated cells piled up on one another to form quite large masses in which the cells became elongated and closely apposed to one another. It is possible that this process is similar to that in the original rats which was responsible for the production of large numbers of small nodules within the peritoneal cavity.

Many cells within the original tumour nodules resembled fibroblasts and all of the cell lines had a morphology *in vitro* resembling that of malignant fibroblasts after some time in culture. It is interesting to note that the piling up of cells to form masses (noted in cultures from 4 out of the 5 tumours), has previously been reported by Sethi *et al.* (19) to occur in approximately half of the cultures established from sarcomas. Furthermore, fibres similar to collagen or reticulin were seen between the cultured cells during electron microscope examination and large amounts of collagen were found in the rat tumour nodules. These observations provide support for the suggestion that mesotheliomas may be derived from connective tissue cells.

This variation and interchangeability of cell structure continued to occur when cell lines from all five tumours were injected into athymic mice. Originally all lines with the exception of some from tumour 3, which produced only ascites, began to grow as multiple small nodules scattered throughout the peritoneal cavity but nodules produced by tumour 1 never became attached to any of the peritoneal surfaces and their central regions quickly necrosed. With tumour 2 some nodules became attached and

vascularised and these remained cellular throughout. Unattached nodules from tumour 2 did show central necrosis but scattered among the necrotic material were spherical masses of whorled collagen indicating that some active fibroblasts had been present before general necrosis had occurred. Tumours 3, 4 and 5 produced nodules in athymic mice which were almost invariably attached and vascularised in the initial stages. They frequently produced central acellular areas of collagen after a time but were never seen with necrotic centres. Tumour 3 which originally had a vesiculated structure showed very little evidence of this on injection into athymic mice, but tumour 4 which originally had a uniform spindle cell pattern did produce some vesicular areas in athymic mice as well as a nodular fibromatous pattern. In general, where athymic mice survived for more than two months after injection, quite large tumour masses were present at autopsy which showed a constant fibrosarcomatous pattern and usually showed evidence of direct invasion of surrounding tissues. This finding fits in with the observations of Enticknap and Smither (11) who reported that human peritoneal mesotheliomas appeared more frequently fibrosarcomatous at autopsy than biopsy specimens taken some time previously.

From all these considerations it seems reasonable to suggest that mesothelioma cells originate from the transformation of multi-potential connective tissue stem cells that can differentiate into most mesenchymal tissue patterns. Most rats with peritoneal mesotheliomas develop blood stained ascites in the terminal stages and it had been assumed that this process was associated with advanced tumour development. However, the fact that some cell lines were associated with the development of ascites in nude mice within weeks of injection while most of the mesothelioma lines tested in nude mice never produce this condition in these animals, suggests that the process may be associated with a particular type of tumour cell differentiation. Greenbaum (20) reported that many inoculated or invading tumours produce leukokinins which greatly increase vascular permeability and so contribute to the development of peritoneal ascites. It may be that the mesothelioma cell lines that rapidly result in the development of blood stained ascitic fluid produce leukokinin-like factors while others do not.

Some attempts were made to passage mesothelioma cell lines through the strain of AF/HAN rats in which they originated, although with this random bred strain, problems of tissue compatibility would have been expected to prevent the growth of all but the most malignant tumours. One typical peritoneal mesothelioma did develop in a rat but this took five and a half months to become detectable. This is an unusually long period for tumour transplantation studies and compares with the nine months needed for tumour production following asbestos injection in the original rats. It may be that mesothelioma cell growth had been held in check for a long period by the host's immune response. If this occurs in man it might explain the extremely long period usually recorded for mesothelioma cases between first exposure to asbestos dust and tumour development. The findings of two sarcomas within the pleural and peritoneal cavities of rats as much as two years after the injection of mesothelioma cells into these same sites supports this suggestion, but since these tumours could not be classified with certainty as mesotheliomas this finding should at present be treated with caution. However, as reported in this paper and previous publications (9, 21), some rat mesotheliomas do exhibit a uniformly sarcomatous histological pattern and it may well be that the injected cells remained alive in the host animals for two years before once again producing a positive neoplasm. This matter will be studied further using larger numbers of animals and the results presented in a later publication.

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Fibrinolysis by rat mesothelial cells *in vitro*: the effect of mineral dusts at non-toxic doses

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Summary. Rat mesothelial cell cultures were shown to have considerable plasminogen dependent and independent fibrinolytic activity *in vitro* using an ^{125}I fibrin degradation assay. At non-toxic doses of the mineral dusts titanium dioxide, quartz and crocidolite asbestos, as assessed by ^{51}Cr release, the fibrinolytic activity of mesothelial cells was inhibited. Quartz had the greatest inhibitory effect and crocidolite asbestos had the least. These results suggests that inhibition of mesothelial cell fibrinolysis does not, on its own, explain pleural fibrosis due to toxic mineral dusts.

Keywords: fibrinolysis, mesothelial cells, plasminogen, titanium dioxide, quartz, crocidolite asbestos

Many agents are toxic to the pleura in humans, resulting in inflammation, adhesions and fibrosis of the pleural lining. These agents have been grouped into three types: (1) infectious agents; (2) systemic drugs; and (3) inhaled inorganic dusts (Bignon & Gee 1986). In addition many agents have been used as experimental models to produce pleural inflammation (Antony *et al.* 1986). Amongst these, asbestos has been the subject of considerable research interest in view of the risk to occupationally exposed individuals. The toxic effects on the pleura caused by asbestos include pleural plaques, pleural calcification, benign asbestos pleurisy and in addition, malignant mesothelioma is also found (Herbert 1986; Becklake 1976). Experimentally, pleural mesothelioma has been produced following deposition of asbestos in the pleural cavity of rats (Wagner *et al.* 1980). Prolonged inflammation in the pleural cavity also causes reactive mesothelial hyperplasia (Herbert 1986), a lesion identified in the pleura of rats exposed to asbestos by inhalation in the long term in our own studies (Davis *et al.* 1986). The mesothelium of the peritoneal cavity is also a target for asbestos toxicity in occupationally exposed humans where, presumably due to translocation of fibres from the thoracic cavity, peritoneal mesothelioma has been frequently described (Becklake 1976). Experimentally, intraperitoneal asbestos injection has also been found to produce mesothelioma in the peritoneal cavity of rats (Bolton *et al.* 1982). The pleura is frequently found to be fibrosed in advanced silicosis (Morgan & Seaton 1984) and we have observed granulomas in the pleura of rats exposed to coalmine dust containing quartz (Robertson *et al.* 1984). However, silica exposure is not associated with mesothelioma development.

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Mesothelial cells in culture have been reported to have potent fibrinolytic activity which is depressed on injury and decreased fibrinolytic activity could lead to increased fibrin accumulation, organisation of fibrin and colonisation by macrophages and fibroblasts, leading to the development of fibrous lesions (Myhre-Jensen *et al.* 1969; Raftery 1979; Whitaker *et al.* 1982*a*; Buckman *et al.* 1976). We therefore set out to determine whether exposure to low, non-toxic levels of mineral dusts, including asbestos and silica could cause inhibition of mesothelial cell fibrinolysis.

Materials and methods

Animals. Female syngeneic HAN/SPF rats, 12 weeks of age, were used.

Primary culture of rat mesothelial cells. Mesothelial cells were isolated from scrapes of the peritoneal cavity by the method of Jaurand *et al.* (1981) modified by use of F10 medium + 10% fetal calf serum (FCS; Gibco, Paisley). Early passage cells were frozen down in liquid nitrogen and used as the source of mesothelial cells for each series of experiments.

Assay of fibrinolytic activity. Fibrinogen (Sigma, Poole) was labelled with ^{125}iodine (Amersham International, Amersham) using the Chloramine T method of McConahay and Dickson (1966) modified by substituting L-cysteine-hydrochloride for sodium metabisulphite. The unbound ^{125}I was removed by chromatography on a Sephadex G25 column (PD10; Pharmacia, Milton Keynes) equilibrated with phosphate buffered saline (PBS) containing 0.01% unlabelled fibrinogen. The fractions containing the ^{125}I fibrinogen peaks were pooled and the counts were >90% trichloracetic acid precipitable. For preparation of ^{125}I fibrin plates the ^{125}I fibrinogen was diluted with 1 mg/ml unlabelled fibrinogen in PBS to yield 1×10^5 ct/min in 40 μl. Aliquots of 40 μl of this solution were then added to microtitre plate wells (Sterilin, Feltham) and dried at 45°C in a hot air oven for 3 days. The wells were incubated with 2.5% freshly thawed foetal calf serum in F10 medium to convert fibrinogen to fibrin, washed three times with 200 μl of PBS and stored at 4°C until use. To measure fibrinolysis, 5×10^4 mesothelial cells were added to wells in a volume of 200 μl of F12 medium + 10% FCS, allowed to adhere for 2 h, then washed with 2×300 μl of PBS. The cells were then incubated either with or without 1.2 μg/well of plasminogen (Kabi, Stockholm) in 200 μl of F12 + 2% bovine serum albumin (Sigma; Poole, Dorset). Plates were incubated for 24 h at 37°C and solubilised ^{125}I fibrin was assessed by counting 100 μl of supernatant in a Gamma Counter. Three replicate wells were set up for each condition. Time and dose responses of cell number were carried out. In experiments where modulation of fibrinolysis by mineral dusts was under investigation the dusts were added in F12 + 2% bovine serum albumin following adherence and washing of the cells.

Dusts. The following dusts were used: (1) Titanium dioxide, rutile form (Tioxide Limited, Stockton-on-Tees); (2) DQ_{12} standard quartz; (3) chrysotile asbestos (UICC standard sample 'A'.)

Cytotoxicity assay. To assess cytotoxicity by mineral dusts the cells were set up as for the fibrinolysis assay and labelled with 74MBq ^{51}Cr (Amersham International, Aylesbury) overnight. The cells were then washed once with sterile PBS and the dust added as above. Following incubation overnight, 100 μl of supernatant were collected and counted in a Gamma Counter as an index of cell lysis.

Statistical analysis. Repeat experiments were examined by analysis of variance and differences in the means of treatment groups were tested for significance using a *t*-test.

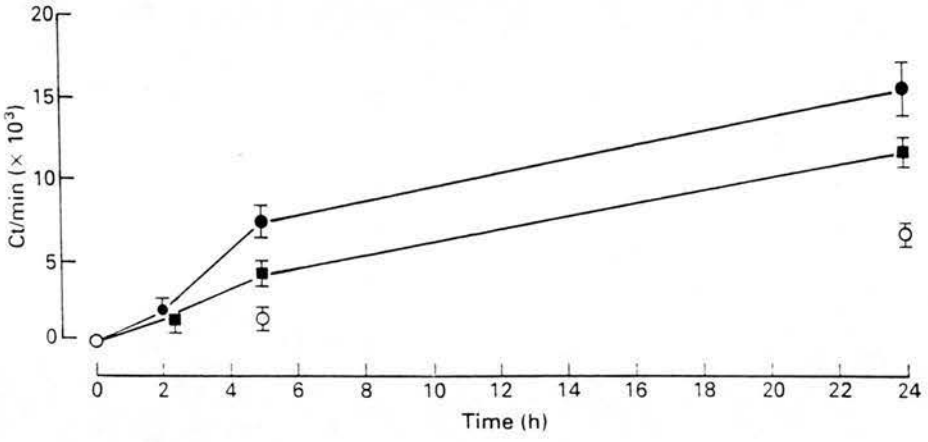


Fig. 1. Time course of fibrinolytic activity by rat mesothelial cells. Significantly more fibrinolysis by cells than spontaneous release ($P<0.001$) and significantly more fibrinolysis in the presence of plasminogen ($P<0.05$) at the 5 h and 24 h time points than in its absence. Each point represents mean ± s.e.m. of three separate wells in two separate experiments. ○, Spontaneous release of label. ●, Plasminogen dependent; ■, plasminogen independent.

Results

Plasminogen-dependent and independent fibrinolysis by rat mesothelial cells

Rat mesothelial cells cause substantial time-dependent fibrinolysis up to 24 h after plating onto ^{125}I fibrin mats (Fig. 1). The fibrinolysis in the presence of cells was significantly ($P<0.001$) more than the slow spontaneous release of ^{125}I shown in Fig. 1. The same level of spontaneous release was present in all experiments and is consequently omitted from subsequent figures. In addition, in the presence of plasminogen, there was significantly ($P<0.05$) more

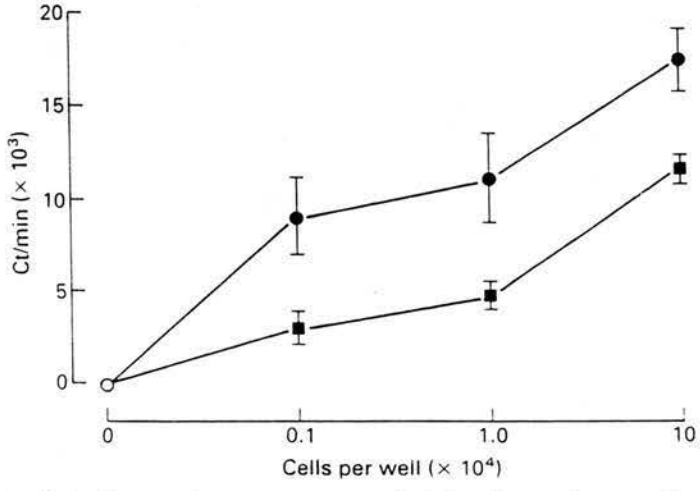


Fig. 2. Increase in fibrinolysis with increasing mesothelial cell number: significant dose response ($P<0.01$). Each point represents mean ± s.e.m. of triplicate wells in two separate experiments. ●, Plasminogen dependent: ■, plasminogen independent.

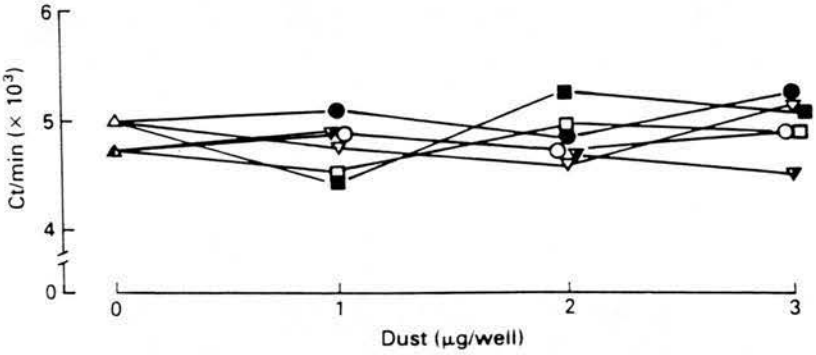


Fig. 3. Release of ^{51}Cr, an index of cytotoxicity, from pre-labelled rat mesothelial cells in the presence of mineral dust. No significant difference from untreated cells with mineral dust treatment. Control cells lysed with detergent released $> 15 \times 10^3$ ct/min. Each point represents the mean of triplicate wells in three separate experiments: s.e.m.'s which were $< 10\%$ of the mean are omitted for clarity. △, Control; ●, TiO_2; ▽, quartz; ■, crocidolite; △, control + p; ○, TiO_2 + p; ▽, quartz + p; □, crocidolite + p (p denotes plasminogen).

fibrinolysis than in its absence at both the 5 h and 24 h time points. With increasing numbers of cells plated onto the fibrin mats there were significantly increasing amounts ($P < 0.01$) of plasminogen-dependent and plasminogen-independent ($P < 0.01$) fibrinolysis (Fig. 2).

The effect of mineral dust on viability, plasminogen-dependent and plasminogen-independent fibrinolysis of rat mesothelial cells

Experiments to assess cytotoxicity and fibrinolysis were run in tandem at doses of dust which preliminary experiments revealed to

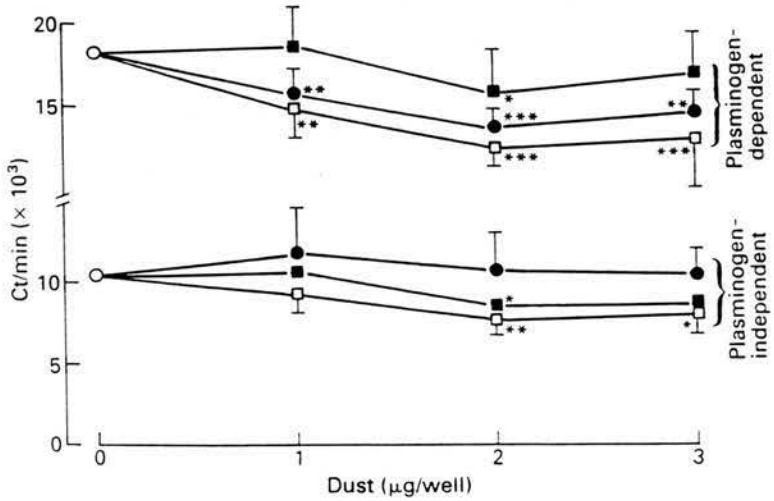


Fig. 4. Effect of mineral dust on fibrinolytic activity of rat mesothelial cells. Significant reduction in fibrinolytic activity compared to controls: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Each point represents mean ± s.e.m. of triplicate wells in three separate experiments. ●, TiO_2; □, quartz; ■, crocidolite.

Table 1. Plasminogen-independent fibrinolytic activity of rat mesothelial cells cultured with 1–3 μg of TiO_2, quartz or crocidolite asbestos. Data represents $\bar{x}$ (s.e.m.) ct/min of solubilized ^{125}I fibrin in three separate experiments

	Dose (μg/well)		
Dust	1	2	3
TiO_2	11 710 (1367)	10 692 (1178)	10 361 (1083)
Quartz	9252 (1162)	7727 (885)**	8035 (1131)*
Crocidolite	9602 (1347)	8360 (938)*	8702 (1026)
		Control 10 283 (1385)	

Significant difference from control: *$P<0.05$; **$P<0.01$.

Table 2. Plasminogen dependent fibrinolytic activity of rat mesothelial cells cultured with 1–3 μg of TiO_2, quartz or crocidolite asbestos. Data represents $\bar{x}$ (s.e.m.) ct/min of solubilized ^{125}I fibrin in three separate experiments

	Dose (μg/well)		
Dust	1	2	3
TiO_2	15 456 (1578)**	13 514 (1148)***	14 602 (1212)**
Quartz	14 716 (1318)**	12 161 (1103)***	12 836 (1756)***
Crocidolite	18 463 (1635)	15 711 (1716)*	16 810 (1593)
		Control 17 916 (1707)	

Significant difference from control: *$P<0.05$; **$P<0.01$; ***$P<0.001$.

be non-toxic—1 to 3 μg/well: in preliminary experiments doses of 10 μg/well and above caused toxicity. As shown in Fig. 3, at doses of 1–3 μg/well there were no significant losses of ^{51}Cr into the medium over the 24 h of the incubation. Over the same doses, however, significant ($P<0.05$–$P<0.001$: see Fig. 4 and Tables 1 and 2 for details) reductions in plasminogen dependent fibrinolysis occurred at 1 μg/well with quartz, 2 μg/well with all three dusts and 3 μg/well with quartz and titanium dioxide: crocidolite at 3 μg/well caused reduced fibrinolysis but this failed to attain statistical significance. The only inhibition of plasminogen independent fibrinolysis occurred with quartz at 2 μg/well and 3 μg/well ($P<0.01$ and $P<0.05$, respectively) and crocidolite asbestos at 2 μg/well ($P<0.05$).

Discussion

The experiments described here confirm the fibrinolytic activity of mesothelial cells reported previously (Myhre-Jensen *et al.* 1969; Raftery 1979; Whitaker *et al* 1982*a*; Buckman *et al.* 1976). In previous studies, however, the fibrinolysis detecting system consisted of squashed tissue preparations in contact with fibrin plates with a zone of lysis around the mesothelium yielding only a semi-quantitative index of fibrinolytic acti-

vity. In the present study isolated rat mesothelial cells were cultured on ^{125}I fibrin mats and a sensitive radioactive fibrin degradation assay was used; this confirmed that both plasminogen dependent and independent fibrinolysis are produced spontaneously by rat mesothelial cells in culture.

Considerable research has been carried out into the likely role of mesothelial fibrinolytic activity in the fibrogenesis associated with adhesion formation following surgery (reviewed in Whitaker *et al.* 1982*b*). Various types of injury to the mesothelium result in loss of fibrinolytic activity which may lead to fibrous adhesions. This hypothesis is supported by the fact that intrapleural inoculations with the anti-coagulant heparin inhibits tetracycline-mediated pleural fibrogenesis (Good *et al.* 1980).

It is also possible that inhibition of mesothelial fibrinolysis has a role in the pleural fibrosis, and possibly other pathology, associated with asbestos exposure and in fibrosis associated with advanced silicosis. Asbestos is known to be cytotoxic to a range of cells including phagocytes (Harington *et al.* 1975) and non-phagocytic cells (Brown *et al.* 1986) including mesothelial cells (Jaurand *et al.* 1983); silica is also markedly cytotoxic (Harington *et al.* 1975). Whilst injured or dead cells would be expected to produce decreased levels of fibrinolytic activity we sought to determine whether low levels of dust, in the non-toxic range, would be inhibitory to fibrinolysis by mesothelial cells.

The experiments using non-lethal doses of mineral dust clearly showed that inhibition of plasminogen dependent fibrinolysis occurred with all three dust types; both quartz and crocidolite asbestos also produced inhibition of plasminogen independent fibrinolysis further confirming the two distinct proteolytic entities as reported in previous studies (Myhre-Jensen *et al.* 1969; Raftery 1979; Whitaker *et al.* 1982*a*; Buckman *et al.* 1976).

The inhibition produced by the mineral dusts may be the expression of generalised, non-lethal toxicity involving reduction in protein synthesis, or specific inhibition of fibrinolytic activity alone. The fact that TiO_2 caused inhibition of plasminogen-dependent fibrinolysis, in the absence of inhibition of plasminogen-independent fibrinolysis, suggests that these two enzyme pathways, at least, are being modulated separately. However, whether there is a generalized inhibition of mesothelial cell function or a specific reduction in fibrinolysis pathways does not alter the conclusions described below.

These findings are of interest because of current views on the mechanism of mesothelial fibrinolysis during regeneration following injury. These suggest that, following mesothelial injury, there is detachment of dead mesothelial cells and localized fibrinogenesis occurs due to the fibrinolytic deficit. However the newly divided replacement mesothelial cells are rich in fibrinolysis activity and the fibrin is dissolved before it can be colonized by fibroblasts and rendered permanent as an adhesion (Whitaker *et al.* 1982*b*). Clearly, as shown here in the case of mineral dusts, if the fibrinolytic function is inhibited but the pleural mesothelial cells are not killed, then there would be no replacement by recently divided cells with enriched fibrinolytic activity; subsequently no dissolution of fibrin will occur and fibrinogenesis will be favoured. Whilst fibrinous adhesions are not found typically in the pleura of asbestos-exposed individuals the commonly found pleural fibrosis could arise through colonisation of fibrin by macrophages and fibroblasts under the particular conditions pertaining in the pleural space with dust exposure and a similar situation could pertain with quartz. Fibrinogenesis could also be directly stimulated through the action of dust on elements of the clotting cascade (Hamilton 1983).

However, in the experiments described above, using non-lethal doses of mineral dust, inhibition of fibrinolysis occurred with all three dusts including TiO_2 which does not cause fibrosis; it was also notable that crocidolite asbestos had least activity.

Following exposure to asbestos, fibres have been detected in the periphery of the

lung and in the pleural tissues (Kannerstein *et al.* 1978; Bignon *et al.* 1979). This property is not confined to fibrous dusts however, and isometric dusts such as quartz, hematite (Heppleston 1963), coalmine dust and titanium dioxide (personal observations) are also found adjacent to the mesothelial layer following inhalation exposure: all three dusts used in the present study could therefore make contact with mesothelial cells. Since inhibitory effects were produced by all three dusts in the present study, then inhibition of fibrinolysis at sub-lethal doses of dust does not explain the development of fibrous pleural lesions in asbestosis and silicosis and not in workers or experimental animals exposed to titanium dioxide (Richards *et al.* 1985). It is not possible, however, to rule out an increase in the effective dose of toxic dust in the pleura compared to inert dust, due to the difficulty of clearing a toxic dust from a region of the pleura where there has been localized fibrinogenesis.

The study suggests that while inhibition of fibrinolysis may contribute to fibrosis in the pleura some other aspects of the biological activity of dusts are almost certainly of importance. The list of products produced by leucocytes in response to mineral dusts and which could be important in this respect is now extensive and includes proteases (Donaldson *et al.* 1988), chemotaxins (Kagan *et al.* 1983), anti-mitotics (Donaldson *et al.* 1984) and interleucin 1 (Schmidt *et al.* 1984).

Stimulated production of cytokines by the leucocytes of the pleural space could therefore have a profound effect in altering the activity of the mesothelial cells and could also affect the pleural extracellular matrix. The general level of pleural inflammation could be enhanced by the actions of these cytokines and could be exacerbated by interaction between the leucocytes and fibrin formed by the mechanism mentioned above and thereby the activation and interactions of the complement, clotting, kinin-generating and fibrinolytic systems (Ryan & Majno 1977). Further work is therefore required to characterize the dust burden, leucocyte populations and the inflammatory milieu of the pleural space generally, following deposition of toxic dusts in the lung. Such studies should help to explain the spectrum of pleural responses found with asbestos, and the pleural fibrosis found in silicosis in contrast to the lack of pleural pathology associated with exposure to inert dust such as TiO_2.

Acknowledgements

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Secretion of Plasminogen Activator Inhibitor by Normal Rat Pleural Leukocytes in Culture

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Abstract. The normal balance between coagulation and fibrinolysis in the pleural cavity is poorly understood despite the critical role of the pleura in the movement of the lungs. To determine the fibrinolytic activity and the interaction between plasminogen activators and their inhibitors in the normal pleural space, we tested normal rat pleural leukocytes, principally macrophages and mast cells, and their supernatants, for activity in an [^{125}I]fibrin degradation assay. It was found that pleural leukocytes did not release plasminogen activator, but the leukocytes and their supernatants inhibited the plasminogen-dependent fibrinolysis caused by both alveolar leukocytes and mesothelial cells. Further experiments demonstrated that pleural leukocytes produce a protein inhibitor primarily against urokinase-induced fibrinolysis in culture and that macrophages are the main source of the inhibitor. The lysate of mast cell-enriched population exhibited high plasminogen activator activity while no such activity could be determined in macrophage-enriched lysate. These data show that normal rat pleural leukocytes contain plasminogen activator inside the cells and synthesize a urokinase-type plasminogen activator inhibitor in culture that may be important in the fibrinolysis/coagulation balance in the pleural space.

Key words: Plasminogen activator inhibitor—Pleural leukocyte—Fibrinolysis.

Introduction

Plasminogen is a proenzyme present in large quantities in the extracellular space, which can be converted into plasmin by plasminogen activators. Plasmin

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is primarily involved in the breakdown of fibrin (fibrinolysis) and directly or indirectly promotes degradation of all components of the extracellular matrix [1]. There are 2 major plasminogen activators (PA): the tissue-type plasminogen activator and the urokinase-type plasminogen activator [23]. Tissue-type PA is believed to be the mediator of vascular fibrinolysis, while the functions of urokinase-type PA appear to be more related to cellular functions and tissue remodeling [10]. Previous studies have shown that many cell lines can synthesize PAs [5–9, 12, 15, 21, 24]. PAs play an important role in the proteolytic events associated with cell migration, tissue destruction, and remodeling, particularly in the context of inflammation [25], including degradation of fibrin, elastin, laminin, and glycoprotein in the extracellular matrix [3, 9]. Moreover, it has been shown that fibrin degradation products may be toxic to the lungs and that peptides derived from fibrin or fibrinogen may potentiate the inflammatory response [12] and the traffic of inflammatory cells.

There are specific inhibitors for plasminogen activators and the activities of PAs are regulated by these inhibitors. PA inhibitors can be classified into at least 3 immunologically different groups: the endothelial cell type PA inhibitor, the placental-type PA inhibitor, and protease nexin-I [23]. Some cell lines secrete PA inhibitors after being stimulated or induced [4, 13, 22].

The connective tissue of the pleura plays an important role in pleural function in health and disease. Under normal circumstances, it helps to distribute mechanical forces evenly over the entire surface of the lung and contributes to the overall elastic properties of the parenchyma. It also provides a secure anchorage for pleural mesothelial cells and modulates the permeability characteristics of the pleura. In the light of these contributions to lung function, it is not surprising that abnormalities in the pleural connective tissue matrix, such as inflammation, pleural fibrosis, adhesions, and tumor, lead to derangements of overall lung function [19]. However, the kinetics of the interaction of PA with PA inhibitor in normal and abnormal situations in the pleural cavity has not been studied.

Exposure to mineral dusts lead to pleural changes. In particular, exposure to fibrous mineral dusts such as asbestos is associated with a range of pleural abnormalities varying from fibrosis to mesothelioma [11]. We have been interested in the ability of fibrous mineral dusts to cause pleural pathological changes via effects on the fibrinolytic activity of the pleural surface [6]. Based on our finding that mesothelial cells lining the pleural cavity can produce PA in culture, we questioned whether pleural leukocytes from normal rats secreted a PA inhibitor.

Materials and Methods

Animals

Male PVG rats, inbred at the Institute of Occupational Medicine Laboratory Animals Unit, were used throughout.

Bronchoalveolar Lavage

The lungs were removed from the thoracic cavity of Nembutal- (Ceva Ltd. Watford) killed rats and were lavaged 4 times with 8 ml aliquots of sterile phosphate-buffered saline (PBS) at 37°C. The pooled cells were spun out at 1000 rpm for 5 min. After that the supernatant was decanted and cells were resuspended in F10 medium (Gibco. Paisley) with 2% bovine serum albumin (BSA. Sigma. Poole).

Pleural Lavage

Five ml aliquots of PBS at 37°C containing 10 u/ml of Heparin (Leo Labs) were injected into the pleural space of Nembutal-killed rats, between the lower 2 ribs, using a round-ended needle. After the rib cage was massaged, the suspended cells were withdrawn. This was repeted 3 times and cells were concentrated by centrifugation and resuspension in F10 + 2% BSA. When cell-free pleural lavage fluid was required, at the very beginning of the above procedure, 2 ml of F10 medium with 2% BSA was injected in the pleural space and withdrawn after massaging, then kept on ice; cells were removed by centrifugation.

Primary Culture of Rat Mesothelial Cells

Mesothelial cells frozen down at early passage were recovered from liquid nitrogen as described previously [6]. The cells were then cultured in F12 medium (Flow Laboratories) containing 10% fetal calf serum (FCS. GIBCO. Paisley) until required when cells were spun down and resuspended in F12 medium plus 2% BSA.

Fibrinolysis Assay

The plasminogen-dependent and -independent fibrinolytic activity of bronchoalveolar leukocytes and mesothelial cells were measured using the assay of Unkeless et al. [24]. Dissolved fibrinogen (Sigma. Poole) was labeled with [^{125}I]Na (Amersham International. Amersham). Free ^{125}I was removed by chromatography on a Sephadex G25 column (PD-10 column. Pharmacia. Milton Keynes) equilibrated with PBS containing 0.01% fibrinogen. ^{125}I-labeled fibrinogen was diluted in PBS with 1 mg/ml unlabeled fibrinogen so that an 80 μl aliquot contained 1×10^5 cpm. Aliquots of 80 μl of this solution were added to wells of Falcon microtiter plates and the plates were dried at 45°C for 3 days. The wells were then incubated at 37°C for 4 hr with 100 μl F10 containing 2.5% freshly thawed FCS to convert fibrinogen to fibrin. The plates were washed twice with 200 μl PBS prior to use.

The cells were incubated either with or without 1.2 μg/well plasminogen (PLG. Kabi. Stockholm and a gift from Dr. I. McGregor. Blood Transfusion Centre. Edinburgh) in 200 μl F10 plus 2% BSA for bronchoalveolar cells and in 200 μl F12 plus 2% BSA for mesothelial cells to measure PA activity. The plates were incubated at 37°C in a 5% CO_2/95% air mixture for 24 hr and 150 μl were sampled from each well to assess the solubilized [^{125}I]fibrin. The assay was carried out in triplicate.

Measurement of Fibrinolysis Inhibitor

To determine whether pleural leukocytes secrete PA inhibitor, supernatant of pleural leukocytes was obtained by culturing cells in F10 medium plus 2% BSA at a concentration of 1×10^6 cells/ml for 24 hr at 37°C and then spinning the cell suspention at 3000 rpm for 10 min. Pleural leukocytes or their supernatant was then added to the culture of bronchoalveolar leukocytes and mesothelial

cells (both at 0.25×10^6/ml) for 24 hr to assess their effects on fibrinolysis. The dose–responses of pleural leukocytes and their supernatant were determined. The background counts were normally about 400 cpm both with and without PLG in the assay.

Separation of Pleural Leukocytes and Preparation of Cell Lysate

Pleural leukocyte suspension at about 6×10^6/ml was mixed thoroughly with Sepracell (Sepracell-MN, Sepratech Corporation, Oklahoma) at a ratio of 1 : 3. The mixture was then spun at 3,000 rpm for 15 min. The fraction at the top of Sepracell was macrophage enriched and the fraction on the bottom was the mast-cell-enriched population. The fractions were transferred into universals and washed 3 times with F10 medium, and resuspended in F10 medium plus 2% BSA.

Whole pleural leukocytes or separated subpopulations, at 1×10^6/ml, were frozen in liquid nitrogen and thawed in 37°C water bath immediately. This procedure was repeated three times and the suspensions were then spun at 3,000 rpm for 10 min to get rid of cell debris. The soluble fraction from the lysed cells will be henceforth referred to as the lysate.

The fibrinolysis assay was used for simultaneously measuring both PA and PA inhibitor in supernatants or lysates. This was achieved by adding urokinase and plasminogen to $[^{125}I]$fibrin plates to a concentration that causes a modest degree of fibrinolysis and then observing if the addition of supernatant or lysate enhanced or decreased fibrinosysis.

Characterization of PA Inhibitor

The concentration of urokinase (UK, kindly provided by Dr. McGregor L., Blood Transfusion Centre, Edinburgh), plasmin, and streptokinase (Sigma, Poole) were used at a concentration causing 50% of total $[^{125}I]$fibrin lysis. The activity of plasminogen activator inhibitor (PAI) in the pleural leukocyte cells and supernatants was determined by titration against UK and PLG. In our assay, the concentrations of PLG and UK were chosen at 1 μg/ml and 0.05 U/ml, respectively. Solubilized $[^{125}I]$fibrin was assessed as described above. For typical fibrinolysis induced by UK and PLG, the counts of spontaneous release were about 4,000 cpm. The counts of trypsin-treated wells gave approximately 25,000 cpm.

The PAI activity was expressed as percentage inhibition of PLG-dependent fibrinolysis caused by urokinase in the presence of diluted supernatants compared with the fibrinolysis in the absence of supernatants. PA inhibitor activity was determined according to the following equation:

$$\text{Percentage inhibition} = \frac{(\text{Fib. without Sup.-blank}) - (\text{Fib. with Sup.-blank}) \times 100}{(\text{Fib. without Sup.-blank})}$$

where units of fibrinolysis = cpm, Fib. = fibrinolysis, and Sup. = supernatant.

To characterize the PA inhibitor partially, the pleural leukocyte culture supernatant was incubated at 50°C or 70°C for 1 hr. The activity of the inhibitor was then compared with unheated supernatant. To assess the molecular weight of the inhibitor, the supernatant was centrifuged using a centricon-10 microconcentrator (Amicon Division, W. R. Grace & Co., Danvers), which has a 10,000 D cut off.

Results

Table 1 shows the populations of the different leukocyte types present in lavage of the bronchoalveolar and pleural spaces. The alveolar leukocytes were mostly macrophages, whereas the pleural leukocytes contained substantial proportions of mast cells and eosinophils.

Table 1. Different cell types present in rat bronchoalveolar and pleural leukocyte populations

	Macrophages	Lymphocytes	Mast Cells	Eosinophils
Bronchoalveolar leukocytes	97 (3.2)[a]	3 (3.2)	0	0
Pleural leukocytes	83 (5.3)	1 (1.0)	11 (5.8)	5 (2.7)

[a] n = 9 rats. $\bar{X}$ (SD)%

No neutrophils were seen in either normal cell population

Studies on the Fibrinolysis Inhibitor Found in Supernatants of Pleural Leukocytes

Our previous experiments [6] have shown that mesothelial cells cause plasminogen-dependent fibrinolysis. To assay for the presence of the inhibitor from pleural leukocytes, both alveolar leukocyte and mesothelial cell fibrinolysis were tested after the addition of pleural leukocytes or their supernatants. When pleural leukocytes or their supernatants were present, the fibrinolytic activity of both cells was inhibited (Figs. 1,2). This reduction in fibrinolytic activity was dose-dependent with both pleural leukocyte number and supernatant dilution. In contrast, plasminogen-independent fibrinolysis by both populations remained at a very low level that was near the background and not inhibited by pleural leukocytes or their superantants.

Interaction of Pleural Leukocytes and Their Supernatants with Different Fibrinolysis Systems

For characterization of the specific point of action of the fibrinolysis inhibitor, i.e., activity on plasmin or plasminogen activator, 3 different fibrinolytic systems were used: urokinase plus plasminogen, streptokinase plus plasminogen, and plasmin alone. The concentrations of urokinase, plasmin, and streptokinase were chosen to degrade about 50% of the [^{125}I]fibrin. Figure 3 illustrates the effect of pleural leukocytes and their supernatants on fibrinolysis induced by PLG and UK. The fibrinolytic activity of urokinase was dramatically inhibited by either pleural leukocytes or their supernatants. Even at high dilution, fibrinolysis was inhibited up to nearly 90%. The fibrinolytic activity of plasmin was less inhibitable than that of urokinase, with less than 40% of total fibrinolysis being blocked (Fig. 4). No inhibition of fibrinolysis by streptokinase was seen after the addition of pleural leukocytes and their supernatants for up to 24 hr (results not shown).

From these preliminary results, we believe that there existed a PA inhibitor(s) in the supernatant of pleural leukocytes. Because of the ability of the inhibitor to act mainly on urokinase and plasminogen, it was presumed that the pleural leukocytes released a urokinase-type PA inhibitor. To substantiate this

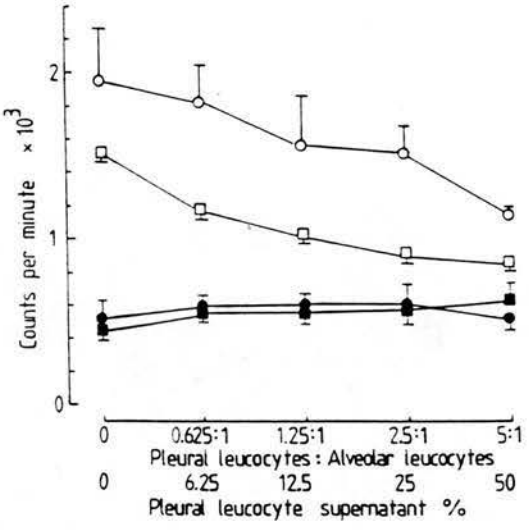


Fig. 1. Effect of increasing concentrations of pleural leukocytes and their supernatants on the fibrinolysis caused by alveolar leukocytes (□ Pleural leukocytes with PLG. ○ Pleural leukocyte supernatant with PLG. ■ Pleural leukocytes without PLG. ● Pleural leukocyte supernatant without PLG). Each point is mean ± SD of triplicate wells in three separate experiments.

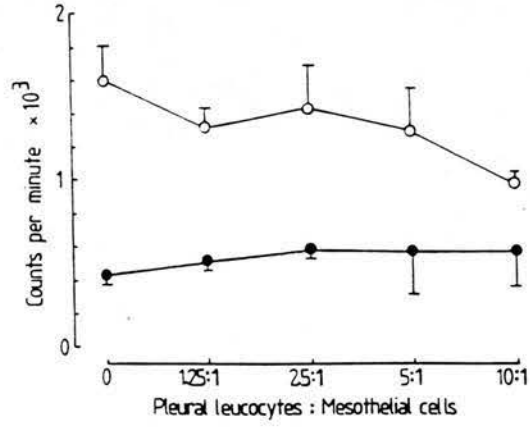


Fig. 2. Effect of increasing concentrations of pleural leukocytes on the fibrinolysis caused by mesothelial cells (○ Pleural Leukocytes with PLG. ● Pleural leukocytes without PLG). Each point represents mean ± SD of triplicate wells in two separate experiments.

hypothesis further, some work to identify the cell source of PA inhibitor and to characterize the inhibitor was carried out using the urokinase/plasminogen fibrinolysis system.

Cell Source of Plasminogen Activator Inhibitor

By using separation techniques, 2 subpopulations were obtained: a macrophage-enriched population (94% macrophages) and a mast-cell-enriched population

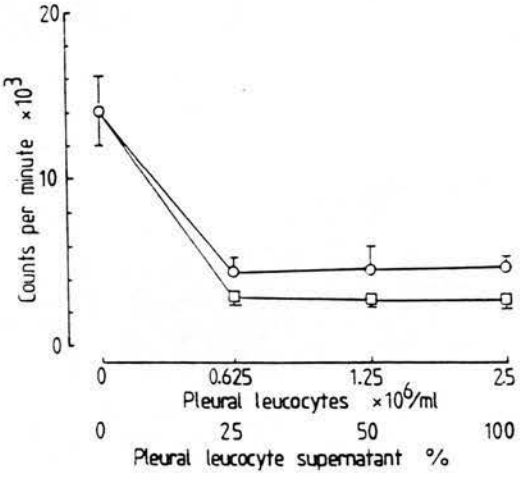


Fig. 3. Effect of increasing concentrations of pleural leukocytes and their supernatants on fibrinolysis induced by UK and PLG (○ Pleural leukocytes. □ Pleural leukocyte supernatants). Each point is mean ± SD of triplicate wells in four separate experiments.

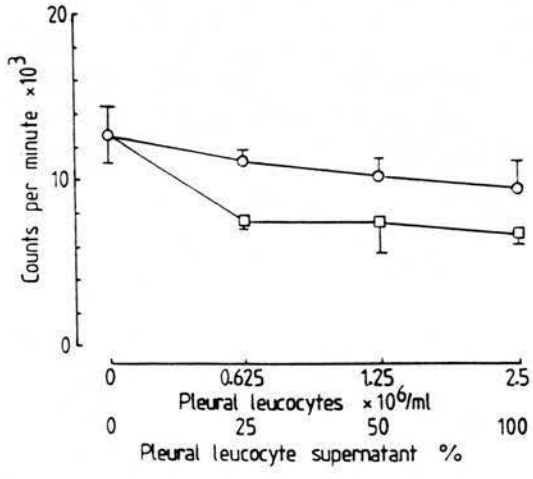


Fig. 4. Effect of increasing concentrations of pleural leukocytes and their supernatants on plasmin-mediated fibrinolysis (□ Pleural leukocytes. ○ Pleural leukocyte supernatants). Each point is mean ± SD of triplicate wells in four separate experiments.

(76% mast cells and 20% macrophages) (Table 2). The inhibition of fibrinolysis caused by supernatants from these subpopulations indicated that macrophages are by far the major source of PA inhibitor release in culture (Fig. 5). There was no significant difference between inhibition caused by whole pleural leukocytes and inhibition caused by the macrophage-enriched population. The fact that there was no increase in PA inhibitor activity corresponding to the increased proportion of macrophages in the macrophage-enriched subpopulation (from 82 to 94%) may be explained by slight damage caused to macrophages during the separation process.

Table 2. Different cell types present in rat pleural leukocyte subpopulations after separation

	Macrophages	Lymphocytes	Mast Cells	Eosinophils
Macrophage-enriched population	94 (2.9)[a]	4 (1.8)	1 (1.4)	1 (0.8)
Mast cell-enriched population	20 (12.5)[b]	0	76 (12.7)	4 (1.4)

[a] n = 6, $\bar{X}$ (SD)%
[b] n = 4, $\bar{X}$ (SD)%

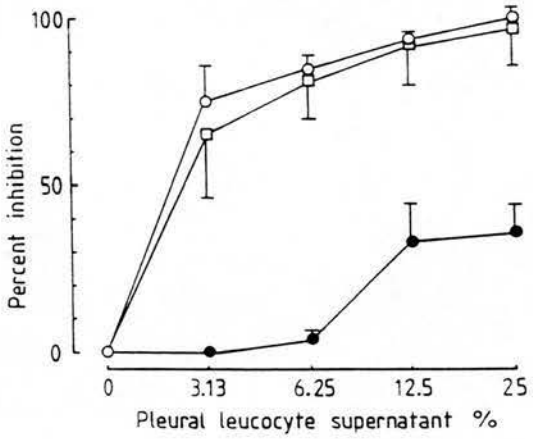


Fig. 5. Inhibition of UK and PLG-induced fibrinolysis by supernatants of separated subpopulations of pleural leukocytes (○ Whole pleural leukocyte supernatant. □ Macrophage-enriched supernatant. ● Mast cell-enriched supernatant). Each point represents mean ± SD of triplicate well in two separate experiments.

Fibrinolytic Activity of Lysates and Pleural Lavage Fluid

In these experiments, a certain level of fibrinolysis was obtained with UK and PLG such that PA could be detected by enhanced fibrinolysis and PA inhibitor could also be detected as reduced fibrinolysis. Studies with lysates illustrated that pleural leukocytes contain high PA activity intracellularly (Fig. 6). This activity seemed to be contained mainly inside mast cells, with very limited intracellular PA activity in macrophages.

Pleural lavage fluid had neither PA inhibitor activity nor PA activity. Even at the highest concentration (50%, which was high enough to demonstrate PA or PA inhibitor activity in pleural leukocyte lysates and supernatants), pleural lavage fluid had no effect on fibrinolysis induced by UK and PLG. There was no significant difference among different dilution points (Fig. 6).

Stability and Specificity of the Inhibitor Secreted by Pleural Leukocytes

The inhibitory activity of supernatants from pleural leukocytes remained constant after incubation at 56°C for 1 hr but was virtually abolished after incubation at 70°C for 1 hr (Fig. 7).

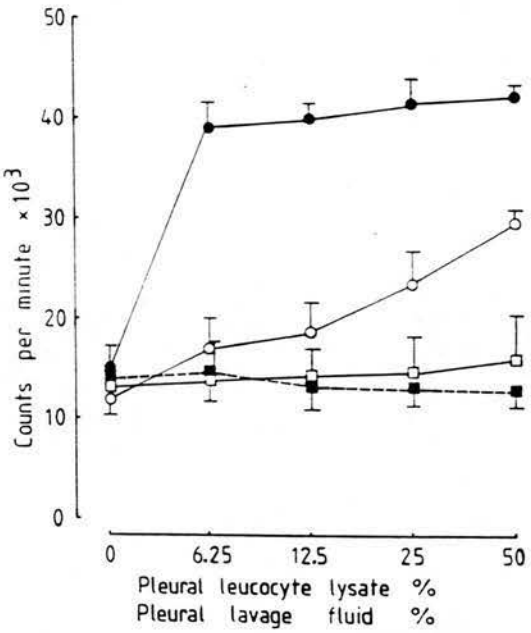


Fig. 6. Effects of lysates of separated subpopulations of pleural leukocytes and pleural lavage fluid on fibrinolysis induced by UK and PLG (○ Whole pleural leukocyte lysate. □ Macrophage-enriched lysate. ● Mast cell-enriched lysate. ■ Pleural lavage fluid). Each point represents mean ± SD of triplicate well in three separate experiments.

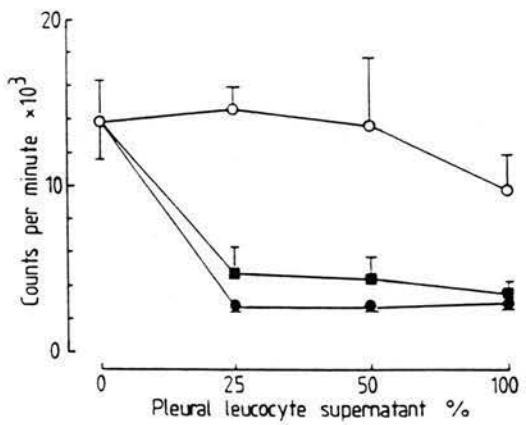


Fig. 7. Inhibitory effects on fibrinolysis induced by UK and PLG of heat-treated pleural leukocyte supernatants (○ Supernatants were incubated at 70°C for 1 hr. ■ Supernatants were incubated at 56°C for 1 hr. ● Unheated supernatants). Each point represents mean ± SD of triplicate wells in three separate experiments.

The supernatant of pleural leukocytes was assayed for fibrinolytic activity after being fractionated into 2 parts: molecular weight greater and less than 10,000 D. The fibrinolytic activity of urokinase/plasminogen was inhibited by the high-molecular-weight (MW) fraction, while no inhibitory effect could be observed in the low-MW fraction (Fig. 8). These results provide evidence that the inhibitor of urokinase in supernatants of pleural leukocytes is a high-MW, heat-labile substance, probably a protein.

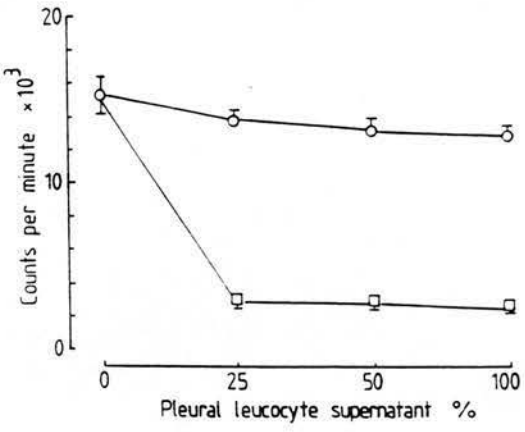


Fig. 8. Inhibition effects of different fractions of pleural leukocyte supernatant on fibrinolysis induced by urokinase and PLG (○ MW less than 10,000 D, □ MW greater than 10,000 D). Each point represents mean ± SD of triplicate wells in two separate experiments.

Discussion

Due to their important role in the pathogenesis of many lung diseases, alveolar macrophages have attracted a great deal of interest and the fibrinolytic activity of these cells has been examined [3, 5, 9, 21]. Little attention, however, has been paid to the functions and characteristics of pleural leukocytes, despite the presence of pleural diseases, particularly among workers in industries in which they are exposed to fibrous dusts [11]. No studies known to us have sought to measure the fibrinolytic activity of pleural leukocytes. Pleural leukocytes may play an important role in pleural responses and subsequent pathologic responses such as inflammation, fibrous adhesions, fibrosis, and neoplasia, which lead to derangements of overall lung function. One factor that could lead to these problems is altered fibrinolytic activity, since disorder of the fibrinolytic enzyme system could be important in leukocyte activation and altered metabolism of extracellular matrix in the pleural wall. Moreover, matrix abnormalities could be central to the pathologic process. Because extracellular matrix degradation is mediated, in part, by PA, the production of PA/PA inhibitor by pleural leukocytes could be an important factor in maintenance of the normal integrity and architecture of the pleura.

The present work arose from the finding that pleural leukocytes, cultured in vitro, do not activate plasminogen via synthesis of plasminogen activators, while alveolar leucocytes and mesothelial cells do [5, 6, 9, 21, 25, 27]. When either alveolar leukocytes or mesothelial cells were cultured together with pleural leukocytes or their supernatants, fibrinolytic activity was inhibited and the effect was dose-dependent. There was very limited plasminogen-independent fibrinolysis by alveolar leukocytes and mesothelial cells, and this was not inhibited by the addition of pleural leukocytes. We hypothesized that the inhibitory activity was caused by a substance(s) secreted by pleural leukocytes. This was confirmed by demonstration of a factor, probably a protein, in supernatants of pleural leukocytes that mediated the inhibition.

The point of action of the inhibitor could be at the level of the plasminogen activator or plasmin, and so, using different model fibrinolysis systems, we attempted to identify the specific effect of the inhibitory substance secreted by pleural leukocytes. Although the pleural leukocyte-derived inhibitor inhibited fibrinolysis by plasmin, this was much less than the inhibitory activity shown against fibrinolysis mediated by urokinase and plasminogen, which could be up to 100% of the total. From the data we conclude that pleural leukocytes produce a plasminogen activator inhibitor(s) that is active mainly against urokinase-type PAs but has some activity against plasmin. By separating the pleural leucocytes into subpopulations enriched for macrophages and mast cells, we found the PA inhibitor to be a product of the macrophage population. The small amount of inhibitory activity in the mast cell population is consistent with the contaminating macrophages in the population.

Many workers have reported that cell lines produce PA inhibitors. However, sometimes stimuli were needed for the production or release of PA inhibitors from these cells. The macrophages lavaged from normal human lungs, for example, do not contain the PA inhibitor and only express and secrete the inhibitor after stimulation with endotoxin in vitro [4]; PA inhibitor can also be secreted by casein-elicited macrophages [22]. It has been suggested that the macrophage PA inhibitors are not a constitutive protein, but instead are expressed in response to activating stimuli. The work of Levin [14] demonstrated that the inhibitor secreted by human endothelial cells is primarily in the inactive form in conditioned medium, whereas, intracellularly, the protein is fully active. The extracellular and intracellular inhibitors have immunochemical similarity that suggests that the inhibitor is synthesized and stored in the cell in its active form and then converted to its latent counterpart during or after release from the cells. It is clear from our studies that nonstimulated rat pleural leukocytes, or cells stimulated in response to the conditions of culture, produce a fully active fibrinolysis inhibitor. However, no intracellular inhibitor can be measured.

Many studies have shown that cell lines release PA and PA inhibitor concomitantly and, after secretion, they tend to form complexes [2, 18, 26]. Due to the formation of enzyme–inhibitor complexes, active enzyme would be rapidly inhibited by the cosecreted ligand: inhibitor had a high affinity for both tissue-type PA and urokinase-type PA. After studying cellular and extracellular PA and PA inhibitor in an experimental tumor system, 1 study reported that, inside tumor cells, PA was the predominant activity that overshadowed PA inhibitor. In the extracellular fluid, however, PA activity was masked by powerful PA inhibitors [7]. The same theory was put forward by other workers who suggested that for some people the levels of fast-acting inhibitor were higher than normal [20]. This was not due to a primary failure of tissue-PA release by the endothelium but to a "masking" of the released tissue-PA by high levels of inhibitor.

In this study we have demonstrated that PA inhibitor released by pleural macrophages was powerful in culture and that intracellular PA activity, which is located mainly in the mast cells, is predominant as confirmed by results of fibrinolytic activity in cell lysates. In view of our failure to detect PA activity in normal pleural fluid, it is possible that, in the pleural cavity, PA inhibitor is

complexed with PA so that no PA inhibitor or PA activity can be measured in pleural lavage fluid. When pleural leukocytes are cultured in vitro, however, macrophages are stimulated by their new environment to synthesize large quantities of PA inhibitor, which leads to the overshadowing of PA activity by the powerful PA inhibitor. In contrast, mast cells do not respond to culture in vitro. However, it is also possible that pleural macrophages are capable of producing both PA and PA inhibitor activity, with the balance of the activities in the conditions used in the present study favoring PA inhibitor activity. The fibrinolysis we measured could therefore depend on macrophage intracellular regulation rather than intercellular regulation between macrophages and mast cells.

The inhibitor was found to block the proteolytic activity of urokinase substantially more than plasmin and this result is consistent with what other workers have found. The PA inhibitor secreted by human macrophages readily inhibited urokinase but had no activity on tissue-type PA [22]. A similar result was found for the inhibitor in ascitic fluid in an experimental tumor [7]. [^{125}I]Urokinase-binding data indicated that the endotoxin-inducible PA inhibitor from mouse macrophages binds to the heavy chain of urokinase, which contains the active site [4]. We therefore propose that the PA inhibitor produced by normal pleural leukocytes is primarily a urokinase or urokinase-type PA inhibitor.

Without doubt PAs play an important role in fibrinolysis. They also participate in other important physiological reactions such as inflammation, malignant invasion, and metastasis [13]. They not only break down plasmin-sensitive proteins (e.g., laminin) but also degrade plasmin-resistant connective tissue elements, such as elastin and glycoproteins [3, 9]. Studies have indicated that human alveolar macrophages, which synthesize a PA similar to active urokinase, degraded an insoluble fibrin matrix in the presence of plasminogen and also degraded insoluble elastin [3]. In the pleural cavity, the metabolism of extracellular matrix could be critical in maintaining the normal functions of the pleura, and the balance between PA and PA inhibitor is likely to play an important role. An illustration of the likely participants in the balance of fibrinolysis in the pleural cavity is given in Fig. 9.

PA inhibitors from different cell lines have different heat stabilities. For the human macrophage-derived PA inhibitor, after the inhibitor preparation was heated at 56°C for 30 min before incubation with urokianse, its functional activity was lost [4]. The pleural leukocyte inhibitor demonstrated in our studies was heat stable, as described by others [22], the activity being stable during incubation at 56°C for 1 hr but completely inactivated after incubation at 70°C for 1 hr.

PA inhibitors have a molecular mass around 40–50 kD [23]. If they form complexes with proteases, their net molecular weight can be greater than 100 kD [10]. Although we cannot give an accurate MW for the inhibitor in the supernatant, the results have confirmed that it is greater than 10,000 D and probably protein.

A large body of evidence has shown the production of PA inhibitor by cells of the mononuclear phagocyte lineage [4, 22, 23, 26], especially for PA inhibitor-type 2. Our results confirm that the cellular source of the PA inhibitor is pleural macrophages.

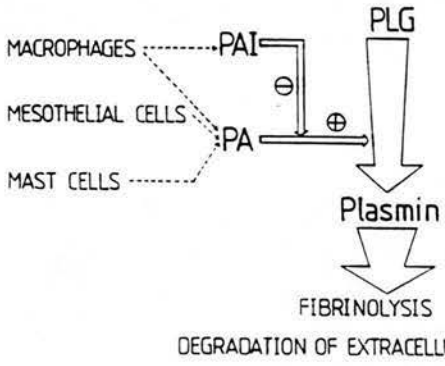


Fig. 9. Control of fibrinolysis in the pleural cavity.

In summary, this study has revealed that pleural macrophages, without stimulation, produce and release a protein inhibitor of urokinase-type plasminogen activator in culture. This finding suggests that a balance of fibrinolysis/coagulation may exist in the pleural cavity between the pleural macrophages and the pleural mesothelial cells or mast cells. Alternatively, the balance may also exist between the pleural macrophages themselves (see Fig. 9). One previous study has suggested that there is an alteration in the number and type of leukocytes present in the pleural space after exposure to asbestos [17]. The effect that this alternative population has on the fibrinolysis/coagulation balance in the pleural cavity and in particular the production of the PAI could be important in the various types of pleural response and pathologic reactions described in persons exposed to fibrous dusts [11] and will be the subject of further study.

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Increased Production of Plasminogen Activator Inhibitor *in Vitro* by Pleural Leukocytes from Rats Intratracheally Instilled with Crocidolite Asbestos

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We have previously reported that normal pleural leukocytes secrete a urokinase-type plasminogen activator inhibitor (PAI) in culture. In view of the pathogenic effects of asbestos on the pleura, in particular pleural fibrosis, we have extended these observations to crocidolite asbestos-exposed rats. Pleural leukocytes from rats exposed to crocidolite asbestos were found to secrete more PAI in culture than controls. The activity of PAI in pleural leukocyte-conditioned medium increased in a dose-dependent manner in relation to the quantity of asbestos injected into the lung. However, with increasing time post asbestos instillation, there was no significant change in the secretion of PAI by pleural leukocytes in culture compared with earlier time points of crocidolite-exposed rats. Plasminogen activator was not detectable in the conditioned medium at any time point. The data derived from this study may help to elucidate the pathogenesis of some pleural disorders caused by exposure to fibrous dusts in the lungs. © 1991 Academic Press, Inc.

INTRODUCTION

The fibrinolysis system is a multicomponent enzyme system composed of plasminogen, plasminogen activators (PA), and plasminogen activator inhibitors (PAI). Two immunologically and biochemically distinct types of PA have been identified, i.e., urokinase-type PA and tissue-type PA. PAs convert the abundant extracellular zymogen, plasminogen, into plasmin, an active protease that promotes degradation of almost all the components of extracellular matrix (Blasi *et al.*, 1987). For the coordinated action of PAs, a precise regulation of their activity is required. In principle, regulation is possible at the level of transcription, mRNA processing, translation, and secretion of PAs, but also through the production of specific inhibitors. Two distinct protenase inhibitors specific for PAs have been identified and purified: PAI type 1, released mainly by endothelial cells, and PAI type 2, released by cultured monocytes–macrophages (Kruithol *et al.*, 1986; Jong *et al.*, 1987). As the regulators of PA activity and function, PAIs control a variety of critical events associated with connective tissue turnover, coagulation, fibrinolysis, and inflammatory reactions. They also participate in other important physiological reactions such as malignant invasion and metastasis (Hart and Rehemtulla, 1988). PAIs have been found in many tissues and have been described in the conditioned medium of endothelial cells (Levin, 1986), blood monocytes (Wohlwend *et al.*1987; Golder and Stephens, 1983), lymphoma cell line U-937

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(Kruithol *et al.*, 1986), hepatoma cells (Zeheb *et al.*, 1987), leukocytes (Kopitar, 1981; Kopitar *et al.*, 1985), and macrophages (Sprengers and Kluft, 1987).

One of the primary functions of PAs is their involvement in fibrinolysis. The fibrinolytic system is not only required for the removal of blood clots within the vascular system, but also for the degradation of extravascular fibrin deposits (Medcalf *et al.*, 1988). Several lines of evidence suggest that fibrin and fibronectin in tissues may serve as a provisional matrix for fibroblast proliferation (Schleef *et al.*, 1988). During inflammation, including that caused by fibrous dusts, inflammatory cells present at sites of inflammation may release inhibitors of fibrinolysis. We have reported that leukocytes from the normal rat pleural space secrete, in culture, a PAI specific against urokinase which is not prostaglandin in view of its large size (Li *et al.*, 1990). It is of importance to understand the regulation of PA by PAI in the pleural space, in particular, after exposure to an agent such as asbestos which causes pleural pathology. If PA activity is abnormally modulated, excessive fibrin deposition may occur leading to pleural fibrosis.

In view of the foregoing, we questioned whether the secretion of PAI by pleural leukocytes was influenced by pulmonary deposition of asbestos.

MATERIALS AND METHODS

Materials

PVG rats of around 12 weeks of age bred in the Institute of Occupational Medicine Animal Unit were used.

Nembutal was purchased from Ceva Ltd, (Watford, UK). F10 medium and fetal calf serum (FCS) were obtained from GIBCO, Paisley. Bovine serum albumin (BSA), fibrinogen, and trypsin were purchased form Sigma, Pool, Dorset. F12 medium and trypan blue were obtained from Flow Laboratories, Rickmansworth, Herts; $Na^{125}I$ was purchased from Amersham International, Amersham. Sephadex G25 columns (PD-10 column) were obtained from Pharmacia, Milton Keynes. Plasminogen was purchased from Kabi, Stockholm, or together with urokinase were kindly supplied by Dr. Ian McGregor, Blood Transfusion Centre, Edinburgh. The crocidolite asbestos sample was the Union Internationale Contre Le Cancer (UICC) standard sample A. Diff-Quick staining solution was purchased from Merz-dade, Dudingen, Switzerland.

Exposure of Animals and Pleural Lavage

Rats were anesthetized with ether and the cervical trachea exposed. Injection of 0.5 ml of phosphate-buffered saline (PBS) containing the required doses of crocidolite asbestos was achieved by instillation via an incision in the trachea; control rats received 0.5 ml of PBS alone. The animals were sacrificed 1, 3, 14, or 30 days after instillation. For pleural lavage, rats were injected into the pleural space with 5-ml aliquots of PBS at 37°C by introducing a blunt needle into the pleural space between the lowest two ribs. The abdominal wall was incised for observation of the position of the needle in the pleural cavity. The pleural lavage fluid was withdrawn and transferred to a universal container kept on ice, this procedure was repeated four times and the fluid pooled.

Preparation of Cells, Cell Supernatants, and Lysates

The pleural lavage fluid was centrifuged at 1000 rpm for 5 min and the cell pellet was resuspended in F10 medium + 2% BSA. The cells were counted using a Neubauer counting chamber with trypan blue. Cytocentrifuge smears of the cell suspension were prepared and stained with Diff-Quik for analysis of the cell types present. The cells were resuspended in F10 medium + 2% BSA at 1×10^6/ml and incubated at 37°C, 5% CO_2 for 24 hr. The cell supernatants were then spun at 3000 rpm for 10 min, aliquoted, and stored at −70°C prior to use.

To prepare cell lysates, pleural leukocytes, at 1×10^6/ml, were frozen in liquid nitrogen and thawed in a 37°C water bath immediately. This procedure was repeated three times and the suspension were then spun at 3000 rpm for 10 min to remove cell debris.

Assay of Plasminogen Activator and Plasminogen Activator Inhibitor

Fibrinolytic activity and inhibitor activity were assayed in ^{125}I-fibrin-coated tissue culture wells. The plates were prepared as previously described (Li *et al.*, 1990). Briefly, dissolved fibrinogen was labeled with ^{125}I and free ^{125}I was removed by chromatography on a Sephadex G25 column. ^{125}I-labeled fibrinogen was diluted with PBS to obtain a final radioactivity of 12.5×10^5 cpm/ml. Aliquots (80 μl) of the solution were added to wells of Falcon 96-well plates and the plates were dried at 45°C for 3 days. A further 4 hr incubation of the wells with 100 μl F10 medium + 2.5% freshly thawed FCS was used to convert fibrinogen to fibrin. The plates were washed with PBS and stored at 4°C until required.

The PA activity in pleural leukocyte supernatants was assayed by overnight incubation of 200 μl diluted supernatant on the ^{125}I-fibrin matrix in the presence of excess plasminogen. Because previous experiments have demonstrated that there is very little plasminogen-independent fibrinolytic activity caused by pleural leukocytes or their supernatants (Li *et al.*, 1990), plasminogen independent fibrinolysis was not measured. The supernatants were tested for PAI by mixing a suboptimal dose of urokinase (i.e., causing 50% of total ^{125}I-fibrin lysis) with excess plasminogen followed by incubation in ^{125}I-fibrin-coated wells at 37°C, 5% CO_2 for 24 hr; 150 μl of supernatant was then harvested from each well and counted by a gamma counter.

Inhibitor activity was expressed as the percentage inhibition of plasminogen-dependent fibrinolysis caused by urokinase in the presence of diluted supernatants compared with fibrinolysis in the absence of the supernatant. PAI activity was determined according to the following equation:

$$\% \text{ inhibition} = \text{Fibrinolysis}$$
$$\frac{(\text{Fib.}) \text{ without supernatant (sup.)} - \text{blank}) - (\text{Fib. with sup.} - \text{blank}) \times 100}{(\text{Fib. without sup.} - \text{blank})},$$

where unit of fibrinolysis = cpm.

For each plate, blank (spontaneous release) was obtained from control wells incubated with medium alone. All assays were performed in triplicate.

Statistical Analysis and Presentation of Results

Results are expressed as the means ± SEM as an index of variation. Analysis of the data on inhibition was performed by comparing control pleural leukocyte supernatants with those of asbestos-instilled rats using Student's t test; a difference of $P < 0.05$ was considered significant.

RESULTS

1. Pleural Leukocytes from Normal and Asbestos-Instilled Rats

Table 1 shows the total cell number and differential count of pleural leukocytes from normal rats. The pleural leukocytes contained substantial proportions of mast cells and eosinophils while more than 80% of cells were macrophages. Mesothelial cells were virtually absent in these preparations.

One day after injection of 1, 5, or 10 mg crocidolite asbestos, there was no change in the total number of pleural leukocytes (Table 2). There was, however, a minimal increase in percentage eosinophils and a consistent decrease in percentage of macrophages with each dose and the appearance of a few neutrophils at the highest dose.

Intratracheal instillation of 5mg crocidolite asbestos did, however, elicit changes in the pleural leukocytes at later time points (Table 3). There was a significant increase in cell number from Day 3 after injection and this persisted until Day 30. The percentage of eosinophils was also significantly increased between Days 3 and 30. This was balanced by slight decreases in the proportions of macrophages and mast cells. Neutrophils and lymphocytes were unaltered.

2. PA Activity in Asbestos-Exposed Rats

We have reported previously that normal pleural leukocytes do not express any PA activity in culture, but intracellularly, pleural leukocytes, mainly mast cells, contain large quantities of PA activity (Li *et al.*, 1990). In the present study, there was no PA activity detectable in either control or asbestos-exposed pleural leukocyte supernatants. Also, there was no significant difference in PA activity in cell lysates of control pleural leukocytes and leukocytes 1 day after intratracheal instillation of 10 mg crocidolite (data not shown).

3. PAI Activity Released by Pleural Leukocytes from Asbestos-Exposed Rat

As shown in Fig. 1, the percentage inhibition of urokinase activity caused by pleural leukocytes from asbestos-exposed rats was significantly greater than con-

TABLE 1
THE PROPORTION OF NORMAL PLEURAL LEUKOCYTES

Total number ($\times 10^6$)	Percentage			
	Macrophages	Lymphocytes	Mast cells	Eosinophils
4.51 (0.29)[a]	82 (1.6)	1 (0.3)	11 (1.5)	6 (0.8)

[a] Mean (SEM), n = 14 rats.

TABLE 2
PLEURAL LEUKOCYTE POPULATIONS 1 DAY AFTER THE INDICATED INTRATRACHEAL DOSES OF CROCIDOLITE ASBESTOS

Dose (mg)	Total No. ($\times 10^6$)	Percentage				
		Macrophages	Neutrophils	Lymphocytes	Mast cells	Eosinophils
1	4.83 (0.22)[a]	79 (1.2)	0	2 (1.0)	11 (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)**

[a] Mean (SEM). n = 5–7 rats. Compared with normal pleural leukocytes. *$P < 0.05$, **$P < 0.01$.

trols. At all of the time points following exposure to asbestos, supernatants exhibited markedly increased PAI activity. However, there was no significant difference in the production of inhibitor between different time points following asbestos instillation. The apparent difference in Fig. 1 may have been masked by experimental variation. Statistical analysis showed that except for points at the 1:64 dilution for the 3-day group and the 1:16 dilution for the 14-day group, all of the other points were significantly different than control ($P < 0.05$ to $P < 0.001$).

Figure 2 shows inhibition of urokinase-induced fibrinolysis caused by supernatants of pleural leukocytes from rats injected intratracheally with different doses of asbestos 1 day previously. It was very clear that higher doses of asbestos stimulated increased secretion of PAI by pleural leukocytes in culture. The cells recovered from rats dosed with 1 mg asbestos did not express significantly higher PAI activity in culture than controls, at least at higher dilutions. In contrast, when the dose of asbestos was increased to 5 mg, significantly higher PAI secretion was observed in the supernatants. In the 10-mg asbestos-instilled group, even at the highest dilution (1:64), the inhibition of fibrinolysis was still nearly 50% showing that the inhibition was present at high concentrations. Statistical analysis showed that compared to controls, there was only a significant difference at dilutions 1:8, 1:4, and 1:2 points for the 1-mg group ($P < 0.05$ to $P < 0.01$). All of the dilution points for the 5- and 10-mg groups were significantly higher in PAI production than controls ($P < 0.01$ to $P < 0.001$). Compared with the 1-mg treatment group, the 5-mg treatment group was significantly higher in PAI production at almost every dilution point, and the same was found for the 10-mg group ($P < 0.05$ to P

TABLE 3
PLEURAL LEUKOCYTE POPULATIONS AFTER THE INDICATED NUMBER OF DAYS FOLLOWING 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)[a]	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)***

[a] Mean (SEM). n = 5–7 rats. Compared with normal pleural leukocytes. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

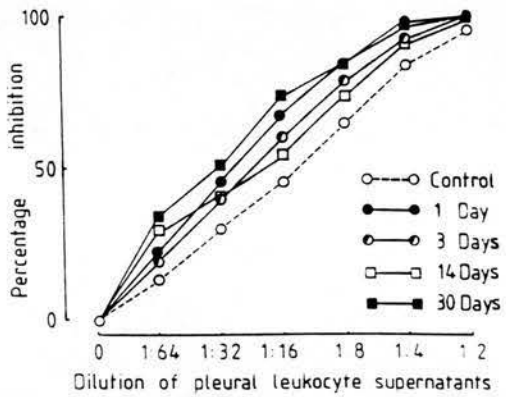


FIG. 1. Inhibition of fibrinolysis by supernatants of pleural leukocytes obtained from rats at different times after intratracheal instillation of 5 mg crocidolite asbestos. Fibrinolysis produced by urokinase plus plasminogen. Control is addition of supernatant of pleural leukocytes from untreated rats. Remainder of points represent the effect of supernatants from pleural leukocytes obtained 1–30 days after intratracheal crocidolite. For clarity, SEM, which was less than 15% of the mean, is not presented in the figures. Each point represents the mean of triplicate wells in 3–14 separate experiments. See Results for the description of significant differences.

< 0.001). At the higher dilution points, the 10-mg treatment group was significantly higher in PAI activity than the 5-mg group ($P < 0.001$).

DISCUSSION

Pathological responses have been found in the pleural cavity of asbestos-exposed individuals. These include pleural adhesions, diffuse fibrosing pleurisy, and pleural fibrosis as well as mesothelioma (Herbert, 1986; Wagner, 1986). However, almost no research has been done to investigate the fibrinolytic situation in the pleural cavity, including the effect of asbestos, despite its propensity to cause pathogenic change there.

Evidence that exposure to asbestos causes abnormalities of the fibrinolytic system has been presented previously. It was shown that the early lower respiratory tract inflammatory response in sheep resulting from chronic asbestos exposure is associated with a marked increase in PA, both at the alveolar macrophage surface and in the alveolar extracellular milieu, and it was suggested that this was correlated with the intensity of the alveolar inflammatory response (Cantin *et al.*, 1989). We have demonstrated that plasminogen-dependent fibrinolysis caused by rat alveolar macrophages was inhibited to varying degrees by a range of particulates tested, including chrysotile asbestos. The results revealed that some stimulations of plasminogen-dependent fibrinolysis precede cell death due to chrysotile asbestos *in vitro* (Donaldson *et al.*, 1987). Exposure of mouse peritoneal macrophages to chrysotile for 2 hr stimulated PA activity and a low concentration of retinoic acid inhibited the asbestos-induced production of PA in macrophages (Lison *et al.* 1989).

In the present study, we concentrated on the control of fibrinolysis in the pleural cavity. Our previous studies (Li *et al.*, 1990), indicated that normal rat

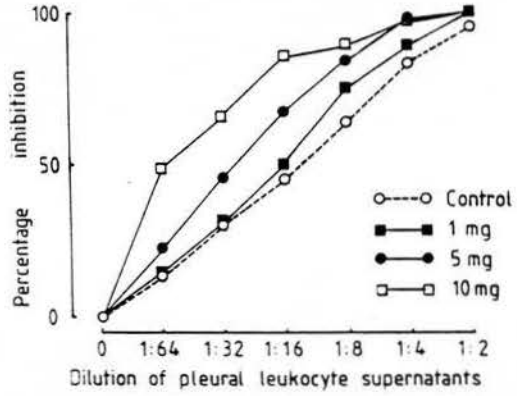


FIG. 2. Inhibition of fibrinolysis caused by supernatants from pleural leukocytes obtained from rats 1 day after intratracheal instillation of different doses of crocidolite asbestos. Model fibrinolysis produced as in Fig. 1. Control shows the effect of supernatant of pleural leukocytes from untreated rats. Remaining points show the effect of supernatants of leukocytes obtained from rats treated with 1–10 mg crocidolite for 1 day. For clarity, SEM, which was less than 15% of the mean, is not presented in the figures. Each point represents the mean of triplicate wells in 5–14 separate experiments. See Results for the description of significant difference.

pleural leukocytes release a urokinase-type plasminogen activator inhibitor in culture and strongly suggested that the cellular source of PAI is the pleural macrophages. We concentrate here on the change in PAI secretion by pleural leukocytes after deposition of asbestos in the airspaces. It was found that pleural leukocytes from rats exposed to crocidolite asbestos released significantly more PAI than controls at time points between 1 and 30 days after asbestos instillation while cell populations changed only very slightly; the PAI accumulation in pleural leukocyte supernatants increased dramatically. The altered secretion of PAI in asbestos-elicited pleural leukocytes cannot be explained by alterations in the proportions of the pleural leukocyte population since there was no directly comparable effect on the differential count.

It has been demonstrated in a previous study that inhalation of asbestos fibers induces a macrophage-rich inflammatory exudate in the alveolar region (Donaldson *et al.*, 1988). An intraperitoneal injection of asbestos fibers also induces an inflammatory exudate characterized by macrophages (Hamilton *et al.*1976). Approximately 70% of the asbestos-induced macrophages incorporated [^{3}H]thymidine *in vivo* during the 4-day period of exudate formation and, therefore, it appears that many of the cells are young cells, having recently divided locally. Macrophages derive from monocytes which leave the microcirculation, enter the tissue, and adopt the characteristics of tissue macrophages. Therefore, recently migrated cells from the blood, i.e., "young" macrophages, may also be present. From our data, it is also clear that 3 days after asbestos instillation, the total number of pleural leukocytes also increases. This very likely indicates that there is an accumulation of newly recruited leukocytes in the pleural cavity of lungs exposed to asbestos. Current concepts of the pathogenesis of extracellular matrix alterations and fibrosis in asbestos-induced interstitial lung diseases suggest that

activated lung inflammatory leukocytes play a critical role in mediating these changes (Crystal *et al.*, 1981). Newly recruited macrophages are normally more active in producing inflammatory mediators (Medcalf *et al.*,1988), which may include PAI, so causing inhibition of degradation of extracellular matrix.

It is reported that fibrin (or fibrinogen) is not a normal constituent of either the pulmonary interstitium or the alveolar space. However, fibrin deposition is a prominent component of a number of acute and chronic inflammatory disorders of the lungs. The sites of early fibrin deposition correlate with the location of the subsequent fibrotic process, suggesting that fibrin has importance for fibroblast attachment, spread, growth, and proliferation (Chapman and Stone, 1985; Chapman *et al.*, 1986). As an active protease, plasmin, which is converted from its zymogen plasminogen by PAs, can promote degradation of all components of the extracellular matrix (Blasi *et al.*, 1987); fibrin (or fibrinogen) is a major substrate of this protease. Because fibrin resorption is virtually dependent on synthesis and expression of a classic plasminogen activator of the urokinase type (Chapman and Stone, 1985), the increased PAI may lead directly to deposition of fibrin and other extracellular matrix components, such as fibronectin, so forming a scaffold for fibroblast adherence and proliferation. Hopper *et al.* (1981) have demonstrated the deposition of fibrin on the surface of peritoneal macrophages *in vivo*. In another study, based on their findings, they proposed that the lymphokine-activated macrophages have thromboplastic properties which would enable local activation of the extrinsic clotting pathway (Geczy and Hopper, 1981). Because macrophages can produce fibrin or fibrinogen on their own surface, although there is no severe pleural inflammation to involve other cells, pleural macrophages are thus able to serve as a nidus for pleural fibrosis by forming the fibrin scaffold around themselves.

The present study provides evidence to suggest that there may be a localized fibrinolytic deficit in the pleura of asbestos-exposed lung which could favor fibrogenesis. Further evidence that PAI may be important in pleural fibrosis comes from quinacrine pleurodesis treatment of malignant pleural effusions (Agrenius *et al.*, 1989). Studies have shown that the concentration of PAI-1 in exudative pleural effusions was four times higher 6 hr after quinacrine instillation and that of D-dimer, a product of lysis of the fibrin, was five times higher before treatment. Another study indicated that exudative pleural effusions from patients with congestive heart failure, loculated pleural effusions, and so on are characterized by relatively increased procoagulant and decreased fibrinolytic activity favoring local fibrin formation. Abnormalities of fibrin turnover favor fibrin deposition in the inflamed pleural space (Idell *et al.*, 1989).

The present finding that pleural leukocytes from rats exposed to asbestos, which may include newly recruited cells, express increased PAI in culture contrasts with previous observations for alveolar macrophages exposed to asbestos in which increased PA activities were observed (Cantin *et al.*, 1989; Hamilton *et al.*, 1976). In these studies, it is likely that macrophages made direct contact with the asbestos, whereas, in our study, the asbestos fibers, as confirmed by a number of approaches (including ultrastructural studies, unpublished data), have failed to transfer from the lung parenchyma to the pleural space in detectable numbers. In

our model, instillation of asbestos into the lungs therefore must be assumed to affect the pleural leukocytes by the transfer of a message to the pleural cavity from the adjacent parenchyma. We have not assessed the levels of plasminogen activator or PAI in alveolar leukocytes following asbestos deposition in our rat model as this was not within the scope of the present study. We have, however, demonstrated alveolar macrophage-derived plasminogen activator activity (not PAI activity) which could be inhibited by pleural leukocyte supernatants (Li *et al.*, 1990). The pleural cavity and the bronchoalveolar spaces are therefore fundamentally different with regard to the fibrinolytic status.

The highest dose of instilled asbestos caused recruitment of neutrophils, a cell type not normally present in the normal pleural leukocyte population. Alveolar macrophages are thought to play an important part in the early stages of asbestosis by modulating the migration of neutrophils to the lung (Schoenberger *et al.*, 1982) and may also influence their accumulation in the pleural space. There was no correlation between neutrophils and PAI production in this study and the neutrophils disappeared quickly with time after instillation of asbestos. Thus it is not clear what role the neutrophils play in asbestos-induced pleural inflammation. Similarly, the role of eosinophils in the inflammatory events occurring in the pleural cavity of asbestos-exposed rat remains unclear. It has been suggested that mast cells may play a role in the deposition of fibrous tissue (Miller *et al.*, 1989). However, we found no correlation between increased fibrinolytic activity and percentage of mast cells in the pleural leukocyte population in the present study. The functions of the mast cells in the inflammatory events therefore remain to be investigated.

In summary, this study has provided evidence of a change in the functional expression of PAI in the pleural region of asbestos-exposed lungs with increased levels of pleural leukocyte PAI following exposure. Increased PAI would favor localized build-up of the normal products of coagulation, including fibrin, which could then serve as a scaffold for fibroblast aggregation and ultimately lead to fibrosis.

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Intratracheal Injection of Crocidolite Asbestos Depresses the Secretion of Tumor Necrosis Factor by Pleural Leukocytes In Vitro

Xiao Yang Li, David Lamb, and Kenneth Donaldson

ABSTRACT: *Tumor necrosis factor (TNF) is a cytokine released predominantly by monocytes/macrophages that has been shown to modulate a variety of different immune and metabolic functions. To understand the regulatory mechanisms of TNF in governing responses in the pleural cavity following deposition of fibrous dust in the airspace of the lung, we studied the capability of leukocytes, lavaged from the pleural cavity, to release TNF in culture. TNF production by lavaged pleural leukocytes was measured using the L-929 TNF-sensitive cell line, after intratracheal instillation of crocidolite asbestos. A high level of TNF activity was found in the supernatants of normal, unstimulated pleural leukocytes; the addition of 100 ng/ml lipopolysaccharide to the culture increased the activity up to threefold. Following intratracheal instillation of 5 mg crocidolite asbestos, the pleural leukocytes secreted less TNF than the control. With increasing mass of intratracheally instilled asbestos, there was a dose-dependent reduction in TNF release. Changes in the population of the pleural leukocytes or their number could not be related to variation in TNF activity. These results suggest that exposure of rat lungs to crocidolite asbestos by intratracheal instillation affects the response of pleural leukocytes without causing changes in the population. Such changes in the bronchoalveolar space may be related to the pleural pathology found in asbestos-exposed individuals.*

INTRODUCTION

Tumor necrosis factor/cachectin (TNF) is a multifunctional cytokine produced by all types of macrophages in response to lipopolysaccharide (LPS). Although characterized by the cytotoxic or cytostatic effect on some tumor cells, TNF affects almost every cell type. It has a profound effect on tissue

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remodeling, repair, and inflammation by coordinating the activities of many other cells—including endothelial cells, granulocytes, fibroblasts, lymphoid cells, and neutrophils—and by production of inflammatory mediators by those cells, such as prostaglandin, interleukin 1 (IL-1), IL-6, as well as inflammatory products, such as superoxide anion and H_2O_2 [1–12].

The pleura plays a crucial role in the movement of the lungs. Any changes in the pleural cavity will affect this function dramatically. For example, the pleura is highly susceptible to different sorts of pathologic changes following pulmonary deposition of fibrous dusts such as asbestos [13]. The pleura is not only a mechanical envelope for the lung but also represents a crossroads for exchange of cells and fluids. Because the normal population of pleural leukocytes participates in immunologic and inflammatory responses, a better understanding of the pleural response to fibrous dusts, such as asbestos, will lead to an explanation of the relationship between inhalation exposure to fibrous dusts and the pleural pathologic changes.

Amphibole crocidolite asbestos has been used extensively in insulation, fireproofing, and soundproofing. This straight, blue fiber has been recognized to be the most dangerous asbestos in causing mesothelioma. It also is associated with a series of pleural dysfunctions, such as pleural plaque, pleural thickening, and pleural fibrosis [14–18].

In this study, we investigated the ability of rat pleural leukocytes to secrete TNF in vitro with or without stimulation, and after rats were intratracheally instilled with crocidolite asbestos.

MATERIALS AND METHODS

Animals

Male or female PVG rats, age 12 weeks or older and inbred at the Institute of Occupational Medicine Laboratory Animal Unit, were used throughout the experiments.

Intratracheal Instillation of Crocidolite Asbestos

Rats were anesthetized with ether. The trachea was exposed and a blunt-ended needle introduced into it. A volume of 0.5 ml of phosphate buffered saline (PBS), containing required doses of Union Internationale Contre Le Cancer (UICC) crocidolite asbestos, was then injected. Control rats were uninjected or injected with PBS alone. No difference was found between these two controls. The injected rats were killed at intervals of up to 1 month and pleural lavage was carried out.

Pleural Lavage and Pleural Lavage Fluid

Rats were killed by intraperitoneal injection of Nembutal (Ceva Ltd, Watford, UK). For lavage, rats were injected in the pleural space with 5-ml aliquots of PBS at 37°C between the lower two ribs using a round-ended needle. The rib cage was massaged gently, and then the suspended cells were withdrawn. This procedure was repeated three times and cells were concentrated by centrifugation and resuspension in F10 medium (Gibco, Paisley, UK) with 2% bovine serum albumin (BSA, Sigma, Poole, UK). Pleural lavage fluid was obtained by injecting 2-ml F10 plus 2% BSA solution into the pleural cavity, then withdrawn after massaging prior to pleural lavage procedure. The fluid was spun at 1000 rpm for 10 min and the fluid retained.

Cell Counts

The total cell number lavaged from each rat was determined by dilution and counting in a Neubauer chamber. Lavage cell viability, assessed by trypan blue exclusion, was always higher than 90%. The cell differential count was obtained from cytocentrifuge smears stained with Diff-Quik (Merz Dade, Dudingen, Switzerland). The total numbers of each cell type were obtained by multiplying differential percentage of each cell type by the total cell number.

Supernatants of Pleural Leukocytes

Lavaged pleural leukocytes were diluted in F10 medium + 2% BSA to a concentration 1×10^6/ml and incubated at 37°C, 5% CO_2 for 24 h. For determining the inducing effect of lipopolysaccharide (LPS, Sigma) to the generation of TNF by pleural leukocytes, LPS was added into the culture at 100 ng/ml according to previous studies. The supernatants were collected, clarified by centrifugation, and stored frozen at −70°C until tested.

L929 Cytotoxic Assay

The presence of TNF in supernatants conditioned by pleural leukocytes in culture was detected by its cytotoxic activity in the cell-lytic assay using L929 cells as targets standardized with TNF alpha (a gift kindly supplied by Dr. J. Symonds). L929 cells were cultured in minimum essential medium (MEM, Gibco) with 5% fetal calf serum (FCS, Gibco).

The assay medium was MEM + 5% FCS without antibiotic. Cells were detached from a semi-confluent status and resuspended in assay medium at 3×10^5/ml. Cells were dispensed into Falcon 96 well plates as 100 μl. The plates were incubated at 37°C, 5% CO_2 for 24 h. The supernatant was then aspirated and discarded. Assay medium, 100 μl, plus 1 μg/ml Actinomycin

D (Sigma) was then added to all wells, and 50 μl of test supernatants and 50 μl of 2 μg/ml Actinomycin D were added to experimental wells in triplicate. They were then double diluted using a multichannel pipette. The plates were then incubated 18–20 h at 37°C, 5% CO_2. Medium was discarded and replaced with 100 μl of a crystal violet solution. The L929 cells were left for a few minutes, washed vigorously with water, and allowed to dry. The plate was then read in a MR650 reader (Dynatech Laboratories Inc, Alexandria, VA) at 540 nm. The addition of medium alone to the L929 cells represented the negative control, while maximal cell lysis was obtained as the wells cultured without L929 cells. The amount of TNF present in the pleural leukocyte supernatants was determined using a TNF alpha standard dilution curve.

Endotoxin in Medium

To find out whether the secretion of TNF by pleural leukocytes in culture was caused by endotoxin contamination in the media or serum used in the assay, a kit (Coatest, Kabi Diagnostica, KabiVitram Ltd, Uxbridge, Middlesex, UK) was used, according to the suppliers' instructions, to detect endotoxin levels in all media used in the study.

To determine the effect of endotoxin in the medium on TNF production by pleural leukocytes, polymyxin columns (Detoxi-Gel Affinitypak Columns, Pierce Chemical Company, Illinosis, IL) were used to remove endotoxin from medium. Our previous experiments demonstrated an endotoxin-depleting efficiency of 99% with these columns.

Inhibitory Effect Caused by Normal Pleural Lavage Fluid on TNF Activity in Pleural Leukocyte Supernatant and TNF Standard

Normal pleural lavage fluid at different concentrations was added into the assay while TNF standard and supernatant of pleural leukocytes were applied in the assay at concentrations 12.5 U/ml and 1:16 dilution, respectively, at the same time to measure possible interaction among them. The results were presented as percent inhibition according to the following equation:

$$\text{Percent inhibition} = \frac{\text{TNF activity (U/ml)} - \text{TNF activity after addition of PLF}}{\text{TNF activity}} \times 100$$

where PLF = pleural lavage fluid.

Statistical Analysis

All experiments were repeated at least three times. The experimental numbers mentioned in figure legends were the numbers of animals used in the experiments. Because of multicomparisons, Bonferroni's correction [19] was used in conjunction with Student's t test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of Intratracheal Injection of Crocidolite Asbestos on Pleural Leukocyte Population

The mean total number of control pleural leukocytes recovered was 4.51×10^6 and the differential count was macrophages 83%, lymphocytes 1%, mast cells 11%, and eosinophils 5%. Figure 1 shows the differential pleural leukocytes from rats at different times following intratracheal instillation of 5 mg crocidolite asbestos: 1 day after instillation of asbestos, the pleural leukocyte population was unaffected except for a modest neutrophil increase. From 3 days up to 1 month after 5 mg asbestos instillation, the total numbers of macrophages increased significantly compared with control pleural leuko-

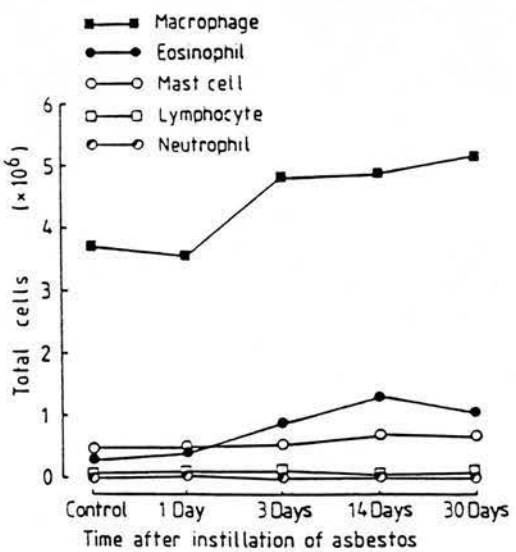


Figure 1 Differential numbers of pleural leukocytes from rats at different times after intratracheal instillation of 5 mg crocidolite asbestos. For clarity, standard error of mean (SEM), which varied from 6 to 40% of the mean, is not shown. Each point represents mean of 5–13 separate experiments. Compared with control, total numbers of macrophages were significantly higher from 3 to 30 days ($P < .05$ to $P < .01$). Total numbers of eosinophils were also significantly higher than control from 3 to 30 days ($P < .01$ to $P < .001$).

cytes. Meanwhile, eosinophils increased markedly, although other pleural leukocytes remained at the same level.

Figure 2 illustrates the population of pleural leukocyte 1 day after injection of different doses of crocidolite asbestos up to 10 mg. The higher doses of crocidolite caused a slight recruitment of neutrophils. However, other types of cells remained the same as controls.

Secretion of TNF by Normal Plural Leukocytes With and Without LPS Treatment

As shown in Fig. 3, normal pleural leukocytes secrete high levels of TNF in vitro in the absence of LPS. The concentration of TNF in supernatants of normal leukocytes is about 600 U/ml using recombinant TNF alpha as a standard. The presence of 100 ng/ml of LPS enhances TNF production by several times compared with the constitutive release.

To elucidate the possible role of endotoxin contamination of medium in the observed TNF secretion, endotoxin levels in media and serum used in the assay were determined. Low levels of endotoxin contamination in the media were found: MEM + 5% FCS contained 29 pg/ml of endotoxin, F10 + 2% BSA contained 106 pg/ml of endotoxin, and in PBS and saline, the concentrations of endotoxin were 16 and 0 pg/ml, respectively. TNF activities in pleural leukocyte supernatants that were prepared in medium

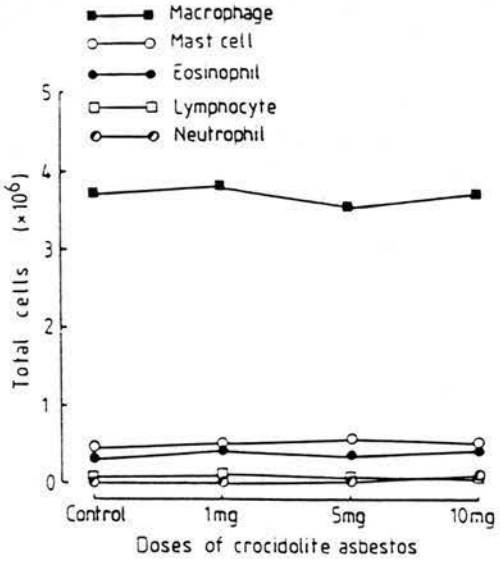


Figure 2 Differential numbers of pleural leukocytes from rats 1 day after intratracheal instillation of different doses of crocidolite asbestos. Each point represents the mean of 6–13 separate experiments. SEM from 3 to 40% of the mean is not shown. Only total number of eosinophils at the 10-mg point was significantly different from control ($P < .05$). No significant difference was found for other cell types.

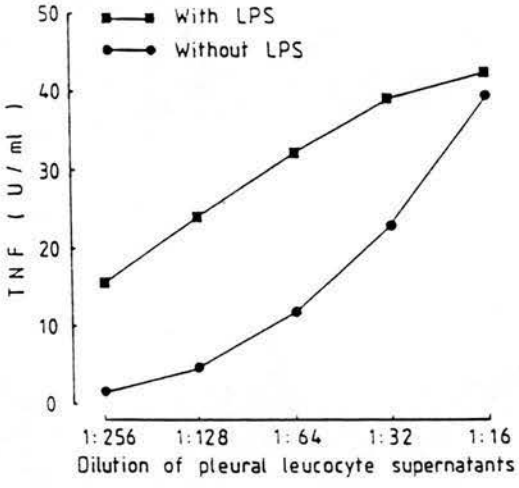


Figure 3 TNF production by normal pleural leukocytes with or without stimulation with LPS. Each point represents the mean of triplicate wells in 5–7 separate experiments. SEM (not shown) is 10–25% of the mean. Except for the highest dilution of supernatant, all other LPS-stimulated points had significantly higher TNF activity than unstimulated points ($P < .001$).

before or after endotoxin removal by polymyxin columns showed no significant difference, TNF activities (in units/ml) calculated from a supernatant dilution of 1 : 16 were for the medium, 1062.72 ± 81.2 (n = 12), and for endotoxin-depleted medium, 1086.24 ± 171.6 (n = 12).

TNF Secretion by Pleural Leukocytes After Deposition of Crocidolite Asbestos in the Bronchoalveolar Space

Up to 30 days after intratracheal instillation of 5 mg crocidolite asbestos, the capacity of pleural leukocytes to secrete TNF in culture was reduced significantly compared with controls. However, there was no detectable time-related effect on this reduction of secretion with TNF activity present at 1 day being similar to that found at 1 month (Fig. 4).

To determine the dose/response relationship between crocidolite deposition in the lung and the secretion of TNF by pleural leukocytes in vitro, doses of 1, 5, or 10 mg of crocidolite asbestos were injected intratracheally into rat lungs for 1 day. Figure 5 illustrates that increasing crocidolite in the lungs caused a gradual decrease in the ability of the pleural leukocytes to release TNF in vitro; this was particularly notable for the 10 mg crocidolite group, where TNF secretion was inhibited almost completely. TNF activity in the pleural lavage fluid was measured at the same time. There was no detectable TNF activity in the fluids from normal and crocidolite instilled rats (data not shown).

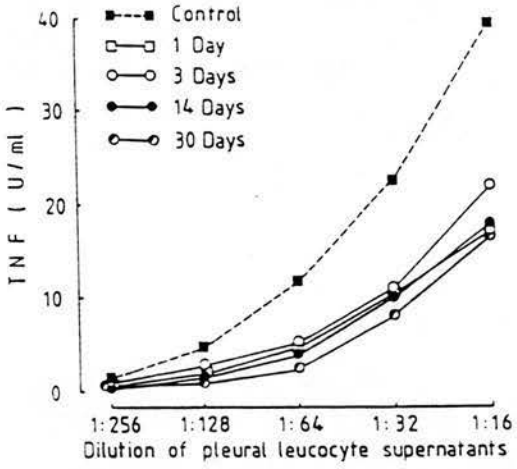


Figure 4 TNF production by pleural leukocytes from rats at different times after intratracheal instillation of 5 mg crocidolite asbestos. Each point represents the mean of triplicate wells in 10–22 separate experiments. SEM (not shown) varied from 10 to 40% of the mean. Results of Student's *t* test using Bonferroni's correction [19] from dilutions 1:16 to 1:64 showed that TNF activity at almost all of dilution points of asbestos-treated groups was significantly higher than the control ($P < .05$ to $P < .01$).

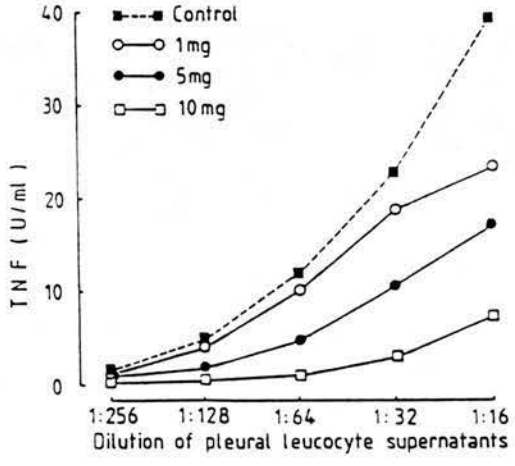


Figure 5 TNF production by pleural leukocytes from rats 1 day after intratracheal instillation of different doses of crocidolite asbestos. Each point represents the mean of triplicate wells in 9–22 separate experiments. SEM (not shown) is from 10 to 70% of the mean. Compared to corresponding dilution points of control, all asbestos-treated groups had significantly higher TNF activity ($P < .05$ to $P < .01$). At dilutions 1:64 to 1:32, TNF activity in the 10 mg group was significantly greater than in the 5 mg group ($P < .05$).

TNF Inhibition Caused by Normal Pleural Lavage Fluid

Figure 6 shows that pleural lavage fluid inhibited TNF activity in a concentration-related manner. At 1:4 dilution, the lavage fluid almost completely abolished TNF activity in both pleural leukocyte supernatant and TNF standard.

DISCUSSION

Exposure to asbestos is associated with diseases of the lung parenchyma, airway, and—of particular interest to the present study—the pleura [14–17]. Although the role of bronchoalveolar leukocytes in pathologic changes to fibrous dusts has been demonstrated [20, 21], little is known of the pleural leukocyte response to deposition of fibrous dust. Therefore, we set out, using the rat model, to investigate pleural leukocyte response to intratracheally deposited asbestos.

In summary, the results presented here indicate that normal pleural leukocytes secrete high levels of TNF in vitro, and that with intratracheal injection of crocidolite asbestos, the secretion of TNF by pleural leukocytes was reduced dramatically. This inhibition was dose dependent in relation to the amount of asbestos instilled into the lung.

Mononuclear phagocytes are a major source of the cytokine TNF, and the release can also be increased by exposure to LPS in vitro [22–24]. Resting alveolar macrophages do not express the TNF mRNA transcripts, but, when activated by LPS, they expressed TNF transcripts and synthesized and released TNF [25]. The production of TNF by LPS-induced alveolar macrophages was as much as 10-fold greater than that by noninduced alveolar macrophages [23]. Human monocytes cultured in a specially prepared me-

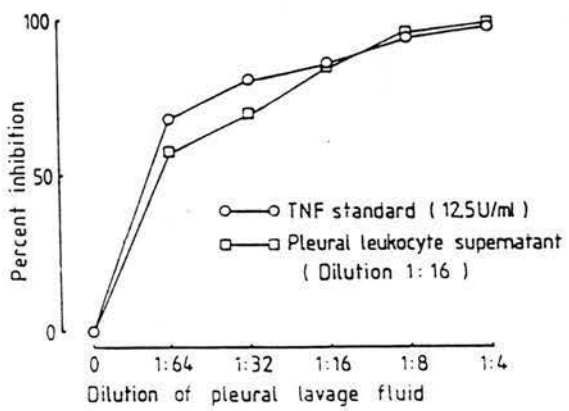


Figure 6 Inhibitory effect of TNF activity caused by normal pleural lavage fluid. Each point represents mean of triplicate wells in 4–7 separate experiments. SEM varied from 7 to 20% of the mean (not shown).

dium free of LPS constitutively produced a small amount of TNF. After addition of LPS, the production of TNF dramatically increased, eventually becoming 100-fold greater than when the LPS concentration was below 1 ng/ml [24].

We presumed that the high TNF activity in some samples in the present study was due to contamination by endotoxin during experimental procedures. Our results confirmed that there were very low levels of endotoxin (0.02–0.1 ng/ml) in the media used in the experiments. To investigate the role of endotoxin in the secretion of TNF, we depleted endotoxin from the medium used to prepare supernatants through the use of polymyxin columns. This procedure had no effect on the high production of TNF by pleural leukocytes, thus ruling out endotoxin in the medium as the explanation. This was supported by the fact that, despite this high spontaneous secretion of TNF, pleural leukocytes could still be stimulated with 100 ng/ml LPS in culture, to release markedly higher TNF activity, consistent with other workers' findings. We also noted that, if these low levels of endotoxin had been stimulatory to TNF production by pleural leukocytes, then this population is much more sensitive to endotoxin than rat alveolar leukocytes since, in our hands, culture in the same medium did not produce such a high background of TNF release by bronchoalveolar leukocytes.

When crocidolite asbestos was intratracheally injected into rat lungs, and different postexposure time points were selected, there were only modest changes to the pleural leukocyte population. Differential cell number of pleural leukocyte changed from 3 days to 1 month: macrophages increased significantly as did eosinophils. The most notable change, however, was the total cell number which increased from 4.22×10^2 to 6.69×10^6. This might suggest that the deposition of asbestos in the lungs caused the active recruitment of pleural leukocytes. The high numbers of eosinophils and mast cells we found in the pleural cavity of rats were described in previous studies: mast cells, 12–30%, and eosinophils, 9–14% [26]. As time after asbestos deposition increased, the total numbers of eosinophils increased significantly. We do not know what role eosinophils play in the events in the pleural space caused by deposition of asbestos in the lung. However, there was no relationship between TNF production and variation of eosinophil number. Meanwhile, mast cell total number remained at the same levels throughout exposure. Although it was suggested that mast cells may play a role in secretion of TNF [10], there was no evidence that mast cell number was association with the production of TNF in our study. Because mast cells may play a role in the development of the response to fibres [26], their functions in the pleural space require investigation.

Normally, processes of inflammation and response to injury are associated with augmented release of TNF [1]. Interestingly, our results demonstrate that deposition of asbestos in the air space causes pleural leukocytes to secrete significantly less TNF than control pleural leukocytes while the pop-

ulation remains relatively constant. One previous study involving intrapleural injection of crocidolite asbestos [27] described the presence of lysosomal enzymes and nonspecific esterase in mononuclear cells and giant cells in asbestos-induced granulomas. This reveals that asbestos may trigger changes in the characteristic of cells and supports the contention that intratracheal injection of asbestos, if some asbestos reaches the pleural space, could change the characteristics of the pleural leukocytes to cause reduced TNF secretion. However, our own studies, which form the basis of a separate publication (manuscript in preparation), suggest that, in our model, asbestos in air space does not lead to substantial transfer of fibers to the pleural cavity. In view of these findings, we suppose that there is likely a communication between the alveolar and pleural spaces through the transfer of inflammatory signals.

Although pleural leukocytes produced great quantities of TNF in culture, there was no measurable biologically active TNF in pleural lavage fluid. This might result from a lack of spontaneous release by pleural leukocytes in vivo. However, it is also possible that, in the pleural space, a special TNF inhibitor exists.

The failure to measure TNF in pleural lavage fluid may result from the interaction and neutralization of TNF by its inhibitor. We have demonstrated here that pleural lavage fluid has an inhibitory effect on TNF activity in pleural leukocyte supernatants and on the activity of the TNF standard. A TNF alpha-specific inhibitor has been purified and characterized by other workers [28]. Because in another study (unpublished data) we found that pleural mesothelial cells are not responsible for the TNF inhibitory effect, it is likely that an inhibitor(s) is produced by pleural leukocytes, which also are the source of TNF.

The normal situation is likely to depend mainly on the balance of activities of TNF and its inhibitor. For normal pleural leukocytes in culture, the production of TNF is predominant. After intratracheal instillation of asbestos, inflammatory signals from the lung may trigger the expression of the TNF inhibitor gene or the release of the inhibitor and lead to the decreased TNF activity in culture. On the other hand, the signals may block the expression of the TNF gene or the release of TNF and also result in a predominance of TNF inhibitor activity in the culture.

As an inflammatory mediator, TNF functions beneficially for the body in many aspects during inflammation. The suppression of TNF activity described here may prevent necessary second-order responses of the pleura to asbestos deposition in the lung. A deficit in these TNF-mediated defensive responses, which could include recruitment of leukocytes and their activation for inflammation and immune defense, if persistent, could lead to injury and subsequent pleural pathology.

In another study we confirmed that pleural leukocytes secrete interleukin 1 (IL-1) in culture. Therefore, the effect of TNF in the L929 assay may be affected by IL-1 [29]. However, following asbestos deposition, IL-1 elabora-

tion by the cells shows an initial fall, which recovers gradually (unpublished data) while TNF production by the cells remains at the same low level. Therefore, it is unlikely that present results are caused by the production IL-1 by the same cells.

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Reactive pleural inflammation caused by intratracheal instillation of killed microbes

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Reactive pleural inflammation caused by intratracheal instillation of killed microbes. X.Y. Li, G.M. Brown, D. Lamb, K. Donaldson.

ABSTRACT: To investigate the pleural leucocyte response to severe alveolar inflammation, heat-killed *Corynebacterium parvum* were instilled intratracheally into the lungs of PVG rats and pleural lavage was performed.

Polymorphonuclear neutrophils are not normally resident in the pleural space but were found transiently after intratracheal instillation of *C. parvum*. Macrophages increased gradually in the pleural space following instillation, reaching a peak at day 5. The activity of plasminogen activator inhibitor in the pleural leucocyte supernatants was increased at day 1, but returned to control levels by day 5. The activities of interleukin-1 and tumour necrosis factor secreted by pleural leucocytes were decreased compared with control pleural leucocytes at day 1 and were further reduced at day 5. The analysis of particle translocation showed that intratracheally instilled *C. parvum* and fluorescent beads with or without *C. parvum* did not reach the pleural space.

We hypothesize that pleural inflammation resulting from *C. parvum*-induced inflammation in the lung is the result of transfer of a diffusible factor from the adjacent parenchyma.

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Lung inflammation has been studied extensively, however, very few experimental studies have been carried out on pleural inflammation [1, 2]. The pleura is a structure adapted to form a lubricated surface that allows the lungs to expand and recoil without resistance, and to resist the accumulation of fluid despite negative intrapleural pressure. Pleural pathological changes, such as pleural effusions, pleural fibrosis and pleural mesothelioma may occur as a result of oral drug treatment, and also inhalation exposure to asbestos and other dusts [3, 4]. In a rabbit study, for instance, intravenous Ethchlorvynol caused a patchy, subpleural, haemorrhagic polymorphonuclear neutrophilic inflammatory response [5]. A reactive pleuritis may also occur when there is adjacent pneumonia in the underlying lung parenchyma. For example, a para-pneumonic effusion is a common complication of bacterial pneumonia [6]. Approximately 60% of patients with pneumococcal pneumonia and 40% of all bacterial pneumonias have associated pleural effusion [7].

Interleukin-1 (IL-1) [8] and tumour necrosis factor (TNF) [9] are cytokines which have effects on recruitment and activation of polymorphonuclear leucocytes (PMNs), on endothelial cell activity, fibroblast proliferation, macrophage activation and immune response. Plasminogen activator (PA) converts the abundant extracellular zymogen plasminogen into plasmin, an active protease that promotes degradation of all components of the extracellular matrix. Plasminogen activator inhibitor (PAI) [10] can influence cellular function and tissue remodelling by finely regulating the location and extent of PA-induced plasminogen activation. We have reported modulation of the production of these inflammatory mediators by pleural leucocytes following deposition of fibrous dusts in the bronchoalveolar space [11]. We previously described the inflammatory response of the lung parenchyma to a range of agents [12] and noted, in preliminary studies, that intratracheal *Corynebacterium parvum* caused a transient pleuritis.

In the present study, in an attempt to study reactive pleural inflammation caused by *C. parvum* pneumonitis, we measured the translocation of particles to the pleura following alveolar deposition. We also assessed the kinetics of the production of the cytokines, TNF and IL-1 by pleural leucocytes and determined secretion of PAI by pleural leucocytes during pleural inflammation.

Materials and Methods

Animals

Syngeneic, male and female PVG rats, aged 12 weeks or more, obtained from the Institute of Occupational Medicine Animal Unit, were used throughout.

Intratracheal instillation of C. parvum *and microspheres*

Anaesthetized rats were instilled intratracheally with 1.4 mg of heat-killed *C. parvum* (Wellcome, Beckenham, UK) in a volume of 0.2 ml. Bronchoalveolar and pleural lavage were carried out at intervals of 1–5 days thereafter. To study the possible transfer of particles from the alveolar to the pleural space, animals were intratracheally instilled with 0.4 ml of phosphate-buffered saline (PBS) containing 1:20 diluted monodispersed fluorescent carboxylated microspheres which have a geometric diameter of 0.770 μm (Polysciences Inc., Warrington, UK), since they are easily seen when viewed by UV illumination. The microspheres were injected together with *C. parvum* when required.

Bronchoalveolar and pleural lavage

Rats were sacrificed by intraperitoneal injection of Nembutal (Ceva Ltd, Watford, UK). The rat trachea was cannulated with a blunt, 18-gauge needle. The lungs were removed from the thorax and bronchoalveolar lavage was performed by instilling 8 ml of PBS at 37°C into the lungs *via* the cannula. PBS was then withdrawn after gentle massage and the entire procedure repeated 4 times.

Pleural lavage was carried out as follows: the rat peritoneal cavity was opened and the skin covering the right chest removed. A round-ended 8-gauge cannula was inserted through the chest, between the lower two ribs, into the pleural space, for injection of 5 ml PBS at 37°C into the space, monitored *via* the opened peritoneal cavity. The PBS was then withdrawn after massaging the rib cage and this procedure was repeated on four occasions.

The cell suspensions were spun at 1,000 rpm for 5 min and resuspended in F10 medium (Gibco, Paisley, UK) containing 2% bovine serum albumin (BSA). (Sigma, Poole, UK). Cell viability, as judged by trypan blue exclusion, was normally more than 95%. For differential counts, cell suspensions were prepared as cytocentrifuge smears and then fixed and stained with May Grünwald-Giemsa stain.

Mesothelial cell characterization

To determine whether there were significant numbers of mesothelial cells present in the pleural leucocyte populations, a monoclonal antibody to rat leucocyte common antigen (CD45) (Antibody MRC OX-1, MCA 43, Serotec, Oxford, UK) was used. Pleural lavage cells obtained from control rats and rats at 16 h and 5 days after intratracheal instillation of *C. parvum* were stained with the antibody. The second antibody was a fluorescein-conjugated F(ab') 2 fragment of rabbit immunoglobulins raised against mouse immunoglobulin (F313, Dako Ltd, High Wycombe, Bucks, UK). The fluorescence was determined in a Coulter Flow Cytometer (Epics-Profile II, Coulter Electronic Ltd, Luton, Beds, UK).

Observation of beads and C. parvum *in bronchoalveolar and pleural lavage leucocytes*

Following instillation of fluorescent beads, 100 PMNs and macrophages of cytospin preparation of bronchoalveolar and pleural leucocytes were observed on each smear by light microscopy under epi-fluorescent illumination. The macrophages and PMNs from both lavaged populations were divided into two groups: with or without beads. In the group with beads, the cells were further divided into three subgroups: cells containing <5 beads, cells containing 6–10 beads and cells containing >10 beads. During bead counting, a differential count was also carried out.

In addition, following *C. parvum* exposure alone, the lavaged leucocytes were also examined for intracellular organisms. Both macrophages and PMNs were divided into one of two groups: with or without intracellular organisms.

Preparation of supernatants

Pleural leucocytes were adjusted to a concentration of $1\times10^6\cdot ml^{-1}$ in F10 medium + 2% BSA and cultured for 24 h at 37°C in a 5% CO_2 atmosphere. For leucocyte stimulation, lipopolysaccharide (LPS), (Sigma, Poole, UK), was added to the culture at 100 $ng\cdot ml^{-1}$. The supernatants were collected and centrifuged at 3,000 rpm for 10 min to remove cell debris, then stored at -70°C prior to assay.

Assay for TNF activity

The assay was performed according to the method of Warner and Libby [13]. In brief, murine L929 cells grown in minimal essential medium (MEM) (Gibco, Paisley, UK), containing 5% foetal calf serum (FCS) were trypsinized and suspended in assay medium (MEM + 5% FCS, no antibiotics), 100 μl aliquots containing 3×10^5 cells were added to each well of microtitre plate and incubated at 37°C for 24 h. The supernatant was then discarded, and 100 μl of assay medium containing 1 $\mu g\cdot ml^{-1}$ actinomycin D (Sigma, Poole, UK), 50 μl of pleural leucocyte supernatants and 50 μl of assay medium with 2 $\mu g\cdot ml^{-1}$ of actinomycin D was added to experimental wells; then double diluted. Following further 18–20 h incubation, the wells were emptied, stained with crystal violet methanol solution, and then washed with water and allowed to dry. Optical density at 540 nm was determined with a MR650 plate reader (Dynatech Laboratories Inc., USA). TNF activity in the supernatant was determined by comparison with a TNF-alpha standard (a gift kindly supplied by J. Symonds, Dept of Rheumatology, Northern General Hospital, Edinburgh, UK) dilution curve.

Assay for IL-1 activity

IL-1 activity in the supernatant was quantified using the C3H/HeJ mouse thymocyte assay [14]. Briefly, thymus glands of mice killed by ether were removed and single cell suspensions of thymocytes were prepared by homogenization. The cells were washed and suspended at $6\times10^6\cdot ml^{-1}$ in RPMI-1640 medium (Gibco, Paisley, UK) with 10% FCS and 20 μM 2-mecaptoethanol (Sigma, Poole, UK). One hundred μl of the cell suspension was added to each well, together with 50 μl of RPMI medium containing 20 $\mu g\cdot ml^{-1}$ of phytohaemagglutinin (PHA) (Sigma, Poole, UK) and 50 μl of dilutions of test pleural leucocyte supernatants (PLS). The cells were then incubated at 37°C in 5% CO_2 for 48 h and pulsed with 0.25 μCi ^{3}H-methyl-thymidine per well (Amersham International, Amersham, UK). After one more day of culture, the cells were harvested onto glass filters, and ^{3}H incorporation was quantitated by scintillation spectrophotometry.

Assay for plasminogen activator inhibitor activity

PAI activity was assayed in ^{125}I-fibrin coated tissue culture wells as described previously [15]. Briefly, dissolved fibrinogen (Sigma, Poole, UK) was labelled with $Na^{125}I$ (Amersham International, Amersham, UK). Free ^{125}I was removed by chromatography on a Sephadex G25 column (Pharmacia, Milton Keynes, UK). ^{125}I-labelled fibrinogen was diluted with PBS and added to microtitre plates so that each well contained 1×10^5 cpm; the plates were then dried at 45°C for 3 days. The fibrinogen was converted to fibrin by treatment with F10 medium containing 2.5% freshly thawed FCS. The plates were washed twice with PBS prior to assay. To determine the PAI activity in the supernatants, the concentrations of urokinase (kindly provided by I. McGregor, Blood Transfusion Centre, Edinburgh, UK) which caused 50% of total ^{125}I-fibrin lysis when cultured with an excess of plasminogen (Kabi, Stockholm, Sweden and a gift from I. McGregor), were used. The activity of PAI in the PLS was determined by titration against urokinase and plasminogen. Solubilized ^{125}I-fibrin degraded by urokinase in the culture was measured after incubation for 24 h at 37°C, 5% CO_2. The PAI activity was expressed as percentage inhibition of plasminogen-dependent fibrinolysis according to the following equation:

$$\text{Percentage inhibition} = \frac{(\text{Fib without PLS} - \text{Blank}) - (\text{Fib with PLS} - \text{Blank}) \times 100}{(\text{Fib without PLS} - \text{Blank})}$$

Where units of fibrinolysis = cpm; and Fib = fibrinolysis.

Statistical analysis

All experiments were repeated at least twice and details of experiments are given in legends to figures and tables. Results of repeated experiments 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-test [16].

Results

Alveolar and pleural leucocyte populations after intratracheal instillation of C. parvum

Normal rat alveolar leucocyte populations contained more than 95% macrophages with a total number of less than 5×10^6. One day after intratracheal instillation of *C. parvum*, large number of PMN were recruited to the bronchoalveolar space and this increased the total cell number to around 30×10^6, although the macrophage number remained at the same level (fig. 1). Three days after instillation, the number of PMNs had decreased dramatically and was minimal by day 5. The number of macrophages was markedly increased by day 3 and remained elevated at day 5.

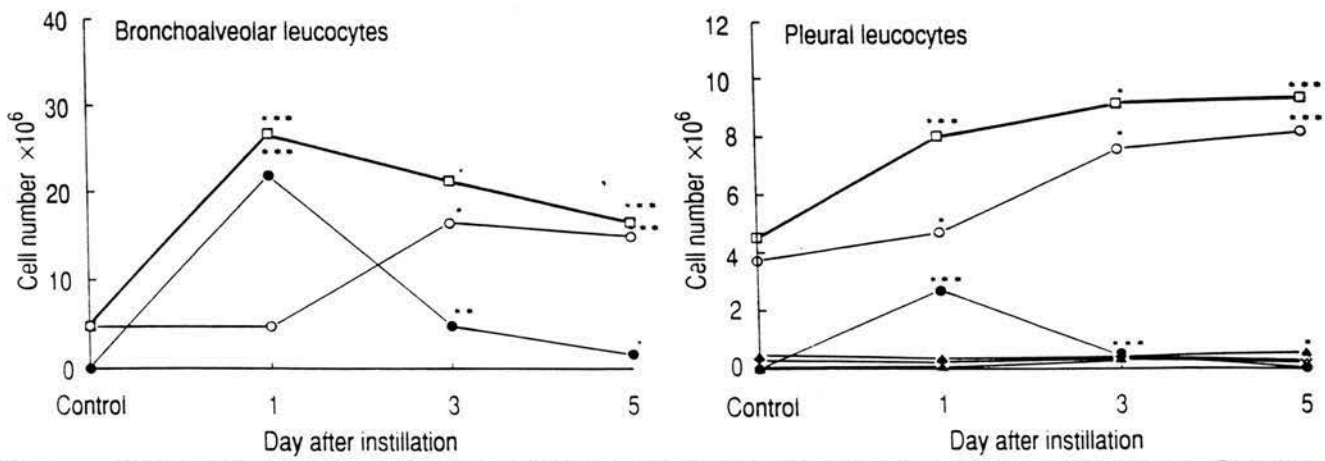


Fig. 1. – Bronchoalveolar and pleural leucocyte populations at various times after intratracheal instillation of *C. parvum*. Each point shows means of 2–13 separate experiments. For clarity, SEM which varies from 8 to 30% of mean, is not presented in the graph. Asterisks denote a significant difference from control: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. —□—: total cell number; —○—: macrophage; —●—: PMN; —△—: lymphocyte; —▲—: mast cell; —◇—: eosinophil.

There were no pleural PMNs detectable in normal pleural leucocyte populations. One day after intratracheal instillation of *C. parvum* there was a slight increase in number of macrophages but a marked increase in number of PMNs in the pleural space (fig. 1). The peak of PMNs at day 1 disappeared quickly and no PMNs were found by day 5 after *C. parvum* instillation. However, number of macrophages increased gradually from day 3 and this was sustained up to day 5. When the components of pleural leucocytes were compared with those of alveolar leucocytes, the pattern of change in the macrophage and PMN populations was similar.

Analysis of beads and C. parvum *organisms in alveolar and pleural leucocytes*

When beads were instilled intratracheally alone, they produced a moderate alveolitis characterized by 24% PMNs in the bronchoalveolar lavage (table 1). A high percentage (72%) of alveolar macrophages engulfed beads and most macrophages (>70%) were found to contain more than 10 beads (table 2). One day after intratracheal *C. parvum* plus beads, the total number of alveolar leucocytes increased up to eight times compared with bead instillation alone. A severe inflammatory response was observed with PMNs accounting for more than 80% of the total alveolar population (table 1). The distribution of beads in the macrophages changed with most macrophages (>80%) containing less than 10 beads. The numbers of PMN with beads reduced from 33 to 4% (table 2).

In the pleural space, little effect on cell components was seen following alveolar deposition of beads alone and beads were negligible in the pleural leucocytes. Intratracheal instillation of *C. parvum* and beads together caused recruitment of PMNs into the pleural space (29% of total population) and the total cell number increased nearly threefold (table 1). In spite of such severe pleural inflammation resulting from

Table 1. – Alveolar and pleural leucocyte populations after intratracheal instillation of beads and/or *C. parvum*

Treatment	Total cell no.	Differential percentage				
	×10⁶	Mac	PMN	Lymph	Mast	Eos
Alveolar leucocytes						
B alone	7.36 (1.26)	75 (3.5)	24 (2.8)	1 (0.7)	0	0
B+*Cp* (day 1)	58.97 (2.95)	16 (2.1)	84 (2.8)	0	0	0
B+*Cp* (day 5)	13.87 (1.76)	81 (9.5)	18 (9.5)	1 (0)	0	0
Pleural leucocytes						
B alone	4.10 (0.70)	76 (6.4)	0	1 (0)	6 (3.5)	7 (2.8)
B+*Cp* (day 1)	11.80 (3.38)	60 (0.7)	29 (11)	0	6 (5.7)	5 (5.7)
B+*Cp* (day 5)	9.47 (0.55)	86 (0)	3 (1.5)	6 (1)	4 (1.7)	1 (0.6)

Results are presented as mean (SD) of two separate experiments. B: beads; *Cp*: *C. parvum*; Mac: macrophages; PMN: polymorphonuclear leucocytes; Lymph: lymphocytes; Mast: mast cells; Eos: eosinophils.

Table 2. – Analysis of beads in the alveolar and pleural leucocytes, following intratracheal instillation of beads and/or *C. parvum*

Treatment	% of cells with beads	Distribution of beads in cells		
		1–5	5–10	>11
Alveolar macrophages				
B alone	72 (7.1)	12 (8.5)	17 (4.2)	71 (12.7)
B+*Cp* (day 1)	37 (6.4)	57 (7.1)	26 (8.5)	17 (1.4)
B+*Cp* (day 5)	15 (1.5)	52 (8.7)	25 (6.2)	23 (2.6)
Alveolar PMNs				
B alone	33 (18.4)	90 (2.8)	9 (1.4)	1 (1.4)
B+*Cp* (day 1)	4 (0.7)	100 (0)	0	0
B+*Cp* (day 5)	0 (0)	0	0	0
Pleural macrophages				
B alone	<1	100	0	0
B+*Cp* (day 1)	<1	100	0	0
B+*Cp* (day 5)	0	0	0	0
Pleural PMNs				
B alone	0	0	0	0
B+*Cp* (day 1)	0	0	0	0
B+*Cp* (day 5)	0	0	0	0

Results are presented as mean (SD) of two separate experiments. For abbreviations see legend to table 1.

C. parvum in the lung, negligible numbers of beads could be observed in the pleural leucocytes. We found that the beads seen very occasionally in cytospins of pleural lavage leucocytes were extracellular. In another study (data not shown), we demonstrated that even one month after intratracheal instillation of beads alone, or together with mineral dusts, there was no detectable translocation of beads from airspace to pleural space.

Our results suggest that there is no direct communication of particles between the alveolar space and the pleural space, even during acute inflammation. To confirm this hypothesis, we extended our observations to assess numbers of cells containing *C. parvum* organisms in both spaces. Table 3 shows that one day after intratracheal instillation of *C. parvum*, numbers of macrophages and PMNs containing organisms intracellularly in the alveolar space, were 26 and 34%, respectively. By day 5, the cells with organisms were negligible in the alveolar space and the percentages of macrophages and PMNs containing identifiable organisms had dropped to 1 and 0%. No organisms could be found in either macrophages or PMNs in the pleural space at any time.

Table 3. – Analysis of *C. parvum* organisms in the alveolar and pleural leucocytes after intratracheal instillation of *C. parvum*

Cells	% of cells with organisms
Alveolar macrophages	
Day 1	26 (3.1)
Day 5	1 (0.6)
Alveolar PMNs	
Day 1	34 (5.6)
Day 5	0
Pleural macrophages	
Day 1	0
Day 5	0
Pleural PMNs	
Day 1	0
Day 5	0

Results are presented as mean (SD) of 3 to 5 separate experiments. PMNs: polymorphonuclear leucocytes.

Characterization of pleural leucocytes

In all treatments the number of leucocyte common antigen positive cells was around 95% (mean (SD) of two experiments: Control: 96.6 (1.2)%; 16 h after intratracheal instillation of *C. parvum*: 94.9 (3.5)%; and 5 days after *C. parvum* instillation: 95.9 (0.9)%). The remainder of the cells (around 5%) was assumed to be exfoliated mesothelial cells. Any difference between the pleural populations was very likely, therefore, to be a result of change in leucocytes and their functions and not the population of mesothelial cells.

Secretion of cytokines by pleural leucocytes in vitro: *effect of intratracheal instillation of* C. parvum

Normal pleural leucocytes produced high levels of IL-1 and TNF in culture and this production was dramatically enhanced whilst the leucocytes were stimulated with LPS (fig. 2). One day after intratracheal instillation of *C. parvum*, the activities of both IL-1 and TNF released by pleural leucocytes decreased compared to control levels. Five days after instillation, this reduction had become even more marked (fig. 3).

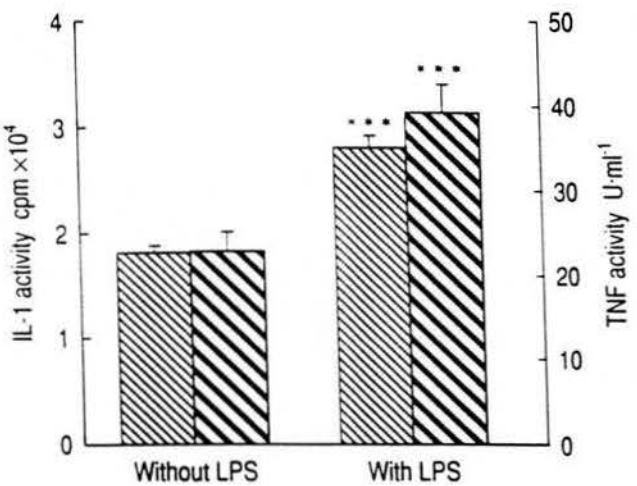


Fig. 2. – IL-1 and TNF production by normal pleural leucocytes with and without stimulation with LPS. Each bar represents the mean and SEM of 4–10 separate experiments performed in triplicate. The dilution of pleural leucocyte supernatant is 1:8 for IL-1 and 1:32 for TNF. ***: $p<0.001$ significantly different from control. ▨: interleukin-1 (IL-1); ◪: tumour necrosis factor (TNF); LPS: lipopolysaccharide.

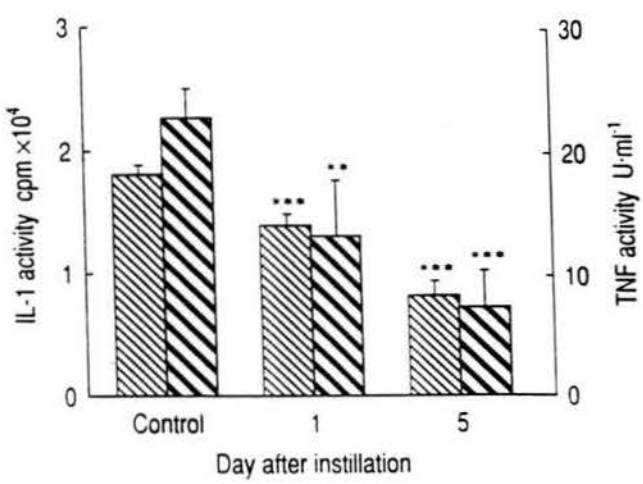


Fig. 3. – IL-1 and TNF production by pleural leucocytes at various times after intratracheal instillation of *C. parvum*. Each bar represents the mean and SEM of 4–9 separate experiments performed in triplicate. The dilution of pleural leucocyte supernatant is 1:8 for IL-1 and 1:32 for TNF. **: $p<0.01$, significantly different from control; ***: $p<0.001$. ▨: interleukin-1 (IL-1); ◪: tumour necrosis factor (TNF).

PAI produced by pleural leucocytes in vitro after intratracheal instillation of C. parvum

Figure 4 shows that the production of PAI by pleural leucocytes lavaged from rats one day after *C. parvum* instillation had increased significantly. However, five days after instillation of *C. parvum*, PAI activity produced by pleural leucocytes had returned to normal levels.

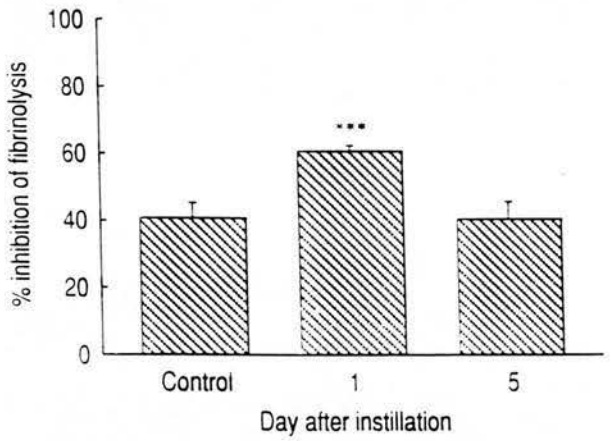


Fig. 4. – Fibrinolytic inhibition caused by pleural leucocytes supernatants at various times after intratracheal instillation of *C. parvum*. Results are presented as mean and SEM of 3–8 separate experiments performed in triplicate. The dilution of pleural leucocyte supernatant is 1:32. ***: $p<0.001$, significantly different from control.

Discussion

This study has shown that intratracheal instillation of *C. parvum* induces severe alveolar inflammation, characterized by transient recruitment of large numbers of PMN. The *C. parvum*-elicited macrophage alveolitis was a relatively long-lived process. The same inflammatory pattern, but of a much lesser magnitude, was found in the pleural space after intratracheal instillation of *C. parvum*. The very similar inflammatory patterns in both alveolar and pleural spaces indicated that there may be some communication of inflammatory messages between the lung and the pleural space. To address this question, we investigated the possibility that particles might be translocated from the lung to the pleural space.

The present study on the translocation of particles (*i.e.* beads and *C. parvum* organisms) indicated that, following deposition in the lung, those particles were rapidly engulfed by alveolar macrophages and PMNs. Meanwhile, in the pleural space, no macrophages or PMNs were found to contain beads. Analysis of macrophages and PMNs containing *C. parvum* organisms also confirmed that no microbial particles reach the pleural space. These findings are consistent with the study of LEHNERT *et al.* [17] which showed that, after instillation of particles with diameter of 2 μ into the lung, there was no detectable translocation of particles to the pleural spaces.

Although there is no translocation of particles to the pleural space, this region is, nevertheless, influenced by inflammation in the lung as shown here by the fact that one day after intratracheal instillation of *C. parvum*, there was an increase in the number of pleural PMNs. The visceral pleura is thick and composed of five different layers, including mesothelial layer, submesothelial layer, external elastic layer, interstitial layer and internal elastic layer [4]. This represents a substantial barrier to passive movement of particles from alveolar to pleural spaces and could explain absence of particle transfer. Since particles do not cross the pleura, we presume that pleuritis is due to the transfer of a diffusible signal(s) to the pleural space from the parenchyma.

Asbestos and other fibres have been found at visceral and parietal sites of the pleura, and in pleural fluid after deposition in airspaces [18, 19], but failure to find fibres has also been reported [20].

MILLER *et al.* [5] found that intravenous injection of Ethchlorvynol (ECV) produced a PMN-predominant exudative alveolitis and pleural effusion in rabbits, and they proposed that ECV-induced lung injury results in migration of PMN into the alveolar and interstitial space. Furthermore, damaged lung tissue was also suggested to stimulate PMN infiltration into and across the adjacent visceral pleura. FLENLEY [6] also suggested that pneumonia, in a subpleural location with an abundance of PMNs, causes increased pulmonary and pleural capillary permeability resulting in a leak of protein-rich fluid into the pleural space. Once the pulmonary endothelium becomes more permeable, the pleura is likely to offer less of a barrier to fluid, protein, and cellular movement, promoted by a pulmonary-pleural pressure gradient that is enhanced by the lung leak [5].

IL-1 levels normally increase during inflammation [21]. Our present study indicated that IL-1 activity released by pleural leucocytes one day after *C. parvum* instillation was decreased and the reduction was even more dramatic by day 5 compared with control levels. Presumably, the initial reduction in IL-1 activity may be due to the accumulation of PMNs in the pleural space, since IL-1 inhibitor is constitutively present in normal PMNs [22]. During the later stages after *C. parvum* instillation, when PMN numbers decreased, the further reduction of IL-1 activity might result from the additional secretion of IL-1 inhibitor by newly recruited macrophages [23]. The same explanation may be extended to the reduction in TNF activity [24]. Our findings, which indicate the existence of IL-1 and TNF inhibitor activity in the normal pleural lavage fluid (data not presented), favour this hypothesis.

RICH *et al.* [25] found that *in vitro* cultivation of blood monocytes resulted in decreased expression of TNF in response to LPS, which suggests that maturation of mononuclear phagocytes leads to a phenotype capable of producing higher levels of TNF. From this point of view, it is likely that the decrease in TNF production by the pleural leucocytes may partly result from the observed recruitment of blood monocytes to

the pleural space. However, IL-1 elaboration does not show this pattern, and in fact decreases with monocyte maturation [25, 26].

Decreased fibrinolytic activity has been reported in oleic acid-induced lung injury [27] and in quinacrine pleurodesis treatment of malignant pleural effusion [28]. We also demonstrated an increased PAI production by rat pleural leucocytes after intratracheal instillation of crocidolite asbestos [11]. The newly recruited PMNs may be activated and release products, such as oxidants, bioactive lipids and proteases, which influence the function of macrophages [29] including production of PAI. The similarity of changes of pleural PMN numbers and PAI production during the study period favours this hypothesis.

An altered balance of coagulation and fibrinolysis, caused by increased PAI, may establish local conditions which could promote acute fibrin deposition in the pleura. This is supported by the findings of WIDSTROM *et al.* [30], that fibrin deposition is associated with increased formation of thrombin in pleural fluid after intrapleural BCG. Fibrin is not a normal protein constituent of either the pulmonary interstitium or the alveolar space. Fibrin deposition commonly accompanies acute lung injury as well as influencing the subsequent repair processes [31], including the formation of a scaffold for fibroblast proliferation leading to fibrosis. Intrapleural urokinase [32] inhibited the development of pleural fibrosis in rabbits treated with high-dose tetracycline, implying that enhanced pleural fluid clotting and inhibition of fibrinolysis are important in drug-induced pleural fibrosis. The rapid return of the PAI levels to normal in the present study supports the observation that pleural fibrosis rarely results from acute, resolving pneumonia.

Our findings suggest that inflammatory pleural macrophages may produce cytokine inhibitors. In addition, their production of PAI, which may cause fibrin deposition and promote an inflammatory reaction in local connective tissue, is also reduced to normal levels as soon as PMN recruitment passes. These findings may represent an important protective role exerted by pleural macrophages. The phenomenon of a mild, long-standing pleural macrophage accumulation in our study may represent a beneficial reaction to the PMN recruitment engendered in the pleural space by inflammation in the adjacent lung parenchyma. By recruiting monocytes, tissue damage may be restricted through their ability to control the production of a series of inflammatory regulators.

In conclusion, after intratracheal instillation of *C. parvum* or beads, there was no penetration of particles from the airspaces to the pleural space. The PMN-macrophage inflammation occurring in the pleura, characterized by egress of PMNs into the pleural cavity, followed by an increase in macrophage numbers, is therefore presumed to result from transfer of a signal(s) from the parenchyma. The effects on the pleural leucocytes are a generalized decrease in cytokine activities and temporary enhancement of PAI activity. The role that these changes play in pleural effusion is not clear but this rat model of reactive pleural inflammation caused by intratracheal instillation of *C. parvum* may be useful in explaining some pleural events resulting from infection in the airspaces or deposition of agents such as asbestos.

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

Xiao Yang Li, David Lamb, Kenneth Donaldson

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 time[7] 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 asebestos 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.

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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

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, Dudingen, 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% CO_2 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 $0{\cdot}6 \times 10^6$ 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% CO_2 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 ^{3}H-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 supplemented 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% CO_2. 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 leucococytes 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 (×10⁶)	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 (×10⁶)	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-1β 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.

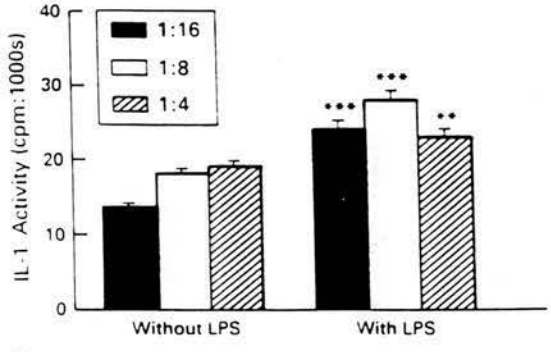


*Figure 1 Production of IL-1 by pleural leucocytes with or without stimulation with LPS. Results are presented as mean (SEM) of 10–14 separate experiments performed in triplicate. Asterisks denote a significant difference from corresponding dilution of controls: **p < 0·01; ***p < 0·001.*

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.*

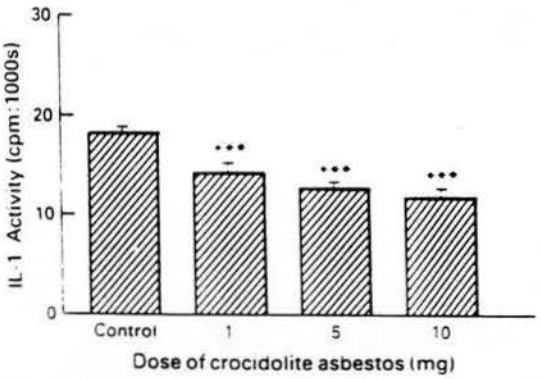


*Figure 3 Production of IL-1 by pleural leucocytes from rats one day after intratracheal instillation of different doses of crocidolite asbestos. Each column represents mean (SEM) of triplicate wells in 9–16 separate experiments. Dilution of pleural leucocyte supernatant is 1:8. Asterisks denote a significant difference from controls: ***$p < 0{\cdot}001$.*

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 treatment[21] 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 E_2 in inhibiting secretion of IL-1[22] 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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Mesothelial Cell Injury Caused by Pleural Leukocytes from Rats Treated with Intratracheal Instillation of Crocidolite Asbestos or *Corynebacterium parvum*

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The pleural and peritoneal mesothelium is a major target in asbestos exposure where mesothelial cell proliferation, exfoliation, and neoplasia have been reported in workers and experimental animals. The objective of this study was to determine the role of pleural leukocytes in mesothelial cell damage caused by asbestos exposure. We therefore investigated detachment and lysis injury to mesothelial cells *in vitro* induced by leukocytes lavaged from the pleural space of rats exposed, by intratracheal instillation, to crocidolite asbestos. Our studies revealed that normal pleural leukocytes were composed of macrophages, lymphocytes, eosinophils, and mast cells. This population showed a small but significant recruitment of mast cells and eosinophils 3 to 14 days after instillation of crocidolite asbestos; there were also modestly increased levels of macrophages present 30 days after low doses of asbestos. One day after intratracheal instillation of 5 mg crocidolite asbestos, the pleural leukocytes caused detachment injury to mesothelial cells *in vitro*. This injury was markedly reduced 3 days after asbestos exposure and was undetectable by Day 14. One month after instillation of asbestos, despite doses of asbestos from 0.5 to 5 mg, pleural leukocytes showed no ability to injure mesothelial cells *in vitro*. In a parallel study, pleural inflammation was induced by intratracheal instillation of heat-inactivated *Corynebacterium parvum*. Transient mesothelial cell-detaching injury was again expressed by pleural leukocytes 1 day after *C. parvum* instillation. This was likely related to an increase in the percentage of neutrophils present on this day. These results show that a single administration of crocidolite asbestos, intratracheally, leads to transient activation of pleural leukocytes in terms of the ability of these cells to detach mesothelial cells from matrix. This finding implies that mesothelial cell proliferation and exfoliation found in individuals exposed to asbestos may result from persistent stimulation of pleural leukocytes caused by the continuous presence of asbestos in the lung. © 1994 Academic Press, Inc.

INTRODUCTION

The pleura is a target organ for inhaled asbestos, and two specific pathologies, fibrosis and cancer (mesothelioma), are produced (Jaurand, 1985). The effects of asbestos on mesothelial cells has been investigated. Bryks and Bertalanffy (1971) observed a marked increase in the proliferation of visceral mesothelium 5–7 days after intratracheal administration of chrysotile in the rat; this was confirmed by Adamson and Bowden in similar experiments (1992). Mesothelial cells treated with chrysotile or crocidolite asbestos *in vitro* showed a significant dose-dependent increase in unscheduled DNA synthesis, indicating a genotoxic effect

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(Renier *et al.*, 1990) as well as chromosome damage (Jaurand *et al.*, 1983). Donaldson and colleagues (1988a) reported that crocidolite asbestos produced inhibition of plasminogen-dependent fibrinolysis by mesothelial cells. This may be related to a fibrinolytic defect contributing to the development of adhesion between the parietal and visceral pleura described in asbestos-exposed individuals (Stephens and Gibbs, 1987). Localized release of protease from inflammatory pleural leukocytes could cause detachment or loosening of the lining cells of the pleural surface (MacDonald *et al.*, 1979; Donaldson *et al.*, 1988b), contributing to protein leak, fibrin formation, and finally fibrosis (Rennard *et al.*, 1985).

The injurious effect of neutrophils toward alveolar epithelial cells has been extensively studied and the mechanism of this injury has been suggested to be the action of granule neutral proteases (Simon *et al.*, 1986; Ayars *et al.*, 1984) and oxidants (Sibille *et al.*, 1986). We have demonstrated that *Corynebacterium parvum*-induced inflammatory bronchoalveolar leukocytes caused nonlethal detachment injury to the alveolar epithelial cells in coculture and the injury was found to be mediated by proteases and oxidants (Donaldson *et al.*, 1987). Although it has been known that pleural mesothelium is susceptible to the toxic effects of asbestos, it is still unclear whether this is the result of direct effect of asbestos fibers or whether it is mediated by other mechanisms such as alterations in pleural leukocyte activity. In the intratracheal instillation model, we have consistently failed to find any evidence that asbestos transports to the pleural space in any significant quantity (Li *et al.*, 1991, 1992). The present study was undertaken to address whether the pleural leukocytes from exposed animals have the ability to damage mesothelial cells.

MATERIALS AND METHODS

Materials

Male or female syngeneic PVG rats (Institute of Occupational Medicine's own Animal Unit, Edinburgh, UK), 12 weeks of age or greater at the commencement of experiments, were used throughout. WISTAR-derived rats of the HAN strain (Western General Hospital Animal Centre, Edinburgh, UK), both male and female and 12 weeks of age or more, were also used.

The Union Internationale Contre Le Cancer (UICC) standard asbestos sample of crocidolite was used in the study. Heat-killed *C. parvum* was obtained from Wellcome Reagents (Beckenham, UK).

Intratracheal Instillation

Rats were anesthetized and the trachea was exposed, a small incision was made, and a blunt-ended needle was introduced. A volume of 0.5 ml of Dulbecco's phosphate-buffered saline (PBS), containing required doses (0.5, 1, and 5 mg) of asbestos or 0.2 ml of *C. parvum* suspension containing 1.4 mg of *C. parvum*, was then injected. The injected rats were killed at intervals up to 1 month and lavage was then carried out.

Pleural Lavage

Pleural lavage was carried out by inserting a round-ended 8-gauge cannula through the parietal pleura between the lower two ribs and injecting 5 ml PBS at

37°C into the pleural space. The PBS was then withdrawn after massaging and this procedure was carried out four times.

Characterization

The differential count of pleural leukocytes was obtained from cytocentrifuge smears stained with Diff-Quik (Merz Dade, Dudingen, Switzerland). The identification of different types of cells was confirmed by transmission electron microscope study.

To determine whether there were significant numbers of mesothelial cells present in the pleural leukocyte populations, a monoclonal antibody to rat leukocyte common antigen (CD45) (antibody MRC OX-1, MCA 43; Serotec, Oxford, UK) was used. The second antibody was a fluoresein-conjugated $F(ab')_2$ fragment of rabbit immunoglobulins raised against mouse immunoglobulin (F313; DAKO Ltd., High Wycombe, Bucks, UK). The fluorescence was determined using Coulter cytometry (Epics-Profile II; Coulter Electronic Ltd., Luton, Beds, UK).

Primary Culture of Rat Mesothelial Cells

Mesothelial cells, which were isolated from scrapes of the peritoneal cavity of normal PVG rats and frozen down at early passage (Donaldson *et al.*, 1988a), were recovered from liquid nitrogen. The cells were then cultured in F12 medium (Flow Laboratories, Rickmansworth, UK) containing 10% fetal calf serum (FCS; Gibco, Paisley, UK) until required.

Mesothelial Cell Lysis and Detachment Assay

Mesothelial cells were grown and serially passaged in F12 medium with 10% FCS. In the assay, the mesothelial cells were placed into 96-well microtiter plates at 5×10^4 cells/well in 200 μl F12 medium plus 10% FCS and labeled with chromium-51 (^{51}Cr; Amersham International, Amersham, UK) at 74 kBq/well. After overnight incubation, supernatants were removed and the monolayers were washed two times with 200 μl of PBS. The lavaged pleural leukocytes were resuspended with F12 medium containing 2% bovine serum albumin (BSA; Sigma, Poole, UK) at different concentrations and 200 μl of cell suspension was pipetted onto mesothelial cell monolayers to achieve final ratios between pleural leukocytes (effectors) and mesothelial cells (targets) of 0:1, 0.1:1, 1:1, 10:1, and 20:1. After coculture of targets and effectors for 4 hr at 37°C, the microtiter plates were centrifuged for 5 min at 1000 rpm. Release of soluble ^{51}Cr, a measure of cell lysis was determined by aspirating 50 μl of supernatant from each well and counting in a gamma counter. Cell lysis was expressed as cpm after being multiplied by four.

The detachment of mesothelial cells was measured by aspirating the remainder of the supernatants and two washes of mesothelial cell monolayers with 200 μl of PBS and counting for ^{51}Cr activity in a gamma counter. The ^{51}Cr activity of detached and loosely adherent cells was determined by subtracting cell lytic ^{51}Cr activity from the count as

$$Y - (X \times 3),$$

where Y is the ^{51}Cr activity in the remaining supernatants of mesothelial cells and two washes and X is the ^{51}Cr activity in 50 μl supernatant of mesothelial cells.

The results of detachment are presented with background (detachment in the absence of leukocytes) subtracted. Because of averaging effects, some detachment results showed a low negative value which was treated as zero.

It was found that after 24 hr incubation, the spontaneous release of ^{51}Cr by mesothelial cells during 4 hr incubation in F12 plus 2% BSA was 18.4% of the total ^{51}Cr uptake as the total ^{51}Cr uptake was determined by lysis with 1% Triton X-100 (Sigma).

Presentation of Results and Statistical Analysis

Results were expressed as the mean and standard error as an index of variation. Results were subjected to analysis of variance and the Student *t* test to determine significant difference.

RESULTS

Normal Pleural Leukocytes

Table 1 shows that normal pleural leukocytes from both PVG and HAN strain rats contained four different cell types, i.e., macrophages, lymphocytes, mast cells, and eosinophils, the majority being macrophages (82 and 71%). HAN rats had more lavagable pleural leukocytes than PVG rats.

Pleural Leukocyte Components after Intratracheal Instillation of Crocidolite Asbestos

Figure 1 shows pleural leukocyte components after intratracheal instillation of 5 mg crocidolite asbestos. Eosinophil numbers increased modestly from 3 days to 14 days after asbestos instillation and then recovered by Day 30. The numbers of mast cells also increased slightly to Day 14 and quickly recovered. Macrophages showed a tendency to increase with elapsed time but this did not attain statistical significance. No neutrophils were observed in the populations throughout the experimental periods.

Figure 2 illustrates that 1 month after intratracheal instillation of various doses of crocidolite asbestos, there was an increase in the macrophage population of the pleural leukocytes. However, this macrophage recruitment only arose with the lower doses of asbestos and was not dose-related; therefore, we attach no biological significance to these changes. Other cell components of the leukocyte showed no marked changes even though the instilled doses of asbestos increased from 0.5 to 5 mg.

TABLE 1
DIFFERENT CELL TYPES PRESENT IN NORMAL PVG AND HAN RAT PLEURAL LEUKOCYTE POPULATIONS

	Total number ($\times 10^6$)	Percentage			
		Macrophage	Lymphocyte	Mast cell	Eosinophil
PVG	4.5 (1.0)[a]	82 (5.6)	1 (1.2)	11 (5.4)	6 (2.8)
HAN	8.7 (3.0)[b]	71 (2.6)	2 (1.6)	15 (2.4)	12 (4.6)

Note. Results are presented as means (SD).
[a] n = 14 rats.
[b] n = 4 rats.

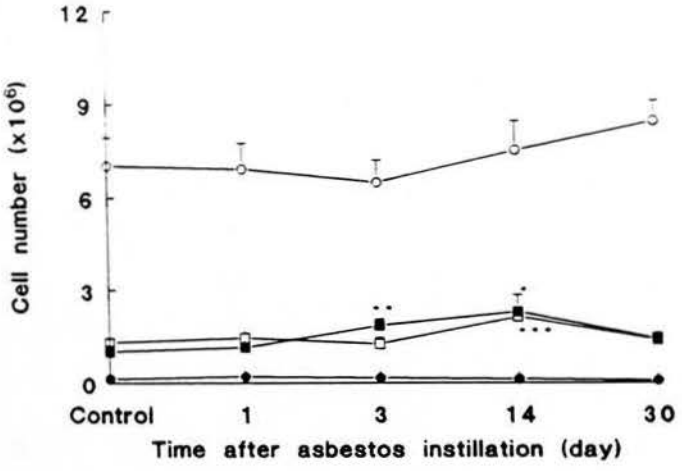


FIG. 1. HAN rat pleural leukocyte components at various times after intratracheal instillation of 5 mg crocidolite asbestos. Each point represents the mean and SEM of results from 7–12 rats. ○, macrophage; ●, lymphocyte; □, mast cell; and ■, eosinophil. Asterisks denote significant differences from control: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Our studies showed that the numbers of leukocyte common antigen present cells were the same for cell populations from both normal and treated rat lungs (around 95%). The remainder of the cells (around 5%) was assumed to be exfoliated mesothelial cells. Any difference between the pleural populations was very likely, therefore, to be a result of change in leukocytes and their functions and not the population of mesothelial cells.

Mesothelial Cell Detachment and Lysis Induced by Protease

We have previously demonstrated that detachment of epithelial cells by inflammatory leukocytes is caused principally by proteases. In the present study, both trypsin and elastase in culture, but not collagenase, caused detachment of mesothelial cells beginning at concentrations of 10 μg/ml, confirming the susceptibilities of mesothelial cells to protease-mediated detachment. All proteases used in the study caused slight lytic injury at high doses (Fig. 3).

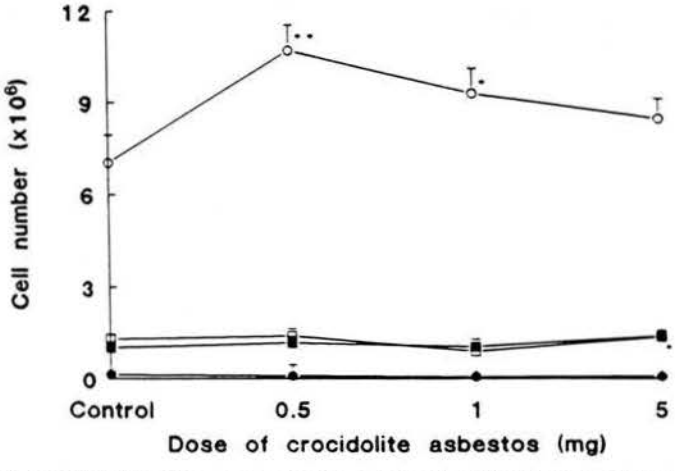


FIG. 2. HAN rat pleural leukocyte components 1 month after intratracheal instillation of various doses of crocidolite asbestos. Each point represents the mean and SEM of results from 6–12 rats. ○, macrophage, ●, lymphocyte; □, mast cell; and ■, eosinophil. Asterisks denote significant differences from control: $*P < 0.05$ and $**P < 0.01$.

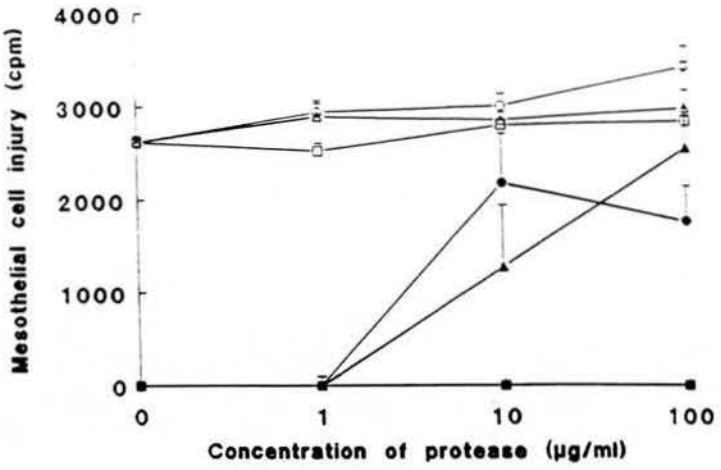


FIG. 3. Mesothelial cell lysis and detachment caused by proteases. Each point represents the mean and SEM of three separate experiments performed in triplicate. Mesothelial cell detachment induced by: ●, trypsin; ▲, elastase; and ■, collagenase. Mesothelial cell lysis induced by: ○, trypsin; △, elastase; and □, collagenase.

Mesothelial Cell Detachment Injury Caused by Pleural Leukocytes after Intratracheal Instillation of Crocidolite Asbestos

Coculture of normal HAN rat pleural leukocytes with mesothelial cells at increasing effector:target ratios induced neither detachment nor lytic injury of mesothelial cells. However, the pleural leukocytes from rats instilled intratracheally with 5 mg crocidolite asbestos 1 day previously caused substantial mesothelial cell detachment injury. This increased in response to increasing effector:target ratios (Fig. 4). By 3 days after instillation of 5 mg crocidolite asbestos, although pleural leukocytes still caused slight detachment of mesothelial cells in culture at the high effector:target ratios, the potency of the effect was much less than that seen at 1 day. By 14 and 30 days after intratracheal instillation of 5 mg asbestos, pleural leukocytes did not cause any detectable detachment of mesothelial cells.

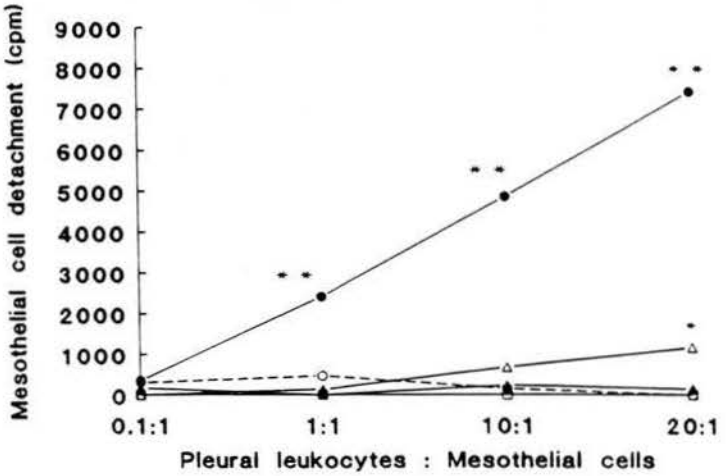


FIG. 4. Mesothelial cell detachment caused by HAN rat pleural leukocytes at various times after intratracheal instillation of 5 mg crocidolite asbestos. Each point represents the mean of results from 3–9 rats. SEM, which varies from 8 to 117% of the mean, is not shown in the graph. ○, control pleural leukocytes; ●, pleural leukocytes from rats 1 day after asbestos instillation; △, 3 days after; ▲, 14 days after; and □, 30 days after. Asterisks denote significant differences from spontaneous mesothelial cell detachment: $^{*}P < 0.05$ and $^{**}P < 0.01$.

Varied doses of asbestos (0.5, 1, and 5 mg) 30 days after intratracheal instillation did not alter the ability of pleural leukocytes to cause mesothelial cell injury compared to control, as confirmed by the results raised from experiments with 6–27 rats (data not shown).

Pleural Leukocyte Populations after Intratracheal Instillation of C. parvum

Figure 5 shows that 6 hr after *C. parvum* instillation, changes of cell components in the pleural space were negligible, except for a slight increase in the number of mast cells and eosinophils. On Day 1 there was a considerable increase in neutrophils, which is not a normal resident population in the pleural space, and a slight increase in macrophages. The numbers of neutrophils had decreased markedly by Day 3 and no neutrophils were found by Day 5. However, numbers of macrophages gradually increased up to Day 5.

Mesothelial Cell Detachment Injury Caused by Pleural Leukocytes Following Intratracheal Instillation of C. parvum

Normal pleural leukocytes of PVG rats did not induce any detectable mesothelial cell detachment (Fig. 6). However, one day after intratracheal instillation of *C. parvum*, the pleural leukocyte, which contained 29% neutrophils, induced a dose-related detachment of mesothelial cells in culture. This effect dramatically decreased 5 days after *C. parvum* instillation.

As a descriptor of direct cell injury, mesothelial cell lysis induced by pleural leukocytes was determined throughout all treatments. No detectable lytic effects were found except at a high ratio of leukocytes from rats 5 days after *C. parvum*, which showed modest but significant lytic injury (data not shown).

DISCUSSION

Mesothelial cells are highly susceptible to damage from agents that they do not normally encounter, including asbestos and silica. All of these cause swelling of the cells, clubbing of microvilli, and separation of the cells from each other and from the basal lamina, resulting in exfoliation (Herbert, 1986).

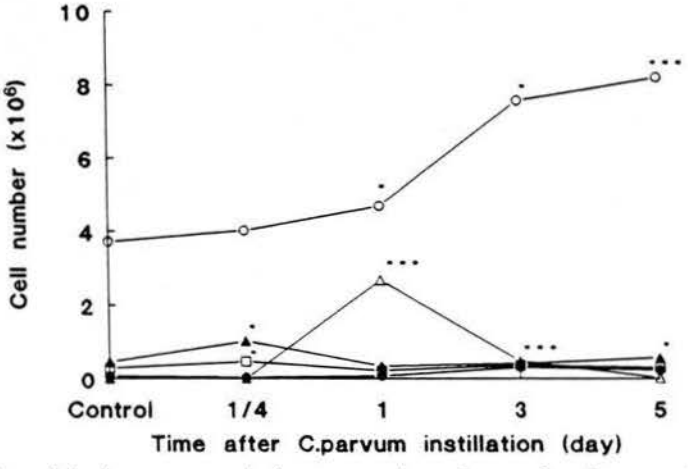


FIG. 5. PVG rat pleural leukocyte populations at various times after intratracheal instillation of *C. parvum*. The points show the mean of results from 2–13 rats. For clarity, SEM, which varies from 8 to 30% of the mean, is not presented in the graph. ○, macrophage; △, neutrophil; ●, lymphocyte; ▲, mast cell; and □, eosinophil. Asterisks denote significant differences from control: $*P < 0.05$ and $***P < 0.001$.

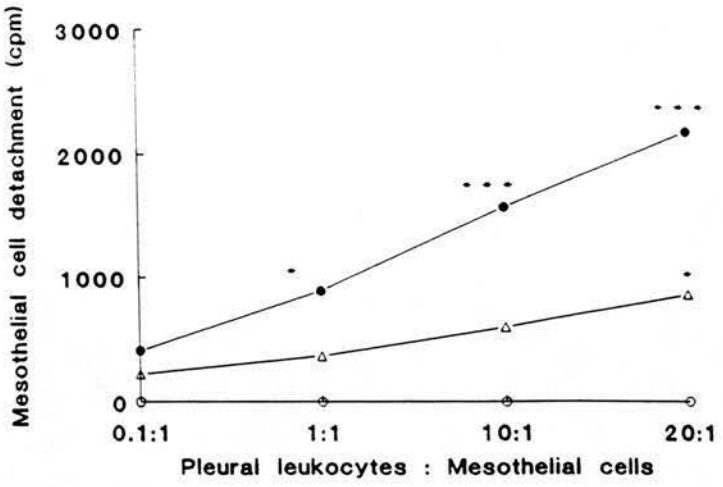


FIG. 6. Mesothelial cell detachment caused by PVG rat pleural leukocytes at various times after intratracheal instillation of *C. parvum*. Each point represents the mean of results from 2–4 rats. SEM, which varies from 7 to 115% of the mean, is not presented in the graph. ○, control pleural leukocytes; ●, pleural leukocytes from rats 1 day after intratracheal instillation of *C. parvum*; △, 5 days after. Asterisks denote significant differences from spontaneous mesothelial cell detachment: $^{*}P < 0.05$ and $^{***}P < 0.001$.

In view of the ability of inflammatory alveolar leukocytes to damage epithelial cells in culture (Donaldson *et al.*, 1987), we formed the hypothesis that pleural leukocytes could be activated to cause mesothelial cell injury following intratracheal instillation of asbestos fibers. Studies were therefore carried out to investigate the interaction between pleural leukocytes and mesothelial cells.

The present study shows that there was modest recruitment of macrophages 1 month after instillation of low doses of asbestos (0.5 and 1 mg) and modest alteration of other components of the pleural leukocyte population during experimental periods. It is notable that there was no increase in neutrophils. We have previously demonstrated detachment injury to epithelial cells by inflammatory bronchoalveolar leukocytes (Donaldson *et al.*, 1987). Using a similar assay for measuring cell detachment, but with mesothelial cells as target cells, in the present study we were able to demonstrate mesothelial cell injury caused by pleural leukocytes from rats intratracheally exposed to asbestos. The mesothelial cell detachment was observed in the absence of neutrophils and there was no lytic injury. Changes in cell components were not the explanation of the findings.

Neutrophil-induced detachment injury of rat pulmonary alveolar epithelial cells has been investigated previously. Ayars *et al.* (1984), Simon *et al.* (1986), and Donaldson *et al.* (1987) showed that stimulated neutrophils produced marked target cell detachment without lysis. However, neutrophils are not involved in mesothelial cell detachment caused by pleural leukocytes from rats exposed to asbestos in this study. It must be presumed that pleural macrophages or other pleural leukocyte components are triggered by inflammatory mediators from adjacent asbestos-exposed parenchyma to release proteases (Henson *et al.*, 1988) which are able to cause mesothelial cell detachment.

Our studies show altered functional changes of pleural leukocytes after intratracheal instillation of asbestos in the absence of evidence of any detectable translocation of asbestos fibers (Li *et al.*, 1991, 1992) and this is in keeping with other reports (Lehnert *et al.*, 1985; Dodson and Ford, 1985). However, although we show no evidence of involvement of asbestos fibers in pleural leukocyte-

mediated detachment of mesothelial cells *in vitro*, it is reasonable to believe that direct interaction of pleural mesothelial cells with asbestos fibers (Kanazawa, 1985), could also be one of causes of mesothelial cell damage *in vivo*.

The exogenous protease-mediated detachment profile of the mesothelial cells provided indirect evidence that an elastase/trypsin-like enzyme may play a role in the mesothelial cell detachment injury caused by inflammatory pleural leukocytes. The detachment is probably due to degradation of extracellular matrix for attachment of the target cells, mediated by elastase or trypsin-like protease (Ayars *et al.*, 1984; Donaldson *et al.*, 1987). Simon *et al.* (1986) suggested that damage to pulmonary alveolar epithelial cells caused by stimulated neutrophils was oxygen metabolite-independent. Brown and Donaldson (1988) found, in an investigation of the role of leukocytes in connective tissue derangements in the lung, that inflammatory cells which contained neutrophils caused consistently and substantially more degradation of fibronectin than controls on a per cell basis and that serine proteases are the major mediators responsible for this matrix degradation.

In vivo protease-mediated attacks on the mesothelial cells could lead to desquamation and exfoliation of mesothelial cells and increased permeability of the pleural barrier as well as damage to the lubrication function of the mesothelium. Exfoliation could stimulate increased mesothelial turnover to replace lost cells in the pleural space following asbestos exposure, as reported by Bryks and Bertalanffy (1971) and Coin *et al.* (1991). Mesothelial cells are not present in large numbers in washings from normal pleural spaces (Spriggs and Boddington, 1968), but are abundant in many forms of effusion (Herbert, 1986). A role for pleural leukocyte-mediated injury in contributing to this phenomenon is to be expected. However, in our own studies, large numbers of pleural mesothelial cells are not found after asbestos or *C. parvum* intratracheal instillation (Li *et al.*, 1995). Therefore the potential of pleural leukocytes to cause injury *in vivo* is likely to be reduced by anti-protease defence mechanisms in the pleura. Under these conditions, however, low-level attacks on the mesothelial cells could still lead to increased permeability of the mesothelial membrane and accumulation of fluid with a high protein content as described for some inflammatory processes and tumors of the pleura (Rennard *et al.*, 1985). Such alteration may finally lead to pathologic changes of mesothelial cells found in human and animals exposed to asbestos (Jaurand, 1985).

This study shows that activation of pleural leukocytes by intratracheal instillation of asbestos occurred in early days only, in terms of their ability to cause the detachment of mesothelial cells *in vitro*. This probably resulted from the single, intratracheal instillation used to administer asbestos to the lung. Presumably, such pleural leukocyte-mediated mesothelial cell alteration may continuously occur in *in vivo* situations where there may be existence of persistent stimulation by asbestos.

As well as asbestos, we utilized intratracheal *C. parvum* as a method of generating pleural inflammation. In contrast to what we found with asbestos, *C. parvum* exposure was characterized by recruitment of neutrophils into the pleural space by Day 1 and recruitment of macrophages by Day 5. Pleural leukocytes from rats 1 day following *C. parvum* exposure, which comprised 29% neutrophils, caused substantial mesothelial cell detachment in an effector:target-related manner. However, the leukocytes from rats 5 days after *C. parvum* instillation, which contained almost no neutrophils, had no effect in causing detachment injury of

mesothelial cells although these cells cause modest target cell lysis. This increased lysis is likely to be an anomalous result since it was not seen at the 1-day time point and was not found with increasing effector:target ratios. Neutrophils have the potential to cause epithelial cell detachment as described by Donaldson *et al.* (1987) and to cause mesothelial cell detachment as found here. The possibility that pleural macrophages or other components play a role in the *C. parvum* pleural leukocyte-mediated mesothelial cell injury cannot be excluded, as indicated by the present findings with asbestos treatment where detachment occurred in the absence of neutrophils.

In summary, intratracheal crocidolite asbestos and *C. parvum* stimulated pleural leukocytes to cause transient detachment injury of mesothelial cells *in vitro* at 1 day postexposure. With asbestos, however, the detachment occurred in the absence of neutrophils while for *C. parvum*, neutrophils were present. Mesothelial cells were detached by exogenous proteases, elastase, and trypsin, suggesting a role of these proteases. Studies involving the separation of pleural leukocytes into purified populations are necessary to clarify the role of these cells and will form the basis of future studies. The present study illustrates that deposition of asbestos in the airspace could result in pleural leukocyte-mediated mesothelial injury and this could be important in pleural pathology found in individuals exposed to asbestos. Studies with the heat-killed bacterium *C. parvum* suggest that pleural leukocyte-mediated mesothelial damage could result from acute bacterial pneumonitis.

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Section 6

Section 6 Neutrophils and oxidative stress in the lung

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Lack of effect of N-acetylcysteine on the release of oxygen radicals from neutrophils and alveolar macrophages

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Lack of effect of N-acetylcysteine on the release of oxygen radicals from neutrophils and alveolar macrophages. E. Drost, S. Lannan, M.M.E. Bridgeman, D. Brown, C. Selby, K. Donaldson, W. MacNee.

ABSTRACT: N-acetylcysteine (NAC) is rapidly de-acetylated *in vivo* to cysteine (CYSH), a precursor of glutathione (GSH) which is an antioxidant in cells and body fluids.

We investigated the effect of oral administration of N-acetyl cysteine for 5 days on the spontaneous and stimulated generation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) from human and rat phagocytic leucocytes. Alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) in control rats and rats given NAC in their drinking water. Neutrophils (PMNL) were harvested from whole blood in normal nonsmoking volunteers before and after NAC was given by mouth.

The stimulated release of H_2O_2 and O_2^- from both rat AM and human PMN was not changed by administration of NAC. However, a small but significant increase was observed in both the spontaneous generation of O_2^- from rat AM and the spontaneous generation of H_2O_2 from human PMNL. Administration of NAC significantly increased cysteine levels in human plasma and rat BAL, but the levels in human PMNL and rat AM after NAC did not differ from control levels. GSH levels were not altered significantly by NAC.

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Phagocytic cells, alveolar macrophages and neutrophils, are present in increased numbers in the lungs of cigarette smokers [1–3] and patients with inflammatory lung diseases [4–6]. Stimulation of these inflammatory cells results in the release of proteolytic enzymes and an increase in the respiratory burst, causing the release of reactive oxygen intermediates [7–9]. Inappropriate or excessive release of these oxygen radicals may cause damage to the lung tissue, either directly, by peroxidation of membrane lipids and proteins, or indirectly by inactivating protease inhibitors, thus favouring proteolysis. The balance between the release of oxidants, and their inactivation by antioxidants could therefore be critical to the prevention of lung damage during the inflammatory response to bacteria and irritant inhalants such as cigarette smoke. Agents capable of reducing the release of oxidants, or inactivating them once released could therefore have a therapeutic value.

Excess reactive oxygen intermediates can be efficiently removed by the actions of superoxide dismutase on the superoxide anion (O_2^-), and by catalase and peroxidases on hydrogen peroxide (H_2O_2). Moreover, reducing the concentrations of H_2O_2 and O_2^- in the extracellular fluid limits the generation of the highly reactive hydroxyl radical (OH), produced by the Fenton reaction, and hypochlorous acid (HOCl), mediated by myeloperoxidase in the presence of halide [10].

Previous work has demonstrated the effectiveness of N-acetylcysteine (NAC) as an antioxidant *in vitro* [11–14]. However, *in vivo*, NAC needs to be present in high concentrations in extracellular fluid to scavenge oxygen radicals. Adequate concentrations of NAC may be difficult to achieve in plasma, since the bioavailability of the drug, following oral dosing, is reported to be less than 5% [12]. However, NAC has been observed to attenuate the injurious effects of endotoxin in the lungs of sheep [14] and to normalize cigarette smoke induced dysfunction in macrophages [2, 15, 16], lung fibroblasts and epithelial cells [12], in both rats and humans. Further *in vitro* work has suggested that NAC can prevent the inactivation of alpha$_1$-proteinase inhibitor (α_1-PI) by scavenging the neutrophil myeloperoxidase derived oxidant, hypochlorous acid (HOCl) [17, 18]. NAC is rapidly de-acetylated to cysteine during its first pass through the liver. Moreover, cysteine is a precursor in the biosynthesis of glutathione, itself and antioxidant [13, 15].

The aims of this study were to measure the effect of NAC, given by mouth, on NAC, cysteine and glutathione levels in human plasma and neutrophils, and in bronchoalveolar lavage fluids and alveolar macrophages from rats. Secondly, we wished to determine if the administration of NAC affected the subsequent generation of H_2O_2 and O_2^- from rat leucocytes.

Subjects and methods

N-acetylcysteine in the form of a water soluble powder containing 200 mg NAC per sachet was provided by Zambon Pharmaceuticals, Milan, Italy.

Rat bronchoalveolar lavage studies

Syngeneic specific pathogen free (SPF) rats (200 g) of the PVG strain were given NAC in their drinking water such that they ingested 10 $mg \cdot kg^{-1} \cdot day^{-1}$ (twice the human dose). After treatment for 5 days the rats were killed, their lungs removed and lavaged with 4×8 ml aliquots of saline as described previously [19]. The resulting bronchoalveolar lavage fluid was spun at 1,600 rpm, at 4°C for 10 min. Bronchoalveolar leucocytes (97% alveolar macrophages and 3% lymphocytes) were resuspended in phosphate buffered saline (PBS) to a concentration of 1×10^7 $cells \cdot ml^{-1}$. Comparisons were made with alveolar macrophages from PVG rats which were not given NAC in their drinking water.

Human neutrophil studies

Eleven normal, nonsmoking volunteers (aged 24–37 yrs) were given 600 mg of NAC by mouth for 5 days. Thirty ml of citrated venous blood were withdrawn from each subject on Day 1, prior to the first dose of NAC, and on Day 5, one and a half hours after the final dose. The samples of whole blood were mixed with Dextran (MW 70,000) (Travenol, UK) and allowed to sediment for one hour. The resulting leucocyte-rich plasma was overlaid on a Plasma/Percoll density gradient (Pharmacia, Sweden) and centrifuged to obtain a leucocyte band which was washed and lysed to remove contaminating erythrocytes, using a technique described previously [1]. The remaining neutrophil population (>95% pure) was resuspended in PBS at a concentration of 1×10^7 $cells \cdot ml^{-1}$. The viability of neutrophils harvested using this technique was >98% by trypan blue exclusion.

Hydrogen peroxide assay

H_2O_2 generation was measured by the method of Pick and Keisari [20]. The reaction mixture consisted of 100 ml 10 mM potassium phosphate buffer (10 mM KH_2PO_4, 7.6 mM K_2HPO_4), containing 1 ml of stock Type II, salt-free horseradish peroxidase 5 $mg \cdot ml^{-1}$ in 0.05 M potassium phosphate buffer; 1 ml of 0.028 M phenol red; 140 mM sodium chloride and 5.5 mM dextrose (Sigma, UK). Alveolar macrophages or neutrophils (2.5×10^5 cells) were added to 1 ml of the reaction mixture. Both the spontaneous and phorbol myristate acetate (PMA, 1 $\mu g \cdot ml^{-1}$) (Sigma, UK) stimulated release of H_2O_2 were measured after 2 h of incubation at 37°C, in 5% CO_2. The reaction was terminated by centrifugation at 2,000 rpm, at 4°C for 10 min and the colour reaction was completed by alkalization with 1 M NaOH. The absorbance of the supernatant fluid was measured on a Pye Unicam SP8-400 spectro photometer at 610 nm and compared with a standard curve derived from dilutions of a reference solution of H_2O_2.

Superoxide anion assay

O_2^- generation was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c* [21]. Inclusion of the enzyme SOD eliminates all cytochrome *c* reduction by agents other than O_2^-. The reaction mixture contained 80 M cytochrome *c* and 0.65 mM dextrose (Sigma, UK) in 50 ml of PBS. Tubes containing 1 ml of the reaction mixture and 2.5×10^5 alveolar macrophages or neutrophils were set up with identical tubes which, in addition, contained 75,000 units SOD (Sigma, UK). Release of O_2^- either spontaneously, or when stimulated with PMA 1 $\mu g \cdot ml^{-1}$, was measured after incubation for 2 h at 37°C in 5% CO_2. O_2^- generation is linear for up to 2 h incubation [21–23]. The reactions were terminated by centrifugation at 2,000 rpm, at 4°C for 10 min. The difference in absorbance of the supernatant fluids, in the presence or absence of SOD, was determined in a Pye Unicam SP8-400 spectrophotometer at 550 nm, and the amount of reduced ferricytochrome *c* was calculated based on an extinction coefficient of 21.0 $mM^{-1} \cdot cm^{-1}$ for cytochrome *c*.

NAC, cysteine and glutathione assays

NAC, cysteine and reduced glutathione concentrations were determined by derivatization with monobromobimane (Thiolyte, Calbiochem) to form fluorescent adducts, which were then separated and quantified using reverse phase high performance liquid chromatography [24, 25].

Catalase assay

Catalase activity was determined using a spectrophotometer assay with titanium sulphate [25].

Statistical analysis

Statistical analyses were performed on the mean values and standard deviations using the Student's t-test for paired samples.

Results

Reduced or free thiol levels measured in human plasma or neutrophils, and rat bronchoalveolar lavage fluid (BALF) or alveolar macrophages are shown in tables 1 and 2. In normal subjects following treatment with NAC, plasma cysteine increased significantly ($p<0.01$) with a trend for plasma GSH to increase, occurring in 5 of the 8 subjects studied. However, neither the level of intracellular cysteine nor free glutathione (GSH) in neutrophils changed significantly. In the rat, cysteine levels increased in BALF without significant change in the GSH levels after treatment with NAC (table 1). NAC was not detectable in human plasma and neutrophils nor in rat macrophages and BALF (data not shown).

In separate experiments, intracellular cysteine and GSH in rat macrophages did not alter following treatment with NAC. However, catalase activity measured in rat macrophages was observed to increase following NAC (control 63.6±16.3, NAC 84.0±13.8 $U \cdot mg^{-1}$ protein; n=6: $p<0.05$), but was not detectable in the extracellular fluid. In these experiments NAC produced a significant increase in BALF GSH but no change in cysteine levels (table 2).

The spontaneous or stimulated generation of H_2O_2 by rat alveolar macrophages did not change following treatment with NAC (spontaneous release H_2O_2: control 6.36±2.4, NAC 6.9±2.8 $nM \cdot 2.5 \times 10^{-5}$ cells; stimulated release H_2O_2: control 15.3±3.8, NAC 14.4±4.2 $nM \cdot 2.5 \times 10^{-3}$ cells; n=9, $p>0.05$) (fig. 1A).

There was a small, but statistically significant ($p<0.001$) increase in the spontaneous generation of O_2^- from rat alveolar macrophage after treatment with NAC, with no change in the stimulated O_2^- release (spontaneous release O_2^-: control 1.17±0.3, NAC 2.14±0.3 $nM \cdot 2.5 \times 10^{-5}$ cells; n=9, $p<0.001$; stimulated release O_2^-: control 9.23±1.1, NAC 9.2±0.6 $nM \cdot 2.5 \times 10^{-5}$ cells; n=9 $p>0.05$) (fig. 1B).

Table 1. – Cysteine (CYSH) and glutathione (GSH) levels in human plasma and neutrophils (PMNL) and rat bronchoalveolar lavage (BAL) before and after N-acetylcysteine (NAC)

	CYSH		GSH	
	Control	NAC 5 days	Control	NAC 5 days
Normal subjects				
Plasma $nM \cdot ml^{-1}$	6.7±1.9 (n=11)	9.8±3.5**	2.9±1.1 (n=8)	4.3±2.4
PMNL $nM \cdot 10^{-6}$ cells	0.1±0.1 (n=11)	0.1±0.0	1.7±0.6 (n=10)	1.9±0.4
PVG rats BAL $nM \cdot ml^{-1}$	0.7±0.6 (n=9)	1.1±0.9*	2.7±2.7 (n=9)	2.5±1.4

**p<0.01; *p<0.05.

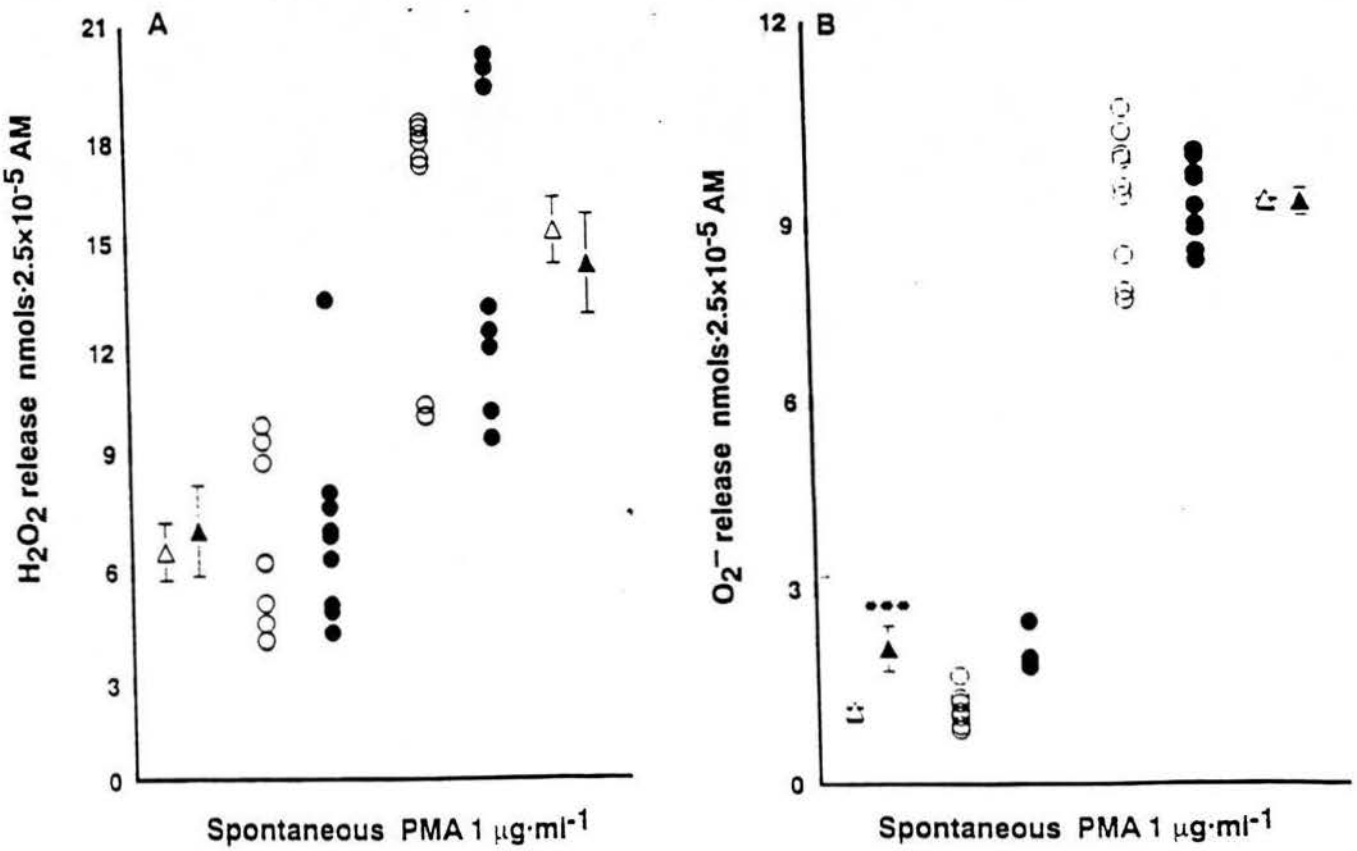


Fig. 1. – (A) Hydrogen peroxide (H_2O_2) and (B) superoxide anion (O_2^-) production by alveolar macrophages obtained from 9 rats after 5 days' treatment with N-acetylcysteine (NAC) (●), and 9 control animals not given NAC (○). Both spontaneous levels and those on stimulation with phorbol-myristate-acetate (PMA) and the mean values (▲) are shown. The bars represent the standard errors of the mean. (***: $p<0.001$).

Table 2. – Cysteine (CYSH) and glutathione (GSH) levels in rat bronchoalveolar lavage (BAL) and alveolar macrophages (AM) before and after N-acetylcysteine (NAC)

	CYSH		GSH	
	Control	NAC 5 days	Control	NAC 5 days
PVG Rats BAL $nM \cdot ml^{-1}$	0.3±0.2 (n=6)	0.3±0.1	3.2±0.9 (n=6)	4.5±1.1*
AM $nM \cdot 10^{-6}$ cells	0.7±0.2 (n=6)	0.6±0.1	3.4±0.8 (n=6)	3.2±0.9

*p<0.05.

statistical significance (stimulated release H_2O_2: control 27.1±10.0 NAC 45.7±27.1 $nM \cdot 2.5 \times 10^{-5}$ cells; n=11, p>0.05).

The spontaneous generation of O_2^- from human neutrophils tended to increase following NAC but this change was not significant (spontaneous release O_2^-: control 1.5±0.4, NAC 2.2±1.4 $nM \cdot 2.5 \times 10^{-5}$ cells; n=11, p>0.05). The PMA stimulated release of O_2^- by contrast tended to fall following treatment with NAC, but again this change was not significant (stimulated release O_2^-: control 18.6±1.6, NAC 9.9±5.2 $nM \cdot 2.5 \times 10^{-5}$ cells; n=11, p>0.05) (fig. 2B).

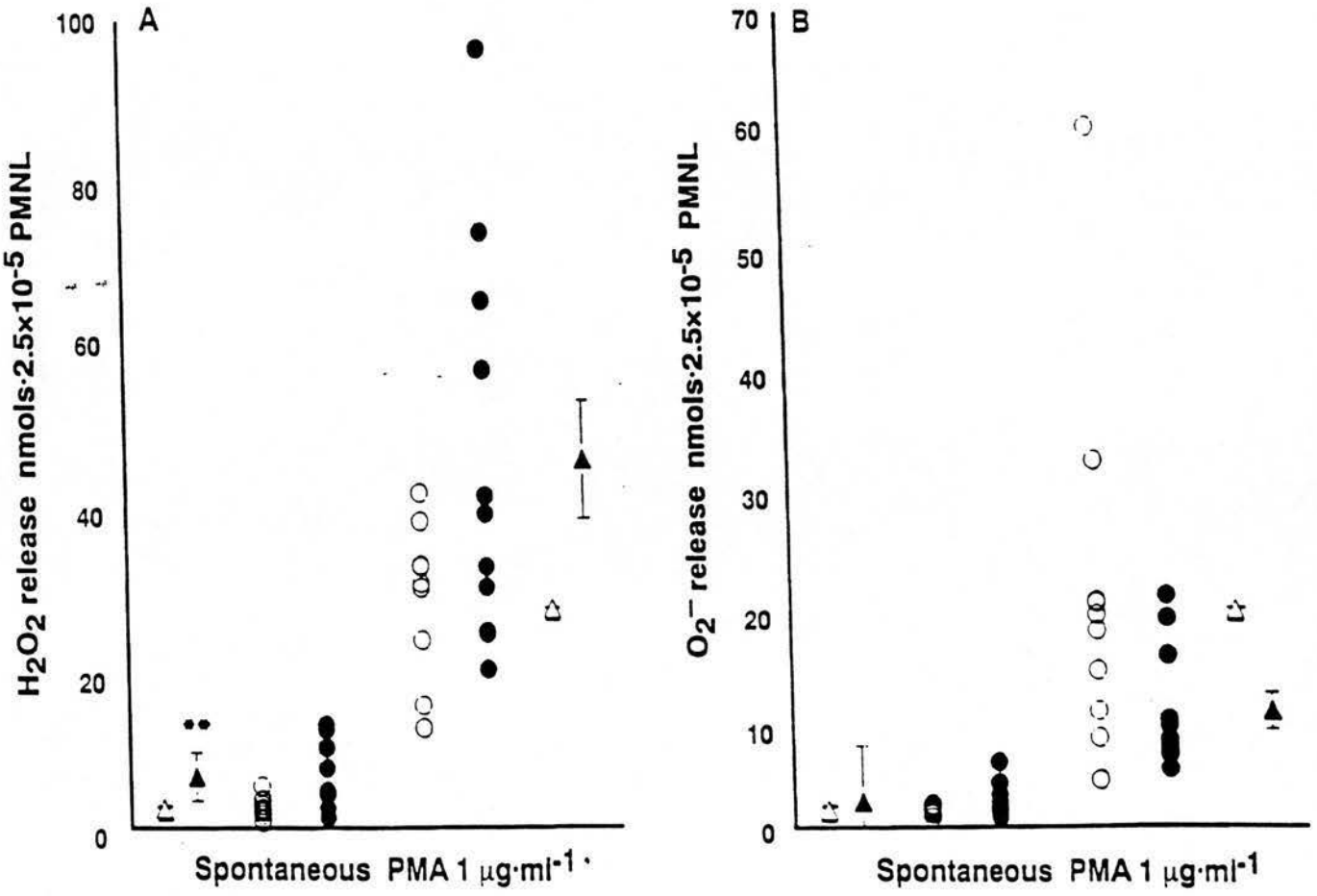


Fig. 2. – (A) Hydrogen peroxide (H_2O_2) and (B) superoxide anion (O_2^-) production by human neutrophils from 11 normal subjects before (○) and after (●) 5 days' treatment with N-acetylcysteine (NAC). Both the spontaneous levels and those on stimulation with phorbol-myristate-acetate (PMA) and the mean values (▲) are shown. The bars represent the standard errors of the mean. **: p<0.02.

Human neutrophils from 11 normal subjects treated with NAC for 5 days showed a small but significant increase in the spontaneous release of H_2O_2 compared with pretreatment values (spontaneous release H_2O_2: control 2.5±1.3, NAC 6.4±4.3 $nM \cdot 2.5 \times 10^{-5}$ cells; n=11, p<0.02) (fig. 2A). Although neutrophils from 7 of the 11 subjects showed an increase in the PMA stimulated generation of H_2O_2, this trend did not achieve

Discussion

This study failed to show any significant decrease in the release of H_2O_2 or O_2^- from human peripheral blood neutrophils and rat alveolar macrophages. On the contrary, small increases in the spontaneous release of O_2^- from rat macrophages and H_2O_2 from human neutrophils was demonstrated.

The thiol compound N-acetylcysteine (NAC) is known to scavenge free electrolytes such as hydrogen peroxide (H_2O_2), and reactive oxygen species such as superoxide anion (O_2^-). *In vitro* studies have previously demonstrated a reduction in the chemiluminescence of stimulated human neutrophils following incubation with NAC [11]. Similarly BERNARD *et al.* [14], employed the chemiluminescence assay to demonstrate the reduced ability of zymosan or PMA stimulated sheep neutrophils to scavenge oxygen metabolites when incubated with NAC. However, the bioavailability of NAC is only 5%, because of its extensive first pass metabolism by the liver [12]. Indeed NAC was not detectable in plasma or BALF in this study, or in our previous study [26]. Thus, it is unlikely that concentrations of NAC in body fluids are high enough to achieve such a scavenging action.

Nevertheless, recent *in vivo* work has shown that NAC protects against smoke induced cellular damage to both the rat [12] and human lung [2, 12, 18], possibly by enhancing cysteine levels and thus GSH biosynthesis [13]. Further *in vivo* studies, such as that by BERGSTRAND *et al.* [15] have also demonstrated a reduction in O_2^- generation by smokers' macrophages after treatment with NAC for 8 weeks. Similarly, treatment with NAC was observed to reduce the chemiluminescence of zymosan stimulated human neutrophils [27]. Furthermore, DE FLORA *et al.* [28] observed increased hexose monophosphate shunt (HMPS) activity in rat macrophage following NAC, and KHARAZMI *et al.* [11] observed an increased oxygen consumption, also suggesting increased HMPS activity, in the face of a reduction in chemiluminescence in neutrophils treated with NAC. The authors of these studies suggest that the increased activity of the HMPS following NAC may be explained by an increase in H_2O_2 catabolism by the glutathione system.

The aim of this study was to determine if NAC given *in vivo* resulted in increased glutathione biosynthesis, thereby preventing free radical generation as suggested in recent reports [29, 30]. However, in this study we have employed specific assays to detect nanomole amounts of H_2O_2 and O_2^-, whereas many of the previously published studies which have investigated the role of NAC as an antioxidant have used the less specific chemiluminescence assay [11, 14, 27].

Our results from human studies indicate that although there was a small increase in plasma cysteine, intracellular thiol levels were not significantly altered by NAC. Although MOLDEUS *et al.* [12] have shown a significant increase in plasma GSH in healthy volunteers following NAC 200 mg twice daily for two weeks, we have previously shown that following 5 days' treatment with a single dose of NAC the GSH levels peak at variable intervals between 2 and 8 h [26]. Plasma cysteine levels, however, peak 90 min after the last dose of NAC [26]. The fact that we sampled blood 90 min after the last dose of NAC may therefore explain the significant increase in plasma cysteine but not plasma GSH levels. It is also possible that any increase in GSH may have been lost as a result of the two hour period required to harvest a pure population of neutrophils. However, intracellular thiol levels which did not increase following NAC, are likely to be more stable than plasma and BALF levels (M. Bridgemen, unpublished observations). We assayed total *i.e.* both oxidised and reduced cysteine and GSH levels, in some of the human subjects (data not shown). These were only marginally higher than the reduced values alone, which is in agreement with other workers [31] and suggests that most of the thiols were present in the free, reduced form.

There was no significant reduction in the release of H_2O_2 or O_2^- from neutrophils following treatment with NAC. Indeed, the spontaneous production of H_2O_2 was significantly increased after NAC. A similar trend for the PMA stimulated release of H_2O_2 and the spontaneous release of O_2^- to increase was also observed. An increase in the spontaneous release of H_2O_2 from neutrophils, following NAC without a significant reciprocal change in O_2^- production, as observed in this study may simply be explained by the individual results in figure 2 which show a similar trend for spontaneous O_2^- to increase following NAC as with H_2O_2. However, the molar ratios observed in this study also suggest that significantly more H_2O_2 than O_2^- was generated from stimulated human neutrophils following treatment with NAC. This appears stoichometrically erroneous since the reduction of molecular oxygen:

$$O_2^- + 2e \rightarrow 2O_2$$
$$2O_2 + 2H^+ \rightarrow H_2O_2 + O_2^-$$

predicts a molar ratio of 2:1 for O_2^-/H_2O_2 production. This apparently contradictory result could be explained by the release of O_2^- from the cell membrane into phagosomes which would prevent its detection in our assay by the scavenger cytochrome *c*. Furthermore, within the phagosome the O_2^- would have more time, both spontaneously and in the presence of SOD, to dismutate to H_2O_2 before its secretion/diffusion out of the cell [32]. This mechanism may also explain the decrease observed in the stimulated release of O_2^- from human neutrophils following NAC occurring in 8 of the 11 subjects studied (fig. 2B). In addition, any catabolism of H_2O_2 by myeloperoxidase in the phagosome, or by catalase or the glutathione system in the cytosol could also reduce the levels of H_2O_2 which were detected. Indeed, plasma myeloperoxidase was observed by EKLUND *et al.* [33] to fall following treatment over 8 weeks with NAC. Yet, despite the possible catabolism of H_2O_2 by myeloperoxidase, an increased H_2O_2 production was evident in most individuals following NAC in this study. Reduced chemiluminescence observed by many workers following treatment with NAC [11, 14, 27] may reflect a reduction in myeloperoxidase activity as a result of myeloperoxidase entrapment in phagosomes and/or its catabolism of H_2O_2, as the chemiluminescence assay is both myeloperoxidase and O_2^- dependent [23]. Intracellular thiol levels in human neutrophils, however, did not increase following NAC.

In the animal study, our results indicate a small increase in the spontaneous O_2^- production from rat alveolar macrophage following treatment with NAC. The animals did ingest NAC, as shown by the significant increase in BAL cysteine, although the changes were small (table 1) and were not sufficient to have a significant effect since intracellular thiols did not alter (table 2). Recent human studies have demonstrated that thiol levels in BAL fluid increase in a dose-dependent fashion [26]. However, in separate experiments in rats, we showed a significant increase in intracellular catalase activity in alveolar macrophages but not in BAL, which may also explain the lack of any increase in spontaneous H_2O_2 generation from alveolar macrophages in spite of a small, but significant, increase in spontaneous O_2^- release following NAC. Interference of the O_2^- assay by catalase is unlikely as cytochrome *c* reduction is not affected by catalase. In this case, in contrast to the data in table 1, GSH but not cysteine levels increased in BALF (table 2). This apparent contradictory result, we believe, may result from a later sampling time of BALF following NAC ingestion in these experiments since our previous work suggests that cysteine peaks earlier than GSH in BALF in man following ingestion of NAC [26]. However, because of the difficulty in accurately determining the time from last dosage of NAC to the time when lavage was carried out in the rat, this hypothesis remains unproven.

In summary, although N-acetylcysteine may act as an antioxidant *in vitro* as a precursor of cysteine and thus glutathione biosynthesis, we found no evidence to suggest that administration of NAC resulted in reduced H_2O_2 and O_2^- generation from lung phagocytes. Indeed, NAC significantly increased the spontaneous oxygen radical production from these cells. This increase in oxygen radical release was small and, therefore, is unlikely to have any clinical significance. An increase in plasma cysteine, however, in the absence of free iron, may scavenge free radicals released into the micro-environment surrounding phagocytic cells, and may help to explain the effect of NAC in protecting the lungs against injury induced by inhaled oxidants such as cigarette smoke, or released from activated phagocytes during inflammation or infection.

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Absence d'effet de la N-acétyl cystéine sur la libération de radicaux d'oxygène à partir des neutrophiles et des macrophages alvéolaires. E. Drost, S. Lannan, M.M.E. Bridgeman, D. Brown, C. Selby, K. Donaldson, W. MacNee.

RÉSUMÉ: La N-acétyl cystéine (NAC) est rapidement désacétylée *in vivo* en cystéine (CYSH), un précurseur du glutathion (GSH) qui est un anti-oxydant dans les cellules et les liquides organiques.

Nous avons investigué les effets de l'administration orale de N-acétyl cystéine pendant 5 jours sur la production spontanée et stimulée de peroxyde d'hydrogène (H_2O_2) et d'anion superoxyde (O_2^-) par les leucocytes phagocytaires de l'homme et du rat. Des macrophages alvéolaires ont été obtenus par lavage broncho-alvéolaire chez les rats contrôle et les rats auxquels NAC avait été administrée dans leur eau de boisson. Les neutrophiles (PMNL) ont été prélevés dans le sang complet chez des volontaires normaux non fumeurs, avant et après administration de NAC par la bouche.

La libération stimulée de H_2O_2 et de O_2^- à partir des macrophages alvéolaires du rat et des polymorphonucléaires humains n'est pas modifiée par l'administration de NAC. Toutefois, une augmentation modeste mais significative a été observée dans la production spontanée de O_2^- par les macrophages alvéolaires du rat et la production spontanée de H_2O_2 par les polymorphonucléaires humains. L'administration de N-acétyl cystéine augmente significativement les niveux de cystéine dans le plasma humain et dans le BAL du rat, mais les niveaux observés dans les PMNL humains et les AM du rat après NAC ne sont pas différents de ceux des contrôles. Les niveaux de GSH n'ont pas été affectés significativement par la NAC.
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Reduction of the Proteolytic Activity of Neutrophils by Exposure to Cigarette Smoke in Vitro

G. M. Brown, E. Drost, K. Donaldson, I. MacGregor, and W. MacNee

ABSTRACT: *Human peripheral blood neutrophils were exposed in vitro, in a tonometer, to two different fractions of cigarette smoke—designated particulate phase and vapor phase. The proteolytic activity of the cells following exposure was assessed by measuring their elastase release and ability to degrade fibronectin. At levels of smoke exposure that were physiologically attainable, neither smoke fraction caused an increase in elastase release or fibronectin degradation. In most experiments, fibronectin proteolysis was suppressed by smoke exposure—an effect that was reversible on treatment with phorbol myristate acetate. These data provide evidence that the proteolytic activity of neutrophils is not enhanced by a direct effect of cigarette smoke on these cells.*

INTRODUCTION

Proteases, secreted by inflammatory leukocytes, have been implicated in causing injury to the cells and connective tissue of the alveolar walls, both in humans [1, 2] and in animal models [3, 4]. Neutrophil elastase in particular is thought to play a major role in the pathogenesis of centrilobular emphysema in cigarette smokers. Support for this hypothesis comes from studies showing increased numbers of neutrophils [5] and enhanced elastolytic ac-

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tivity [6, 7] in the bronchoalveolar lavage fluid of smokers. There may also be an increased burden of elastase in the alveolar capillaries due to increased retention of neutrophils in the pulmonary microvasculature during smoking [8]. This is supported by the finding of increased levels of fibrinopeptide-A, a specific product of the cleavage of fibrinogen by elastase, in the plasma of cigarette smokers [9].

Elastase is released by neutrophils during phagocytosis, in response to specific stimuli such as chemotaxins and, of course, on cell death [10]. Cigarette smoke exposure could conceivably cause increased elastase release by any of the foregoing mechanisms since it is toxic for cells [11], can activate complement to generate chemotaxins [12], and contains a particulate component that may activate neutrophils following phagocytosis. Blue and Janoff [13] demonstrated elastase release from neutrophils exposed to cigarette smoke condensate in vitro at levels that were toxic to the cells. However, the concentrations of condensate that proved to be toxic were calculated to be in excess of those occurring in lung tissue, even in heavy smokers. We recently repeated this work and also found increased elastase release only at excessive concentrations of cigarette smoke condensate [14]. The aim of the present study was to investigate the ability of cigarette smoke to activate neutrophils in vitro at concentrations that might be physiologically attainable.

The three sites where neutrophils present in the lungs may be exposed to cigarette smoke are in the bronchoalveolar space, the interstitium, and the pulmonary capillaries. Therefore, we prepared two different fractions of cigarette smoke. The first, which we designated particulate phase smoke, was chosen to represent the type of cigarette smoke to which neutrophils might be exposed in the bronchoalveolar space. The other, which we designated vapor phase smoke, was chosen as representative of the fraction of smoke that might gain access to the interstitium and to the capillary blood. We exposed human peripheral blood neutrophils in vitro to each cigarette smoke fraction and then measured two key aspects of their proteolytic activity. First, we measured the release of elastase as an indicator of the potential protease burden that might be attained in the lung. Second, we assessed the ability of intact neutrophils to damage lung connective tissue by measuring their ability to degrade fibronectin, a major component of the connective tissue of the alveolar walls [15]. Protein kinase C is part of the second messenger system of the cell, which governs secretion of enzymes and other molecules [16]. We therefore measured fibronectin proteolysis by control or smoke-exposed cells in the presence of phorbol myristate acetate (PMA), which is a potent stimulator of protein kinase C activity. In addition, the role of a phagocytic burden in eliciting increased proteolytic activity was assessed by measuring fibronectin proteolysis in the presence of zymosan.

MATERIALS AND METHODS

Cell Preparation

Venous blood from healthy, nonsmoking volunteers was withdrawn into a syringe containing acid citrate dextrose. A leukocyte-rich plasma (LRP) was obtained by Dextran (Travenol, Thetford, Norfolk; MW 70,000) sedimentation, followed by centrifugation on a discontinuous plasma-percoll (Pharmacia, Uppsala, Sweden) (42% and 65%) gradient, as previously described [8], to obtain a neutrophil band, which was then aspirated. Contaminating erythrocytes were lysed with 0.2% hypotonic saline. The neutrophils were washed with phosphate-buffered saline (PBS) and resuspended to a concentration of 1×10^6/mL in PBS containing 0.5% bovine serum albumin (PBS/BSA) (BSA-Sigma, Poole, Dorset). This technique yielded neutrophil populations that were always greater than 94% pure and were 99% viable as measured by trypan blue exclusion.

In Vitro Cigarette Smoke Exposure

Neutrophils were exposed to five puffs of smoke generated from a single medium-tar cigarette using an animal respirometer (Model 607, Harvard Apparatus Co., Dover, MA). The cigarette smoke was passed through a loosely packed cotton wool plug to remove the largest particles and was then cooled and humidified by bubbling through water. This procedure was designed to be representative of the filtering and moisturizing effects of the conducting airways of the lung. The particulate smoke fraction produced in this way was visible to the naked eye and was composed of both particulate- and vapor-phase smoke. Vapor-phase smoke was produced in the same way, except that particulate material was removed by inserting a Cambridge filter (0.3-μm pore size) in line immediately before the smoke was passed into the tonometer. This smoke fraction was invisible to the naked eye. The leukocyte suspensions (2×10^6 in 2 mL PBS/BSA) were placed in the clean, siliconized flask of a tonometer system (Vitalograph, England). The tonometer flask is spherical and of 5 cm diameter. Agitating the cell suspension in the flask created a thin film of cells over the surface. The smoke fractions (or air in the case of control samples) were passed into the flask, which was then agitated gently for 4 min at ambient temperature (22°C). Neither air nor smoke was bubbled through the cell suspension. This method ensured that the cells received maximum exposure to gaseous smoke and not a smoke condensate. In the dose/response experiments PMN were exposed to only three or one puff of smoke, but the duration of exposure remained constant. Diluted smoke was obtained by puffing it through an air-filled glass bottle prior to passing it into the tonometer. To assess the effect of a

reduced exposure time, one puff of smoke was passed into the tonometer and the cells were removed after only 2.5 min.

Carboxyhemoglobin Levels

To check that the levels of smoke exposure in our experiments were constant, we measured, in each subject, the change of carboxyhemoglobin (CoHb) in whole blood before and after exposure for 4 min to particulate- or vapor-phase cigarette smoke in the tonometer, as described above. CoHb was measured by a co-oximeter (Instrumentation Laboratory UK Ltd., Kelvin Close, Birchwood, Warrington, Cheshire).

Elastase Radioimmunoassay

Immunoreactive elastase was measured following smoke exposure in the tonometer by the method of Greer et al. [16], in aliquots of whole blood and in the supernatants obtained from the neutrophil suspension, both before and after smoke exposure. The antibody was specific for neutrophil elastase; it did not cross-react with platelet or pancreatic elastase.

Fibronectin Proteolysis

Proteolysis of fibronectin was measured before and after smoke exposure in a solid phase assay, which we previously described [4]. Briefly, ^{125}I-labeled fibronectin was adhered in microtiter plates. Smoked and unsmoked neutrophils (1×10^5 cells) in 200 μL of medium (F10 + 2% BSA) (F10, Gibco, Paisley) were cultured on the matrix for 4 h at 37°C and proteolysis was assessed by measuring the ^{125}I counts released into the supernatant medium. Proteolysis was measured in the presence or absence of phorbol myristate acetate (PMA) or the particulate trigger, zymosan (Sigma, Poole, Dorset).

Transmission Electron Microscopy

To detect changes in neutrophil morphology following smoke exposure, control and smoke-exposed cells from six separate experiments were fixed for 24 h in 10 mL of a 2% glutaraldehyde solution (pH 7.4). Thereafter, they were washed in normal saline, fixed with 1% O_5O_4 for 1 h, and then washed again in both cacodylate buffer and in distilled water in preparation for transmission electron microscopy.

Table 1 Effects of in Vitro Smoke Exposure on Elastase Release from Neutrophils and Whole Blood

		Smoke exposure			
		Neutrophils		Whole blood	
Smoke fraction	n^a	Unexposed	Smoke-exposed	Unexposed	Smoke-exposed
Particulate phase	13	8.7(6.8)[b]	5.3(2.9)	37.7(23.4)	37.2(17.0)
Vapor phase	8	1.8(0.7)	2.2(0.7)	47.6(28.7)	44.3(26.4)

[a] n = number of individual neutrophil preparations in each group.

[b] mean (SD) elastase units in ng/mL.

No significant difference in any group following smoke exposure ($P > .05$).

Statistical Analysis

Results were analyzed by paired comparisons using Student's t test.

RESULTS

The level of smoke exposure was consistent between experiments for both particulate- and vapor-phase smoke as judged by increases in CoHb in whole blood. The increase in CoHb engendered by vapor-phase smoke was less than that produced by particulate-phase smoke, possibly due to differences in the techniques of preparing the two smoke fractions. The mean carboxyhemoglobin (CoHb) in whole blood smoked in the tonometer rose from 1.6 (0.6) to 8.5 (2.6) CoHb [$\bar{x}$ (SD)] following exposure to particulate-phase smoke (n = 13), and from 1.6 (0.9) to 5.2 (3.8) CoHb for vapor-phase smoke exposure (n = 8).

Elastase Release

Total immunoreactive elastase released by neutrophils did not increase following exposure in vitro to either particulate- or vapor-phase cigarette smoke. Similarly, there was no increase in the level of elastase in whole blood following in vitro smoke exposure (Table 1).

Proteolysis of Fibronectin

The ability of neutrophils to degrade fibronectin was minimally increased by exposure to particulate-phase cigarette smoke in only 5 of 17 experiments, and in only 1 of 11 experiments with vapor-phase smoke. In these six experiments the mean increase in fibronectin degradation was 6.4% (range

2–14%) of the baseline value. In the majority of experiments (22/28) fibronectin proteolysis was suppressed following smoke exposure. The level of suppression, as a percentage of the control value, was variable, ranging from 2% to 62% overall, with a mean of 23% for both particulate- and vapor-phase smoke exposure. The suppressive effect of particulate phase cigarette smoke exposure on neutrophil proteolysis was significant (mean reduction in fibronectin degradation 33 ± 5.4%, $\bar{x}$[SEM], $n = 12$) $P < .005$ in contrast to vapor-phase smoke exposure where the reduction was not statistically significant (8 ± 3, $n = 10$) (Figs. 1 and 2). Reducing the smoke exposure from five puffs to three or one puff did not ameliorate the suppression of proteolysis (Table 2). The suppressive effect of one puff of smoke was also unaltered when the cells were exposed for only 2.5 min (control, 5714 (156) [mean (SEM) counts per minute of degraded fibronectin]; smoke, 4524 (142) [significant difference, $P < .005$]). Diluting the one puff exposure by mixing it in a 1:1 ratio with room air did, however, lead to abrogation of suppression [control, 7866 (480); one puff, undiluted, 5264 (309); one puff, 50% diluted, 8196 (58)]. Triggering the neutrophils with phorbol myristate acetate (PMA) caused increased proteolysis of fibronectin (Fig. 1). However, the increase was statistically significant only in the experiments using particulate-phase cigarette smoke ($P < .005$). Zymosan had no significant effect on the proteolytic activity of either the control or smoke-exposed neutrophils (Fig. 2).

Morphology

Transmission electron micrographs demonstrated clear differences in morphology between control and cigarette smoke-exposed polymorphonuclear leukocytes (Fig. 3). Those exposed to cigarette smoke showed extensive blebbing of the cell membrane, which appeared to be intact, without obvious cellular degranulation.

DISCUSSION

Several studies have presented evidence indicating increased proteolytic activity in the lungs of cigarette smokers [5–9]. It has been suggested that this may be due to the toxic effects of cigarette smoke, resulting in neutrophil death and consequent elastase release [13]. In addition, neutrophils may become activated in the inflammatory milieu of smokers' lungs [17] or in response to a direct effect of components of cigarette smoke. The present study investigated neutrophil activation by cigarette smoke but found no evidence that cigarette smoke alone induces enhanced proteolytic activity of neutrophils.

Human peripheral blood neutrophils were exposed in vitro to either particulate- or vapor-phase cigarette smoke. The dose of smoke used in these

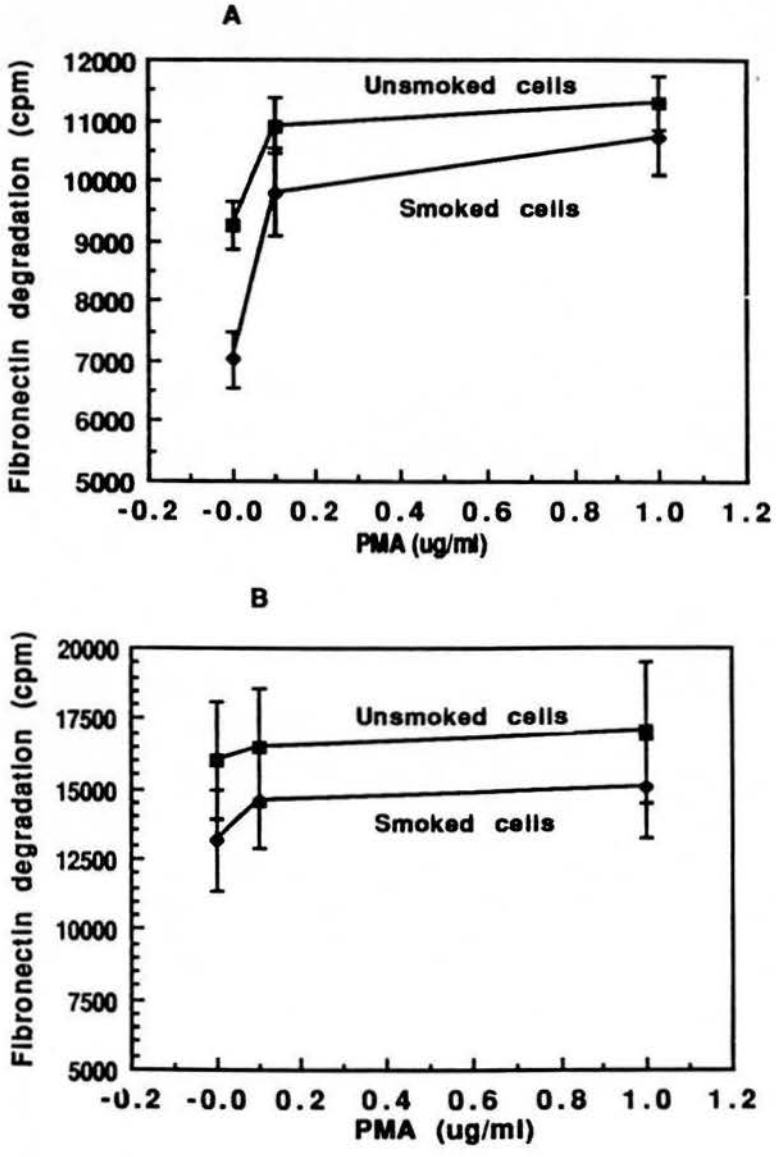


Figure 1 Fibronectin proteolysis by unexposed, particulate-phase (*a*), or vapor-phase (*b*) smoke-exposed neutrophils in response to PMA triggering. Results are expressed as the mean (SEM) counts/min of ^{125}I-fibronectin released in triplicate wells in at least seven experiments with vapor-phase and eight with particulate-phase cigarette smoke.

experiments was chosen to be representative of a physiological level of smoke exposure and is similar to that used in previous work [18]. There is no information on the actual dose of cigarette smoke that PMN experience intravascularly or in the alveolar lining fluid. However, an approximate measure of smoke exposure can be obtained by measuring levels of CoHb in the peripheral blood of smokers. In this study, therefore, we used a smoke-

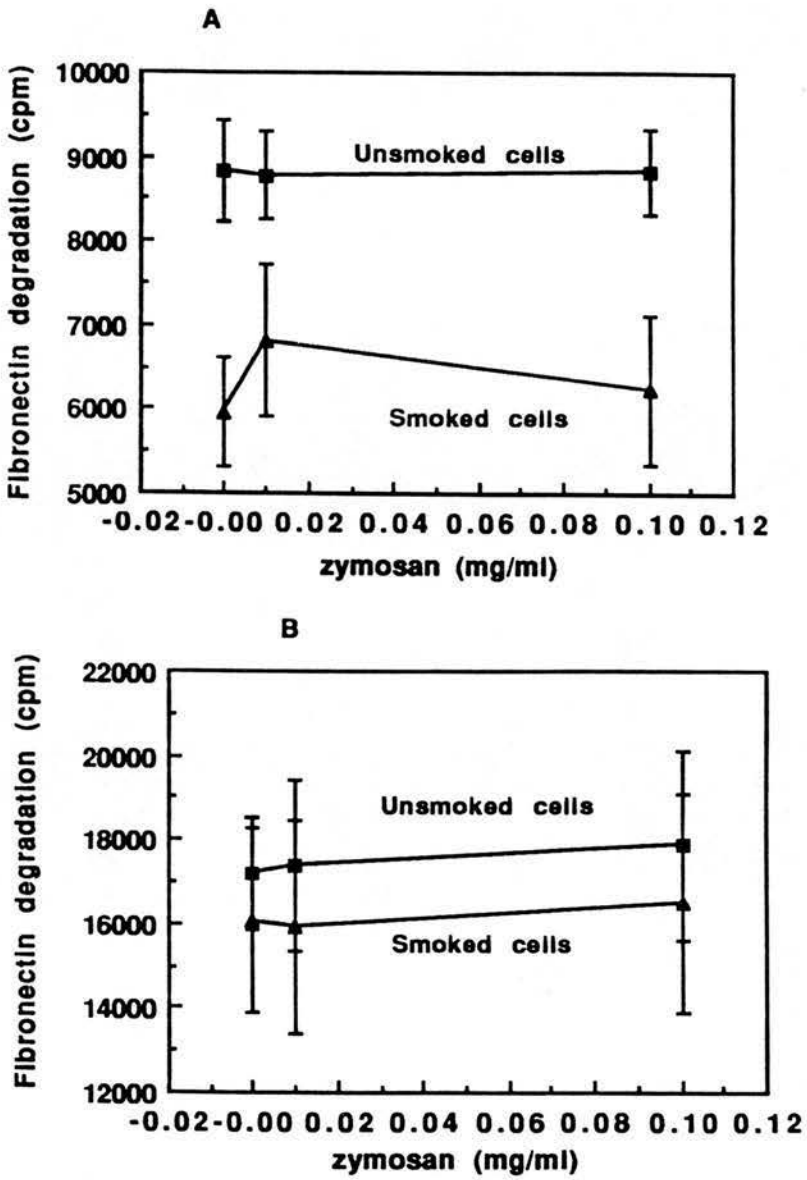


Figure 2 Fibronectin proteolysis by control, particulate-phase (*a*), or vapor-phase (*b*) smoke-exposed neutrophils in response to zymosan triggering. Results are expressed as the mean (SEM) counts/min of ^{125}I-fibronectin released in triplicate wells in at least seven experiments with vapor-phase and eight with particulate-phase cigarette smoke.

exposure regimen that produced levels of CoHb in whole blood smoked in vitro that were similar to those pertaining in the blood of heavy smokers [19]. Measurements of peripheral blood CoHb may markedly underestimate actual levels in the pulmonary microvasculature since Doerschuk et al. [20] reported that in rabbits, following one puff of cigarette smoke, there is a

much higher peak of intravascular carbon monoxide in the aorta than is measured downstream in arterial blood. Similarly, measurement of blood gases may underestimate the level of smoke exposure in the alveolar space since there may be dilution effects during diffusion of smoke across the blood air barrier. Thus, the cigarette smoke exposure used in this in vitro study is likely to represent the lower levels to which PMN may be exposed during cigarette smoking. This treatment failed to stimulate elastase release or increase proteolysis of fibronectin by the neutrophils. Our data indicate that, at the concentrations used in this study, neither phagocytosis of cigarette smoke particles nor exposure to cigarette smoke vapor enhance the proteolytic activity of neutrophils in vitro. To ascertain that the separation procedure we used was not inhibitory to the release of elastase by neutrophils, we exposed whole blood to both smoke fractions, but also failed to provoke increased elastase release.

The failure of cigarette smoke exposure to elicit elastase release from viable neutrophils may have been due to a dose effect since only one dose of smoke was used. However, using the same dose of smoke as that used in the present study, we demonstrated alterations in the deformability [21], chemotaxis [22], and adhesiveness [22] of PMN. In addition, in the present study, data on the proteolytic activity of intact neutrophils indicate that the concentration of smoke was sufficient to modulate a separate aspect of neutrophil function, that is, fibronectin proteolysis.

Interestingly, we observed a reduction in fibronectin proteolysis in the present study in 22 out of 28 experiments (minimum 2%, maximum 62% suppression) following both particulate- and vapor-phase cigarette smoke exposure. The reduction in proteolysis of fibronectin was not due to cytotoxic effects of the cigarette smoke, since post-smoke exposure the PMN were >99% viable by trypan blue exclusion. In addition, the ability of the cells to respond to PMA triggering is a further indication that the smoke effect was sublethal. A similar effect was reported by Hutchinson et al. [23], who found reduced lysosomal enzyme release by leukocytes exposed to a solution of cigarette smoke in vitro.

Table 2 Dose Response of Cigarette Smoke on Fibronectin Proteolysis by PMN

	Agitated control	1 puff	3 puffs	5 puffs
Mean	11,230[a]	8166[b]	9120[c]	8272[b]
SEM	490	194	382	108

[a]Counts per minute of degraded fibronectin released.

[b]Significantly less than control, $P < .01$.

[c]Significantly less than control, $P < .05$.

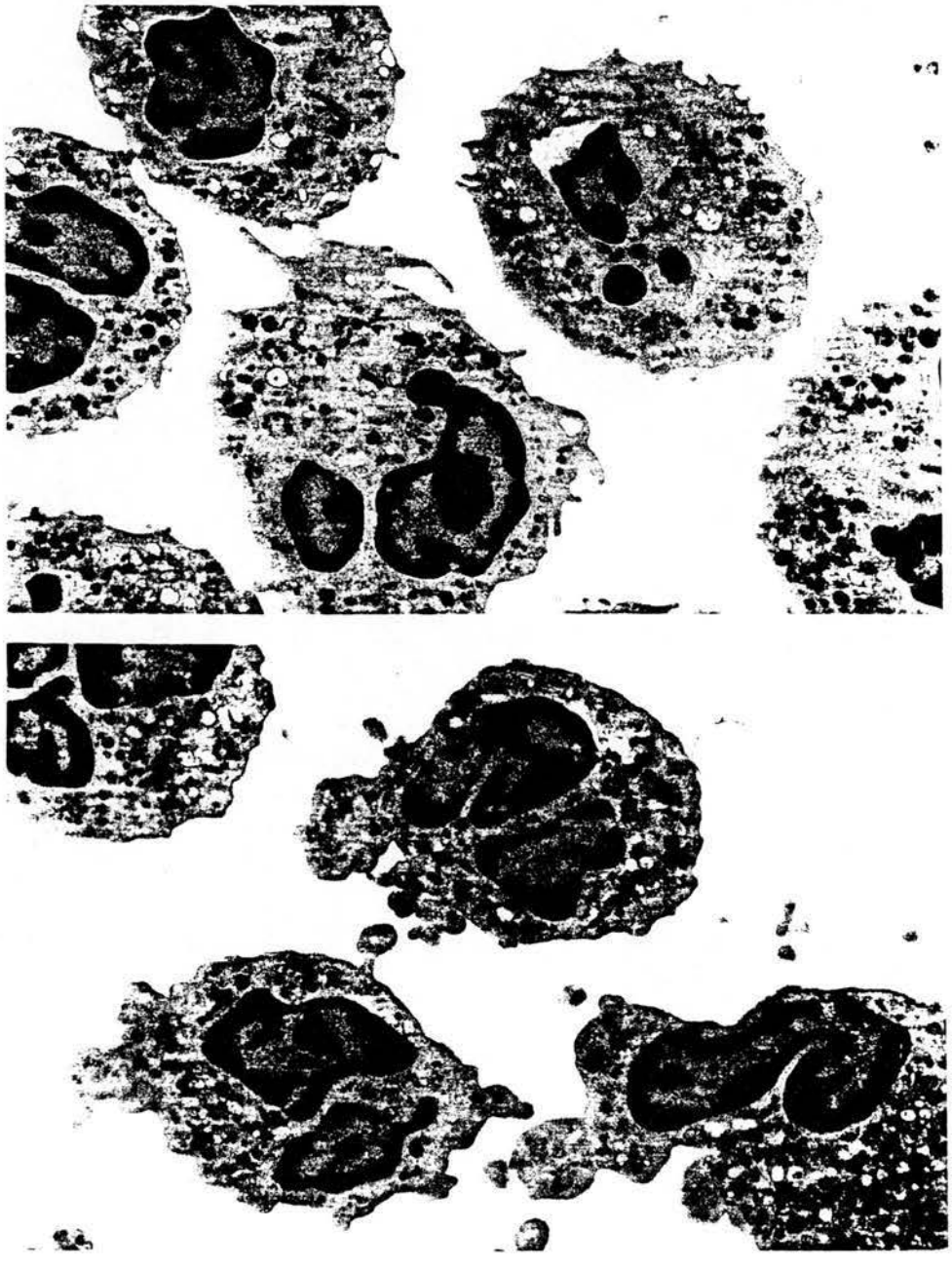


Figure 3 Morphology of (*a*) control and (*b*) particulate-phase smoke-exposed neutrophils from the same subject assessed by transmission electron microscopy. Smoke-exposed neutrophils exhibit altered morphology with membrane blebbing but no evidence of degranulation.

The action of smoke in suppressing fibronectin proteolysis by PMN appeared to be an all or none effect since reducing the smoke concentration by up to one fifth did not alter fibronectin proteolysis. Halving the duration of exposure to one puff of smoke also failed to abrogate the suppressive effect of the smoke. However, when the smoke exposure was reduced by mixing the lowest previous dose (i.e., one puff) with 50% air, the suppression of fibronectin proteolysis was abruptly lost. This indicates that there may be a threshold level of smoke exposure required before modulation of PMN function can occur. The proteolytic activity of the PMN was not restored by culturing the cells for up to 8 h at 37°C in culture medium or by maintaining them in 50% autologous plasma in PBS for up to 2 h prior to use in the fibronectin proteolysis assay (results not shown). These results are in agreement with those of Churg et al. [18], who reported an effect of smoke on airway epithelial cells that persisted for up to 48 h. In the present study, only PMA triggering restored the ability of PMN to degrade fibronectin. PMA acts to stimulate the second messenger system of the cell, one of the cellular systems that governs lysosomal secretion [24], and so cigarette smoke may suppress proteolysis by interfering with this system. Although zymosan triggering failed to restore the proteolytic activity of smoke-exposed PMN, this does not indicate that smoking rendered the cells unresponsive to a particulate stimulus since even the control cells did not respond. This effect is a feature of the solid-phase assay system since we observed a similar inability of zymosan to trigger increased fibronectin proteolysis by rat inflammatory PMN and macrophages [25]. Werb et al. [26] also showed that zymosan triggering elicited minimal stimulation of proteolysis by mouse macrophages in a similar solid-phase assay. An alternative mechanism for the reduced proteolytic activity of smoke-exposed neutrophils is suggested by the changes in cell morphology following smoke exposure. Cells exposed to cigarette smoke showed marked changes in shape, as assessed by transmission electron microscopy, with extensive blebbing of the plasma membrane compared with unexposed cells. Several studies described zeiosis (or blebbing) of cells in the absence of cell degranulation or death [27, 28]. The changes in cell shape that we observed in this study may thus represent a sublethal and reversible cell injury. In P388D1 cells exposed to H_2O_2, blebbing is associated with altered F-actin polymerization [29]. Similar effects may also occur in smoke-exposed cells since smoke exposure interferes with cell motility [22, 30]. The reduced fibronectin proteolysis that we observed in the present study may thus represent altered ability of the cells to traverse and so digest the fibronectin matrix.

Our findings that neither of the smoke preparations used in this study could elicit enhanced proteolysis of fibronectin or elastase secretion from neutrophils suggests that cigarette smoke does not directly activate neutrophils in the alveolar space, within the interstitium or in the capillaries. Our results are in apparent contradiciton to those of Blue and Janoff [13] who

observed elastase release by neutrophils exposed to cigarette smoke condensate in vitro. However, in that study, elastase release occurred only at concentrations of condensate that were likely to be not physiologically attainable and cause cell death. At lower concentrations, where no cell death occurred, there was no neutrophil activation as measured by elastase release. Our results, taken together with those of Blue and Janoff [13], suggest that increased elastase release by neutrophils may not be the primary mechanism in the development of emphysema associated with cigarette smoking. Other mechanisms may operate instead in smokers' lungs whereby even normal levels of elastase could lead to emphysematous change. Tissue proteolysis may be due to inactivation of α1-proteinase inhibitor by oxidant gases in cigarette smoke, thus reducing inhibition of elastase [31, 32], although this has been disputed [33–45]. Alternatively, cigarette smoke may interfere with elastin resynthesis, thus interfering in the normal connective tissue turnover in the lungs and leading to emphysema [36, 37]. However, data suggestive of increased levels of elastase in the air spaces [6, 7] and pulmonary vasculature [9] of smokers, in association with a concomitant increase in the numbers of neutrophils in the lung [5, 8], are compelling. Although in vivo experiments indicating release of protease by smoke-stimulated neutrophils and macrophages in the lungs are not supported by the effects on neutrophils of in vitro cigarette smoking, cigarette smoke could still act indirectly in vivo to trigger elastase secretion. Elastase release from neutrophils in vivo may be mediated by cellular messengers secreted by other lung cells, such as alveolar macrophages and pulmonary epithelial and endothelial cells in response to cigarette smoke. Our findings that control and smoke-exposed neutrophils have increased proteolytic activity in response to PMA indicates that human neutrophils do have the potential to respond to this and possibly other physiological triggers. Interleukin-1 (IL-1) [38] and tumor necrosis factor (TNF) [39] are two such inflammatory mediators that are found at sites of inflammation and can stimulate the secretion of proteases by neutrophils. Alveolar macrophages from smokers secrete increased amounts of IL-1 [40] and thus the potential for such stimulation exists. These cytokines could thus induce increased elastase secretion by neutrophils in transit through the pulmonary microvasculature, in the interstitium or in the alveolar space, but would not be active in the in vitro system used in this study. In addition to TNF and IL-1, a plethora of other potent mediators accumulate at inflammatory sites and act in concert to modulate the inflammatory response [41]. This study suggests that multifactorial nature of the signals operating to modify neutrophil responses in smoke-exposed lungs and points the way for further studies to elucidate these events.

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The Effects of N-Acetylcysteine and Glutathione on Smoke-Induced Changes in Lung Phagocytes and Epithelial Cells

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We studied the mechanism of the delay in neutrophil traffic in pulmonary microvasculature previously observed during cigarette smoking, the effect of cigarette smoke on lung phagocytes and epithelial cell function, and augmentation of the glutathione (GSH) antioxidant system using the thiol drug N-acetylcysteine. Using a micropore membrane system to mimic the dimensions of the average pulmonary capillary, we showed that cigarette smoke reduces cell deformability, increasing the difficulty experienced by the larger neutrophils in negotiating the smaller capillary segments, so delaying their passage during smoking. This effect is both diminished and recoverable by the addition of plasma, and by GSH in concentrations found in plasma.

Cigarette smoke induces oxidative changes in both the cell membrane and the cell cytoskeleton, and diminishes the ability of neutrophils to release reactive oxygen intermediates. The injurious effect of oxidants can be measured in vitro by the detachment of ^{51}Cr-radiolabeled alveolar epithelial cells grown in monolayers, an effect also diminished by the addition of GSH. Such epithelial cell detachment in vitro may be reflected as the epithelial permeability that occurs at an early stage in asymptomatic smokers. N-Acetylcysteine given orally (600 mg/day) increases both plasma and bronchoalveolar lavage GSH in normal subjects, but a sustained increase in plasma GSH requires higher dosage regimens in patients with chronic obstructive pulmonary disease (600 mg three times daily). Thus, the potential exists to enhance the antioxidant status of both plasma and the airspaces of the lungs against oxidant-induced injury.

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The lungs are constantly exposed to oxidants such as cigarette smoke [1] and ozone [2] that are inhaled into the airspaces, or those released from airspace inflammatory leukocytes [3]. These oxidants cause direct damage to the lung interstitium [4], inactivate antiproteases (α_1-proteinase inhibitor [5]), and affect airspace leukocytes directly. Airspace epithelial injury, present in asymptomatic smokers [6], may result in enhanced epithelial permeability. Oxidation of α_1-proteinase inhibitor is thought to be a central event in the protease/antiprotease imbalance that results in centrilobular emphysema [7,8]. Inflammatory leukocytes, obtained from bronchoalveolar lavage in smokers, release more reactive oxygen intermediates and proteases than in nonsmokers [9–11].

Cells in transit in the pulmonary capillary bed, when activated, may release reactive oxygen intermediates, which damage the endothelium, an effect that may be important in the pathogenesis of the adult respiratory distress syndrome (ARDS) [12]. However, neutrophils passing through the pulmonary capillary bed may also be exposed to components of inhalants, such as cigarette smoke, containing 10^{16} oxidant molecules per puff [1]. Airspace and intravascular oxidant stress may also be prolonged by redox reactions in smoke condensate in the alveolar airspace [1]. Furthermore, the acute injurious effects of smoke inhalation create a secondary inflammatory milieu with the influx of neutrophilis and macrophages [13].

The lung is protected from oxidant stress by various antioxidants, including the glutathione (GSH) system [14]. Smokers may adapt to the constant inhalation of oxidants by increasing the levels of antioxidants in the lungs [15], although these changes vary depending on the antioxidant. Whereas there is evidence of a decrease in vitamin E [16] and catalase [17] in the bronchoalveolar lavage (BAL) in smokers, exposure to cigarette smoke induces an initial fall in total lung GSH following acute exposure [18]. However, GSH is increased in BAL after chronic exposure to cigarette smoke [19].

Using cigarette smoke as our model of an inhaled oxidant, we studied the effect of cigarette smoke on

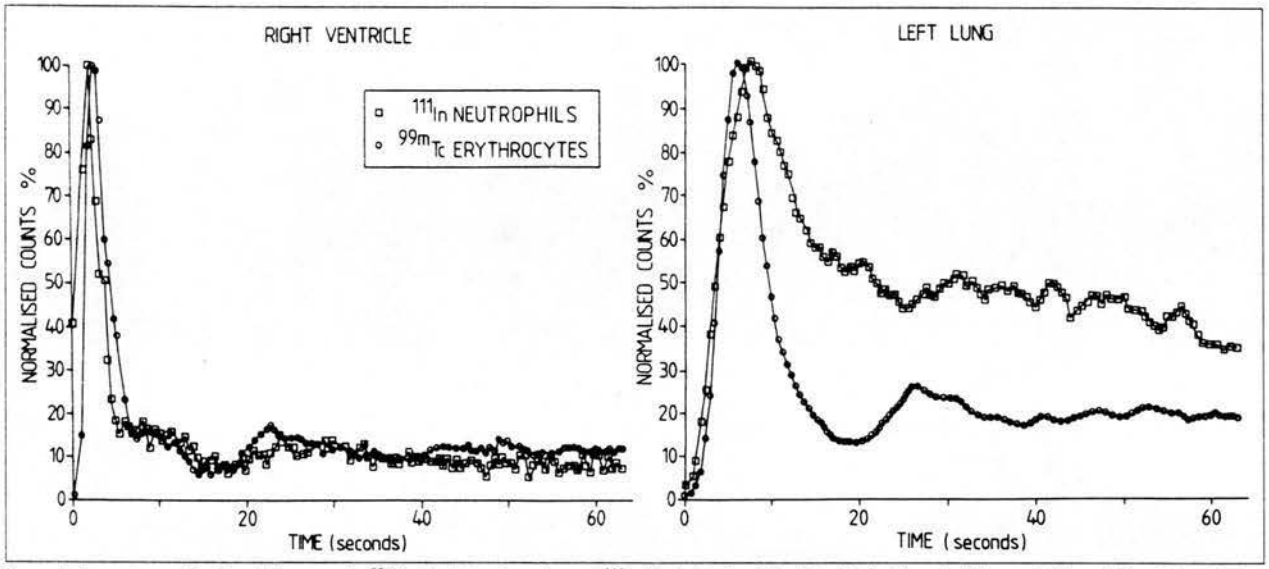


Figure 1. Comparison of time–activity curves for ^{99m}Tc-labeled erythrocytes and ^{111}In-labeled neutrophils during the first transit of these cells through the right ventricle or the lung. All curves are normalized to peak counts for comparison. Whereas ^{99m}Tc-labeled erythrocytes and ^{111}In-labeled neutrophils pass through the right ventricle identically, neutrophils are delayed within the lungs relative to erythrocytes. First-pass neutrophil retention relative to erythrocytes can be calculated from these curves.

the passage of neutrophils in transit in the pulmonary circulation in humans in vivo, and in a model of the pulmonary microcirculation in vitro. The effects of cigarette smoke on neutrophils and airspace epithelial cell function were assessed. The ability of GSH to protect against the effects of cigarette smoke and the potential of N-acetylcysteine (NAC) to augment the levels of GSH in both plasma and BAL were also studied.

INTRAVASCULAR NEUTROPHIL KINETICS IN THE LUNGS

The lungs contain a pool of "marginating" or "noncirculating" neutrophils [20,21], and recent evidence suggests that they are not truly marginating within postcapillary venules as in the systemic circulation venules, but delayed or even stationary within the pulmonary capillary segments [22]. This has been demonstrated in animal experiments using radiolabeled cells [23], and by morphometric techniques [24]. In addition, direct visualization of fluoroscein-labeled neutrophils in the dog, through a window inserted into the periphery of the lung, indicates that neutrophils are delayed exclusively within the capillary bed [22]. Although the expression of CD11/CD18 surface adhesion proteins appears to be important in the sequestration of activated neutrophils, the normal sequestration of unactivated neutrophils is independent of these adhesive-promoting glycoproteins [25].

This normal delay in neutrophil passage in the lungs can be measured in humans by reinjecting ^{111}In-labeled neutrophils, harvested from whole blood, together with ^{99m}Tc-labeled erythrocytes, as a bolus, into a peripheral vein and comparing their passage through the right ventricle and lungs [26]. Comparison of the time–activity curves for both cell types from regions of interest over the right ventricle and the lung shows that neutrophils are delayed in the lungs relative to erythrocytes (**Figure 1**). Calculations from these curves of the first-pass neutrophil retention in the lungs are between 5 and 15% in normal subjects [26,27]. Thereafter, the cells recirculate, and the neutrophil activity over the lungs describes an exponential curve [27]. The later retention of neutrophils, for example 10 minutes following reinjection, correlates with the transit time of erythrocytes in the lungs [23,27], a measure of local blood velocity [28], so that the longer the erythrocyte transit time, the slower the blood velocity and the more neutrophils are sequestered in the lungs [23,27] (**Figure 2**). However, the first-pass retention does not correlate with the erythrocyte transit time [29].

When considering the factor that influence neutrophil transit in the lungs, it is important to remember that all of the circulating neutrophils must pass through the pulmonary capillary bed, which consists of a complex branching system of 2.8×10^{11} capillary segments of varying dimensions, with a mean length of 11 μm, and a mean diameter of 5 μm [30]. Since the average diameter of the neutrophil is 7 μm [31], a proportion of neutrophils must deform in order to negotiate the narrower capillary segments. One factor that influences cell deformability is the cell surface to volume ratio, so that the neutrophil has an 84% excess surface area above that necessary to contain its volume as a sphere [32]. This excess surface area can be used to enable the cell to deform. Neutrophil deformability also

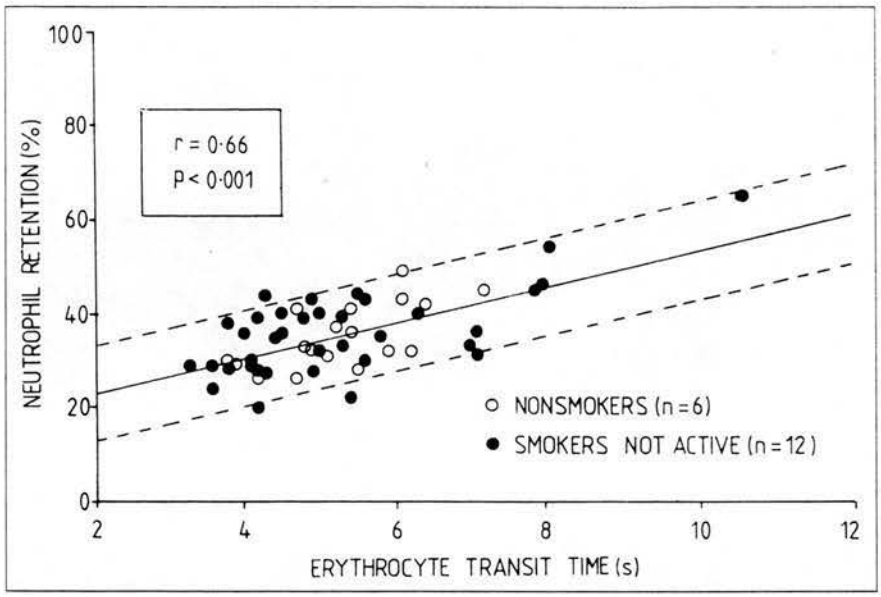


Figure 2. Linear-regression slope between neutrophil retention of three lung regions and erythrocyte transit time in the same regions. Values for six nonsmokers and those for 12 smokers who were not actively smoking are not significantly different. The values for these 18 subjects fall within the 95% confidence limits (dashed lines) of the equation $y = 15.51 + 3.73 \times$ transit time ($R = 0.006$; $p < 0.001$). Thus, the longer the erythrocyte transit time, the more neutrophils are retained in the lungs.

depends on the viscoelastic properties of the cell membrane, and the viscosity of the interior of the cell [32]. Since the neutrophil has a viscous cytoplasm, and contains a nucleus, it is 700 times less deformable than an erythrocyte [32].

Neutrophil deformability can be measured in vitro both by micropipette aspiration of individual cells [33] or by driving neutrophils at a constant flow through a micropore membrane [34] whose dimensions mimic those of the average pulmonary capillary segment. As cells enter and plug the pores of the membrane, they increase the pressure developed within the filter system over time, developing a plateau pressure after 6 minutes of filtration. This pressure–time curve is a measure of the deformability of the population of neutrophils under study, and has been correlated with more direct measurements of cell deformability [35]. Recently we have shown that the first-pass neutrophil retention of reinjected radiolabeled neutrophils in the lungs in vivo correlates with the deformability of aliquots of the same cells, measured by the cell filtration technique in vitro [36]. Thus, any factor that alters neutrophil deformability may also influence neutrophil passage in the lungs.

THE EFFECT OF CIGARETTE SMOKING IN VITRO ON NEUTROPHIL KINETICS IN THE LUNGS

The relationship we have shown between the erythrocyte transit time in the lungs, as a measure of local blood velocity, and neutrophil retention in young healthy smokers and nonsmokers [27] has also been demonstrated in animal experiments [23]. This relationship is disturbed in healthy smokers who actively smoked during the injection and subsequent passage of radiolabeled neutrophils through the lungs. Thus, the erythrocyte transit time was shorter during cigarette smoking [27], reflecting faster blood velocity, which would increase the shear forces dispersing cells through the pulmonary circulation. In fact, the reverse was true so that neutrophils were delayed in the lungs during active smoking, but not in chronic smokers who were not actively smoking. This was true both during the first passage of these cells through the lungs and during their later sequestration [27]. Preliminary data also suggest that intravascular neutrophil sequestration is greater in patients with chronic obstructive pulmonary disease (COPD), particularly during exacerbations of their condition [29]. During active smoking and during exacerbations of COPD, there is increased sequestration of intravascular neutrophils, presumably in the capillary bed, with the potential to increase the elastase burden in the lungs. Whether this intravascular sequestration is followed by migration of these cells into the distal airspaces of the lungs awaits further study.

THE MECHANISM OF THE INTRAVASCULAR SEQUESTRATION OF NEUTROPHILS IN THE LUNG DURING SMOKING

We hypothesized that components of cigarette smoke, inhaled into the alveoli, may affect neutrophils in transit in the pulmonary capillaries, altering their deformability, resulting in increased capillary sequestration. To test this hypothesis, we exposed neutrophils harvested from the blood of normal subjects to cigarette smoke produced by a smoking machine (35 ml/puff, 1 puff/min). The cells were exposed in phosphate-buffered saline to which 1% bovine serum albumin was added in a tonome-

ter, which was agitated in a water bath at 37°C. Following exposure to both particulate and vapor phase cigarette smoke, there was an increase in the filtration pressures of neutrophils reflecting a decrease in neutrophil deformability (**Figure 3**) [37]. We also showed that this effect of cigarette smoke can be diminished if the cells are exposed to plasma or to GSH in concentrations present in plasma [38]. This smoke-induced change in deformability is associated with a change in cell shape, resembling oxidant injury to the cell membrane, with cell membrane blebbing, rather than the shape changes that follow cell activation [39]. The shape changes are similar to those of alveolar macrophages from chronic smokers [40]. The change in neutrophil deformability following smoke exposure can be reduced by exposing the cells in the presence of cytochalasin B [38], suggesting that smoke produces changes in the microfilaments of the neutrophil, resulting in the polymerization of actin, which is reduced by GSH. In the microenvironment of the pulmonary capillaries, neutrophils wedged within the smaller diameter capillary segments may be in a vulnerable and unprotected site, in terms of the amount of surrounding antioxidants. Moreover, variations in plasma and intracellular antioxidants, such as GSH, may result in a variable response to the inhalation of cigarette smoke.

Evidence supporting the hypothesis described above comes from preliminary animal data that indicate that rat neutrophils exposed to cigarette smoke in vitro show increased lung sequestration when reinjected compared with control unexposed cells [41]. Cigarette smoking also results in an acute reduction in erythrocyte GSH, suggesting that oxidants in cigarette smoke do reach the intravascular space and could potentially affect neutrophils in transit in the pulmonary capillary bed (unpublished data). A similar decrease in erythrocyte GSH has also been demonstrated in humans, following the inhalation of the oxidant ozone, a component of both air pollution and cigarette smoke [42].

In addition to an effect on neutrophil rheology, other membrane functions, such as the release of reactive oxygen intermediates, and also the proteolytic activity of neutrophils, are reduced following acute cell exposure to cigarette smoke in vitro. These cells can still be stimulated to increase the production of reactive oxygen intermediates and proteases by the additional stimulus of phorbol myristate acetate [43]. These effects of in vitro smoke exposure on neutrophil function contrast with the function of leukocytes obtained by BAL in chronic smokers in whom the cells are reported to be more activated than those from nonsmokers [6,10]. However, the data from studies of BAL may result from a secondary inflammatory response to chronic smoking rather than from an acute effect of cigarette smoke.

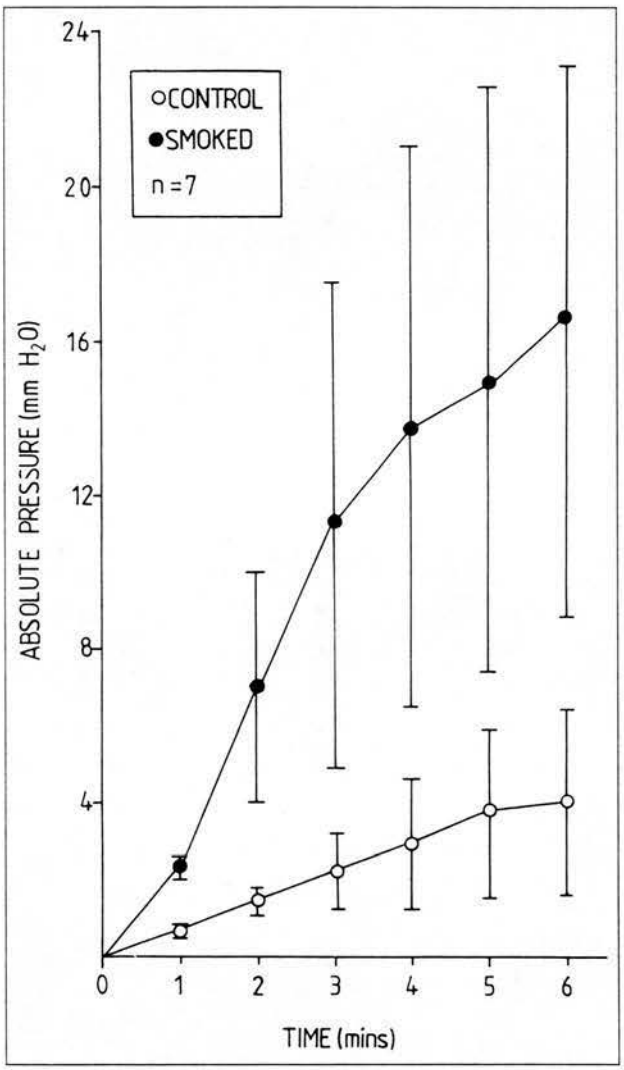


Figure 3. The effect of in vitro vapor phase cigarette smoke exposure on the filterability of plasma/Percoll-harvested neutrophils from 15 subjects. The results are expressed as the pressure developed during 6 minutes of filtration before and after exposure to vapor phase cigarette smoke at 37°C for 4 minutes in vitro. Mean ± SD (bars). Smoke-exposed cells had significantly ($p < 0.05$) higher pressures than control cells after 2 minutes of filtration.

EFFECTS OF CIGARETTE SMOKE ON ALVEOLAR EPITHELIAL CELLS

The detachment of type II alveolar (A549) epithelial cells labeled with ^{51}Cr from a monolayer culture can be used as an in vitro model of epithelial injury [44]. Using this technique, we showed that increased cell detachment occurs following treatment with hydrogen peroxide, an effect that is reduced in the presence of GSH (**Figure 4**). Preliminary studies also indicate a similar effect with cigarette smoke exposure in vitro, which reduces intracellular GSH. This epithelial detachment may be

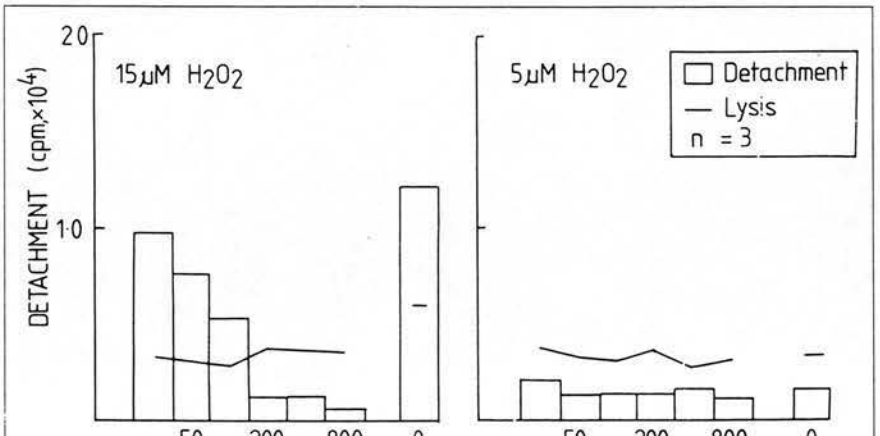


Figure 4. The effect of reduced glutathione (GSH) on the detachment and lysis of type II alveolar epithelial cells (A549 cells) following exposure to hydrogen peroxide (H_2O_2). H_2O_2 (15 μM) increases the detachment of A549 cells measured as the cell-associated counts per minute (cpm) in the supernatant, which is reduced by the presence of reduced GSH (>100 μM).

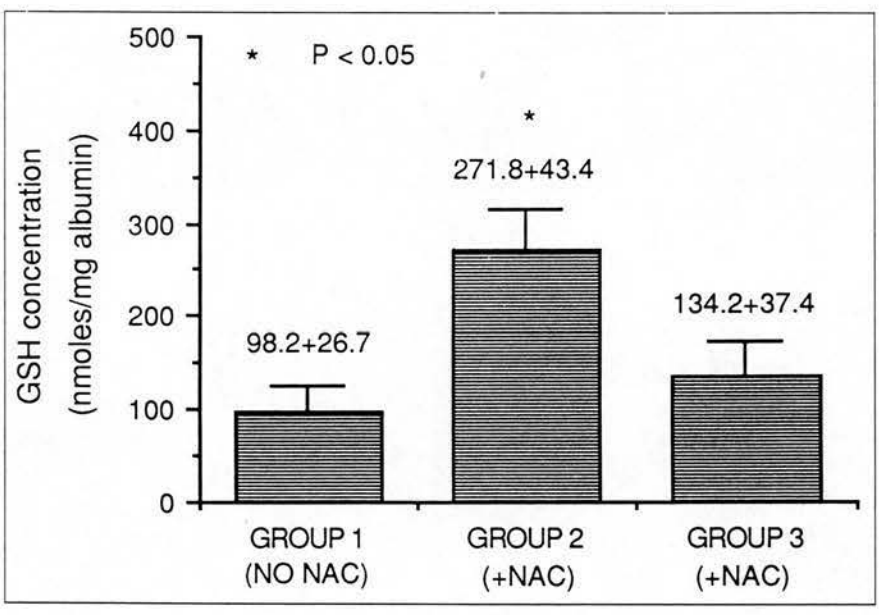


Figure 5. Bronchoalveolar lavage glutathione (GSH) concentrations with respect to albumin in three groups of patients undergoing diagnostic bronchoscopy. Group 1 is the control, without N-acetylcysteine (NAC): NAC was given orally (600 mg/day for 5 days) to two of the groups and samples were obtained 1–3 hours (group 2) and 16–20 hours (group 3) after the last dose of NAC. All results were quoted as mean ± SEM. Bronchoalveolar lavage GSH concentrations were significantly higher in group 2 than in other groups.

reflected as the increased epithelial permeability occurring as an early event in asymptomatic cigarette smokers.

AUGMENTATION OF GLUTATHIONE IN PLASMA AND BRONCHOALVEOLAR LAVAGE BY N-ACETYLCYSTEINE

Over the past 20 years, NAC has provoked much interest as a therapeutic agent in lung disease [45,46]. Although some studies have shown it to be efficacious in reducing the number of exacerbations of chronic bronchitis [47,48], other studies have not been so positive [49]. These contrasting data may be due to several factors, including inappropriately low dosage regimens, differences in patients studied, and inconsistencies in the definition of an exacerbation of COPD. The effect of NAC on exacerbations of COPD may be due to its mucolytic properties, due to the thiol group of NAC and to its reducing and antioxidant properties [50]. It has been postulated that NAC may protect lung cells from inhaled oxidants, or oxidants produced by inflammatory leukocytes by increasing intra- and extracellular GSH [45].

We gave NAC to patients undergoing diagnostic bronchoscopy and thiols were measured in the plasma and BAL [51]. At a dose of 600 mg/day for 5 days, plasma cysteine and GSH concentrations were higher than in a similar group of patients not receiving NAC. The levels of reduced GSH in BAL of patients receiving NAC within 1–3 hours of the bronchoscopy were higher than those in whom the last dose of the drug was given 16–20 hours prior to bronchoscopy (**Figure 5**).

We also compared plasma thiols in normal subjects and in patients with COPD, following NAC at a dose of 600 mg/day and in a higher dose of 600 mg three times daily over 5 days in patients with COPD [52]. A short-lived increase in plasma cysteine peaked at 30 minutes in normal subjects and

at 60 minutes in COPD patients following NAC at 600 mg/day. The levels of plasma cysteine were lower in COPD patients than in normal subjects. Hence, we studied the effects of a higher dose of NAC (600 mg three times daily) given for 5 days to patients with severe COPD. Preliminary results indicate that such a dose regimen produces a sustained increase in both plasma cysteine and GSH [52]. Whether such increases in plasma GSH produce corresponding further increases in thiols in BAL or increases the intracellular GSH in lung cells awaits further study. In contrast to other studies, we have not demonstrated that NAC given to either animals or humans significantly changes the production of reactive oxygen intermediates from either the rat alveolar macrophages or from peripheral blood neutrophils [53].

Thus oxidants in cigarette smoke may affect neutrophil traffic and function in the lungs, and have a direct injurious effect on airspace epithelial cells. These events are important in the pathogenesis of COPD. The potential exists with thiol derivative drugs, such as NAC, to enhance the antioxidant status of both the plasma and the airspaces of the lungs against oxidant-induced injury.

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Neutrophil Sequestration in Rat Lungs[a]

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The pulmonary microvasculature contains a large marginated pool of neutrophils (PMNs)[1] whose passage through the pulmonary circulation is delayed during cigarette smoking.[2] They may become activated by exposure to cigarette smoke and thus contribute to lung damage. We previously showed that exposure to cigarette smoke *in vitro* reduces the deformability of PMNs,[3] and we questioned whether this might influence the sequestration of PMNs in the lung. We therefore developed a rat model to investigate the factors that influence the passage of PMNs through the pulmonary circulation.

MATERIAL AND METHODS

Rats were transfused with a plasma expander (Hetastarch) to increase the blood yield. Peripheral blood PMNs (PBNs) were obtained by separation using Percoll and Sepracell gradients. Inflammatory PMNs (INs) were obtained by bronchoalveolar lavage of rat lungs inflamed with *C. parvum*. Red blood cells (RBCs) were obtained by heartstab from a control rat. PBNs and INs were then labeled with ^{51}Cr and RBCs with ^{99m}Technetium. Radiolabeled cells (6×10^6) were exposed to smoke for 1 minute in a tonometer system which agitated the cells at 37°C in a 1-ml volume of phosphate-buffered saline solution containing 1% bovine serum albumin. The radiolabeled PMNs and RBCs were mixed and injected via the iliac artery of anesthetized rats. After 10 minutes the chest cavity was opened, a small volume of blood was obtained by heartstab, and the heart and major blood vessels were immediately snared to prevent blood loss. The lungs were fixed overnight in gluteraldehyde, and then both blood and lungs were counted by gamma counter. PMN sequestration was expressed as the fold increase in the PMN : RBC ratio in lungs compared with peripheral blood. As a measure of cell deformability, the pressure developed by PMNs (1×10^5 in 2 ml) during filtration at constant flow (1.5 ml/m) through micropore membranes (pore dimensions 5×11 μ) was assessed over 6 minutes.

[a] This research was supported by the Chest, Heart and Stroke Association (England).

RESULTS

The sequestration of INs (90.5 [10.5], mean [SE]) in the lung was significantly greater than that of PBNs (8.1 [2.5]; $p < 0.001$). A trend towards a greater-fold increase of PBN sequestration (16.2 [6.7]) in inflamed rat lungs compared with control lungs was also noted, but the difference was not statistically significant. Exposure to cigarette smoke caused significantly increased PMN sequestration in control rat lungs (27.2 [2.4]) compared with untreated PBNs ($p < 0.001$) but not in comparison with PMNs agitated in the tonometer (34.5 [6.7]). The filtration pressure of INs (9.95 [1.6]), mean [SD]) was greater than that of smoke-exposed PBNs (6.42 [0.96]), agitated PBNs (1.47 [0.24]), and untreated PBNs (0.56 [0.3]).

DISCUSSION

We have shown that the increase in PMN sequestration is associated with alterations in PMNs caused by exposure to the inflammatory milieu of the lung (IN) or by treatment *in vitro*. The increased sequestration may be explained by decreased deformability of the PMN, reducing its ability to negotiate the pulmonary capillaries. Although alterations in the inflamed lung caused a modest increase in sequestration, the change was insignificant compared with increases due to changes in the PMN. These initial results suggest that changes in the neutrophil may contribute more to cell sequestration than do alterations in the pulmonary microvasculature.

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Does Cigarette Smoke Enhance the Proteolytic Activity of Neutrophils?

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Neutrophils are found in increased numbers in the alveolar region of individuals who smoke cigarettes, and the release of proteinase by these cells in the alveolar region is considered to play an important role in the subsequent development of emphysema in a proportion of individuals who smoke.[1] We therefore undertook a series of studies to determine whether cigarette smoke, or its components, could directly stimulate human neutrophils to release elastase and degrade fibronectin.

MATERIALS AND METHODS

Human neutrophils were obtained from the peripheral blood of normal subjects by sedimentation through dextran and separation of the granulocyte fraction over plasma Percoll. Cigarette smoke exposure was accomplished in a tonometer system which allowed the neutrophils to be agitated in a 2-ml volume of phosphate-buffered saline solution with bovine serum albumin, in the presence of fresh cigarette smoke. Elastase levels in cells and supernatants were measured by radioimmunoassay using antibody specific for human neutrophil elastase. Fibronectin-degrading activity of the neutrophils was assessed by incubating the smoke-exposed cells on a matrix of radiolabeled fibronectin and measuring the release of radioactivity into the supernatant.[2]

RESULTS

Elastase Release

On exposure to vapor phase cigarette smoke in the tonometer system, neutrophils did not show an increased release of elastase, whereas intracellular elastase was reduced by smoke exposure; phorbol myristate acetate (PMA) was able to stimulate a modest release of elastase (TABLE 1). Cigarette smoke condensate, at a concentration of 1%, did not stimulate release of elastase in the tonometer system (TABLE 1). Exposure to a solution of 10% condensate was associated with a significant release of elastase into the supernatant, but this was a result of a

TABLE 1. Effect of Cigarette Smoke, PMA, and Condensate on Elastase Content and Secretion by Neutrophils[a]

Neutrophil Elastase Treatment	Neutrophils	Neutrophil Supernatant
Control	3,853 (456)	1.8 (0.7)
Smoke exposed	1,085 (590) $p < 0.01$	2.2 (0.7) NSD
Control	ND	3.7 (4.0)
PMA exposed	ND	12.2 (8.0) $p < 0.01$
Control	2,771 (1,370)	5.1 (3.3)
Condensate exposed	2,090 (1,188) NSD	3.6 (2.2) NSD

[a] *Upper panel,* mean (SD) of six experiments; *middle panel,* mean (SD) of six separate experiments; *lower panel,* mean (SD) of seven experiments. All data are ng/ml of human neutrophil elastase. NSD = no significant difference from control. PMA = phorbol myristate acetate 1 μg/ml.

direct toxic effect of the condensate because it was accompanied by a substantial decrease, from 98% to 43%, in the proportion of neutrophils able to exclude trypan blue.

Fibronectin Degradation

Neutrophils that had been exposed to particulate phase cigarette smoke in the tonometer system and subsequently incubated on a fibronectin matrix did not show an increased ability to degrade this extracellular matrix component but, in fact, displayed reduced activity (TABLE 2).

DISCUSSION

The release of increased amounts of neutrophil proteinase in smokers' lungs could be a result of (1) normal release of enzyme by a much expanded population, (2) stimulation of enzyme release, and (3) death of neutrophils with subsequent release of granule contents. In the present study we examined the likelihood of direct stimulation and toxicity of cigarette smoke and its components. The results suggest that cigarette smoke is not capable of directly stimulating elastase release or fibronectin-degrading activity in peripheral blood neutrophils. In experiments with condensate designed to repeat the conditions of Blue and Janoff,[3] the release

TABLE 2. Degradation of ^{125}I-Labeled Fibronectin by Control and Smoke-Exposed Neutrophils[a]

Treatment	Fibronectin Degradation
Control	9,262 (405)
Smoke exposed	7,032 (467) NSD

[a] Data given as mean (SD) counts per minute in eight separate experiments. NSD = no significant difference from control.

of elastase was accompanied by loss of ability to exclude trypan blue and so reflects a toxic effect of the smoke as opposed to a stimulatory one. The increased proteolytic activity seen in the lungs of smokers may, therefore, be a result of release from smoke-damaged cells or a result of the inflammatory response arising from it. Modification of neutrophil response by macrophage cytokines, however, cannot be ruled out and the role of $alpha_1$-protease inhibitor also needs to be considered.

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ACTIVATED HUMAN PERIPHERAL BLOOD NEUTROPHILS PRODUCE EPITHELIAL INJURY AND FIBRONECTIN BREAKDOWN IN VITRO

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Abstract—The ability of peripheral blood neutrophils to injure elements of the alveolar septum was assessed. Experiments consisted of activating previously isolated neutrophils (PMNs) with a soluble (phorbol myristate acetate, PMA) and a particulate (zymosan) trigger, and measuring detachment of ^{51}Cr-labeled epithelial cells. In addition, the mechanisms of epithelial injury were investigated by including antiproteinase and antioxidants in the system. Untriggered PMNs produced only slight detachment injury to epithelial cells at two effector–target cell ratios, this effect being dramatically increased after triggering with PMA; zymosan caused triggering but less than was produced by PMA. Alpha-1-protease inhibitor produced a decrease in detachment when both PMA- and zymosan-triggered cells were the effectors; superoxide dismutase did not significantly reduce detachment. The ability of triggered PMNs to cause proteolysis of fibronectin, as measured in a radiolabeled fibronectin matrix degradation assay, was related to their triggerability in the detachment assay, zymosan being largely ineffective in triggering enhanced proteolysis. These findings suggest that fibronectin is important in maintaining the integrity of the alveolar epithelial surfaces. Furthermore, in inflamed alveoli, activated PMNs can release proteinases, which cause degradation of this matrix component leading to compromise of the alveolar epithelial barrier.

INTRODUCTION

The resident population of leukocytes in the peripheral airspaces of the normal lung is composed largely of alveolar macrophages. Polymorphonuclear neutrophils (PMNs) are, however, recruited to inflamed lung and, although they function as defensive cells, many studies have shown their potential for injuring elements of the lung parenchyma by release of proteinases or oxygen metabo-

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lites (1–4). In disease states such as adult respiratory distress syndrome (ARDS), idiopathic pulmonary fibrosis (IPF), and pneumoconiosis, increased numbers of PMN can be found in bronchoalveolar lavage (BAL) (5). When recruited to the alveolus, these PMNs have been implicated in alveolar epithelial cell injury and connective tissue proteolysis (3). In the short-term these effects contribute to changes in permeability, enhanced inflammation, and type-II cell hyperplasia and in the long-term to pathological change in the parenchyma of the lung (6).

We have previously studied the ability of PMNs from the rat lung to injure elements of the alveolar septum (3, 4). In the present study, as a model for epithelial cell injury by PMNs in the human lung, we used an in vitro assay in which peripheral blood PMNs were cocultured with ^{51}Cr-labeled cells of an alveolar epithelial cell line [A549] (1, 3, 7). The potential of PMNs to damage these target cells and hence release the radiolabel was assessed by culturing different ratios of peripheral blood PMNs to target cells with and without activation by a soluble trigger (PMA) or a particulate trigger (zymosan). The mechanisms of epithelial injury were investigated by assessing the effects of antiproteinase and antioxidants using this model. Since fibronectin, a component of the extracellular matrix of the lung is thought to be an important factor in the attachment between epithelial cells (8), we related the ability of PMNs to injure epithelial cells to their ability to cause proteolysis of fibronectin, in addition to studying the preference of epithelial cells for fibronectin-coated surfaces.

MATERIALS AND METHODS

Isolation of PMNs. Two separate 30-ml volumes of venous blood from normal subjects were withdrawn into 4 ml of acid citrate dextrose. To one aliquot, 12 ml dextran (Blood Transfusion Service, Edinburgh) were added, and this was allowed to sediment for 1 h at room temperature. The remaining 30-ml aliquot was centrifuged (1600 rpm, 10 min) and the plasma removed. This fraction was further centrifuged (3000 rpm, 30 min) to provide a platelet-poor plasma (PPP). The plasma was used to prepare discontinuous 42% and 65% density gradients of Percoll from a 90% stock solution (Pharmacia, Milton Keynes, U.K.). The leukocyte-rich plasma from the dextran sedimentation was centrifuged (1200 rpm, 10 min) and the pellet washed in HBSS (Gibco, BRL, Paisley, U.K.) (calcium-, magnesium-free), before being resuspended in 8ml PPP. Four milliliters of this suspension was layered onto the previously prepared gradients. Centrifugation (1200 rpm, 10 min) at room temperature produced a PMN layer that was carefully removed, washed twice in HBSS, and resuspended in serumless medium (Gibco, BRL). The cells were counted and cytocentrifuge smears prepared and stained with May Grunwald-Giemsa.

Detachment Assay. We have previously described this assay in detail (3). Briefly, A549 cells (7) were removed from routine culture in MEM + 10% heat-inactivated fetal calf serum (FCS) (Gibco, BRL) with trypsin–EDTA (Gibco, BRL). The concentration of cells was adjusted to 2.5×10^5 cells/ml, 200 μl of which was added to each well of 96-well plates (Flow Labs) to yield a final concentration of 5×10^4 cells/well. Seventy-four kBq of ^{51}Cr (Amersham International, Amersham, U.K.) was placed in each well in a 10-μl volume of PBS. The plates were incubated overnight in a humidified 5% CO_2 atmosphere at 37°C. After incubation, the A549 target cells

were washed twice with PBS and PMNs were added to give PMN–A549 ratios of 1:1 and 5:1. Triggers and/or inhibitors were added to wells as described below, and the plates were then incubated for a further 4 h.

The following triggers were prepared: PMA (0.1 μg/ml) (Sigma, Poole, Dorset, U.K.) and zymosan A (0.1 μg/ml) (Sigma) and wells were set up with and without alpha-1- proteinase inhibitor (0.1 μg/ml) (Sigma) or the antioxidant superoxide dismutase (0.1 μg/ml) (Sigma). In addition, wells containing target cells alone in medium with or without inhibitors were included as controls. To control for any detachment attributable to the triggers, PMA Triton X-100 and zymosan were included at the concentrations described above. Control experiments of serumless medium alone to measure spontaneous release of label, and 0.1% Triton X-100 to measure total ^{51}Cr uptake were undertaken.

After an incubation period of 4 h, the plates were centrifuged at 1000 rpm for 5 min and 50 μl of the supernatant was sampled from each well. The remaining 150 μl of supernatant was pipetted into a tube and each well washed twice with 200-μl aliquots of PBS. Washings from each well were pooled into the corresponding tube containing the original 150 μl of supernatant. ^{51}Cr activity in each tube was assessed by a gamma counter. Counts attributable to cell lysis in the first 50 μl of supernatant were multiplied by four and subtracted from the counts obtained from the remaining 150 μl of supernatant plus 2 × 200-μl washes, giving the counts due to cell detachment.

Assessment of Proteinase Activity. The assay used was that described by Brown and Donaldson (4). Briefly, the method of McConahey and Dixon (9) was employed to label fibronectin with ^{125}I (Amersham International). After labeling, fibronectin was diluted in PBS to give an activity of 10,000 cpm in 100 μl of solution. This was dried onto microliter plate wells, 100 μl/well by incubating at 37°C for three days. Before use, the wells were preincubated for 2 hr with F10 medium (Gibco) + 2% BSA (BDH, Poole, U.K.), and then washed once with 300 μl PBS to reduce background counts.

The assay consisted of adding 10^5 cells plus appropriate triggers + 2% BSA in 200 μl of F10 to triplicate groups of wells. A positive control consisting of trypsin at a concentration of 1 mg/ml in the above medium was also included (200 μl/well) in triplicate. Background counts were assessed by incubating wells in medium alone. The plates were incubated for 4 h at 37°C, after which 150 μl of the supernatant from each well was sampled and measured by gamma counter. This assay has been validated extensively as one that measures the proteolytic degradation of fibronectin without the involvement of "iodase" or oxidant (4, 10).

Epithelial Cell Attachment Assay. A549 cells at a concentration of 2.5 × 10^6/ml were incubated in 1 ml MEM + 10% FCS containing 200 μCi ^{51}Cr for 1 h at 37°C in a shaking water bath. After labeling, cells were spun and washed twice in fresh MEM + 10% FCS before resuspending in the same medium at a concentration of 2.5 × 10^6 cells/ml. This suspension was placed in triplicate groups of fibronectin-coated or plastic wells in 200-μl aliquots, and the plates were spun at 1000 rpm for 5 min. At 10, 30, 60, and 90 min after incubation at 37°C, triplicate groups of wells corresponding to a particular time point were washed twice with 200-μl aliquots of PBS and 200 μl Triton X-100 added to lyse attached cells. Finally, 150 μl of each cell lysate was assessed for ^{51}Cr release by a gamma counter.

Statistical Analysis. Data were analysed using Student's *t* test.

RESULTS

Assessment of Detachment with PMNs. Figure 1 illustrates detachment of A549 cells by peripheral blood PMNs, which in the untriggered condition increased as the ratio of PMN to target cells increased. At an effector–target

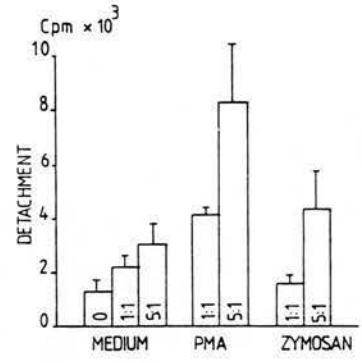


Fig. 1. The effect of detachment of ^{51}Cr-labeled A549 cells cocultured at increasing effector-target cell ratios. Results show the effect of soluble and particulate stimuli and represent the mean (*SEM*) of triplicate wells untriggered compared with triggered conditions in four separate experiments. ($P < 0.001$ within experiments.)

ratio of 5 : 1, detachment is more than two fold greater than with medium alone. The increase compared with medium alone is less marked at a ratio of 1 : 1. In the presence of the soluble trigger PMA, detachment at a ratio of 1 : 1 shows almost a two fold increase, compared with the same ratio without the trigger. Medium containing PMA alone caused some detachment, but this was less than half of that produced when PMNs at a ratio of 1 : 1 were added. The particulate trigger, zymosan, caused some increased detachment ($P < 0.01$) at the 5 : 1 ratio, but this was markedly less than PMA. Compared with the untriggered cells, zymosan produced a decrease in detachment of A549 cells by neutrophils in a ratio of 1 : 1. Medium containing zymosan produced detachment similar to that observed with medium but less than half the value with PMA.

Inhibition of Detachment by Alpha-1-Proteinase Inhibitor (PI) and Superoxide Dismutase. As in the noninhibited experiments, we observed an increase in epithelial cell detachment with increasing ratios of PMNs to target cells and in the presence of PMA and zymosan (Figure 2). The proteinase inhibitor alpha-1 PI produced a marked decrease in detachment of triggered cells. Using PMA as the trigger at a ratio of five PMNs to one A549 cell, detachment was reduced from 2135 $\pm$ 356 (*SEM*) cpm to 642 $\pm$ 135 cpm ($P < 0.01$). A similar effect was observed in the zymosan-treated wells with marked inhibition being produced by alpha-1 proteinase inhibitor, although this effect was not statistically significant ($P > 0.05$). SOD did not significantly reduce detachment (Fig. 3) but did, however, increase the amount of detachment, particularly with PMA.

Proteolytic Effects of PMNs. Table 1 summarizes the effect of PMNs on fibronectin degradation, measured as ^{125}I released from fibronectin into the medium. Addition of PMNs substantially increased proteolysis ($P < 0.001$). The

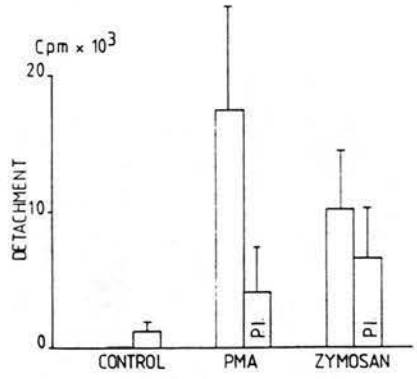


Fig. 2. The effect of detachment of ^{51}Cr-labeled A549 cells cocultured at increasing effector-target cell ratios. Results show the effect of soluble and particulate stimuli and include the inhibitory effect produced by the proteinase inhibitor alpha-1-PI. Data show the mean (*SEM*) of triplicate wells in four separate experiments.

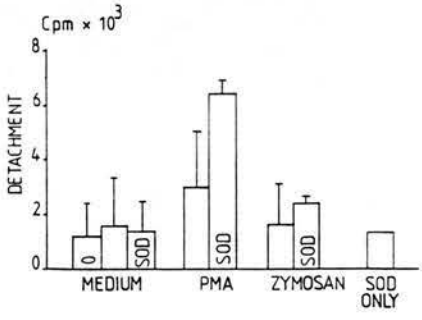


Fig. 3. The effect of detachment of ^{51}Cr-labeled A549 cells cocultured with peripheral blood PMNs and soluble and particulate stimuli. Results show the effect of superoxide dismutase (SOD) and represent the mean (*SEM*) of triplicate wells in four separate experiments. (No significant difference.)

Table 1. ^{125}I-Labeled Fibronectin Degradation by PMNs[a]

	Counts per minute		Trypsin
	Medium	Cells	
$\bar{X}$	3,875	14,961	17,463
SEM	705	1,971	1,602

[a] Results show cpm released into the medium after 4-h incubation. Highly significant differences were noted for cells compared with the medium control. Mean (*SEM*) of triplicate wells in six separate experiments.

effect of PMNs on fibronectin degradation was similar to that produced by trypsin.

Table 2 shows the effect of triggered PMNs on fibronectin degradation. An increase in proteolysis was noted for PMA-triggered neutrophils, with no significant difference in fibronectin breakdown observed when PMNs were triggered with zymosan. Individual experiments show significant differences between untriggered cells and PMN-stimulated neutrophils ($P < 0.05$), but no significant differences for untriggered and zymosan-triggered PMN.

Attachment Assay. Figure 4 shows the rate of attachment of A549 cells to fibronectin-coated and plastic substrata. Over the time course of the experiment, A549 cells show a preferential adherence to fibronectin compared with

Table 2. ^{125}I-Labeled Fibronectin Degradation by PMNs triggered with 0.1 μg/ml PMA or 0.1 μg/ml Zymosan[a]

	Counts per minute				
	Medium	Untriggered PMN	PMA-triggered PMN	Zymosan-triggered PMN	Trypsin control
$\bar{X}$	1,579	7,618	9,616	7,601	12,402
SEM	176	1,173	1,857	711	629

[a] Results show cpm released into the medium after 4-h incubation. Significant differences ($P < 0.05$) were noted for PMNs compared with PMA-triggered cells, no significant difference being shown between untriggered and zymosan-triggered neutrophils. Mean (*SEM*) at triplicate wells in three separate experiments.

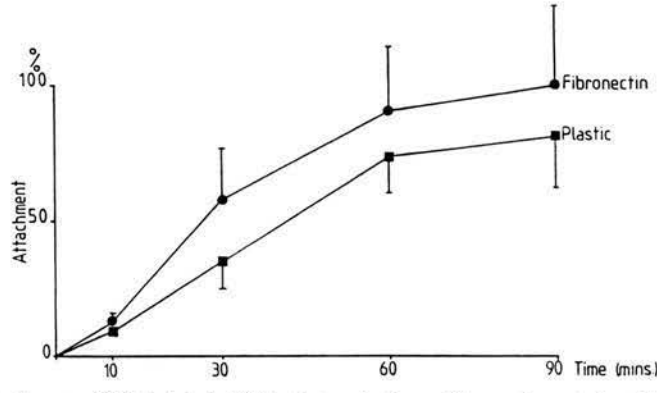


Fig. 4. Attachment of ^{51}Cr-labeled A549 cells to plastic- or fibronectin-coated wells. Results are expressed as a percentage of total attachment and represent the mean (*SEM*) of triplicate groups of wells in three separate experiments. ($P < 0.01$ within experiments.)

plastic. Significant differences ($P < 0.01$) between preference for fibronectin or plastic were noted for data within experiments.

DISCUSSION

Neutrophils (PMNs) play an important role in the defense of the respiratory tract against infection (10). However, various studies have suggested that persistent inflammation, with increased numbers of PMN, can result in damage to epithelial cells and other elements of the alveolar septum (1, 11). Studies demonstrating that PMNs derived from the inflamed rat lung have the ability to injure epithelium and attack extracellular matrix suggest that activation in vivo enhances the ability of PMNs to produce proteolytic damage (3, 4). The present study supports the view that resting human peripheral blood PMNs do not injure epithelial cells to any significant extent (1). However, treatment with leukocyte triggers in vitro enhances the ability of neutrophils to cause proteinase-mediated epithelial injury.

These experiments also confirm that untriggered peripheral blood PMNs produced only modest detachment injury to cells of an alveolar epithelial cell line, there being no lytic damage as previously shown by Ayars et al. (1). However, treatment with substances that can trigger leukocyte secretion did cause substantial detachment injury. By implication, therefore, substances that are present in the inflammatory milieu may be involved in priming the PMN to reach a state of activation where they can cause detachment injury to epithelial cells. The PMN-induced injury has been reported to be determined largely by the type of toxic product produced by the PMN (13). In previous studies, target cell lytic injury has been found to be mediated by oxygen metabolites. In our study, PMNs triggered with PMA and zymosan in the presence of the superoxide anion scavenger SOD produced no significant decrease in detachment injury. This supports the view that oxygen metabolites are not involved in detachment injury to the A549 epithelial cell line and supports previous work (1, 3). By contrast, the proteinase inhibitor alpha-1-PI produced a significant reduction in epithelial cell detachment by PMNs triggered with PMA. However, the reduction was not significant when zymosan was the trigger.

Support for our hypothesis that the detachment injury produced by PMNs is due to proteinase release comes from the fibronectin-degradation experiments. Increased proteolysis of fibronectin by triggered PMNs confirmed a previous finding that inflammatory cells cause detachment of epithelial cells by digesting fibronectin (14). It can be inferred that, following activation at an inflammatory site, PMNs have the potential to release proteolytic enzymes that can attack fibronectin, enhancing epithelial cell injury. Fibronectin, present in

basement membranes, is the likely target for proteinases released from activated PMN. The importance of the fibronectin component of basement membrane was shown by enhanced affinity of A549 epithelial cells for a fibronectin compared to a plastic surface.

There is alpha-1-PI present in the normal airspaces of the lung, and this will be increased during inflammation by exudation from plasma and the presence of raised levels due to the acute phase response. It is clear from the experiments in this study that the level of active alpha-1-PI would be crucial to the expression of PMN-mediated injury.

We have shown here that peripheral blood PMNs degrade fibronectin and cause epithelial detachment, and we have demonstrated the affinity of epithelial cells for a fibronectin matrix. It has been suggested that fibronectin is important in cell-to-cell and cell-to-matrix adhesion (8, 15) and that fibronectin is also an important component of the alveolar septum (16). During inflammation, the likelihood of enhanced proteolysis of fibronectin, as described in this pap by the inflammatory leukocytes, could lead to a reduction of this importan rix glycoprotein.

Proteolytic destruction producing a net loss of fibronectin could be important in destructive lung diseases such as emphysema and bronchiectasis. In this regard, peripheral blood PMNs from patients with emphysema or bronchiectasis appear to degrade significantly more fibronectin than control PMN (17). This suggests that there is some signal for the spontaneous production of the proteinase that can be received by circulating PMNs.

A second possible pathological sequel of increased fibronectin proteolysis is the epithelial injury that we have demonstrated here in vitro. Desquamation, or a lesser degree of epithelial injury, which may be stopping short of detachment, could lead to increased permeability, further enhancing inflammation. Evidence for epithelial injury has been found in diverse inflammatory lung conditions where an obvious epithelial toxin has not been identified. Epithelial abnormalities and type-II hyperplasia are often a feature of idiopathic pulmonary fibrosis (18), a condition where neutrophils are thought to play an injurious role. Increased epithelial cell permeability has also been demonstrated in smokers who are known to have increased numbers of PMNs in the distal airspaces (19). Type-II cell hyperplasia also occurs in a diverse range of inflammatory lung conditions (20), where substances toxic to the epithelium could be implicated but where the inflammatory leukocyte may also have a role. The pneumoconioses are an example of this; type-II cell hyperplasia and alveolar lipoproteinosis are frequent findings in silicosis (21), and desquamated epithelial cells have been described in the bronchoalveolar lavage of silicotics (22). The same type of response to epithelial injury is found in asbestosis (23).

The present study provides evidence to support the contention that fibronectin is an important factor in maintaining the integrity of the epithelial sur-

faces and that degradation of fibronectin by PMN proteinase may result in injury to the alveolar wall in man. Thus, in any condition where PMNs accumulate in the alveolar airspaces, the balance between the release and destruction of this vital matrix component could lead to diverse pathological changes with important consequences for change such as occur in interstitial lung disease, pneumoconiosis, and in the lungs of smokers.

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Changes in neutrophil morphology and morphometry following exposure to cigarette smoke

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Summary. Acute cigarette smoking delays neutrophils within the pulmonary circulation in some smokers. Evidence from an in-vitro Micropore filter model of the pulmonary capillaries indicates that this may be due to a smoke induced decrease in cell deformability. In order to determine whether changes in cell shape are associated with the observed decrease in neutrophil deformability following smoke exposure, cell morphology, using scanning electron microscopy, and morphometric measurements, made using transmission electron microscopy, were performed on aliquots of neutrophils harvested from whole blood in non-smoking subjects before and after exposure *in vitro* to cigarette smoke. Smoke exposure increased the maximum diameter and circumference of neutrophils, without changing their area. There was also a change in the maximum to minimum cell diameter ratio, which indicated that the cells had become less spherical. Scanning electron microscopy showed that smoke exposed cells had developed blebbing of their surface membranes, suggestive of an oxidative injury to the cell membrane rather than the shape changes associated with cell activation. These changes in the morphology and morphometry of smoke exposed neutrophils may contribute to the reduction in cell deformability induced by cigarette smoke.

Keywords: cigarette smoke, neutrophil, morphology, morphometry

Cigarette smoking is a major risk factor in the development of pulmonary emphysema (Auerbach *et al.* 1972). Neutrophils are present in increased numbers in both the airspaces (Hunninghake & Crystal 1983) and alveolar walls (Ludwig *et al.* 1985) of smokers. Acute cigarette smoking delays the passage of polymorphonuclear leucocytes within the pulmonary circulation in some smokers (MacNee *et al.* 1989a). In order to traverse the pulmonary microcirculation neutrophils must deform, as their average diameter of 7 μm (Schmid-Schonbein *et al.* 1980) is greater than that of the average pulmonary capillary segment diameter of 5 μm (Weibel 1963).

Studies using an in-vitro Micropore filter, whose dimensions mimic those of the average pulmonary capillary segment (MacNee *et al.* 1989b), suggest that neutrophils

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exposed *in vitro* to either vapour or particulate phase cigarette smoke have decreased cell deformability. It is reasonable to expect that neutrophils which are less deformable will take longer to traverse the microcirculation and may consequently have more time to increase the proteolytic burden in the lungs.

A number of factors influence the deformability of a cell. These include the fluidity of the cell membrane, the turnover of the cytoskeleton, the viscosity of the cytoplasm, the size of the nucleus and the surface area: volume ratio of the cell (Chien *et al.* 1984). The neutrophil is a multi-nucleate cell with a cell membrane area calculated to be between 40 and 90% in excess of that necessary to contain its volume (Schmid-Schonbein *et al.* 1980; Bagge 1976). In its resting state the membrane of the neutrophil is ruffled, exhibiting folds. During deformation within the capillary segments a proportion of the excess surface area of the membrane is utilized to allow a passive change in cell shape. The aim of this study was to assess the smoke induced changes in neutrophil morphology and morphometry which may contribute to the reduced filterability of the cells *in vitro* following smoke exposure.

Methods

Polymorphonuclear leucocyte harvesting

Neutrophils were harvested from 30 ml of venous blood obtained from six healthy, normal non-smokers. Whole blood was mixed with 12 ml of dextran MW 70000 (LOMODEX 70 Fisons P/C Pharmaceutical Division, Loughborough, UK) and allowed to sediment for one hour to separate the majority of the erythrocytes so as to obtain a leucocyte-rich plasma (LRP). This LRP (approx 20 ml) was washed twice with phosphate buffered saline (PBS, pH 7.4, 10 ml) at 100 *g* for 10 min. The resulting LRP cell pellet was resuspended in platelet-poor plasma (PPP) (4 ml), previously prepared by centrifuging 20 ml of blood for 10 min at 400 *g*, aspirating the plasma, followed by centrifugation at 1000 *g* for 30 min. This was overlaid on a discontinuous plasma Percoll (Pharmacia, Uppsala, Sweden) 42 and 65% density gradient and centrifuged at 200 *g* for 10 min. The resultant neutrophil band which settled at the interface between the 65 and 42% plasma–Percoll gradient was aspirated and washed with PBS at 200 for 10 min. Any contaminating red blood cells were lysed with 0.2% saline for 20 s. The tonicity of the solution was re-established immediately with an equal volume of 1.6% saline. The neutrophils were washed and then resuspended in PBS to give a final concentration of 1×10^6 cells/ml.

Smoke exposure

Two-millilitre aliquots of whole blood or harvested neutrophils at a concentration of 1×10^6 cells/ml, were placed in the silicon ized flask of a tonometer and exposed to four puffs (37 ml/puff) of vapour phase cigarette smoke over 4 min. Vapour phase cigarette smoke was produced by drawing particulate cigarette smoke, generated by a smoking machine, through a Cambridge filter. Control blood and cell samples were agitated in the tonometer for the same length of time but were not exposed to smoke. Carboxyhaemoglobin (CoHb) was measured in the whole blood samples using a co-oximeter (Co-oximeter 282 IL, Instrumentation Laboratory) as an indication of smoke exposure. The neutrophils were washed after exposure in PBS at 200 *g* for 10 min, then fixed in freshly prepared 3% gluteraldehyde and processed for electron microscopy.

Morphometry

Samples of neutrophils post fixation were placed in 1% osmium tetroxide in sodium cacodylate buffer for 1 hour, dehydrated through to absolute alcohol (10, 50 and 100%, 15 min per change), then transferred to propylene oxide (10 min, twice) and impregnated overnight in Araldite resin

This preparation was finally embedded in Araldite resin and polymerized at 50°C. The sections were cut on a LKB Ultrotome Nova to a thickness of 60 nm and stained with uranyl acetate and lead citrate. Eight random fields were then photographed on a Jeol 100 s electron microscope at a magnification of 2.1×10^3. Morphometric analysis was done using a Tektronix 4050 series computer linked to a digitizing tablet (GIS Blairgowrie, Scotland), on a total of 200 cells for each condition, taken from 6 paired experiments. The following parameters were measured: minimum diameter, maximum diameter (perpendicular to the minimum diameter), circumference and area. Two other parameters were then calculated from these measurements:

$$\text{diameter ratio} = \frac{\text{minimum diameter}}{\text{maximum diameter}}$$

$$\text{form factor} = \frac{\text{area}}{\text{circumference}^2} \times 4\,\pi$$

The form factor of a perfect circle is 1 representing the maximum area bound by a given circumference. This tends towards zero where the area bound by the circumference is not maximal, due to increases in ellipticality and/or undulations of the circumference. It differs from the diameter ratio in that it is little affected by minor undulations of the circumference.

Morphology

Neutrophil morphology was assessed before and after smoke exposure in 30 cells randomly selected from two subjects using scanning electron microscopy. The changes in cell shape were categorized as:

(1) Neutrophils with a normal surface membrane ruffling and a spherical shape.
(2) Neutrophils producing lamella podia.
(3) Cells with membrane blebbing.
(4) Cells which have lamella podia and surface blebbing.

An aliquot of neutrophils activated with 1 μg/ml of phorbol myristate acetate (PMA) was processed for scanning electron microscopy for comparison with the smoke exposed cells. Cell viability was assessed before and after smoke exposure by trypan blue exclusion.

Statistical analysis

Results were analysed using Kruskal–Wallis one-way ANOVA.

Results

Carboxyhaemoglobin in whole blood increased from 1.1%±0.3% to 4.5%±2.2% following smoke exposure ($P<0.05$). Cell viability remained >98% after smoke exposure, as assessed by trypan blue exclusion.

Morphometry

The maximum diameter and circumference of smoke exposed neutrophils increased following smoke exposure, and consequently the diameter ratio and form factor decreased, compared to control cells (Table 1). However, there was no significant change in either the minimum diameter or area following smoke exposure. The maximum diameter of the smoke exposed neutrophils was approximately 10% larger than that of the control neutrophils, with a shift in the frequency of distribution of these cells towards the higher values (Fig. 1). Neither the skewness nor the kurtosis of these frequency distributions changed after smoke exposure ($P>0.05$). This suggests an increase in maximum diameter in the majority of cells, as opposed to an increase in the size of only the smaller cells. The mean circumference of the neutrophils also increased by approximately 14% whilst the shape of the frequency distribution of the circumference remained the same (Fig. 2).

Morphology

Scanning electron microscopy indicated that

Table 1. Comparison of the morphometry of control and in-vitro smoke exposed neutrophils

Measurement	Control cells	Smoke exposed cells
Minimum diameter (μm)	6.02 ± 1.18	6.08 ± 1.25
Maximum diameter (μm)	7.33 ± 1.88	8.09 ± 1.79*
Diameter ratio	0.84 ± 0.12	0.76 ± 0.12*
Circumference (μm)	24.14 ± 5.58	27.43 ± 5.19*
Area (μm^2)	32.44 ± 11.72	34.08 ± 10.84
Form factor	0.71 ± 0.15	0.58 ± 0.14*

* $P < 0.001$ compared with control cells. Results represent mean ± s.d. of 200 cells.

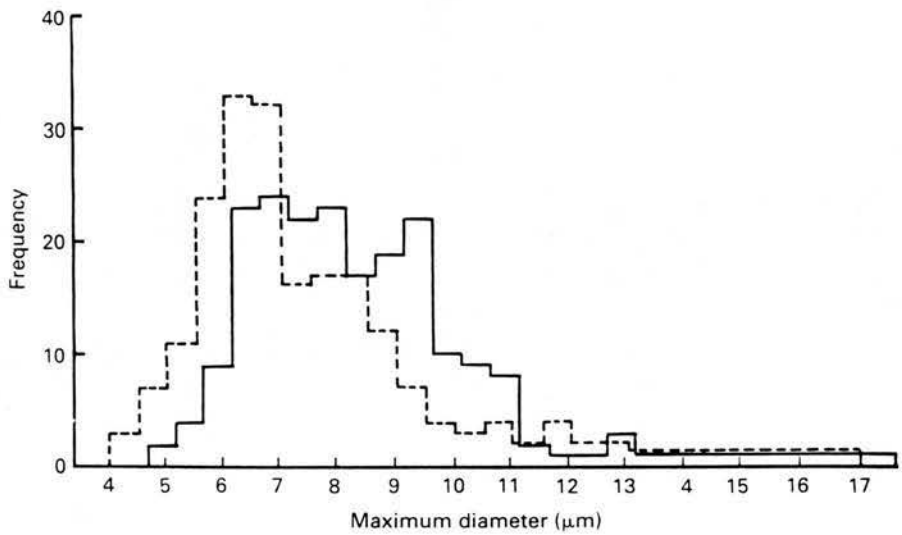


Fig. 1. The effect of in-vitro smoke exposure on the maximum diameter of human neutrophils. – – Control cells: ——. smoke exposed cells.

a greater proportion of smoke exposed cells had developed surface blebbing after exposure to smoke (Table 2). Examples of control, smoke exposed and PMA stimulated neutrophils are shown in Figs 3–5.

Discussion

The acute effect of cigarette smoke on neutrophils has not been previously studied. This study has shown that exposure to vapour phase cigarette smoke *in vitro* causes changes in cell shape. These result increased cell circumference and maximu diameter with accordingly decreased di meter ratio and form factor so that the ce become less spherical. The presence of su face blebbing indicates that smoke exposu produces a change in cell shape which dissimilar to that which occurs when ce are triggered with PMA.

The effects of cigarette smoke on t morphology of alveolar macrophages ha been more widely studied than the effects

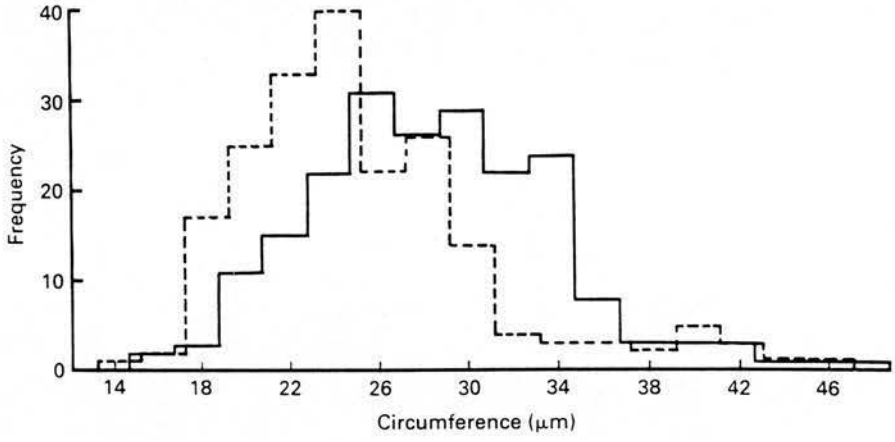


Fig. 2. The effect of in-vitro smoke exposure on the circumference of human neutrophils. – – –, Contro cells; ——, smoke exposed cells.

Table 2. Analysis of the morphology of neutrophils from two subjects before and after smoke exposure in-vitro by scanning electron microscopy

	Number of neutrophils	
Morphological category	Control	Smoked
Normal	49	6
Lamella podia (1)	11	0
Surface blebbing (2)	0	49
Combined (1 + 2)	0	5

neutrophils but have produced conflicting results. Some studies indicate that macrophages from smokers are larger than those from non-smokers (Warr & Martin 1978; Finch *et al.* 1980) whereas others found no differences (Harris *et al.* 1970). The use of differing culture systems with or without the presence of serum and a lack of standardization of the smoking history immediately prior to the bronchoalveolar lavage may explain some of these contradictory results. Since we were interested in assessing the acute effect of components of cigarette smoke which might affect cells in transit in the pulmonary capillaries, we studied vapour phase cigarette smoke. Guarnari (1978 found that alveolar macrophages recovere from lavage and then exposed to in-vitr smoke did not become less viable, which is i agreement with our observations using neu trophils.

Smith *et al.* (1986) found that alveola macrophages from healthy cigarett smokers generated a greater pressure durin filtration than cells obtained from non smokers, associated with an increase in ce size and loss of the ruffles on the surfac membrane. They concluded that the loss c the excess surface membrane available fo deformation, due to cell shape change, ma be responsible for the impaired filtration. Th changes in neutrophil morphometry an morphology following smoke exposure i this study may also act to reduce the ability c cells to passively deform in a Micropor membrane (MacNee *et al.* 1989b). Thes shape changes are dissimilar to thos induced by stimulation with PMA (Fig. 5 and thus do not support the hypothesis tha smoke exposed neutrophils are activated.

Studies of the effects of smoking on alveo lar macrophages may also help to explain th mechanism of the changes induced in neu trophils by smoke. Rasp *et al.* (1978) foun that up to 60% of alveolar macrophages fro

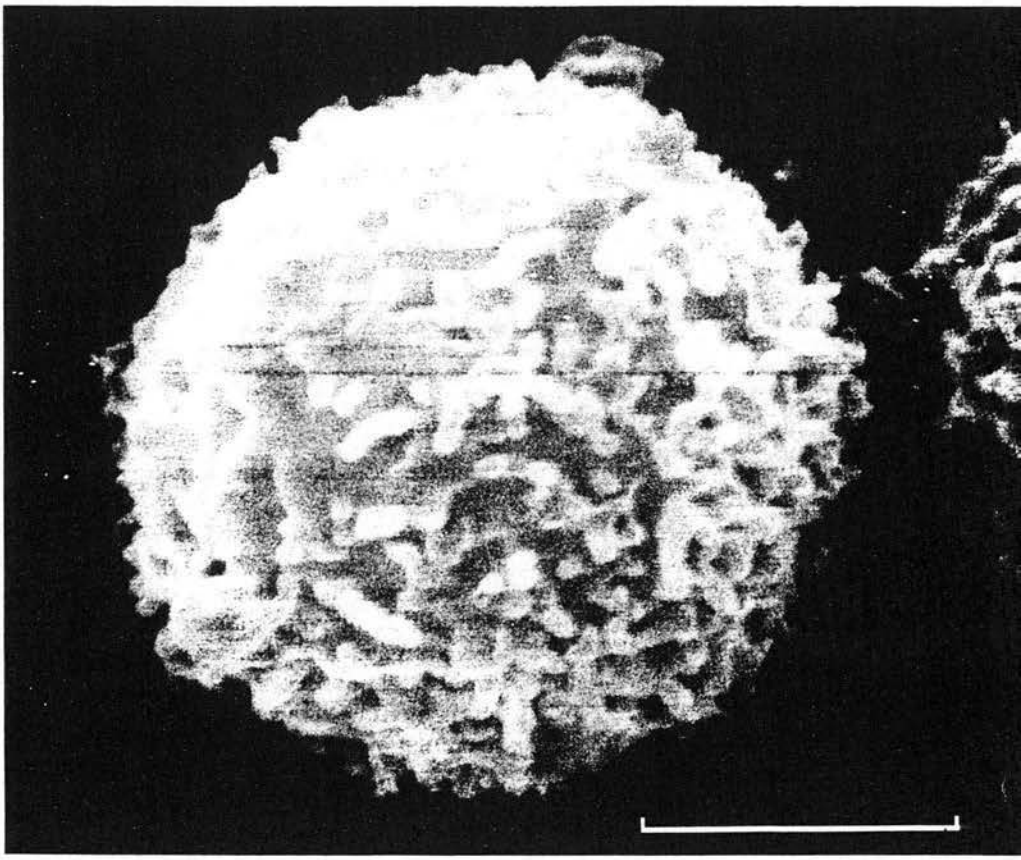


Fig. 3. Scanning electron micrograph of an untreated human neutrophil showing a spherical shape a normal membrane ruffling. The line represents 2.31 μm.

smokers had a blebbed surface, which was not the case in non-smokers. Cell membrane blebbing or zeinosis has been observed in Type I pneumocytes after oxidative injury (Hayashi *et al.* 1987). This may result from oxidation of the sulphydryl groups in actin (Mirabelli *et al.* 1988) producing an increase in F-actin. Oxidative stress produced by quinones, a component of cigarette smoke, has been shown to deplete intracellular glutathione, followed by oxidation of protein sulphydryl groups which in turn leads to dissociation of the cytoskeleton from the plasma membrane (Billamo & Mirabelli 1987–88). This results in disruption of the active binding sites of the actin binding proteins which attach the cytoskeleton to the plasma membrane, resulting in surface bl bing. Tulenko *et al.* (1988) also suggest that chronic cigarette smoking produ reorganization of the phospholipid bilayer smooth muscle cell membrane. Cigare smoke has an immense oxidant potent since it is estimated to contain 10^{17} oxida molecules per puff (Church & Pryor 198 Thus oxidant induced changes in both t cytoskeleton and the cell membrane likely to have resulted in the shape chan which we noted in neutrophils after ex sure to cigarette smoke.

If cigarette smoke does lead to a change the neutrophil membrane with the li bilayer becoming less fluid, such cells v take longer to change shape and may

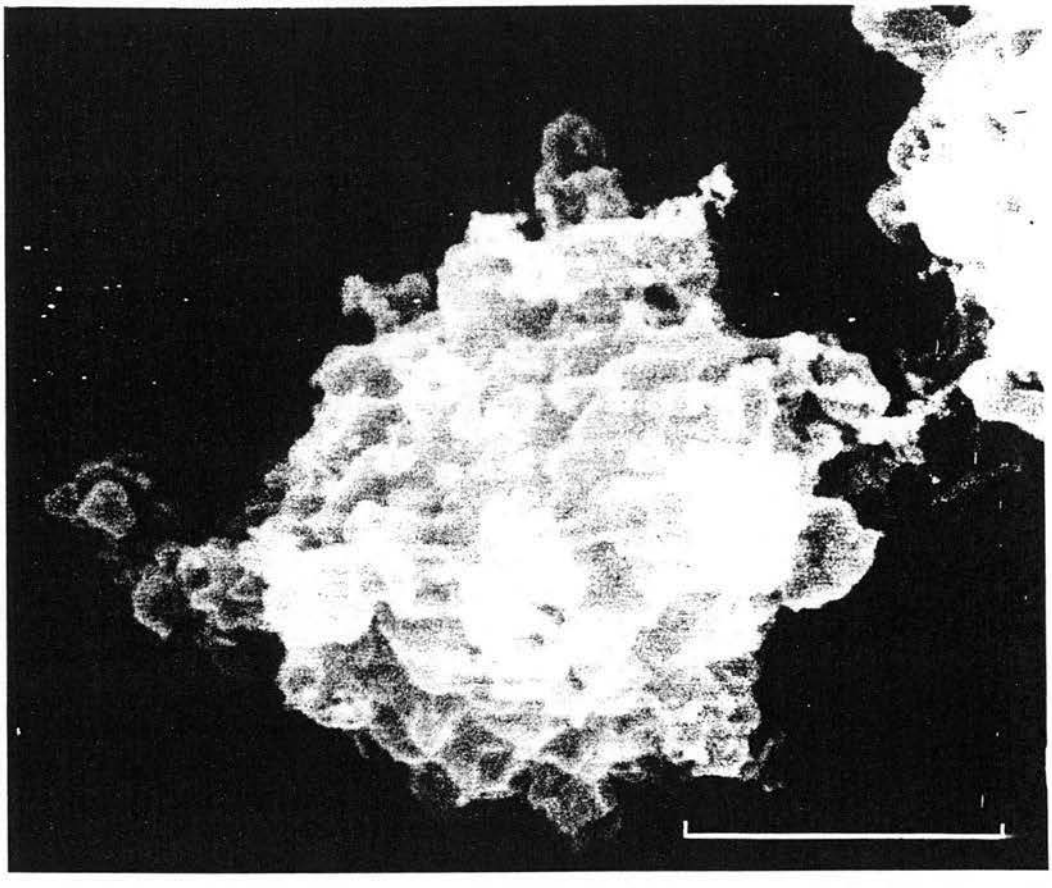


Fig. 4. Scanning electron micrograph of a human neutrophil exposed to cigarette smoke for 4 min. The cell has undergone zeinosis shown by membrane 'blebbing'. The line represents 2.7 μm.

slowed when attempting to deform in order to negotiate the smaller pulmonary capillary segments. This change in cell deformability will be compounded by the changes in the cytoskeleton, induced by smoke, due to alteration in the configuration of actin and destruction of the active sites of the membrane binding proteins.

A prerequisite for the hypothesis that neutrophils are delayed in the pulmonary microcirculation by a direct effect of cigarette smoke on the cell is that oxidative products of inhaled cigarette smoke must reach the intravascular space in the pulmonary capillaries. The fact that inhalation of ozone, a component of cigarette smoke, causes intravascular oxidative stress as shown by a decrease in erythrocyte glutathione and an increase in erythrocyte fragility (Buckley *et al.* 1975) is at least supporting evidence that cigarette smoking could produce a similar effect *in vivo*.

We conclude that in-vitro exposure of PMN to vapour phase cigarette smoke leads to zeinosis, a cell surface change which is regarded as a response to oxidative injury. Smoke probably also produces concurrent changes in the cytoskeleton and associated proteins which, taken together, result in a change in both cell shape and cell size. These changes could be major contributory factors in the increased pressures observed during

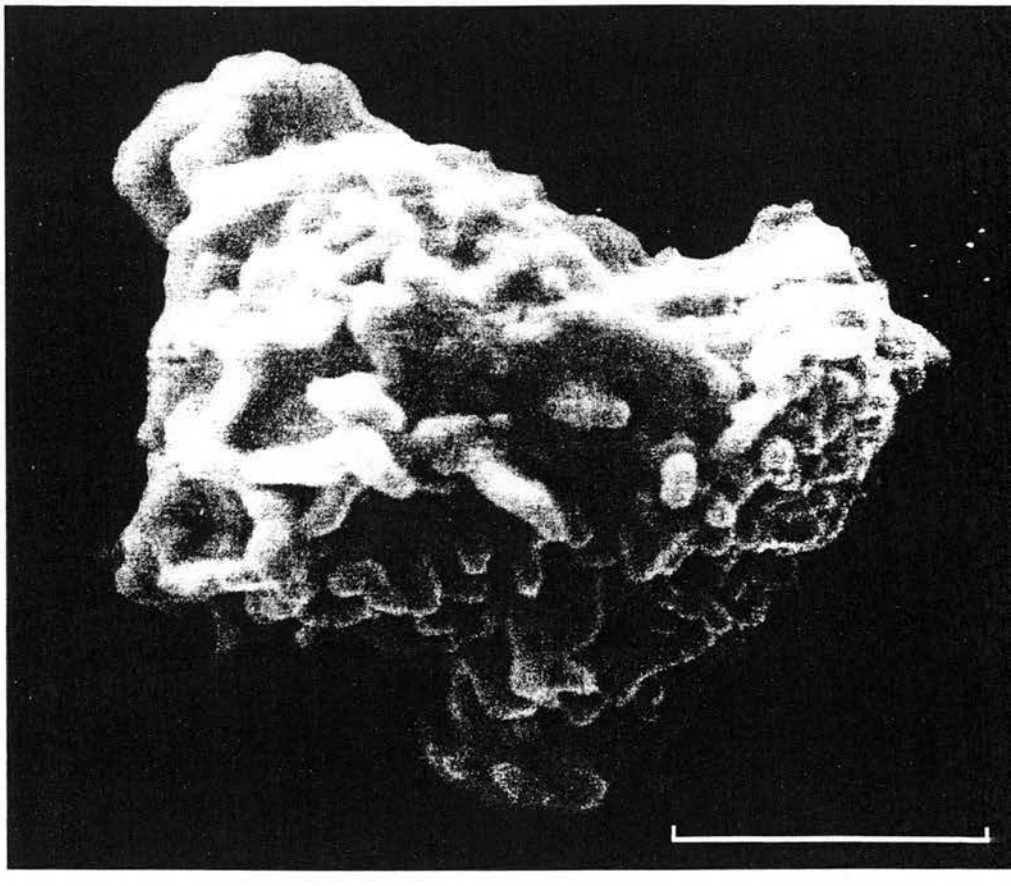


Fig. 5. Scanning electron micrograph of a human neutrophil exposed to PMA for 4 min. The cell has its spherical configuration and the surface has developed folds. The line represents 2.31 μm.

filtration of smoke exposed cells *in vitro* in a model of the pulmonary capillary bed (MacNee *et al.* 1989b) and help to explain the increase in the normal delay of neutrophils in transit in the pulmonary microcirculation which occurs during smoking *in vivo* (MacNee *et al.* 1989a).

Acknowledgements

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CHARACTERISTICS OF BRONCHOALVEOLAR LEUKOCYTES FROM THE LUNGS OF RATS INHALING 0.2–0.8 ppm OF OZONE

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The study was aimed at investigating the likely role of the bronchoalveolar leukocytes in the epithelial injury arising from exposure to ozone at low concentrations. Rats were exposed to 0.2–0.8 ppm of ozone for 7 hr/day for up to 4 days. At the selected time points the lungs were lavaged and the bronchoalveolar lavage cell profile was assessed by differential counting. Selected activities of the bronchoalveolar leukocytes, which could contribute to epithelial injury in vivo, were assessed in vitro, including (1) spontaneous and phorbol-stimulated release of superoxide anion, (2) proteolysis of fibronectin, and (3) ability to damage epithelial cells. Exposure to ozone at the highest concentrations caused inflammation in the bronchoalveolar region as shown by an increase in the proportion of neutrophils in the lavage. There was adaptation to the ozone-induced injury as shown by the fact that this inflammation was reduced as the time of exposure increased. No increases in any of the indicators of ability to injure epithelial cells in vivo were seen. There was evidence of decreased ability to mount an oxidative burst in the bronchoalveolar leukocytes from rats inhaling 0.6 ppm of ozone but this was not present in those rats that were inhaling 0.8 ppm. The macrophages from the lungs of rats inhaling the highest concentrations of ozone showed blebbing and this was reflected in an increase in the size compared to controls. We conclude that ozone, at the time points and concentration utilized here, causes inflammation but that the inflammatory cells are unlikely to mediate injury to the epithelial cells.

INTRODUCTION

Ozone is an atmospheric pollutant whose oxidizing activity makes it potentially injurious to the human lung, and epidemiological studies tend to confirm this potential (reviewed by Bresnitz and Rest, 1988). In experimental studies, ozone is without doubt injurious to the lung of laboratory animals if the dose is sufficiently high. In the short-term (hours to days) there is type 1 alveolar epithelial cell injury with compensatory type 2 epithelial cell hyperplasia (Tepper et al., 1990; Wright et al., 1987). There are substantial alterations to the epithelial cells of terminal

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bronchioles including hyperplasia even at levels as low as 0.5 ppm (Tepper et al., 1990) or 0.25 ppm (Barry et al., 1988). With longer term exposure there is an increase in interstitial mesenchymal cells and their products (Barry et al., 1985). Increased collagen synthesis has been described in rats exposed to 0.5–2.5 ppm (Last et al., 1983) but was not detected in another, similar study (Wright et al., 1988).

Recruitment of inflammatory macrophages and neutrophils to the lung has been reported in several studies with severity of inflammation depending to a large extent on the exposure concentration (Esterline et al., 1989; Holtzman et al., 1983; Hotchkiss et al., 1989a; Tepper et al., 1989). Recent experiments in humans tend to support these suggestions from animal experimentation. Healthy human volunteers who inhaled 0.4 ppm of ozone were lavaged prior to and following exposure and showed dramatic increase in elements indicating inflammation (Koren et al., 1989).

It has also been suggested that in experimental animals chronic inhalation of ozone causes an excess of lung tumors (Hassett et al., 1985; Mustafa et al., 1988). This could be related to the hyperplasia mentioned above.

Our previous work examined the relationship between inflammation and epithelial injury. Using pathogenic dusts we demonstrated that leukocytes recruited to dust-exposed lung have the ability to injure epithelial cells by a protease-mediated mechanism (Brown and Donaldson, 1988; Donaldson et al., 1988). We therefore set out to determine whether the inflammatory leukocytes recruited to rat lung on exposure to low levels of ozone could mediate injury to epithelial cells and add to the direct ozone-mediated epithelial injury.

MATERIALS AND METHODS

Animals

Twelve- to sixteen-week-old male syngeneic, SPF-maintained rats of the PVG strain, weighing approximately 200 g, obtained from the Institute of Occupational Medicine's own breeding unit, were used in these studies. Both control and ozone-exposed rats had access to food and water ad libitum.

Ozone Exposure

Ozone was generated using a Wallace Tiernan laboratory ozonator type BA.023. This instrument was tested and found to produce a stable ozone output and it was ascertained that the output could be controlled by interfacing with a BBC microcomputer and an ozone monitor. The ozone monitor was an Analysis Automation 427 u/V photometric ozone analyzer. The ozone analyzer was connected to a BBC microcomputer and the ozone levels analyzed were stored on a floppy disk. The level of

ozone in the exposure chamber was monitored by the ozone analyzer connected to the computer and this was fed back to a solenoid valve that corrected the percentage of the time the valve was open to allow ozone to bleed into the chamber air supply (15 l/min). The ozone exposure chamber comprised a sealable 0.12-m^3 perspex box containing three stainless steel cages in which the rats were placed two to a cage. Groups of six rats were placed in the primary ozone exposure chamber at 0900 hr and were exposed to the required ozone concentration for 7 hr; such an exposure is hereafter referred to as 1 day. The rats remained in the chamber overnight and were exposed for further days if required. On the morning following the end of the requisite number of days exposure, groups of three rats were killed and subjected to bronchoalveolar lavage to obtain the bronchoalveolar leukocyte population.

Bronchoalveolar Lavage of Rats

Animals were killed by intraperitoneal overdose with phenobarbitone and the chest cavity opened. The trachea was cannulated with a blunt 16-gauge needle through a small incision. Lungs were dissected free of the chest cavity and lavaged with 4 × 8 ml aliquots of sterile saline warmed to 37°C; lungs were massaged gently during each lavage to increase the cell yield. The pooled cells were placed immediately on ice before washing with phosphate buffered saline and dividing into aliquots that were delivered into different media for the different assays as described below.

Cell Counting

Bronchoalveolar leukocytes were counted in a Neubauer chamber and then prepared for differential counting using a cytocentrifuge prior to staining with May Grunwald Giemsa stain.

Superoxide Anion

As a measure of oxygen production the release of superoxide anion in the presence or absence of the leukocyte trigger PMA (phorbol myristate acetate, Sigma, Poole) was measured. Superoxide anion was measured according to the method of Johnston (1981), which measures the oxidation of cytochrome C.

Fibronectin Proteolysis

To measure neutral protease, a functional assay of fibronectin degradation was used as described by Brown and Donaldson (1988). The method utilizes fibronectin labeled with ^{125}I, coated onto the bottom of microtiter plate wells. Whole cells are added to the wells and the breakdown of fibronectin is assessed by the release of counts into the supernatant over 4 hr.

Epithelial Injury

To assess alveolar epithelial cell detachment and lysis caused by bronchoalveolar leukocytes, 5×10^4 cells of the A549 epithelial cell line (Lieber et al., 1976) were labeled overnight with ^{51}Cr in microtiter plate wells. The labeled cells were washed to remove free ^{51}Cr and lavaged cells were added at lavage cell/epithelial cell ratio of 10 : 1 in serumless medium (Newmann and Tyteil, Gibco Paisley). After incubation at 37°C in 5% CO_2 for 4 hr, the extent of cell lysis was assessed as free ^{51}Cr; cell detachment was measured by washing off the detached cells followed by centrifugation.

Assessment of Macrophage Size

Macrophage size was assessed by the method of Donaldson et al. (1984) modified to count the size of 200 cells on a cytocentrifuge preparation. Using this technique the maximum diameter of 200 May Grunwald Giemsa-stained macrophages is assessed using a microscope interfaced with a digitizing board and microcomputer.

Overall Design of the Study

The main study took the following design:

1. Groups of three rats were exposed to ozone and groups of three rats were maintained in the animal house as controls (in four experiments, a single control group of three rats acted as control for two treatment groups; this was accounted for in analyses of variance).
2. Animals were exposed to ozone for 7 hr/day for 1, 2, or 4 days.
3. Rats were killed and lavaged on the morning following the last day of exposure.
4. Lavaged cells from each animal were kept separate for total cells, percentage leukocytes, protease assay, and detachment/lysis assay; cells from all exposed or all controls were pooled for the assay of superoxide anion.
5. All experiments were repeated three times on separate occasions.

Statistical Analysis

For each response variable the measured data were analyzed by analysis of variance, as incomplete blocks, with experimental run in the role of block. The mean square for interaction between experiments and treatments was used as an estimate of error. These response variables, which took the form of percentages (the differential leukocyte count), were analyzed on the logistic scale. Examination suggested that all other responses were best analyzed on the log scale, except for radioactivity count measuring cell detachment, which was left untransformed. F tests based on an analysis-of-variance table identified those factors, and the

levels of interaction between them, which significantly affected the response variable at the 5% level. Estimated means obtained from fully parameterized models (i.e., including all interaction terms between treatment factors although these were never significant at the 5% level) were tabulated by these significant factors in order to explore specific contrasts in detail using *t* tests. For this purpose the estimates were arbitrarily normalized to the level of the earliest experiment of the series.

Presentation of Data

All data are presented in the tables as the raw form. When there are important significant treatment effects of ozone treatment, these are clarified as figures using data based on the retransformed estimated treatment means.

RESULTS

Attained Ozone Exposure

Concentrations of ozone produced at the concentrations used over 22 consecutive days of experimentation were (mean [SD] ppm) 0.2 (0.01), 0.4 (0.03), 0.6 (0.05), and 0.8 (0.04) from more than 2500 measurements made at 10-sec intervals throughout the 7 hr of exposure each day.

Total Lavageable Leukocyte Counts

There was no overall change in the total number of lavageable leukocytes with any of the ozone concentrations or time treatments, with the number remaining between 3 and 4 $\times$ 10^6 cells/rat throughout the study (Table 1).

TABLE 1. Total Numbers of Leukocytes Obtained by Bronchoalveolar Lavage from Lungs of Rats Exposed to Ozone for Up to 4 Days[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	3.6 (1.2)	4.0 (1.4)	3.0 (0.6)	3.6 (0.5)	2.8 (0.6)	3.3 (0.8)
0.4	3.2 (0.6)	3.6 (0.6)	3.9 (0.7)	3.2 (0.6)	3.4 (0.6)	3.7 (0.8)
0.6	2.8 (0.4)	2.6 (0.4)	3.4 (0.3)	3.6 (0.3)	2.5 (0.3)	3.3 (0.5)
0.8	3.7 (0.3)	3.2 (0.8)	4.0 (1.0)	4.6 (0.9)	2.4 (0.3)	3.7 (0.2)

[a]Data as mean (SD) millions of leukocytes per rat in 3 experiments, 3 rats per experiment.

Percentages of Different Leukocyte Types

The proportion of macrophages in the bronchoalveolar lavage of control rats was 95–98% throughout the study. However, the proportion of macrophages was significantly decreased compared to controls, with 0.8 ppm ozone at 1 (89.9%) and 2 (93.6%) days (Table 2). This was a result of an increase in the proportion of neutrophils, which reached a mean of 8.7% and 4%, respectively, on 1 and 2 days (Table 3). Analysis of variance showed significant effects of ozone exposure concentration (0.6 and 0.8 ppm) and duration (1 and 2 days) (Figs. 1 and 2). There were no significant effects of ozone treatment on lymphocyte numbers.

Production of Superoxide Anion

Treatment of the lavaged cells with PMA caused a stimulation of superoxide anion production in every case. There was, however, variability in the production of superoxide anion as we reported previously (Table 4). There was no increase in either unstimulated or PMA-stimulated oxidant production with any treatment but there was a reduction in superoxide anion production in bronchoalveolar leukocytes from rats exposed to ozone at 0.6 ppm (Fig. 3). There was a suggestion from the raw data that this was principally present on days 1 and 2 but the effect of time failed to attain statistical significance.

Proteolysis of Fibronectin

There was apparent variability in the ability of bronchoalveolar leukocytes to breakdown fibronectin, but this was mostly due to half-life effects in the iodinated fibronectin matrix (Table 5). There was no evidence of any systematic effects of treatment when comparisons were made with the controls on each day. Although no positive control leukocyte populations were used in the ozone study, experiments carried out using the same [^{125}I]fibronectin plates over the same time period showed that

TABLE 2. Percentage of Macrophages in Bronchoalveolar Leukocyte Populations from Rats Exposed to Ozone for up to 4 Days[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	95.2 (4.2)	96.4 (1.3)	98.8 (1.5)	98.2 (1.3)	95.8 (4.9)	96.9 (2.2)
0.4	98.4 (0.8)	97.6 (0.8)	97.9 (1.2)	96.7 (1.5)	98.0 (1.0)	97.9 (0.8)
0.6	98.9 (0.4)	97.2 (2.1)	98.6 (0.2)	95.7 (2.3)	99.1 (0.2)	98.7 (0.7)
0.8	97.4 (1.6)	89.9 (7.2)	95.7 (2.3)	93.6 (2.3)	98.1 (0.4)	98.2 (0.8)

[a]Data as mean (SD) of 3 experiments, 3 rats per experiment.

TABLE 3. Percentage of Neutrophils in Bronchoalveolar Leukocyte Populations from Rats Exposed to Ozone for Up to 4 Days[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	2.6 (4.1)	2.0 (2.0)	0.1 (0.2)	0.4 (0.8)	2.6 (4.1)	1.6 (2.4)
0.4	0.2 (0.4)	0.9 (0.8)	0.4 (0.5)	1.3 (1.5)	0.7 (0.7)	0.1 (0.2)
0.6	0.0 (0.0)	2.0 (1.5)	0.0 (0.0)	3.1 (2.2)	0.0 (0.0)	0.1 (0.2)
0.8	0.9 (0.8)	8.7 (7.0)	0.8 (0.5)	4.0 (1.8)	0.2 (0.4)	0.3 (0.0)

[a]Data as mean (SD) of 3 experiments, 3 rats per experiment.

inflammatory populations containing 80% neutrophils caused a 10- to 20-fold increase over control bronchoalveolar cells, thus validating the assay.

Epithelial Injury

Bronchoalveolar leukocytes from rats inhaling ozone did not have altered ability to cause lytic injury to the target epithelial cells compared to controls (Table 6). Neither was there any evidence that epithelial cells

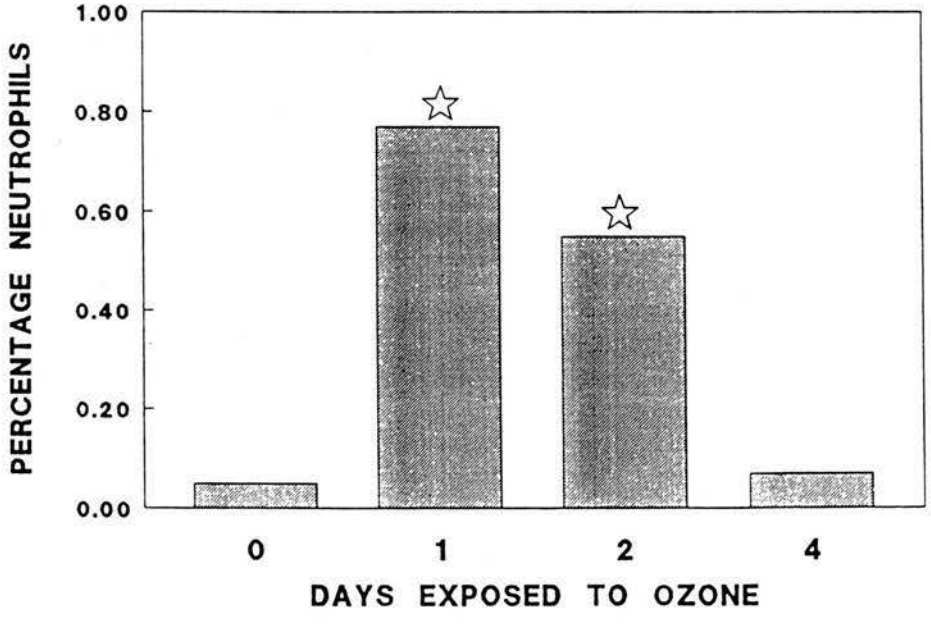


FIGURE 1. Percentage neutrophils present in lavage cells from rats exposed to ozone for 1–4 days. Retransformed estimated means across all exposure concentrations. Stars indicate significant ($p < .05$) differences from controls.

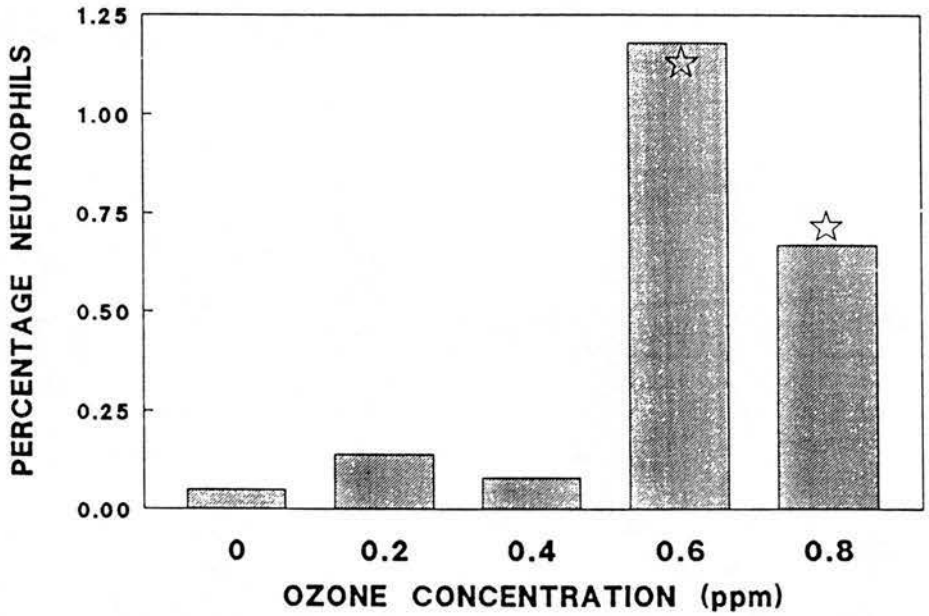


FIGURE 2. Percentage neutrophils present in lavage cells from rats exposed to 0.2–0.8 ppm ozone. Retransformed treatment means across all time points. Stars indicate significant ($p < .05$) differences from controls.

TABLE 4. Production of Superoxide Anion (nmol/10^5 cells/2 hr) by Bronchoalveolar Leukocytes from Control Rats and Rats Exposed to Ozone

		Days exposed to ozone					
Ozone conc.		1		2		4	
(ppm)	PMA	Control	Ozone	Control	Ozone	Control	Ozone
0.2	No	5.8 (2.7)	6.4 (2.9)	8.9 (1.3)	8.3 (1.4)	6.1 (3.0)	6.0 (1.7)
	Yes	21.4 (6.7)	25.5 (11.1)	28.4 (3.5)	27.3 (1.2)	23.9 (8.2)	24.3 (6.4)
0.4	No	6.0 (0.6)	5.3 (1.7)	7.8 (0.5)	7.1 (1.5)	9.7 (6.0)	9.8 (4.0)
	Yes	23.0 (2.7)	16.3 (2.2)	35.6 (4.8)	30.5 (2.6)	21.9 (4.0)	26.2 (9.8)
0.6	No	5.2 (0.5)	4.8 (0.5)	5.8 (0.2)	3.9 (1.0)	7.7 (1.1)	6.6 (0.4)
	Yes	26.2 (1.5)	15.4 (2.3)	28.6 (3.7)	16.0 (3.7)	33.3 (9.1)	31.4 (9.9)
0.8	No	4.4 (6.4)	3.5 (4.1)	2.7 (3.2)	2.3 (1.9)	6.6 (4.8)	5.1 (3.9)
	Yes	15.4 (19.0)	13.2 (15.4)	10.5 (9.1)	9.4 (6.7)	14.5 (7.8)	14.8 (9.4)

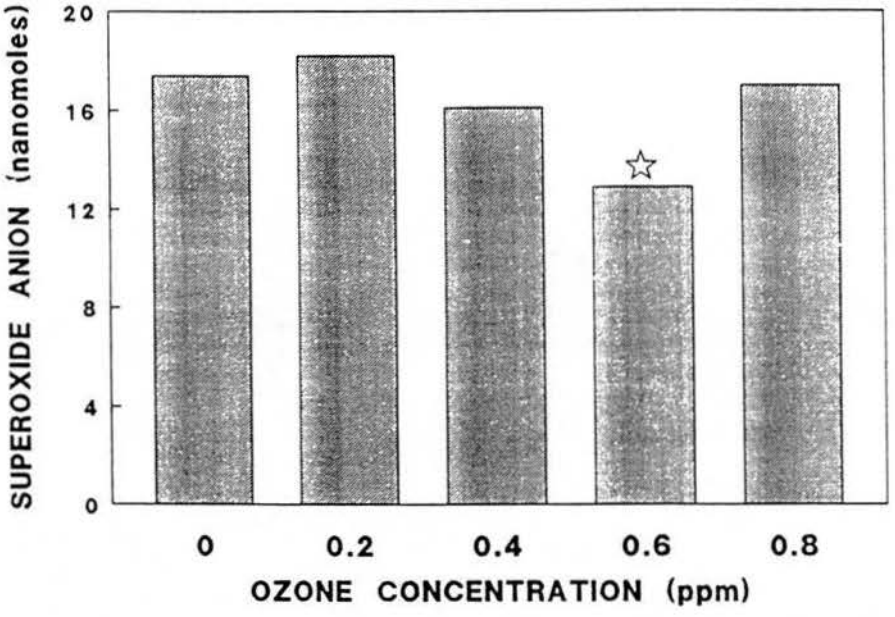


FIGURE 3. Production of superoxide anion by lavaged cells from rats exposed to 0.2–0.8 ppm ozone. Retransformed treatment means across all time points, for non-PMA and PMA-stimulated cells. Stars represent significant ($p < .05$) difference from controls.

were detached by coculture with bronchoalveolar leukocytes from ozone-exposed lung (Table 7). Although no positive control leukocyte populations were used in this study, experiments carried out over the same time period with inflammatory populations containing 80% neutrophils showed detachment injury at 5–8 times the amount caused by control populations.

TABLE 5. Proteolysis of [^{125}I]Fibronectin Matrix by Cells from Control and Ozone-Exposed Lung[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	4247 (3168)	3872 (2559)	2210 (810)	2305 (498)	4133 (2427)	3741 (2427)
0.4	3833 (1123)	4034 (877)	4322 (689)	4422 (1195)	5668 (1605)	5691 (857)
0.6	3880 (121)	4094 (369)	4173 (609)	4296 (739)	3628 (177)	3390 (284)
0.8	6418 (2849)	6830 (3258)	6165 (2783)	6042 (2998)	4059 (2491)	4119 (2689)

[a]Results are cpm mean (SD) of triplicate wells in 3 separate experiments.

TABLE 6. Lytic Injury to Epithelial Cells Caused by Coculture with Bronchoalveolar Leukocytes from Control and Ozone-Exposed Rats[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	3765 (508)	3937 (615)	8115 (1261)	7754 (1384)	5839 (3152)	5787 (3142)
0.4	4469 (2359)	4565 (3073)	8500 (4808)	6996 (3013)	6764 (1149)	6826 (1189)
0.6	4032 (381)	4419 (835)	2987 (413)	3152 (473)	3309 (430)	3277 (419)
0.8	6438 (3055)	7190 (3493)	5493 (3200)	5445 (3102)	6172 (1120)	6035 (894)

[a]Data as mean (SD) cpm of released ^{51}Cr.

Macrophage Size and Shape

Macrophages obtained from the lungs of rats inhaling ozone at 0.8 ppm, when viewed by light microscopy, were large, blebbed, and vacuolated compared to control macrophages (Fig. 4). When size estimations were done there was a significant effect of ozone treatment in causing an increase in the size of lavaged macrophages (Table 8 and Fig. 5) but no significant effect of days of exposure. Although there was a suggestion of a trend of return to normal size with increasing length of exposure to 0.8 ppm ozone, this did not attain statistical significance.

DISCUSSION

The main aim of the study was to assess possible epithelial injuring activities of the bronchoalveolar leukocytes from the lungs of rats inhaling ozone at low levels. The study has revealed that ozone had the ability to cause inflammation in the lung, detectable in the bronchoalveolar lavage leukocyte profile as increased proportions of neutrophils, at the

TABLE 7. Detachment Injury to Epithelial Cells Caused by Coculture with Bronchoalveolar Leukocytes from Control Rats and Rats Exposed to Ozone[a]

Ozone conc. (ppm)	Days exposed to ozone					
	1		2		4	
	Control	Ozone	Control	Ozone	Control	Ozone
0.2	756 (1051)	927 (1166)	4860 (4319)	4667 (3201)	4002 (5138)	4273 (5391)
0.4	1168 (2023)	978 (1463)	4243 (2929)	3723 (2624)	3085 (2193)	2217 (1547)
0.6	1271 (1623)	1272 (1517)	125 (69)	92 (90)	153 (205)	160 (229)
0.8	1705 (1114)	2148 (960)	2065 (2170)	1842 (1780)	1613 (1311)	2357 (1293)

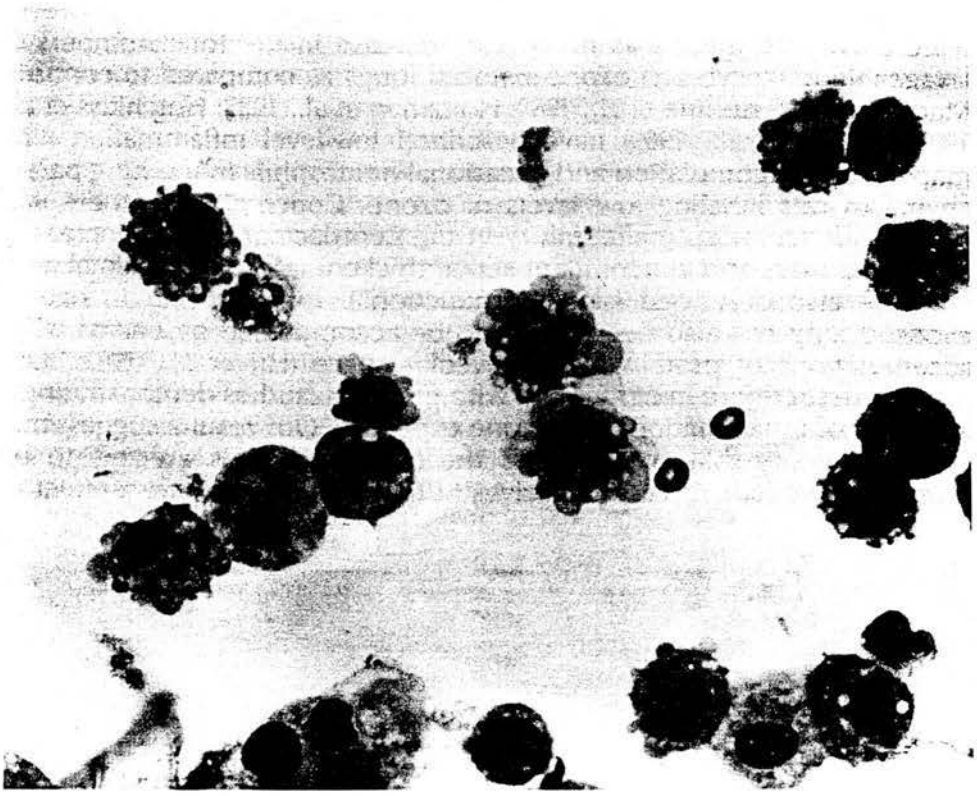
[a]Data as mean (SD) cpm of ^{51}Cr in detached cells in triplicate wells in 3 experiments.

highest concentrations and earliest time points. These effects, however, were small and there was no overall increase in the total numbers of lavageable leukocytes in ozone-exposed lungs as compared to controls. Many studies (Esterline et al., 1989; Holtzman et al., 1983; Hotchkiss et al., 1989; Tepper et al., 1989) have described low-level inflammation with macrophage accumulation and occasional neutrophils in the lung parenchyma of rats inhaling low levels of ozone. Concomitantly, there was often evidence of epithelial injury in the centriacinar region; increased interstitial mass and concomitant septal thickening of the proximal alveoli were also described (see "Introduction"). Inflammation in ozone-exposed lung has also been found to be accompanied by edema, with accumulations of protein in the alveolar space (Hu et al., 1982). Our results are therefore in agreement with previous studies demonstrating a low level of inflammation with ozone exposure. Our results suggest that epithelial injury that accompanies the inflammation is unlikely to be caused by the leukocytes themselves but is most likely a direct effect of



(*a*)

FIGURE 4. (*a*) Light microscopic appearance of control bronchoalveolar leukocytes.



(*b*)

FIGURE 4. (*Continued*) (*b*) Light microscopic appearance of ozone-exposed bronchoalveolar leukocytes showing increased size and blebbing; a neutrophil is present at the top left.

TABLE 8. Macrophage Size in Bronchoalveolar Lavage Samples from Rats Exposed to 0.8 ppm Ozone for Up to 4 Days[a]

	Days exposed to ozone		
	1	2	4
Control	16.25 (2.61)	19.31 (1.35)	18.04 (2.55)
Ozone	19.61 (2.55)	21.96 (3.77)	18.96 (1.93)

[a]Expressed as mean (SD) of major diameter in μm (data from 3 experiments).

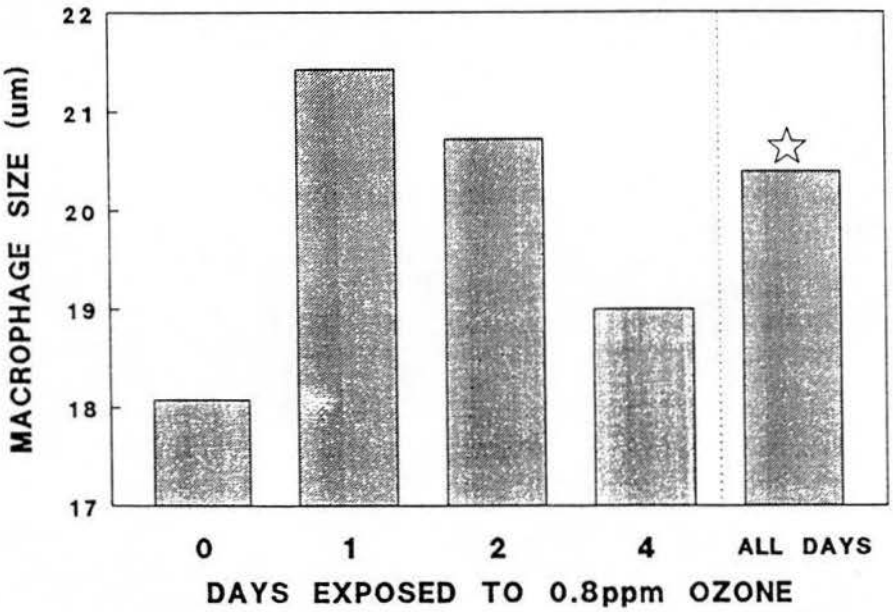


FIGURE 5. Macrophage size after exposure to 0.8 ppm of ozone for 1–4 days or for all days. Star indicates significant ($p < .05$) difference from the control for all days when taken together. Retransformed estimated means.

ozone. Compared to controls the bronchoalveolar leukocytes from ozone-exposed lung showed no increased ability to release oxidants, degrade fibronectin, or damage epithelial cells in vitro.

At the low levels of inflammation that were detected in ozone-exposed lung, no substantial leukocyte-mediate epithelial injury would be anticipated since our own previous studies have indicated that leukocyte-mediated epithelial injury only occurs when there is substantial neutrophil recruitment (Donaldson et al., 1988) and an overall expanded leukocyte population. Neutrophil-enriched inflammatory leukocyte populations have the ability to cause detachment injury when the proportion is greater than approximately 5% (unpublished observation) although the injury is only substantial at much higher proportions of neutrophils. The relatively low level of neutrophil recruitment seen with ozone exposure in the present studies are therefore unlikely to lead to neutrophil-mediated epithelial injury. However, macrophages may possess the ability to cause protease-mediated injury (Brown et al., 1989) mediated by the uptake and release of neutrophil elastase (Campbell et al., 1979). Since there was no substantial increase in neutrophils in the ozone-exposed lung, this was unlikely to be a mechanism in any injury expressed by alveolar macrophages from ozone-exposed lung.

The modest neutrophil recruitment found with ozone may be pro-

duced as a result of neutrophil chemotaxin released by alveolar macrophages treated with ozone (Driscoll and Schlesinger, 1988). The accumulation of protein in the alveolar space described by Hu et al. (1982) in rats exposed to ozone may also contribute to chemotactic attraction of neutrophils found in our study, although we did not measure protein accumulation in the alveolar space.

There was clear evidence of adaptation to the harmful effects of ozone, inflammation being present at the early time points with a return to the normal bronchoalveolar lavage profile by day 4. This is in agreement with previous reports of adaptive responses to ozone in terms of protein accumulation (Hu et al., 1982) and other indices of lung injury (Boorman et al., 1980; Wright et al., 1987). The basis of this adaptive response may be the induction of antioxidant defenses (Menzel, 1984; Wright et al., 1987) but this was not investigated in the present study.

A striking feature of the alveolar macrophages from rats exposed to the highest concentration of ozone at short time points was their increased size and vacuolated/blebbed appearance. A similar increase in size and morphology has been described by other workers in alveolar macrophages from ozone-exposed rats (Hotchkiss et al., 1989). In studies from our own laboratory on the exposure of leukocytes to cigarette smoke in vitro, which also contains oxidizing gas, we noted similar shape change and decreased deformability (Lannan et al., 1992), which is abolished by the microfilament-disrupting agent cytochalasin B. It seems possible therefore that the altered size and structure of alveolar macrophages produced by ozone in the present study may be related to oxidant-induced cytoskeletal changes, although this remains to be shown.

The finding of decreased ability of bronchoalveolar lavage leukocytes from ozone-exposed lung to produce superoxide is a phenomenon that has been described previously by Amaruso et al. (1981) and Ryerpowder et al. (1986) who described this phenomenon in alveolar macrophages exposed to ozone in vitro and in vivo. This effect appears to be related to the loss of activity of cytochrome b558 caused by the direct effect of ozone demonstrable in vitro (Ryerpowder et al., 1986). Although both Amaruso and Ryerpowder described ozone dose-dependent inhibition, we found no significant inhibition at 0.8 ppm and significant inhibition at 0.6 ppm. We can identify no reason for the lack of a dose response, although altogether the control and experimental production of superoxide was less at 0.8 ppm than at any of the other doses. This is likely to be variability in the assay that we have encountered before and is unlikely to mask any effect of ozone on the cells. Interestingly, in the study of Ryerpowder et al. (1986), 0.8 ppm also failed to cause inhibition but the ozone concentration was then increased to reveal inhibition at higher doses, and not downward. It is possible that 0.8 ppm in our study caused protein leakage into the alveolar space (Hu et al., 1982) sufficient to pro-

tect the leukocytes or induce antioxidant defenses. It was not, however, possible retrospectively to further examine this anomalous finding. The diminished oxidative potential could be related to the detrimental effect of ozone exposure on the antibacterial defenses (Aranyi et al., 1983), although oxidant damage to the epithelial cells of the mucociliary escalator, or the diminished ability of macrophages to migrate alluded to above, could interfere with clearance of bacteria and contribute to increased infection.

We conclude that at the exposure levels used injury to epithelial cells lining the airspaces of the lung is unlikely to be caused by inflammatory bronchoalveolar leukocytes and is more likely to arise as direct ozone-mediated damage to the epithelial cells.

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Effect of cigarette smoke and its condensates on alveolar epithelial cell injury in vitro

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Lannan, S., K. Donaldson, D. Brown, and W. MacNee. Effect of cigarette smoke and its condensates on alveolar epithelial cell injury in vitro. *Am. J. Physiol.* 266 (*Lung Cell. Mol. Physiol.* 10): L92–L100, 1994.—The oxidant-antioxidant balance in the airspaces of the lungs may be critical in protecting the lungs from the effects of cigarette smoke. We studied the effect of cigarette smoke and its condensates on the detachment, attachment, and proliferation of the A549 human alveolar epithelial cell line, in an in vitro model of cell injury and regeneration and the protective effects of antioxidants. Whole and vapor phase cigarette smoke decreased ^{51}Cr-labeled A549 cell attachment, increased cell detachment, and decreased cell proliferation, as assessed by [^{3}H]thymidine uptake. Freshly isolated rat type II alveolar epithelial cells showed an enhanced susceptibility to smoke-induced cell lysis when compared with the A549 cell line. Reduced glutathione (GSH) (400 μM) protected against the effects of cigarette smoke exposure on cell attachment, proliferation, and detachment. Depletion of intracellular GSH with buthionine sulfoxamine enhanced the epithelial cell detachment injury produced by smoke condensates. We conclude that cigarette smoke and its condensates cause an oxidant-induced injury to A549 human type II alveolar epithelial cells. Both intra- and extracellular GSH have important roles in protecting epithelial cells from the injurious effects of cigarette smoke.

epithelial cells; oxidants; antioxidants

CIGARETTE SMOKING produces an increased oxidant burden on the lungs. This has implications for the pathogenesis of emphysema by oxidative inactivation of α1-proteinase inhibitor (28) and may also contribute to injury and inflammation of the terminal bronchioles in healthy smokers (26). Cigarette smoking has been shown to result in increased airspace epithelial permeability to radiolabeled macromolecules (17) in healthy subjects, an effect which is rapidly reversible on cessation of smoking (23). Acute exposure to cigarette smoke in animal models also produces increased epithelial permeability (6). This is thought in animal models to occur from damage to the type I epithelial cells, at the level of the bronchiolar-alveolar junction (6). Cigarette smoke is potentially injurious to airspace epithelial cells, since it has been calculated to contain 10^{17} oxidant molecules/puff, of which 10^{14} are oxygen radicals (8). These oxygen radicals are short-lived. However, cigarette smoke condensate, formed in the airspaces, continues to produce oxidants (27), which may result in a more persistent oxidant injury. Antioxidants in the epithelial lining fluid may be critical in protecting epithelial cells from smoke-induced oxidant injury (29). Reduced glutathione (GSH) is a major intra- and extracellular antioxidant, which is present in higher concentrations in the epithelial lining fluid in smokers than in nonsmokers (7), possibly in response to repeated oxidant stress.

In this study we have used a human alveolar epithelial cell line (A549) to study the ability of cigarette smoke to injure epithelial cells. We have also assessed the role of GSH and other antioxidants in protecting these epithelial cells from the effects of cigarette smoke.

METHODS

A549 Epithelial Cells

The alveolar type II epithelial cell line, A549, was maintained in continuous culture, in 75-ml flasks (Falcon, NJ) incubated at 37°C, 5% CO_2 in Eagle's minimum essential medium (MEM, GIBCO, Paisley, Scotland), which contained penicillin (100 $\mu g \cdot ml^{-1}$, Glaxo, Greenford, UK), streptomycin (50 $\mu g \cdot ml^{-1}$, Evans, Horsham, UK), L-glutamine (20 mM, GIBCO), sodium bicarbonate (GIBCO), and 10% fetal calf serum (FCS, GIBCO).

When required for the assays, confluent monolayers of A549 epithelial cells were washed twice with Dulbecco's phosphate-buffered saline (PBS, GIBCO). Thereafter, trypsin-EDTA (GIBCO) solution was added to detach the cells. The cells were then washed with MEM, containing 10% FCS at 200 g for 10 min, to neutralize the trypsin, and resuspended in MEM with 10% FCS. This cell suspension was then used in the attachment, detachment, and proliferation assays.

Attachment Assay

A suspension of A549 epithelial cells (2.5×10^6 cells $\cdot ml^{-1}$) was incubated with ^{51}Cr (5 MBq $\cdot ml^{-1}$) at 37°C, for 1 h, in a shaking water bath. The cell suspension was then washed twice with serum-free MEM and resuspended in serumless MEM, or in certain experiments, MEM containing varying amounts of GSH (50–400 μM, Sigma Chemical, St. Louis, MO), to give a final concentration of 1×10^6 cells $\cdot ml^{-1}$.

Triplicate aliquots of smoke-exposed ^{51}Cr-labeled cell suspensions (5×10^4 cells in 200 μl), together with appropriate controls, were added to 96-well microtiter plates and incubated at 37°C in 5% CO_2. At varying time points the supernatants were aspirated and the wells washed twice with PBS (200 μl). Triton X-100 (0.1%, 100 μl) was then added to lyse the cells that had attached, and the counts present in the Triton X-100 were measured in a gamma well counter (LKB, Wallac, Finland). The total cell-associated counts were measured in triplicate at each time point.

^{51}Cr Release

To determine the level of spontaneous ^{51}Cr release from A549 cells with time in the attachment assay, ^{51}Cr epithelial cells were plated out in MEM as in the attachment assay. At set time points the supernatant was collected and each well was then washed twice with PBS (200 μl). These washings were combined with the initial supernatant and centrifuged at 400 *g* for 10 min. The supernatants from these washes were

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decanted and the cell pellet of unattached cells was lysed with Triton X-100 (200 μl). Cells attached to the plate were lysed with Triton X-100 (200 μl) and were then combined with the lysate of the unattached cells. The counts in the cell lysates were used as a measure of the ^{51}Cr label remaining on the cells. The counts on the supernatant combined with the washings were used as a measure of the ^{51}Cr label released over the time course of the experiment.

Detachment Assay

Confluent monolayers of A549 epithelial cells were trypsinized off the 75-ml flasks as previously described. The cells were resuspended in MEM containing 10% FCS and labeled with 0.37 MBq·ml^{-1} ^{51}Cr in PBS to give a final concentration of 5×10^5 cells·ml^{-1}. These labeled cells were plated out in a 96-well microtiter plate (5×10^4 cells·well^{-1}) and allowed to adhere overnight in an incubator at 37°C, in a humidified atmosphere with 5% CO_2 added.

After plating the cells out overnight in MEM plus 10% FCS, the supernatants were removed and the wells washed twice with PBS (200 μl). Serumless medium (200 μl) containing varying concentrations of cigarette smoke condensate (CSC) or, in certain experiments, hydrogen peroxide, was added to the required number of wells. To one set of triplicate wells, 0.1% Triton X-100 (200 μl) was added to produce 100% lysis as a positive control; this gives the total cell-associated counts per minute. The plates were incubated for up to 4 h at 37°C with 5% CO_2 and then centrifuged at 100 *g* for 5 min. Fifty microliters of the supernatant were removed and counted to give a measure of cell lysis. The remainder of the supernatant was aspirated and combined with two washes of PBS (200 μl) from each well; this gave a measure of cell detachment (10).

Cigarette smoke extracts (10%), combined with the antioxidants superoxide dismutase (SOD, 50 μg·ml^{-1}, Sigma), catalase (50 μg·ml^{-1}, Sigma), GSH (200–800 μM), or α_1-proteinase inhibitor (α_1-PI, 100 μg·ml^{-1}, Sigma) were incubated with the cells. Cell lysis and detachment were measured as described above. The effect of hydrogen peroxide (10–50 μM) in combination with GSH (50–800 μM) was also assessed in the detachment assay. Pretreatment with buthionine sulfoxamine (BSO, 10 μM, for 16 h) was used to reduce the intracellular GSH of A549 cells and study the effect on smoke-induced changes.

Uptake of [^{3}H] thymidine

Alveolar epithelial cells (A549) were incubated overnight at 5×10^4 cells·well^{-1} in a microtiter plate at 37°C, with 5% CO_2, to allow adherence. The wells were washed twice with PBS (200 μl·well^{-1}) to remove any nonadherent cells. Medium containing varying concentrations of CSC (180 μl·well^{-1}) or the appropriate controls was added to triplicate wells along with [^{3}H]thymidine (0.01 MBq, 20 μl). The cells were harvested 16 h later with a cell harvester (Dynatech) onto glass fiber paper. The incorporation of [^{3}H]thymidine was measured by a scintillation counter (Phillips, Netherlands). As described in *Detachment Assay*, antioxidants were combined with CSC to examine their effect on the changes in the uptake of [^{3}H]thymidine produced by CSC.

Smoke Exposure

For the attachment assay (see *Epithelial Cell Attachment*), aliquots of ^{51} Cr-labeled cells in 2 ml of MEM were exposed to an increasing number of puffs of cigarette smoke for 5 min while being agitated in a tonometer (4) consisting of a siliconized glass flask in a water bath maintained at 37°C. This technique allows a thin layer of cells to be exposed to cigarette smoke obtained from a medium-tar cigarette using a smoking machine (37 ml/puff). Vapor phase cigarette smoke was produced by drawing whole smoke through a Cambridge filter to remove particulate material. Control cells were sham exposed in the tonometer, in room air, for the same length of time. The cell samples were washed twice immediately after smoke exposure, in MEM at 200 g for 10 min and resuspended at 2.5×10^5 ml^{-1} in MEM with 10% FCS.

Cigarette smoke condensate was produced by passing whole or vapor phase cigarette smoke from three cigarettes over 3 ml of PBS in the tonometer. The condensate was made up fresh on the morning of each experiment. This solution was then diluted to give concentrations of 0.1, 1, 2, 5, 10, and 20% of the original extract and used in the detachment and proliferation assays.

Rat Type II Epithelial Cells

Type II alveolar epithelial cells were isolated using a modification of the method of Richards et al. (30), from specific pathogen-free, adult male, Wistar rats (200–250 g, Bantin & Kingman Universal, Hull). The rats were anesthetized intraperitoneally with Nembutal (50 mg·kg^{-1}·body wt^{-1}), and the lungs perfused in situ with 0.15 N saline (30 ml, 37°C). The lungs and heart were then removed and the bronchoalveolar leucocytes retrieved by repeated pulmonary lavages with 0.15 N saline. Proteolytic digestion of the lungs was carried out by intratracheal instillation of 0.25% (wt/vol) trypsin in Hanks' buffered salt solution (HBSS, GIBCO) for 30 min at 37°C. The lung parenchyma was then dissected free of the trachea and bronchi and chopped into fine pieces. Digestion of the lung was stopped by the addition of 10% FCS in HBSS (Ca^{2+}, Mg^{2+} free, GIBCO) containing deoxyribonuclease (DNase, 250 μg·ml^{-1}, Sigma) while being shaken in a water bath at 37°C for 4 min. The lung debris was separated out using a 150-μm filter followed by a 30-μm filter. The resulting cell suspension was layered on to a Percoll discontinuous density gradient (10 ml, 1.089 g·ml^{-1} Percoll; 10 ml, 1.04 g·ml^{-1} Percoll; 10-ml cell suspension) and centrifuged at 250 *g* for 20 min at 4°C. The cells aspirated from the 1.089/1.04 density interface were washed with HBSS (Ca^{2+}, Mg^{2+} free) containing DNase (50 μg·ml^{-1}) and resuspended in MEM with the addition of gentamycin M (50 μg·ml^{-1}, Roussel Lab, Uxbridge, UK) with 10% FCS. Viability was assessed by trypan blue exclusion and the percentage of type II cells was checked using Papanicolaou staining. The cells were plated out at 5×10^4 cells·well^{-1} in a 96-well tissue culture plate (Flow Laboratories, Rickmansworth, UK) and incubated at 37°C, with 5% CO_2. After 3 days the cells were washed twice and the medium replaced with MEM and 10% FCS containing ^{51}Cr (0.37 MBq^{-1}, 200 μl). The plate was then incubated overnight and used in the detachment assay.

Statistical Analysis

The data were analyzed using analysis of variance with Bonferroni correction where applicable.

RESULTS

Epithelial Cell Attachment

Cigarette smoke exposure. Epithelial cell viability remained >95% after smoke exposure, as assessed by trypan blue exclusion. A549 epithelial cells demonstrated a decreased ability to attach to plastic over a period of 24 h, after exposure to four puffs of cigarette smoke (Fig. 1). This effect was significant after 45 min

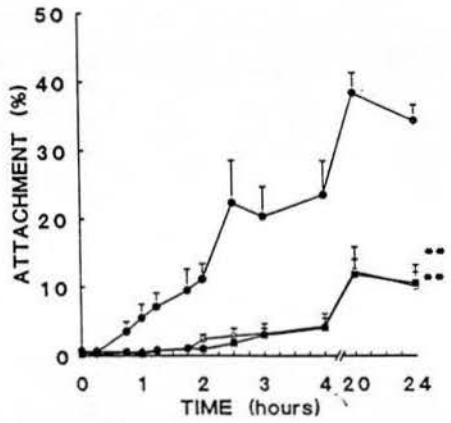


Fig. 1. Attachment to culture plates of control (●), whole smoke- (○), and vapor phase smoke (■)-exposed A549 cells. Vertical axis shows number of cells attached expressed as a percentage of total cells plated. Smoke-exposed cells show significantly reduced attachment from 45 min. Data represent means ± SE of 6 experiments. $^{**}P < 0.01$.

and was similar ($P > 0.05$) following exposure to both whole and vapor phase smoke, when compared with sham-exposed cells. At 4 h, 23.5 ± 4.9% of the unexposed cells had attached, whereas only 4.3 ± 1.8% of the whole and 4.1 ± 1.4% of the vapor phase cigarette smoke-exposed cells had attached (Fig. 1).

Increasing doses of vapor phase cigarette smoke produced a progressive decrease in cell attachment. A single puff of smoke reduced attachment to 19.4 ± 3.8% compared with the control, whereas six puffs of cigarette smoke almost eliminated attachment over 24 h. The difference in attachment following smoke exposure became significant ($P < 0.05$) after 2 h following four and six puffs of smoke (Table 1).

Over the 24-h time period of the assay there was a gradual loss of ^{51}Cr label from both control and smoke-exposed cells. There was no significant difference in the percent of ^{51}Cr loss at 24 h between control cells (55.7 ± 7.7%) and those exposed to whole (60 ± 4.5%) or vapor phase cigarette smoke (46.4 ± 6.6%, $P > 0.05$). Moreover, increasing doses of vapor phase cigarette smoke did not result in significant loss of ^{51}Cr at 24 h (1 puff 55.7 ± 5.4%, 6 puffs 42 ± 2.8%), when compared with control cells and was not great enough to account for the effect of cigarette smoke on cell detachment. All subsequent attachment assays were assessed after a maximum of 4 h.

Table 1. *Attachment after 24 h to culture plates of radiolabeled A549 cells exposed to increasing number of puffs of vapor phase smoke over 5 min*

No. of Puffs of Cigarette Smoke	%Attachment at 24 h
0	24.6 ± 3.6
1	19.4 ± 3.8
2	14.4 ± 4.2
4	7.8 ± 2.1*
6	2.35 ± 0.7*

Values are means ± SE of 6 experiments. Results are expressed as the cells attached as a percentage of total cells plated. $^{*}P < 0.05$.

Effect of reduced glutathione. Glutathione alone, at concentrations of up to 400 μM, did not affect cell attachment (Fig. 2). The reduction in attachment following exposure to cigarette smoke was not significantly altered by the addition of GSH at a concentration of 100 μM (Fig. 2). However, cells suspended in medium to which GSH (150 μM-400 μM) had been added did not have their attachment compromised by vapor phase cigarette smoke.

Hydrogen peroxide. Hydrogen peroxide (H_2O_2), at concentrations of ≥ 15 μM, produced a decrease in cell attachment at 4 h (Fig. 3*A*). When added to A549 epithelial cells at these concentrations, > 90% of the cell attachment was abolished. Although there was a trend for decreasing cell attachment with increasing concentrations of H_2O_2 between 1 and 10 μM, this trend did not reach statistical significance.

Epithelial Cell Detachment

Whole CSC, when added to A549 cells, increased detachment over baseline values at concentrations of ≥ 1%, without significant change in the level of cell lysis (Fig. 4*A*). By contrast, vapor phase CSC increased both detachment and lysis (Fig. 4*B*). In this case increased cell detachment was significant at concentrations of > 5% ($P < 0.05$), whereas cell lysis developed at concentrations ≥ 10% (Fig. 4*B*). Freshly isolated rat type II epithelial cells, when treated with aqueous CSC, lysed following exposure to both the whole and vapor fractions (Fig. 5).

BSO pretreatment. Pretreatment of A549 epithelial cells with the GSH-depleting agent BSO, enhanced cell detachment at all concentrations of CSC (Fig. 6, shows data for 10% CSC). There was increased cell lysis following exposure of BSO-treated A549 cells to concentrations of 1 and 5% vapor phase CSC but no alteration in lysis when exposed to whole phase CSC.

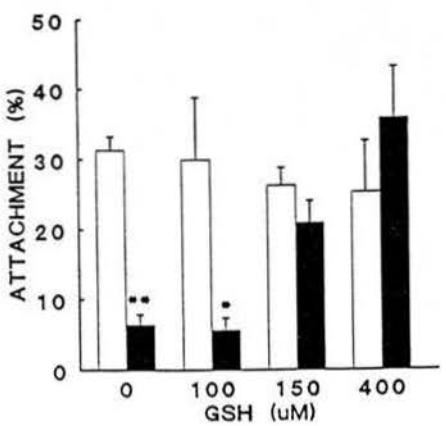


Fig. 2. Attachment of control (open bars) and vapor phase smoke-exposed A549 cells (closed bars), with and without reduced glutathione (GSH) at increasing concentrations. Vertical axis shows number of cells attached expressed as a percentage of total cells plated. Data represent means ± SE of 5 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

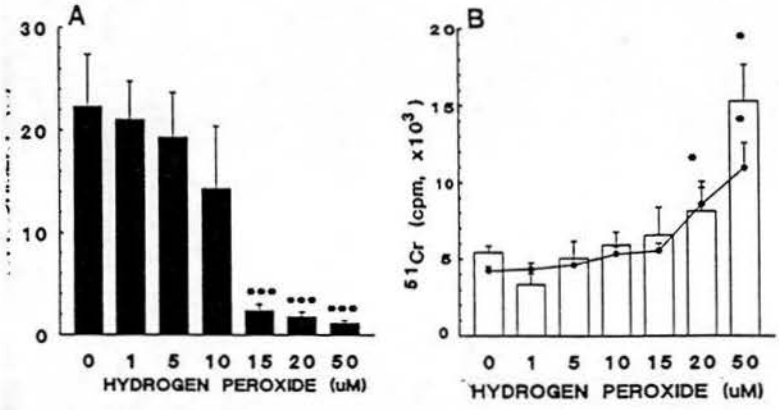


Fig. 3. *A*: attachment of radiolabeled A549 cells to culture plates when treated with increasing concentrations of H_2O_2 (1–50 μM). Vertical axis shows number of cells attached expressed as a percentage of total number of cells plated. Horizontal axis shows increasing concentration of H_2O_2. Data represent means ± SE of 6 experiments. ***$P < 0.001$. *B*: detachment of radiolabeled A549 cells from culture plates, treated with increasing concentrations of H_2O_2 (1–50 μM). Vertical axis represents number of cells detached (closed bar) and those lysed (solid line) expressed as counts per minute (cpm) of radiolabel. Horizontal axis shows increasing concentrations of H_2O_2 used. Data represent means ± SE of 6 experiments. *$P < 0.05$; ***$P < 0.001$.

Antioxidants. Incubation of epithelial cells with the ntioxidants SOD or catalase or the anti-protease α_1-PI ecreased the cell lysis caused by vapor CSC (10%) Table 2), but did not prevent epithelial detachment roduced by exposure to whole or vapor CSC. However, SH prevented cell detachment produced by whole or apor CSC (Fig. 7). Cell detachment was similar to that f control values when GSH (200–800 μM) was added to lls exposed to whole or vapor CSC (10%).

H_2O_2. H_2O_2 at concentrations of ≥20 μM increased pithelial cell detachment and lysis (Fig. 3*B*). With njurious concentrations of H_2O_2 (≥20 μM), GSH (≥200 M) reduced both cell lysis and cell detachment to levels imilar to control values (data not shown). The cell etachment produced by higher concentrations of H_2O_2 ≥50 μM) was not prevented by the addition of GSH 5–800 μM). The highest concentration of GSH (800 M) did reduce cell lysis, but under these conditions here was a small increase in the detachment of A549 lls.

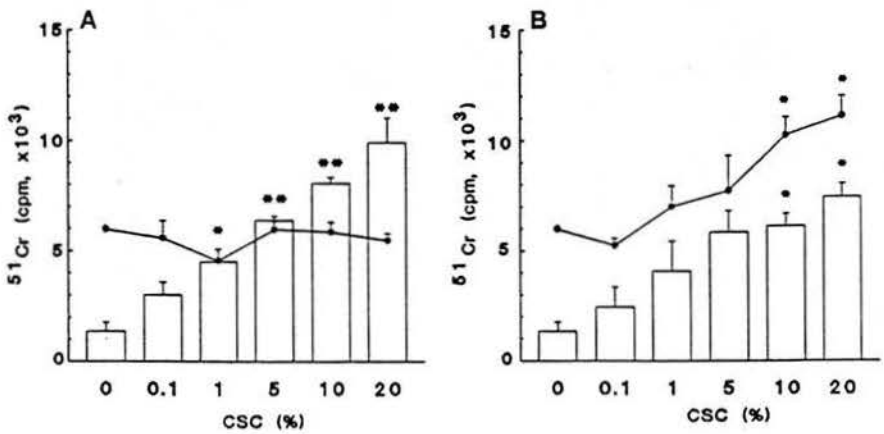
hymidine Uptake

The uptake of [^{3}H]thymidine, as a measure of cell roliferation, was unchanged when A549 cells were exposed to low concentrations (<10%) of both types of aqueous CSC compared with control values. Higher concentrations of CSC (≥10%) produced a fall in uptake of the label into the cell (Fig. 8).

The decreased [^{3}H]thymidine uptake following exposure to CSC could be prevented by the addition of GSH. However, superoxide dismutase, catalase and α1-PI had no significant effect on the reduction of [^{3}H]thymidine uptake in epithelial cells exposed to CSC (Fig. 9).

DISCUSSION

Epithelial cells are important in maintaining the integrity and fluid balance of tissue and in the control of inflammation. Injury to the epithelium may be an important early event following exposure to cigarette smoke and other oxidant gases, manifesting as an increase in epithelial permeability. Following smoke-induced injury to type I alveolar epithelial cells there may be marked type II cell hyperplasia which occurs as an early event following exposure to oxidant gases such as ozone and NO_2 (34). Isolation of type I pneumocytes has proved to be problematic. The majority of investigations published on alveolar pneumocytes have either involved cell lines or primary culture type II cells from



ig. 4. *A*: whole cigarette smoke condensate (CSC); *B*: vapor phase CSC-induced epithelial detachment from culture ates. Vertical axis represents number of cells detached (closed bar) and those lysed (solid line) expressed as cpm of diolabel. Horizontal axis shows increasing percentages of CSC. Data represent means ± SE of 3 experiments. *$P <$ 05; **$P < 0.01$.

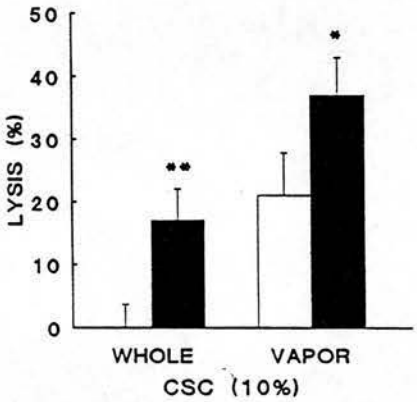


Fig. 5. Comparison of effects of whole and vapor phase CSC (10%) on lysis of A549 epithelial cells (open bars) or freshly isolated rat type II alveolar epithelial cells (closed bars). Vertical axis shows percentage of total cells above baseline that have lysed. Data represent means ± SE of 6 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

nonhuman tissue. We have utilized an alveolar epithelial cell line as a model to study the injurious effects of cigarette smoke. This allows us to use cells derived from human tissue, which technically are identical. We also carried out a limited number of experiments on freshly isolated rat type II pneumocytes. We have used assays of epithelial cell attachment and detachment to model the adverse effects of cigarette smoke on the integrity of the pulmonary epithelial barrier, which might underlie the increased epithelial permeability found in smokers. Epithelial injury may also be a factor in the terminal bronchiolitis that is a feature of the lungs in asymptomatic smokers (26). The results of these studies show that fresh, whole cigarette smoke and vapor phase smoke, together with their condensates, have a profound adverse effect on both the attachment, detachment, and proliferation of epithelial cells in vitro. These effects may be mediated by oxidants in cigarette smoke since the antioxidant, reduced glutathione, protected against the effect, and the oxidant H_2O_2 mimicked the effect of smoke. It is not possible to determine the exact concentration of cigarette smoke to which epithelial cells are exposed to in vivo. However, calculations by Holden et al. (15) suggest that the concentration of condensate in the epithelial lining fluid would be 0.01–1% when one cigarette was smoked. However, in chronic smokers the concentrations will be much higher and its injurious effects may depend on the age of the condensate (27). Four puffs of cigarette smoke in our tonometer system to which epithelial cells were exposed to in this study increase the carboxyhemoglobin of blood to levels found in chronic smokers (5–8%). Thus we believe we are studying the effects of cigarette smoke and its condensates within the dose range found in vivo.

Support for the use of cultured epithelial cells as a model of in vivo oxidant-induced epithelial cell injury comes from experiments using ^{51}Cr-labeled tracheal explants. In this model the addition of both O_2^- and H_2O_2 (10–300 μM) (21) resulted in the release of lactate dehydrogenase (LDH) from the explant, indicating epithelial cell injury. Buckley and co-workers (5) have suggested that cell detachment is an earlier and more sensitive indicator of oxidant injury than the release of LDH. They have also used the release of ^{51}Cr into the

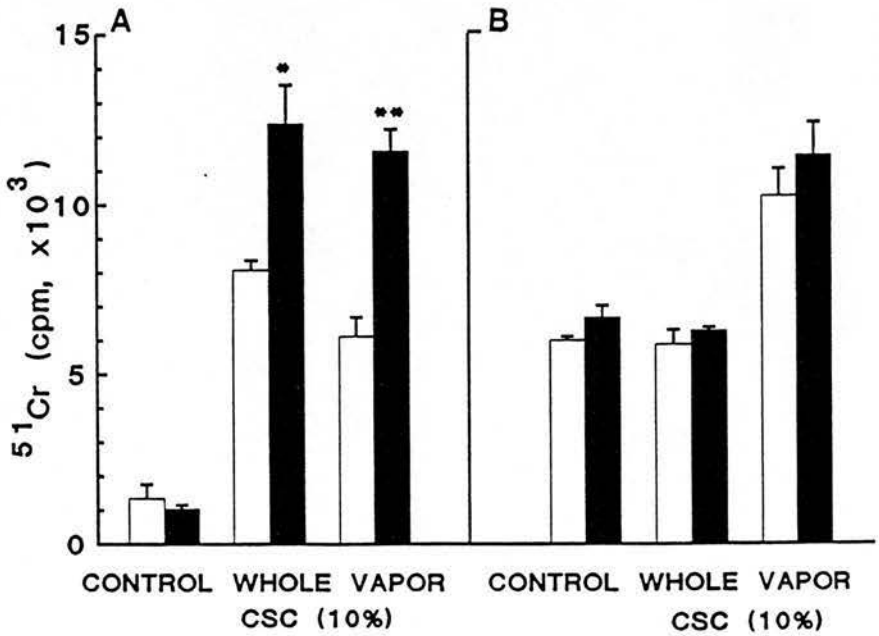


Fig. 6. Detachment and lysis of radiolabeled A549 cells pretreated with buthionine sulfoxamine (BSO) for 18 h and then treated with CSC for 4 h. Vertical axis shows number of cells detaching or lysed expressed as cpm of radiolabel. Open bars are without and closed bars with BSO. Data represent means ± SE of 3 experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 2. *Detachment and lysis of radiolabeled A549 cells treated with CSC (10%) combined with either SOD, CAT, or α_1-PI (50 $\mu g \cdot ml^{-1}$)*

	Control	SOD	CAT	α_1-PI
		Detachment		
Control	1,032 ± 220	1,158 ± 360	1,979 ± 363	1,061 ± 227
Whole CSC	8,043 ± 1,519	6,697 ± 1,686	8,066 ± 2,309	7,484 ± 2,838
Vapor CSC	4,000 ± 514	3,971 ± 993	5,473 ± 1,251	5,853 ± 1,377
		Lysis		
Control	3,773 ± 386	3,597 ± 278	3,414 ± 243	3,783 ± 312
Whole CSC	3,165 ± 250	3,440 ± 264	3,293 ± 105	3,537 ± 220
Vapor CSC	7,092 ± 605	4,472 ± 142*	3,767 ± 133†	4,130 ± 244*

Values are means ± SE of 3 experiments. CSC, cigarette smoke condensate; SOD, superoxide dismutase; CAT, catalase; α_1-PI, α_1-proteinase inhibitor. *$P < 0.01$; †$P < 0.001$.

culture medium as a marker of cytotoxicity, a technique which is comparable with the measurement of cell lysis in this study.

We observed that nonlytic injury could be induced in epithelial cells in culture by exogenous H_2O_2 in low concentrations (< 15 μM), whereas higher concentrations (> 15 μM) produced lytic injury. Similar oxidant-induced cell injury has also been observed following exposure of rat type II epithelial cells to H_2O_2 (5). In the studies reported here, vapor phase CSC was more toxic to epithelial cells, producing both a detachment and a lytic injury, whereas whole CSC produced only detachment. It has been suggested that the carbon particles in whole smoke can absorb a proportion of the toxins present including oxidants, making them unavailable for cell injury (19). Morphologically, cells exposed to CSC show cell surface blebbing (unpublished observations) which would support an oxidant-mediated effect of CSC, since such shape change occurs in other cells following exposure to oxidants (16).

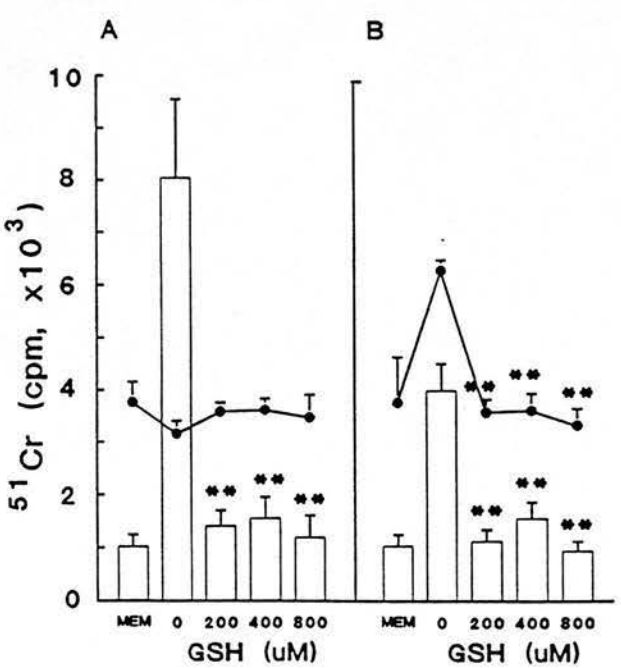


Fig. 7. Detachment (bars) and lysis (solid line) of radiolabeled A549 cells treated with CSC (10%) with and without the GSH (200–800 μM) present in medium. Vertical axis shows number of cells detached or lysed expressed as cpm of radiolabel. Horizontal axis shows increasing concentrations of GSH. Data represent means ± SE of 3 experiments. **$P < 0.01$.

The lung contains a large variety of both intra- and extracellular antioxidants among the most important being superoxide dismutase, catalase, and GSH. An imbalance between oxidant and antioxidant may result in tissue injury (13) and this could well occur in smokers, since cigarette smoke contains 10^{17} oxidant molecules/puff (8). The antioxidant, GSH, is present both extracellularly, in the lung epithelial lining fluid (7), and intracellularly, in the type II epithelial cell (18); the level of GSH is increased twofold in the epithelial lining fluid of chronic cigarette smokers (7). This suggests an adaptation of the lung's antioxidant system in response to oxidant stress. In addition the erythrocytes of cigarette smokers contain more GSH than those of nonsmokers, and thus have an enhanced ability to protect cultured endothelial cells from oxidative stress (31). When added in physiological concentrations to isolated epithelial cells treated with CSC, GSH conferred protection, as shown by the decrease in cell detachment and the abolition of cell lysis. Taken together these results suggest that a proportion of the effects of cigarette smoke are oxidant mediated.

Although there is a persistent increase in GSH in epithelial lining fluid and erythrocytes in chronic cigarette smokers (7, 29), sufficient to protect against the in vitro effects of smoke, acute exposure to cigarette smoke has the opposite effect. GSH decreased by 70% in the isolated perfused rat lung exposed to cigarette smoke (25) and Bridgeman et al. (3) have shown that following exposure of a suspension of A549 cells to only two puffs of cigarette smoke, GSH fell to 10% of the control value. We have demonstrated here that an increased susceptibility to smoke-induced detachment injury occurred when epithelial cells were pretreated with BSO to reduce intracellular GSH, supporting the role of GSH in protecting against smoke-induced oxidant injury.

Other important antioxidants in the lungs are the superoxide anion scavenger, SOD, and catalase, which scavenges hydrogen peroxide. In this study the addition of SOD and catalase extracellularly to epithelial cell cultures gave no protection against the cell detachment

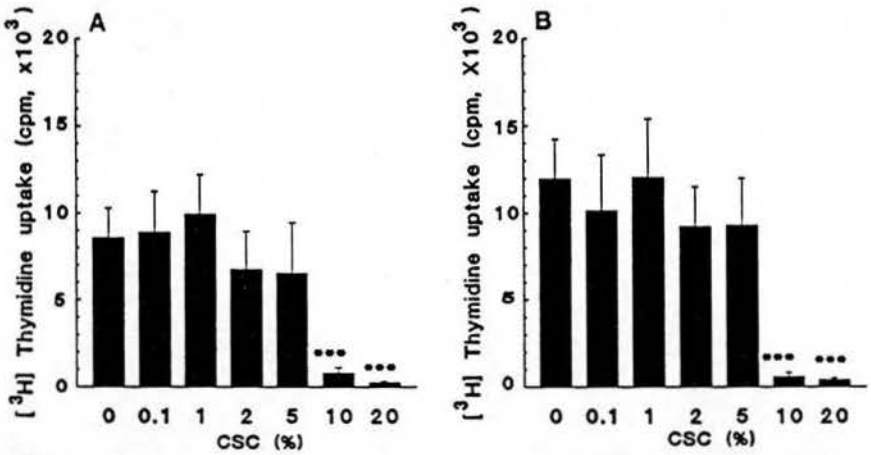


Fig. 8. Proliferation of ^{3}H-labeled A549 cells treated with increasing concentrations of whole (*A*) and vapor CSC (*B*). Vertical axis shows proliferative potential of cells expressed as cpm of [^{3}H]thymidine uptake. Horizontal axis shows increasing concentrations of CSC. Data represent means ± SE of 6 experiments. ***$P < 0.001$.

induced by whole CSC, but did prevent cell lysis when the A549 cell cultures were exposed to vapor phase CSC. However, the intracellular concentrations of these antioxidants may be more important in preventing oxidant injury to cells. McCusker and Hoidal (22) found that the activities of SOD and catalase in alveolar macrophages were higher in cigarette smokers than in nonsmokers. Furthermore, the increased activities of SOD and catalase in the lungs of hamsters exposed to smoke returned to that of control animals after cessation of the smoke exposure (22). Both SOD and catalase are unable to penetrate cell membranes in epithelial type II cell culture, but the levels of these enzymes can be enhanced by the addition of SOD and catalase encapsulated in liposomes or covalently linked to monomethyoxypolyethylene glycol (PEG). Walther and colleagues (32) found that increasing both intracellular SOD and catalase, by the addition of PEG-SOD and PEG-catalase, induced an increased resistance to oxidant stress in isolated alveolar type II cells. The lack of penetration of SOD and catalase into cell membranes may at least partly account for the lack of a protective effect of these antioxidants against the effects of CSC shown here in vitro. In addition, cigarette smoke is known to contain at least 3,000 different compounds (12). Among these are toxic electrophilic and reactive compounds, as well as

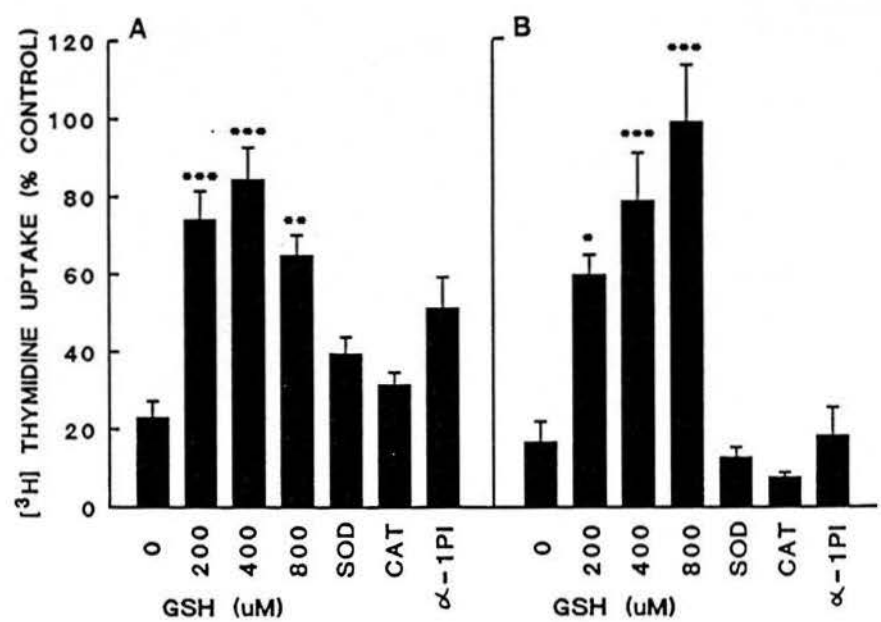


Fig. 9. Effect of GSH, superoxide dismutase (SOD), catalase (CAT) or α1-proteinase inhibitor (α-1PI) on thymidine uptake of whole (*A*) or vapor phase (*B*) CSC-exposed A549 cells. Vertical axis shows proliferative potential of cells expressed as cpm of [^{3}H]thymidine uptake. Data represent means ± SE of 6 experiments. ***$P < 0.001$.

reactive oxygen species. Thus it is unlikely that scavengers of reactive oxygen intermediates would give total protection against the injury caused by CSC.

It is necessary to consider the likely in vivo significance of the epithelial detachment and loss of attachment in culture caused by components of cigarette smoke. The integrity of airspace epithelium is dependent on specific cell surface adhesion molecules or integrins (2). These are a family of glycoproteins and as such will be vulnerable to oxidants. Thus the effects of cigarette smoke on epithelial cell attachment and detachment may be mediated through an effect on cell integrins. Preliminary work from our laboratory suggests that epithelial cell detachment is partly mediated via the specific tripeptide sequence RGD (arginine-glycine-aspartic acid) which is present on many matrix proteins such as fibronectin (9). Thus cigarette smoke may modify the activity or expression of an RGD-binding integrin.

In addition the membrane of the cell may show decreased fluidity following cigarette smoke exposure, as has been demonstrated in alveolar macrophages (14). This may impair the ability of the cell to spread, which is a prerequisite of cell attachment. Other in vitro studies have described oxidant-mediated effects on the cytoskeleton of alveolar cells which could culminate in cell detachment (33). Cigarette smoke may affect the cytoskeleton of the cell by disrupting the continuous turnover of F-actin (11). We found that cigarette smoke inhibited both cell attachment and proliferation which may be relevant to the process of epithelial repair. When the type I pneumocytes are damaged, the type II cells divide and differentiate into replacement type I cells (1). If the ability of the type II epithelial cell to attach is diminished, this may interfere with epithelial repair and may be compounded by a decrease in cell proliferation. This process may be relevant to the alveolar destruction which leads to emphysema.

The demonstration that CSC has a detrimental effect on the proliferative potential of the A549 epithelial cells concurs with work by Miyashita and co-workers (24), who found that cigarette smoke inhibited the proliferation of both normal and malignant human bronchial epithelial cells, although the malignant cells were more resistant to the effects of smoke. We have also found that the effects of CSC on epithelial cells were more pronounced with freshly isolated type II cells than the A549 cell line, which was derived from type II alveolar epithelial cell carcinoma (20). A reduction in the proliferative capacity of the A549 cells produced by smoke can be protected against by extracellular GSH in physiological concentrations. Similarly intracellular GSH has been found to be important in cell proliferation (25). A reduction in intracellular GSH levels with BSO results in inhibition of A549 cell division. Since cigarette smoke has been shown to decrease A549 GSH (3), this may also contribute to the effect of CSC on epithelial cell proliferation.

In summary this study shows that cigarette smoke has a detrimental effect on cultured epithelial cells causing cell lysis, detachment, and a reduction in ability of the cells to attach and also to proliferate. These effects of cigarette smoke are probably oxidant mediated, although the exact mechanism has not been elucidated. These effects, which have important implications for epithelial injury and repair in vivo following smoke exposure, can be ameliorated by the presence of GSH in concentrations present in the epithelial lining fluid of chronic smokers. It remains to be proven whether repeated oxidative insults from acute smoking could result in a local and temporary GSH deficiency in the lung airspaces resulting in direct oxidative injury to airway epithelium.

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Neutrophil sequestration in rat lungs

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Abstract

Background – The transit of neutrophils through the pulmonary microvasculature is prolonged compared with red blood cells and is increased further during cigarette smoking and in exacerbations of chronic obstructive pulmonary disease. The increased residence time (sequestration) of neutrophils in the pulmonary capillaries in these conditions may be the first step leading to the accumulation of cells within the lung interstitium and in the bronchoalveolar space, so potentiating lung damage. A rat model has been developed to investigate the factors which may influence neutrophil transit through the lung microvasculature.

Methods – Intratracheal instillation of the heat killed organism *Corynebacterium parvum* was used to induce an acute neutrophil alveolitis. Neutrophils and red blood cells were isolated from donor rats, labelled with two distinct radioisotopes, and then reinjected into recipient rats to assess their transit through the pulmonary circulation. To ascertain whether peripheral blood neutrophils were minimally altered by the isolation procedure their functional status in vitro was compared with that of inflammatory neutrophils in a number of assays commonly used as descriptors of neutrophil activation. The influence of neutrophil activation on the accumulation of cells in the lungs was assessed by comparing the lung sequestration of control neutrophils, isolated from peripheral blood, with that of inflammatory neutrophils obtained from bronchoalveolar lavage of inflamed rat lungs. Lung sequestration of neutrophils was defined as the fold increase in the ratio of neutrophils labelled with chromium-51 to red blood cells labelled with technetium-99m in lung tissue compared with the same ratio in peripheral blood.

Results – Sequestration of peripheral blood neutrophils occurred in control rat lungs as shown by a 17·5 (2·1) fold increase in the ratio of neutrophils to red blood cells in the pulmonary circulation compared with the ratio of these cells in the peripheral circulation. When inflammatory neutrophils, obtained by bronchoalveolar lavage from *C parvum*-treated animals, were injected into control rats, the increase was 90·6 (11·0) fold. Induction of an inflammatory response in the lung tissue of the recipient rat also caused an increase in the sequestration of control neutrophils compared with the same cells in control rat lungs which was, however, less marked than when inflammatory neutrophils were used (34·7 (4·7) fold). The mean (SE) pressure developed on filtration of inflammatory neutrophils in vitro through a millipore filter (7·53 (0·2) cm H_2O) was greater than that of peripheral blood neutrophils (1·18 (0·2) cm H_2O). Increased filtration pressure indicates a decrease in cell deformability and suggests that this may be a contributory factor to the increased sequestration of inflammatory neutrophils in the pulmonary vasculature.

Conclusions – This study shows that there is sequestration of neutrophils in the pulmonary vasculature in normal rat lungs which increases in acute lung inflammation and when inflammatory neutrophils are injected into control animals. In this model changes in the neutrophil, such as cell deformability, may have a more important role in inducing increased neutrophil sequestration than the inflammatory response in the lungs.

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Keywords: neutrophils, lung inflammation, deformability, radionuclide.

The lungs contain a large so called "marginated" pool of neutrophils[1 2] which are largely located in the capillary bed.[3 4] While the average diameter of the pulmonary capillary segments is only 5 μm,[5] that of the neutrophil is about 7 μm.[6] This size differential is likely to influence neutrophil sequestration in the lungs since most neutrophils must therefore deform to pass through the smaller pulmonary capillaries. The effect of such size differential is that, in normal lungs, the transit of neutrophils through the pulmonary microvasculature is delayed compared with the passage of erythrocytes.[7 8]

Delay in neutrophil transit within the pulmonary microcirculation allows interaction between capillary endothelial cells and the neutrophil and is likely to be a prerequisite for cell adhesion to the vascular endothelium and subsequent diapedesis and emigration. In inflamed lungs the slow transit of neutrophils through the pulmonary capillaries may potentiate their interaction with the capillary endothelium, so increasing their accumulation in the lung tissue.

There are many factors which can influence neutrophil sequestration in the lungs,[3] amongst which are alterations in blood flow,[9] adhesion molecule expression,[10] and cell deformability.[11] We have shown in humans that the sequestration of neutrophils labelled with indium-111 10 minutes after their injection correlates with local blood velocity,[12] whereas

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the initial first pass sequestration in vivo correlates with neutrophil deformability in vitro.[11] Decreased neutrophil deformability is probably related to alterations in the cytoskeleton and can be oxidant-mediated or can result from cell activation.[13] Thus, decreased deformability of neutrophils may contribute to the increased sequestration which occurs during cigarette smoking[12] and in exacerbations of chronic obstructive pulmonary disease (COPD).[14]

Sequestration of neutrophils in the lungs has been the subject of considerable research interest for many years,[1–4 9] with human studies being augmented by experimental studies in dogs and rabbits. We now report the development of a model of neutrophil sequestration in rat lungs in which we demonstrate increased sequestration in acute inflammation, which is influenced both by changes in neutrophil function and by the effects of lung inflammation.

Methods

CELL PREPARATIONS

Rat peripheral blood neutrophils were obtained as previously described by Williams and coworkers.[15] Briefly, male HAN rats of around 250 g in weight were anaesthetised with intraperitoneal nembutal and transfused via the iliac vein with 40–50 ml of plasma expander (Hetastarch; Dupont, Stevenage, UK) to increase the total volume of blood retrieved from the animals. The blood/Hetastarch mixture was allowed to sediment for 40 minutes at room temperature and the leucocyte-rich plasma so formed was decanted and separated in one of two ways.

In the initial experiments a two-step separation was used in which 20 ml of leucocyte-rich plasma was layered over a double gradient of Percoll (top layer, 1·04 g/ml; bottom layer, 1·089 g/ml). The gradients were spun at 1050 rpm for 30 minutes at 10°C. A leucocyte-rich band was retrieved from the interface of the two Percoll layers and the cells were then washed with saline and resuspended in 10 ml ice-cold Hank's balanced salt solution (HBSS) (Gibco, Paisley, UK) containing 1% bovine serum albumin (BSA) (Sigma, Poole, Dorset, UK). One ml of the cell suspension was mixed with 3 ml of Sepracell (Sepratech Corp, Ohio, USA) and the neutrophils were isolated by centrifugation at 2600 rpm for 10 minutes, whereafter the neutrophils were located at the bottom of the centrifuge tube and the mononuclear cells at the top of the separation medium. This technique yielded a mean (SD) neutrophil population of 10·5 (5·0) × 10^6/rat with a purity of 86 (12)%, the remaining cells being predominantly lymphocytes.

In later experiments a modification of the separation technique was used. In this procedure 4·5 ml leucocyte-rich plasma was mixed with 8 ml Percoll whose specific gravity had been adjusted to 1·1120 at 23°C. The leucocyte-rich plasma/Percoll mixture was centrifuged at 2600 rpm for 30 minutes at room temperature. The red blood cells (RBCs) sedimented to the bottom of the tube, with a buffy coat of neutrophils on top; mononuclear cells migrated to the top of the separating medium. By removing the supernatant, Percoll, and mononuclear cells the neutrophils could be cleanly pipetted off the RBC layer. This technique gave a markedly improved cell yield of 34·0 (23·7) × 10^6 per rat with a greater purity of 94 (3)%, viability of >99% (as assessed by trypan blue exclusion), and no RBC contamination. In these experiments all in vitro procedures were carried out at room temperature.

Inflammatory neutrophils were obtained by bronchoalveolar lavage, as previously described,[16] from rats which had been dosed intratracheally 16 hours previously with 1·4 mg in 0·2 ml of the heat-killed *Corynebacterium parvum* (Wellcome, London, UK). This procedure yielded 45·0 (9·4) × 10^6 neutrophils per rat, with a purity of 78 (9)%. The remaining cells in this population were alveolar macrophages and these were removed by differential centrifugation through Percoll as described above, giving a >95% pure neutrophil population.

ASSESSMENT OF IN VITRO FUNCTIONAL ACTIVITY OF CONTROL AND INFLAMMATORY NEUTROPHILS

Chemotaxis

Neutrophil chemotactic response was measured by assessing the migration of the cells towards zymosan-activated serum, through nucleopore filters of 3 μm pore size in Boyden chambers as previously described.[17] 6·0 × 10^5 neutrophils in 400 μl were added to each chamber. Chemotaxis was assessed, following 30 minutes incubation at 30°C, by counting the number of migrated cells per high power field in 10 fields per filter.

Epithelial injury

Cells of the human type II alveolar epithelial cell line A549 were prepared at a concentration of 5 × 10^5/ml in microtitre plates, allowed to form a monolayer, and radiolabelled with chromium-51 (^{51}Cr); 5 × 10^4 A549 cells were added to each well. Peripheral blood and bronchoalveolar lavage neutrophils were incubated on the A549 monolayers at a ratio of 10 neutrophils to one A549 cell for four hours at 37°C without further stimulation. Epithelial injury was assessed as cell detachment from the monolayer by measuring the cell associated ^{51}Cr counts released into the supernatant medium as previously described.[17]

Fibronectin proteolysis

The proteolytic activity of unstimulated neutrophils was tested by their ability to degrade fibronectin labelled with iodine-125 as previously described.[16] Briefly, 1 × 10^5 neutrophils were cultured on a solid phase matrix of ^{125}I-labelled fibronectin in microtitre plates for four hours at 37°C. Proteolysis of fibronectin was assessed as the ^{125}I counts in the supernatant medium.

Superoxide anion production
Superoxide anion (O_2^-) production was measured in the presence of the phorbol ester, phorbol myristate acetate (PMA; 1 μg/ml) using the cytochrome C reduction method. The release of O_2^- from $2{\cdot}5 \times 10^5$ neutrophils was assessed spectrophotometrically as the absorbance at 550 nm as previously described.[18]

Neutrophil deformability
Neutrophils were diluted to a concentration of 1×10^5/ml and filtered at constant flow (1·5 ml/min) through a nucleopore polycarbonate membrane in a filter chamber with a pore diameter of 5 μm and length of 11 μm. These dimensions mimic the average dimensions of human pulmonary capillary segments.[5] Deformability was assessed by measuring the pressure which developed within the chamber over a period of six minutes as previously described.[13]

RADIOLABELLING OF CELLS
Red blood cells were labelled with technetium-99m (^{99m}Tc) using the BNL red blood cell labelling kit (Cadema Inc, New York, USA). Four ml of blood was withdrawn by cardiac puncture from a control rat and anticoagulated with heparin. The blood was then mixed with stannous tin reagent and rotated for four minutes at room temperature. One ml of 4·4% disodium EDTA was added to the tube and inverted five times. The tube was then spun at 2500 rpm for five minutes and the plasma and buffy coat removed and discarded. Technetium (12 MBq) was added to the cell pellet and the tube was rotated for 10 minutes at room temperature. The cell pellet was then diluted to the original blood volume and the labelling efficiency estimated by spinning an aliquot of the cells and measuring the counts remaining in the supernatant. Labelling efficiency of RBC using this system, measured as the uptake of label, was >95%.

Neutrophils were labelled with chromium-51 (^{51}Cr) by incubating 15×10^6 neutrophils with 15 MBq of ^{51}Cr in 0·2 ml saline. Following incubation for 20 minutes at 37°C the cells were washed twice with HBSS/0·2 rat plasma to removed unbound ^{51}Cr. After labelling the neutrophils were recounted and adjusted to a concentration of 10×10^6/ml in HBSS/0·2% rat plasma. The labelling efficiency of neutrophils was >70%. Cell viability was always >99% as assessed by trypan blue exclusion.

The cell association of radiolabel and the ability of the cells to retain the radiolabel in vitro was assessed for both RBCs and neutrophils over a two hour period. Aliquots of each cell suspension were taken at 10 minute intervals, centrifuged, and counts per minute (cpm) in the supernatant were measured. There was no loss of label from the RBCs or neutrophils during this time (RBC T_0 $6{\cdot}5 \times 10^5$, T_{120} $6{\cdot}4 \times 10^5$ cpm; neutrophils T_0 $9{\cdot}2 \times 10^4$, T_{120} $8{\cdot}9 \times 10^4$ cpm).

IN VIVO FUNCTION OF ^{51}CR-LABELLED NEUTROPHILS
Rats of approximately 250 g in weight were anaesthetised with 0·1 ml hypnorm (10 mg/ml) (Janssen, Lamberhurst, Kent, UK) intraperitoneally and 0·2 ml diazepam (10 mg/2 ml) (Roche, Welwyn Garden City, UK) was given intramuscularly as a muscle relaxant. The radiolabelled RBC and neutrophil suspensions were mixed gently in a ratio of 1:1 by volume immediately prior to reinjection. A 200 μl bolus of this cell mixture was then injected into an anaesthetised rat via the iliac vein. Ten minutes later the thoracic cavity was opened and 200 μl peripheral blood was obtained by cardiac puncture. The heart and major blood vessels were immediately ligated to prevent blood loss from the lungs which were then dissected free of the thoracic cavity. The lungs were inflated with 3% gluteraldehyde in saline and fixed overnight to prevent blood loss on dissection. The samples of blood and lung were counted the following day in a gamma counter to determine the amount of ^{51}Cr and ^{99m}Tc in each sample.

To check that the radiolabel remained associated with the cell, and to assess the persistence of radiolabelled cells in the circulation, 200 μl samples of venous blood were taken from three rats at 5, 15, 30, 60, and 120 minutes after the injection of radiolabelled RBC/neutrophil via the iliac vein. These samples were anticoagulated with EDTA, the cells were pelleted by centrifugation, and the ^{99m}Tc and ^{51}Cr counts in the supernatant and pellet were then measured.

The ability of the radiolabelled neutrophils to function as native unlabelled cells was assessed in two experiments in which their ability to respond to an inflammatory stimulus was determined by measuring their accumulation in the bronchoalveolar space in control and inflamed rat lungs. In these experiments a 200 μl bolus of RBC/neutrophil was injected into anaesthetised rats, followed immediately by an intratracheal injection of *C parvum*. Twenty four hours later the lungs were excised and lavaged and the cell-associated radioactivity in the bronchoalveolar lavage fluid was measured.

Measurement of neutrophil sequestration in the lungs
Sequestration of ^{51}Cr-labelled neutrophils (PMN) in the lungs was calculated by dividing the ratio of PMN:RBC counts in peripheral blood by the ratio of PMN:RBC counts in the lung tissue samples. This provides a measure of the fold excess of WBC:RBC in the pulmonary vasculature. The calculation is as follows:

$$\frac{\text{PMN (cpm in blood)}}{\text{RBC (cpm in blood)}} = X$$

$$\frac{\text{RBC (cpm in lung)}}{\text{PMN (cpm in lung)}} = Y$$

$$\text{Fold increase in PMN:RBC ratio} = \frac{X-Y}{X}$$

$$= \frac{Y}{X} - 1$$

STATISTICAL ANALYSIS

Significant differences between treatment groups were assessed by analysis of variance followed by a *t* test.

Results

IN VITRO FUNCTIONAL ACTIVITY OF CONTROL AND INFLAMMATORY NEUTROPHILS

There were no differences in the activity of control peripheral blood neutrophils harvested by the one-step or two-step techniques as measured by epithelial cell detachment (2410 (843) cpm *v* 2780 (515) cpm respectively, n = 4, p>0·05).

The data on the functional status of control peripheral blood neutrophils are summarised in the table. These cells were quiescent compared with inflammatory neutrophils obtained by bronchoalveolar lavage from rat lungs exposed to *C parvum* as judged by significantly lower proteolytic activity, ability to detach epithelial cells, and chemotaxis. The exception to this was the ability of neutrophils to release superoxide anion (O_2^-). Both cell populations released O_2^- in the presence of the phorbol ester, PMA. Although PMA-stimulated O_2^- production by control peripheral blood neutrophils was lower than that of neutrophils obtained by bronchoalveolar lavage after *C parvum* instillation, the difference was not statistically significant.

There was a progressive increase in cell activity between peripheral blood neutrophils from control rats and those from *C parvum* treated animals. Neutrophils in bronchoalveolar lavage fluid from *C parvum* treated animals were the most active as measured by fibronectin degradation and epithelial cell detachment (table). This trend was not present for O_2^- release. The pressure developed after six minutes filtration of peripheral blood neutrophils through a millipore filter was significantly lower (0·94 (0·28) cm H_2O) than that produced by inflammatory neutrophils obtained by bronchoalveolar lavage (8·89 (2·01) cm H_2O, p<0·01) (fig 1), indicating the greater deformability of the peripheral blood neutrophils.

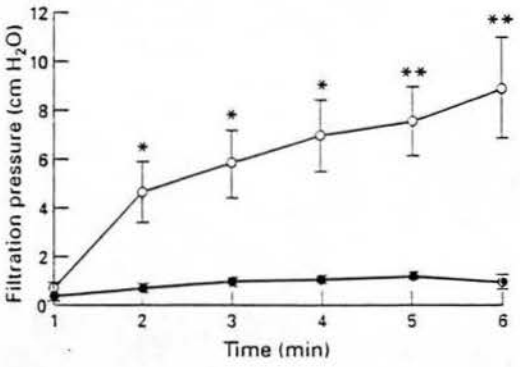


*Figure 1 Mean filtration pressures of peripheral blood neutrophils from control animals (●) and inflammatory neutrophils obtained from bronchoalveolar lavage (BAL) of rats treated with Corynebacterium parvum (○) (n = 4). Data are given as the pressure developed during filtration of 1 × 10^5 cells/ml through a millipore filter of 8 μm pore size. The bars represent the SE in each case. * p<0·05; ** p<0·01 compared with time zero.*

IN VIVO EXPERIMENTS WITH CONTROL AND INFLAMMATORY NEUTROPHILS

Persistence of neutrophils in the bloodstream

The intravenous retention of radiolabel on neutrophils and RBCs was assessed in three control rats in a single experiment over a time course which extended from five to 180 minutes. The proportion of ^{99m}Tc which remained associated with RBCs during this time was 98 (1)%, and the proportion of ^{51}Cr associated with neutrophils was 88 (1)%.

Cell-associated counts due to ^{51}Cr in 200 ml aliquots of peripheral blood were also measured over a period of 5–180 minutes. During this time there was a decrease in counts of ^{51}Cr from 1272 (100)/blood sample to 683 (17)/blood sample (fig 2).

To determine if radiolabelled neutrophils functioned as native cells, the ability of the labelled neutrophils to migrate into a site of inflammation was assessed. In a single experiment the total neutrophil-associated counts in the bronchoalveolar lavage fluid were 51 cpm

Mean (SE) in vitro function of peripheral blood (PB) and bronchoalveolar lavage (BAL) neutrophils (PMN) from control and C parvum treated animals

	Control PB-PMN (Group 1)	C parvum PB-PMN (Group 2)	C parvum BAL-PMN (Group 3)	Number of experiments*	Comparison	p
Fibronectin proteolysis (cpm fibronectin degraded)	2452 (80)	3702 (154)	12367 (1129)	3†	1 *v* 2	<0·05
					1 *v* 3	<0·001
					2 *v* 3	<0·01
A549 epithelial cell detachment (cpm detached cells)	2410 (487)	4150 (500)	7597 (1880)	4†	1 *v* 2	<0·05
					1 *v* 3	<0·01
					2 *v* 3	>0·05
PMA-stimulated superoxide anion release (nmol)	8·16 (1·4)	19·73 (2·2)	14·4 (1·2)	3†	1 *v* 2	>0·1
					1 *v* 3	>0·1
					2 *v* 3	>0·25
Chemotaxis (cells/high power field)	7·4 (1·8)	ND	19·2 (3·0)	6‡	1 *v* 3	<0·05

†Analysis of triplicate sample in each experiment.
‡Duplicate samples in six separate experiments.
ND = not done; PMA = phorbol myristate acetate.

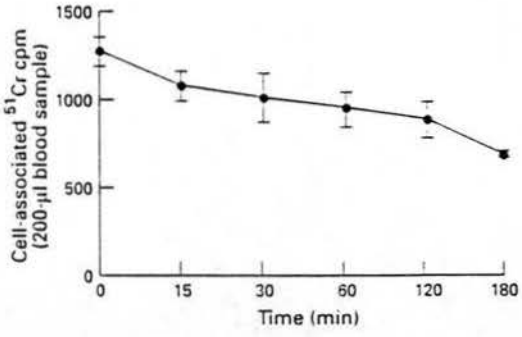


Figure 2 Mean (SE) cell-associated counts of ^{51}Cr in 200 µl blood samples over a three hour period.

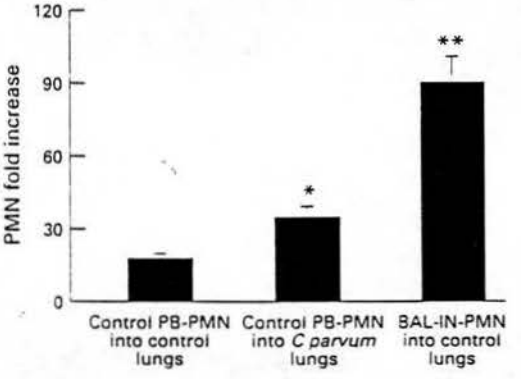


*Figure 3 Sequestration of ^{51}Cr-labelled neutrophils in the lungs 10 minutes after reinjection of peripheral blood (PB) neutrophils from control rats (PB-PMN) (n=12), inflammatory neutrophils from bronchoalveolar lavage fluid (BAL-IN-PMN) (n=11) into control rat lungs, and control PB-PMN into C parvum treated animals (n=10). The histograms represent the mean and the bars the SE of the fold increase of the PMN:RBC ratio in the lungs over that in peripheral blood. * p<0·05; ** p<0·01.*

and 42 cpm for two control rats, and 4680 cpm and 2415 cpm for two rats with lung inflammation induced by *C parvum*.

NEUTROPHIL SEQUESTRATION

Effect of neutrophil activation

The role of neutrophil activation in modulating the sequestration of neutrophils in the lung was assessed by comparing the pulmonary sequestration of untreated peripheral blood neutrophils obtained from control animals and inflammatory bronchoalveolar neutrophils when injected into control rats. The increased sequestration of inflammatory neutrophils in the lungs of control rats was significantly greater than for control peripheral blood neutrophils (fig 3).

Effect of lung inflammation

To investigate the contribution which lung inflammation might have on neutrophil sequestration in this model we compared the lung sequestration of control peripheral blood neutrophils when reinjected into control rats with that of control neutrophils injected into rats in which an acute alveolitis had been elicited by the intratracheal instillation of *C parvum* 16 hours previously. An increased sequestration of control neutrophils in the inflamed lungs compared with control lungs was seen (fig 3).

Discussion

The role of intravascular neutrophil sequestration in the pathogenesis of pulmonary disease has been of interest for a number of years.[1,2] Much of the work in previous experimental studies of neutrophil sequestration was carried out using rabbits[3,10] and dogs.[9,19] In view of the large database of information on rat models of lung inflammation[20,21] we have developed a rat model to investigate the mechanisms which govern neutrophil sequestration in the lungs. The rat has previously been considered unsuitable for studies of neutrophil kinetics because of the large numbers of neutrophils which need to be harvested in such experiments, and the difficulty of obtaining sufficient numbers and purity of cells which maintain their functional integrity from such a small animal. In addition, rat neutrophils sediment with RBCs in dextran which further complicates standard isolation procedures. Rats have been used in previous studies of neutrophil sequestration but, in order to obtain sufficient numbers of cells, the influx of neutrophils into the peritoneal cavity was induced by an intraperitoneal injection of thioglycollate medium or into the bloodstream following an intravenous injection of endotoxin.[22,23] However, the nature of the methods to elicit these cells will induce cell activation producing a population of cells with different properties from normal circulating neutrophils.[23]

The method of cell isolation which we have utilised in the present study permitted us to obtain large numbers of non-activated peripheral blood neutrophils from a single rat. The most important aspect of the separation technique is the use of Hetastarch (hydroxyethyl starch) exchange transfusion.[15] This has several benefits. Firstly, by transfusing the animal the largest possible yield of plasma was obtained. Secondly, the increased time that is taken to bleed the animal permits recovery of the marginated pool of neutrophils from organs such as the lungs and liver. Finaliy, while rat RBCs sediment in Hetastarch, neutrophils remain in suspension so Hetastarch is a suitable alternative separating medium to dextran. The use of Hetastarch exchange to obtain peripheral blood neutrophils from rats was originally described and validated by Williams and coworkers.[15] The functional activity in vitro of the isolated neutrophils in the experiments reported by Williams *et al* was not significantly different from that of neutrophils obtained by simple phlebotomy; in addition, radiolabelled neutrophils isolated with Hetastarch retained normal transit and adherence capacities in vivo.[15]

In our experiments we have extended the work of Williams and colleagues by comparing the in vitro chemotactic activity, fibronectin proteolysis, superoxide anion production, and the injurious effects on epithelial cells of peripheral blood neutrophils prepared by the Hetastarch method with that of exudated neutrophils obtained by bronchoalveolar lavage from inflamed rat lungs. In three of the parameters tested – fibronectin proteolysis, chemotaxis, and epithelial detachment – the control

peripheral blood neutrophils were significantly less active than exudated neutrophils from bronchoalveolar lavage fluid obtained from *C parvum* treated animals. The results of these experiments suggest that the peripheral blood neutrophils were not activated by the isolation procedure and are in agreement with a previous study in which we demonstrated increased proteolytic activity and injurious effects on epithelial cells of inflammatory bronchoalveolar neutrophils.[24] Furthermore, peripheral blood neutrophils in the rat could be activated in vitro by PMA.[25] Increased chemotaxis of inflammatory neutrophils has also been shown previously in rabbits.[26]

These results were not due to the suppressive effect of the Hetastarch on the peripheral blood neutrophils since in vitro exposure of the exudated neutrophils to Hetastarch did not alter their functional activity (data not shown). In addition, exposure to hydroxyethyl starch in vitro has previously been shown to have no adverse effect on human peripheral blood neutrophils.[15] Furthermore, in vivo transfusion with Hetastarch has no adverse effects in humans, rats, or several other laboratory animals.[27 28]

The superoxide anion released by inflammatory neutrophils in bronchoalveolar lavage fluid was greater (although not significantly greater) that that of the control peripheral blood neutrophils. This may be due to downregulation of the oxidant activity of the inflammatory neutrophils, which has been reported previously in neutrophils lavaged from lungs of rats exposed to asbestos[29] and in human bronchoalveolar neutrophils obtained from a patient with acute respiratory failure.[30] It has been proposed that such downregulation of oxidant responses may be due to negative feedback by hydrogen peroxide.[31]

Interestingly, the activity of peripheral blood neutrophils obtained from *C parvum* treated animals was also higher than peripheral blood neutrophils obtained from control animals, but not as high as the activity from cells obtained by bronchoalveolar lavage from inflamed lungs. This result was significant for fibronectin proteolysis, although a similar but non-significant trend was present for epithelial cell detachment. These data suggest that neutrophils become more activated in migrating from the blood to the air spaces, possibly due to the action of cytokines.

The later stages of the separation procedure involved differential separation of the leucocyte-rich plasma using Percoll, a colloidal silica preparation. This type of separation medium is preferable to the usual alternative, Ficoll Hypaque, which may activate the cells during the isolation procedure.[32] The rapid one-stage separation technique which we used in all of the later experiments enabled us to obtain larger numbers of cells, of high purity, in about half the time required for our original method. No differences in the activity of neutrophils prepared by either techniques were observed in vitro or in vivo. This novel technique will permit the use of rats in research programmes where peripheral blood neutrophils are required and where larger animals would previously have been used to provide sufficient numbers of neutrophils. The techniques which we utilised to radiolabel the erythrocytes and neutrophils were standard procedures which have been used previously and which do not alter cell function or viability.[19 23 25] In the present study we have shown that the radiolabel remains associated with the cells both in vitro and in vivo. In this study 54% of neutrophils injected into untreated rats remained in the circulation for up to three hours after the injection. This suggests that the cells were in a condition which was similar to that of neutrophils in previous studies where the half life of radiolabelled neutrophils in the circulation was claculated to be seven hours in humans[33] and six hours in dogs.[34] The number of neutrophils remaining in the circulation in the present study compares favourably with one previous study using peripheral blood neutrophils in dogs where only 17% of radiolabelled neutrophils remained in circulation at four hours.[9] Moreover, in the present study the neutrophils migrated into the air spaces in response to the inflammogen *C parvum*, which is further evidence of their ability to respond like native circulating cells.

These studies of radiolabelled neutrophils confirm in the rat, as has been shown in animal studies in dogs,[19] rabbits,[10] and in humans,[11] that there is sequestration of neutrophils in the normal pulmonary vasculature. We calculated neutrophil sequestration as the fold increase of intravascular neutrophils in excess of that predicted by the local blood volume (estimated by the RBC counts). This calculation produced a 17·4 (2·1) fold excess of neutrophils in the normal pulmonary circulation, which compares with the 15 fold increase obtained using a similar technique when radiolabelled cells were injected into patients undergoing lung resection.[35] These data therefore support the use of this model in the rat as a surrogate for human studies.

Several mechanisms may contribute to the delayed passage of neutrophils within the capillary bed. In this study we have investigated the relative roles of neutrophil activation and acute lung inflammation on the transit of neutrophils through the pulmonary vasculature. We have also investigated, in a preliminary way, the effect of neutrophil deformability on neutrophil sequestration in the lungs. The most marked neutrophil sequestration was observed in experiments where cells obtained by bronchoalveolar lavage from rat lungs inflamed with *C parvum* were reinjected into control animals. The sequestration of these cells in control lungs was six times greater than that of the sequestration of control peripheral blood neutrophils. We have previously shown that these inflammatory neutrophils are activated as defined by a number of criteria.[24 36] In this and previous studies we have shown that neutrophil activation is associated with altered deformability of the cell[8 14] which is probably due to polymerisation of F-actin.[13] The association between the reduced deformability of the inflammatory neutrophils in the present study and their increased intravascular retention

strongly suggests that cell deformability may be a major factor contributing to neutrophil sequestration in the normal lung microvasculature, and is supported by studies in humans where a correlation has been shown between neutrophil sequestration in the lungs of healthy subjects in vivo and neutrophil deformability in vivo.[11] However, the increased sequestration of control peripheral blood neutrophils in inflamed rat lungs in the absence of any difference in cell deformability implicates other mechanisms which enhance the sequestration of neutrophils during lung inflammation.[8] Neutrophil sequestration in the lungs has been shown previously to be variously dependent on blood flow.[9 12] Expression of adhesion molecules on the surface of the neutrophil and the capillary endothelial cells can alter cell adhesion and hence neutrophil sequestration.[10] Such integrin-mediated neutrophil sequestration in the lungs is thought to be important in conditions of neutrophil or lung endothelial cell activation,[37] whereas cell deformability alone may account for the sequestration in normal lungs.[8 11] The differences in lung sequestration of neutrophils under the various conditions in this study demonstrate the multifactorial nature of the signals which can elicit increased neutrophil sequestration in the lungs. The use of this rat model of neutrophil sequestration will enable further studies to be carried out to elucidate the role of the various factors which can influence neutrophil sequestration in lung inflammation and injury.

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The Role of Tumor Necrosis Factor in Increased Airspace Epithelial Permeability in Acute Lung Inflammation

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Increased airspace epithelial permeability is an early event in lung inflammation and injury. In this study, we have developed a rat model to study the mechanisms of the epithelial permeability to ^{125}iodine-labeled bovine serum albumin (^{125}I-BSA), instilled intratracheally during acute lung inflammation. Epithelial permeability was measured as the percentage of instilled ^{125}I-BSA appearing in the blood. The increase in epithelial permeability induced by intratracheal instillation of heat-killed *Corynebacterium parvum* produced a peak influx of neutrophils into the bronchoalveolar space at 16 h, which occurred after the peak increase in epithelial permeability (8 h). The increased epithelial permeability induced by *C. parvum* did not appear to be protease- or oxidant-mediated. Depletion of peripheral blood neutrophils was achieved by an intravenous injection of anti-neutrophil polyclonal antibody. The consequent profound reduction in neutrophil and macrophage influx into the airspaces 8 h after instillation of *C. parvum* reduced the epithelial permeability to control values. Bronchoalveolar lavage (BAL) leukocytes from rats 8 h, but not 16 h, after treatment with *C. parvum* caused a modest increase in epithelial permeability when re-instilled intratracheally into control rat lungs. Separation of the leukocytes before re-instillation indicated that macrophages rather than neutrophils were predominantly responsible for the increased epithelial permeability. The presence of dramatically increased levels of tumor necrosis factor (TNF) in BAL 8 h in contrast to a slight increase in BAL 16 h after *C. parvum*, the release of TNF from 8 h macrophages, the increased epithelial permeability induced by TNF in epithelial monolayers *in vitro*, and the inhibition of *C. parvum*–induced epithelial permeability by TNF antibody support the premise that TNF is a major player in the increased epithelial permeability that occurs during *C. parvum*–induced acute alveolitis.

The barrier between the blood and airspaces has a vital function in restricting the free passage of solutes between the two compartments. This barrier is composed of the alveolar epithelium, capillary endothelium, and interstitial space. The alveolar epithelium appears to be considerably less permeable than the capillary endothelium (1), suggesting that the epithelium plays a major role in limiting the passage of molecules between the airspaces and the blood.

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Abbreviations: adult respiratory distress syndrome, ARDS; bronchoalveolar lavage, BAL; bovine serum albumin, BSA; buthionine sulfoximine, -BSO; fetal calf serum, FCS; glutathione, GSH; lipopolysaccharide, LPS; -Eagle's minimum essential medium, MEM; superoxide anion, O_2^-; ozone, O_3; phosphate-buffered saline, PBS; phorbol myristate acetate, PMA; polymorphonuclear leukocyte(s), PMN; superoxide dismutase, SOD; trichloroacetic acid, TCA; tumor necrosis factor, TNF.

Increased lung epithelial permeability is present and may be important in the pathogenesis of many lung diseases, such as pulmonary sarcoidosis (2), idiopathic pulmonary fibrosis (3), and the adult respiratory distress syndrome (ARDS) (4). It also occurs in smokers (5), in asthmatics (6), and on exposure to ozone (O_3) (7). However, the precise mechanisms by which inflammatory responses initiate and sustain epithelial permeability are not known.

Acute inflammation in the lungs, such as acute bronchitis (8) and ARDS (9), is associated with increased neutrophil sequestration in the lung microvasculature and migration into the airspaces. Neutrophils may therefore play an important role in the initiation of the increased epithelial permeability that occurs in many forms of acute lung inflammation. Indeed, depletion of neutrophils prevented the increased tracheal permeability that occurred in O_3-exposed rats (10). Altered permeability of the alveolar-epithelial barrier may occur at the time of neutrophil sequestration in the pulmonary microvasculature, during their emigration through the alveolar capillary membrane, or when they accumulate in the alveolar airspace. Neutrophils may affect epithelial cells in several ways by the releasing oxygen radicals, neutral proteases, and lipid mediators (11). However, macrophages

ay also affect epithelial permeability through the release of flammatory cytokines. In particular, tumor necrosis factor NF) has been reported to participate in a wide range of flammatory responses in the lungs (12), to modulate functns of neutrophils (13), and to increase the permeability of dothelial cells (14, 15).

Thus, the aims of this study were to assess the role of ukocytes, their products, and TNF in the development of e increased alveolar epithelial permeability that occurs in ute lung inflammation. We used *in vivo* and *in vitro* models study the increased alveolar epithelial permeability that curs in the rat in an acute and resolving neutrophil alveoli- induced by intratracheal instillation of a heat-killed organn, *Corynebacterium parvum*, which has been studied exasively in our laboratory (16, 17).

aterials and Methods

ngenetic Wistar-derived rats of the HAN strain greater than wk of age were used in the study. Heat-killed *C. parvum* s obtained from Wellcome Reagents (Beckenham, UK).

diolabeling of Bovine Serum Albumin with ^{125}Iodine

vine serum albumin (BSA; Sigma, Poole, UK) was iodied according to a method we have previously described). In brief, BSA was linked with ^{125}I from Na^{125}I (Amerm International, Amersham, UK) by chloramine T and ysteine hydrochloride (Sigma). The reaction was then terated by the addition of potassium iodide (Sigma). Free was removed by chromatography on a Sephadex G25 mn (Pharmacia, Milton Keynes, UK). The ^{125}I-BSA then eluted with 0.01% BSA solution. The efficiency of iodination was examined by trichloroacetic acid (TCA; H Chemical Ltd., Poole, UK) precipitation. The bound BSA complex accounted for > 85% of the total counts.

surement of Rat Lung Epithelial Permeability

reviously described (18), rats were anesthetized by an peritoneal injection of sodium pentobarbital (5 mg/100 dy weight). A "butterfly" cannula (G25) attached to a l syringe with 100 U/ml heparin phosphate-buffered sa- (PBS) solution was placed in the femoral vein. A blunt- needle was inserted through a hole cut in the trachea. l-ml volume of 0.01% BSA solution containing 555 KBq ^{5}I-BSA was instilled intratracheally followed by 0.5 ml ir. Through the femoral vein, 0.2 ml of heparin (100 l) was injected before the withdrawal of each (0.1-ml) d sample. Samples were removed 5, 7, 9, 11, and 13 min the intratracheal instillation of ^{125}I-BSA. Before each le, 0.2 ml of a heparin and blood mixture was with- n and discarded. Blood samples were then counted ^{25}I-BSA radioactivity in a gamma counter (Cobra II -gamma Counting System; Packard Instrument Co., den, CT). Free ^{125}I was measured in the blood samples ccounted for < 50% of the counts and was similar in ol and *C. parvum*–treated animals. The blood samples corrected for free ^{125}I based on these results. Measures of permeability were calculated as the percentage of ^{5}I-BSA counts originally instilled that had penetrated he blood. For each blood sample, the ^{125}I count in the l volume was calculated. The blood volume of the rat ot measured but calculated from standard values as previously described (18). Percentage permeability was expressed in two ways: as the mean of the pooled counts of the blood samples 5, 7, 9, 11, and 13 min after administration of ^{125}I-BSA since the change from control values was similar at each of these time points or as the percentage increase in treated animals compared with control values. In some experiments, total protein in BAL fluid was measured to assess global permeability of the airspace blood barrier (see below).

Intratracheal Instillation of *C. parvum*/Lipopolysaccharide

The trachea of anesthetized rats were exposed, and a small incision was made through which a blunt-ended needle was introduced. A volume of 0.2 ml of *C. parvum* solution containing 1.4 mg of *C. parvum* or, in limited experiments, 1 μg of lipopolysaccharide (LPS) was then injected. Epithelial permeability was measured at various time intervals thereafter.

Epithelial permeability was also measured 8 h after simultaneous instillation of rabbit anti-TNF serum (kindly supplied by Drs. Joseph Fantone and Steven Kunkel, Ann Arbor, MI) (20 or 100 μl/rat) with *C. parvum* to assess the role of TNF in the increased epithelial permeability induced by *C. parvum* instillation. Pre-bled rabbit serum was used as the control.

To study the role of airspace leukocytes in increasing airspace epithelial permeability, mixed leukocytes, obtained by bronchoalveolar lavage (BAL) 8 and 16 h after instillation of *C. parvum*, or neutrophils and macrophages separated on a Percoll gradient (see below), were re-instilled. A volume of 0.5 ml PBS containing 33 $\times$ 10^6 cells was used for reinstillation. This cell number was chosen to mimic the cell count in BAL obtained from rats 8 h after intratracheal instillation of *C. parvum*. These cells were then instilled intratracheally into the lungs of control rats, and epithelial permeability was measured 6 h thereafter. The viability of these cells, obtained by re-lavage 6 h after they were instilled intratracheally, was > 95%, as measured by trypan blue exclusion.

Bronchoalveolar Lavage

Rats were killed by an intraperitoneal injection of Nembutal (Ceva Ltd., Watford, UK). The lungs were removed from the thorax, and BAL was performed by injecting and withdrawing 8 ml PBS at 37°C from the lungs, repeated 4 times as previously described (17, 18).

In order to determine the antiprotease activity in BAL fluid, 4 ml of F10 medium (GIBCO, Paisley, UK) containing 2% BSA was injected into the lungs and withdrawn prior to the normal lavage procedure.

Separation of Bronchoalveolar Lavage Leukocytes from Rats Treated with *C. parvum*

BAL leukocytes were spun down and resuspended in hetastarch (Du Pont (UK) Ltd., Herts, UK). The cell suspension was mixed with Percoll (Pharmacia LKB, Uppsala, Sweden) made up to a specific gravity of 1.112 with Hanks' balanced salt solution (GIBCO) in a ratio of 4:6. The cell suspension was then spun at 2,600 rpm for 25 min. After centrifugation, there were four cell bands in the tubes: macrophage-enriched, Percoll, neutrophil-enriched, and red blood cell bands. The

were pipetted, washed with Hanks' balanced salt solu-
and resuspended in assay media as required.

onectin Degradation

iodination of fibronectin with ^{125}I was similar to the io-
tion of BSA. ^{125}I-labeled fibronectin was diluted in sa-
with 1 mg/ml of unlabeled fibronectin so that a 100-μl
uot contained 6×10^4 cpm. Aliquots (100 μl) of this
tion were added to the wells of microtiter plates, and the
es were dried at 45°C for 3 days. All of the wells were
presoaked with F10 medium (GIBCO) plus 2% BSA for
washed with saline, and dried in an incubator.
he proteolytic activity of 50 μl of trypsin or elastase
ma) diluted with F10 medium plus 2% BSA was assessed
dding it to wells containing [^{125}I]fibronectin-labeled mi-
iter plates to obtain final concentrations of 0, 1.25, 2.5,
nd 10 μg/ml. Fifty microliters of BAL fluid was then
d to the wells, which were incubated at 37°C with 5%
for 5 h. To assess the proteolytic activity, a 70-μl ali-
was sampled from each well and the free ^{125}I was
ated in a gamma counter. In experiments using BAL leu-
tes, 50 μl of the mixed or separated leukocyte suspen-
from 8 or 16 h treated rats were pipetted into each well
otain a final concentration of 0.5×10^6 cells/ml with or
out trypsin or elastase. Before sampling to determine
^{125}I counts as a measure of proteolysis of fibronectin,
lates were spun at 1,000 rpm for 5 min. The supernatant
sampled after incubation at 37°C with 5% CO_2 for 5 h
easure the activity of free ^{125}I (17).
o measure the direct proteolytic activity of inflammatory
ocytes on ^{125}I-fibronectin, 100 μl of the mixed or sepa-
l leukocyte suspensions from 8 and 16 h treated rats
added to ^{125}I-fibronectin–coated wells in concentra-
of 0, 0.5, 1, 2, and 5×10^6/ml. This was followed by
f incubation at 37°C with 5% CO_2. The free ^{125}I in the
rnatant was then measured in a gamma counter.

roxide Anion Assay

d leukocytes obtained by BAL 8 and 16 h after *C. par-*
instillation were assessed for their ability to release su-
xide anion (O_2^-) as the superoxide dismutase (SOD)-
itable reduction of cytochrome C using a standard assay
The reaction buffer was made by dissolving 100 mg
ose (BDH, Poole, UK) and 49.5 mg cytochrome C
na) in 50 ml PBS. Five milliliters of the reaction buffer
nixed with 7.5 μl SOD (Sigma). Fifty microliters of the
suspension at a concentration of 5×10^6 cells/ml was
d to tubes with 1.5 ml of reaction buffer, both with or
out SOD. Fifteen microliters of phorbol myristate ace-
PMA; Sigma) at a concentration of 100 μg/ml in PBS
dded to tubes when required. This was followed by in-
tion for 2 h at 37°C with 5% CO_2. The cells were then
at 3,000 rpm for 10 min. The supernatant was sampled
read against a reagent blank at 550 nm in a scanning
rophotometer (SP8-400 UV/Visible spectrophotome-
Pye Unicom Ltd., Cambridge, UK).

urement of Lung Glutathione

pulmonary circulation of the rat lungs was perfused 3
with 10 ml of saline, followed by exhaustive BAL. The
was carefully separated from the other tissues, blotted,
weighed, cut, and placed in a universal container. Thereafter, 6.5% (wt/vol) TCA was added and the lung tissue homogenized. The suspension was then centrifuged at 2,500 rpm for 5 min. The supernatant was neutralized with $NaHCO_3$ to a pH of 7.0 and mixed with 8 mM monobromobimane (Thiolyte; Calbiochem, Nottingham, UK). The sample was kept in the dark at room temperature for 5 min and then acidified to stop the reaction by the addition of 100% (wt/vol) TCA. The precipitated protein was removed by centrifugation for 10 min at 2,500 rpm. The glutathione (GSH) concentration in the supernatant was then measured by high pressure liquid chromatography as previously described (20).

Measurement of Bronchoalveolar Lavage Protein

BAL fluid was mixed with Biorad solution (Bio-Rad Lab, Munchen, Germany) and incubated at room temperature for 10 min. The absorbance of the fluid was then read at 595 nm on a spectrophotometer. The protein concentration was determined by comparison with a standard curve using BSA.

Epithelial Permeability *In Vitro*

Five hundred–microliter aliquots of Eagle's minimum essential medium (MEM) plus 10% fetal calf serum (FCS) were added to a 24-well plate. Nunc tissue culture inserts (GIBCO) were then placed into the wells. Five hundred microliters of a human type II alveolar epithelial cell suspension (A549 cells) at a concentration of 0.5×10^6/ml was seeded to the inserts, followed by incubation at 37°C with 5% CO_2 for 1 to 2 wk with medium changes at 2- to 3-day intervals, until cell monolayers were formed. One day before the assay, the medium was replaced with 400 μl MEM plus 10% FCS and 100 μl of medium containing ^{51}Cr at 7.4 MBq/ml, followed by overnight incubation. The A549 cell monolayer was then washed with MEM, and the medium was replaced with MEM plus 2% BSA. TNF-α was added at various concentrations and incubated for 4 h. Five microliters of ^{125}I-BSA was added to the inserts 30 min before the termination of the incubation. The inserts were then removed, and 250-μl aliquots of the medium in the wells were sampled to measure the activity of the penetrated ^{125}I-BSA (18). The final result was presented as follows:

$$\text{Permeability (\%)} = \frac{\text{cpm of aliquot} \times 2}{\text{cpm of 5 } \mu\text{l } ^{125}\text{I-BSA}} \times 100.$$

The ^{51}Cr activity in a 250-μl aliquot (aliquot 1) of the supernatant in the inserts was measured in the gamma counter and used to assess A549 cell lysis as follows:

$$\text{Lysis (\% of } ^{51}\text{Cr uptake)} = \frac{\text{cpm in aliquot 1} \times 2}{^{51}\text{Cr cpm uptake by cells}} \times 100.$$

The A549 cell detachment was determined by sampling the remaining 250 μl of supernatant (aliquot 2) plus two cell washes with saline, measuring the radioactivity in the gamma counter:

$$\text{Detachment (\% of } ^{51}\text{Cr uptake)} = \frac{\text{cpm [(aliquot 2 + washes) - aliquot 1]}}{^{51}\text{Cr cpm uptake by cells}} \times 100.$$

otal ^{51}Cr uptake by the cells was determined by re-
ng medium with 500 μl of 0.5% Triton for 10 min. A
l sample was then taken, and the ^{51}Cr counts mea-
and multiplied by 2.

tion of Lung GSH or Neutrophils

nilliliters of saline containing 75 mg buthionine sulfox-
(BSO; Sigma) was injected intraperitoneally in rats.
after, the time course of GSH depletion in rat lungs was
ed. BSO (75 mg) was also injected on two occasions
ated by an 8-h interval to maximize the reduction in
GSH. Animals were treated with both BSO and *C. par-*
h before measurements of epithelial permeability were
in order to study the interaction of BSO and *C. par-*
on GSH levels and epithelial permeability in the rat

order to deplete neutrophils, rabbit antibody (0.9 ml)
st rat neutrophils (Accurate Chemical and Scientific
, Westbury, NY) was given intravenously and *C. par-*
reatment was given 16 h thereafter. BAL leukocyte
s and epithelial permeability were measured 8 h after
ation of *C. parvum.*

r Necrosis Factor Assay

activity in BAL and in the supernatant from mixed and
ated leukocytes (neutrophil- or macrophage-enriched
ations) obtained from BAL in rats instilled with *C. par-*
vas measured using the L929 cell-lytic assay (21).
urine L929 cells (a gift from Dr. Julian Symonds,
ern General Hospital, Edinburgh, UK) were grown
EM plus 10% FCS. When used in the TNF assay,
ent monolayers of the cells were trypsinized and sus-
d in assay medium (MEM plus 10% FCS without anti-
s) at a concentration of 0.3×10^6/ml. One hun-
microliter aliquots of cell suspension were added to the
of a 96-well microtiter plate (Greiner Labortechnik
Dursley, UK), and the plates were incubated at 37°C
5% CO_2 for 24 h. The medium was discarded and
ed by 50 μl of BAL or leukocyte conditioned superna-
nd 150 μl of assay medium containing 1 μg/ml ac-
ycin D (Sigma) and then double-diluted using a mul-
nelled pipette. After 18 to 20 h of incubation, the
were emptied and stained with 100 μl/well of crystal
methanol solution. The cells were washed with water
sed in 20% acetic acid. The optical density at 540 nm
letermined with an MR650 plate reader (Dynatech
atories, Alexandria, VA). TNF activity in the superna-
vas determined by comparison with a TNF-α (kindly
ed by Dr. Julian Symonds) standard dilution curve.
confirm the specificity of TNF activity measured in
and leukocyte supernatant, a rabbit anti-TNF serum or
l serum was added to wells with L929 cells before the
on of BAL or supernatant. The results indicated that
activity in the supernatants was decreased by 90% with
ent of anti-TNF serum but not with control serum
not shown).

ics

esults are expressed as means and SEM. Differences
en the means were assessed by a one-way analysis of
ce using Tukey's method.

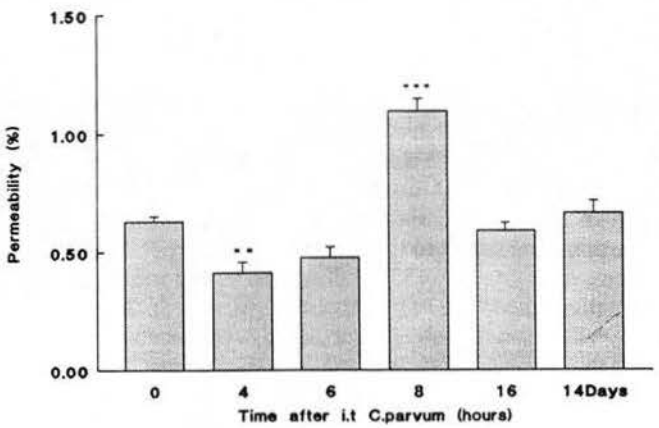


Figure 1. Epithelial permeability in the rat lung at the times indicated after intratracheal instillation of *C. parvum* is expressed as the percentage of ^{125}I-BSA instilled intratracheally that appeared in the blood. Each histogram shows the mean and the bars the SEM of six to 26 rats. Significant differences from control values: ** $P < 0.01$ and *** $P < 0.001$.

Results

Increased Epithelial Permeability and Neutrophil Influx in the Rat Lung Induced by *C. parvum*

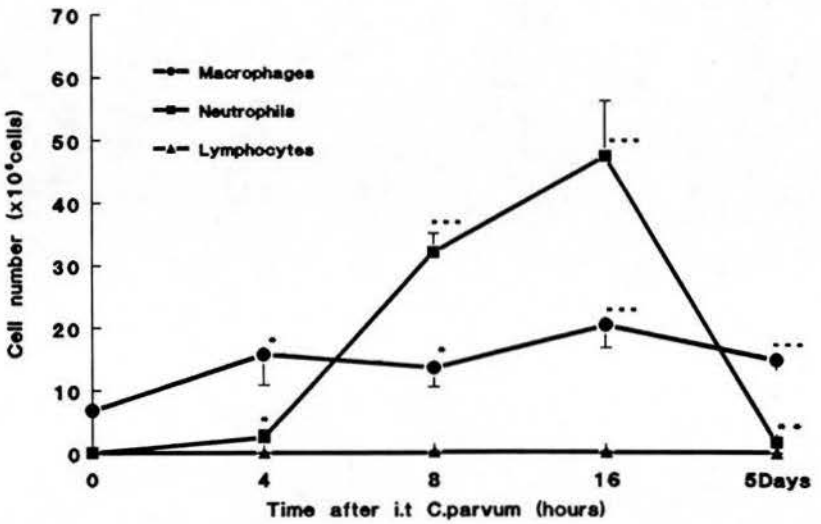
Four hours after intratracheal instillation of *C. parvum*, there was a small but significant decrease in epithelial permeability to ^{125}I-BSA, followed by a dramatic increase in permeability 8 h after *C. parvum* instillation. Epithelial permeability had returned to control levels by 16 h and remained at this level for up to 2 wk (Figure 1).

Intratracheal instillation of *C. parvum* was associated with increased neutrophil numbers in BAL, which was maximal 16 h after *C. parvum* instillation (Figure 2), by which time epithelial permeability had returned to control levels (Figure 1). *C. parvum* also induced an increase in the numbers of macrophages in BAL, without a significant change in lymphocyte numbers.

Six hours after intratracheal instillaton of LPS, there was a similar increase in epithelial permeability to ^{125}I-BSA (control, 0.33 ± 0.02%; LPS, 0.49 ± 0.02%; $P < 0.001$) and in total protein in BAL (control, 0.33 ± 0.02 mg/ml; LPS, 0.64 ± 0.02 mg/ml; $P < 0.001$).

Mechanisms of Increased Epithelial Permeability Induced by *C. parvum*: Role of Neutrophils

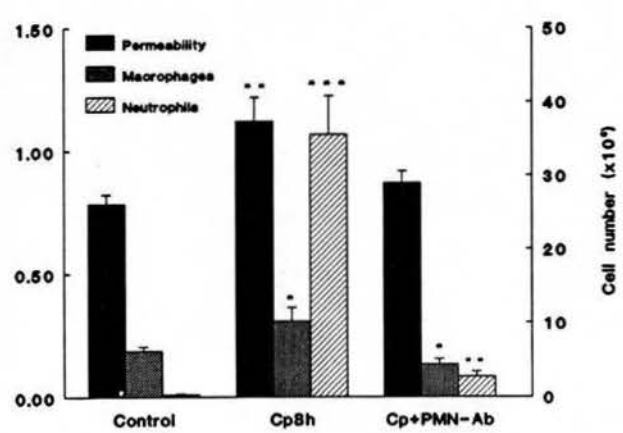
An intravenous injection of neutrophil antibody depleted peripheral blood neutrophils completely between 16 and 24 h after the antibody was given (data not shown). Therefore, animals were instilled intratracheally with *C. parvum* 16 h after the injection of neutrophil antibody. Measurements of epithelial permeability and BAL cell counts were carried out 8 h after treatment with *C. parvum*, at the time of maximal epithelial permeability. Depletion of neutrophils in peripheral blood produced a dramatic reduction in neutrophil numbers in the BAL 8 h after *C. parvum* (92% reduction compared with the cell population from rats with *C. parvum* treatment only; $P < 0.001$; Figure 3). This was associated



ure 2. BAL leukocyte counts in the rat lung at times indicated after intratracheal instillation *C. parvum*. Each point represents the mean and bars the SEM from three to 16 animals. nificant differences from control values: * $P <$, ** $P < 0.01$, and *** $P < 0.001$.

h a return of the increased epithelial permeability to con- values. However, the small increase in the numbers of crophages in BAL after *C. parvum* was reduced to a level er than control values by treatment with anti-neutrophil ibody ($P < 0.05$ compared with *C. parvum* only) (Fig- 3).

We also studied the ability of inflammatory BAL cells ob- ned from rats 8 and 16 h after *C. parvum* treatment to in- ce epithelial permeability when re-instilled in normal rat gs. As shown in Table 1, there were no statistically nificant differences in the differential cell counts between se two populations of cells, although the total cell counts were greater in the 16 h BAL (Figure 2). However, when instilled intratracheally into the lungs of control rats, mixed leukocytes (33×10^6) from BAL 8 h after *C. parvum* treatment significantly increased epithelial permeability, whereas similar numbers of mixed leukocytes from BAL 16 h after *C. parvum* treatment had no such effect (Figure 4). BAL leukocytes from *C. parvum*–treated animals were separated through Percoll to produce neutrophil- or macrophage-enriched cell populations. Both enriched neutrophil and macrophage populations from BAL 8 h after *C. parvum* induced increased epithelial permeability, but only enriched macrophages from rat BAL 16 h after *C. parvum* induced increased epithelial permeability when re-instilled into the lungs of control rats (Figure 4). The cell components in the different subpopulations after separation are presented in Table 1.



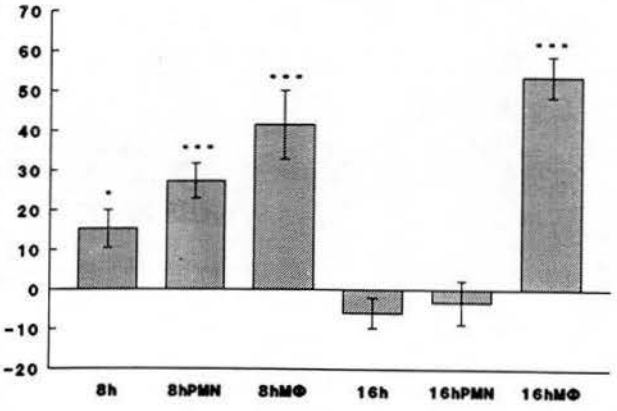
ure 3. Epithelial permeability and BAL cell counts after in- racheal instillation of *C. parvum* and an intravenous injection neutrophil antibody. Each histogram represents the mean and the s the SEM of five animals for cell number or epithelial perme- lity. For clarity, the lymphocyte counts have been omitted be- se they were small and did not change. Cp8h = 8 h after in- racheal instillation of *C. parvum*; Cp+PMN-Ab = intravenous ection of neutrophil antibody plus instillation of *C. parvum*. nificant differences from control values: * $P < 0.05$, ** $P < 0.01$, d *** $P < 0.001$.

TABLE 1

Cell components of BAL leukocytes as a percentage of total cell counts from rats 8 and 16 h after C. parvum *instillation. Cell differential counts from neutrophil- and macrophage-enriched populations are also shown**

Population	Macrophage (%)	Neutrophil (%)	Lymphocyte (%)
Control	99 (0.3)	0 (0)	1 (0.3)
Cp8h	21 (1.3)	79 (1.3)	0 (0.1)
Cp8hPMN	1 (0.3)	99 (0.3)	0 (0)
Cp8hMØ	42 (2.5)	57 (2.5)	1 (0)
Cp16h	24 (1.2)	76 (1.2)	0 (0.2)
Cp16hPMN	3 (0.5)	97 (0.5)	0 (0)
Cp16hMØ	53 (1.9)	46 (2.0)	1 (0.2)

* The results are presented as mean (SEM) of five to 31 animals. Cp8h = BAL cells from rats 8 h after *C. parvum* instillation. Cp8hPMN = PMN-enriched population of BAL cells from rats 8 h after *C. parvum* instillation. Cp8hMØ = macrophage-enriched population of BAL cells from rats 8 h after *C. parvum* instillation. Same symbols for 16 h population. Control represents the BAL differential cell counts from control animals.



gure 4. Effect on epithelial permeability in control rat lungs of ratracheal instillation of either mixed or separated leukocytes obned from BAL 8 or 16 h after treatment with *C. parvum*. Epithel permeability is calculated as in Figure 1 but expressed as a pertage change from control values. Each histogram represents ean and the bars SEM of two to five rats. 8h or 16h = mixed leucytes from rats 8 or 16 h after instillation of *C. parvum*; 8h or hPMN = PMN-enriched leukocytes from rats 8 or 16 h after inlation of *C. parvum*; 8h/16hMØ = macrophage-enriched leukoes from rats 8 or 16 h after *C. parvum*. Significant differences m control values: * $P < 0.05$ and *** $P < 0.001$.

le of Oxidants/Antioxidants

determine if the increased epithelial permeability after *parvum* could result from a reduction in antioxidants in the gs, total lung GSH as a measure of oxidant stress was assed at various times after an intraperitoneal injection of O. As shown in Table 2, 8 h after a single intraperitoneal ection of 75 mg BSO, there was a 32% reduction in total g GSH, compared with control levels; however, this had effect on epithelial permeability. Two 75-mg injections of O separated by an 8-h interval reduced total lung GSH by ean of 92% compared with control values, which was ac-

TABLE 2

ffect of BSO and C. parvum *on GSH concentrations and epithelial permeability in the rat lung**

	GSH (*nmol/g lung wet wt*)	Reduction in GSH (% *of control*)	% Increase in Permeability from Control
trol	670 (44)		
i.p. 4h	541 (53)†	19	ND
i.p. 8h	456 (43)‡	32	2 (8)
i.p. 16h	580 (67)	13	ND
BSO i.p. 8h	52 (7)§	92	24 (8)‡
.t. 8h	596 (40)	11	60 (6)§
+ BSO	351 (65)‡	48	35 (6)§

he results are presented as means (SEM) of three to nine animals for GSH ninations and two to nine animals for permeability measurements. The of BSO for each injection was 75 mg per rat. i.t. = intratracheal instilla-.p. = intraperitoneal injection. Cp = *C. parvum*. BSO = buthionine suline. Cp + BSO = measurements made 8 h after *C. parvum* and 75 mg 2 × BSO i.p. 8h = double intraperitoneal injections of 75 mg BSO separby 8-h intervals. ND = not done. Significant differences from control : † $P < 0.05$, ‡ $P < 0.01$, and § $P < 0.001$.

TABLE 3

Superoxide anion production (nM/2 h/2.5 × 10^6 cells) by leukocytes from rats 8 and 16 h after C. parvum *instillation*

Cell Population	Spontaneous	PMA Stimulated
Cp8h	1.50 (0.35)	11.92 (0.78)
Cp8hPMN	0.10 (0.05)	12.00 (2.23)
Cp8hMØ	1.66 (0.43)	7.57 (1.69)
Cp16h	1.02 (0.18)	7.05 (1.40)†
Cp16hPMN	0.29 (0.13)	4.76 (0.14)†
Cp16hMØ	0.93 (0.27)	3.88 (0.44)‡

* The results are presented as mean (SEM) of six experiments. For definition of cell populations, see Table 1.

† $P < 0.01$ and ‡ $P < 0.05$ comparing similar populations between 8 and 16 h *C. parvum* cells for spontaneous and PMA-stimulated superoxide anion release.

companied by a modest increase in epithelial permeability in the rat lung, an effect that was less than half the magnitude of that produced by *C. parvum* (Table 2).

Eight hours after intratracheal instillation of *C. parvum*, there was a nonsignificant reduction in GSH in the lungs (11% of control values), although at this time the increase in epithelial permeability was at its greatest (Table 2). The combination of treatment with BSO and *C. parvum* produced a further reduction in total lung GSH (48% of control values) compared with *C. parvum* or BSO treatment alone. However, this was not associated with a further increase in epithelial permeability compared with *C. parvum* treatment alone (Table 2).

Spontaneous O_2^- release by mixed or separated leukocyte populations (macrophage-enriched and polymorphonuclear leukocyte [PMN]-enriched populations) obtained by BAL 8 and 16 h after *C. parvum* instillation were not different, comparing similar cell populations at both time points (Table 3). However, after stimulation with PMA, 8 h leukocytes, mixed or separated, produced higher O_2^- than did 16 h leukocytes ($P < 0.01$) (Table 3).

Role of Proteases and Antiproteases

Cells from BAL of rats 8 h but not those from rats 16 h after instillation of *C. parvum* enhanced the activity of both trypsin and elastase to induce lysis of fibronectin, compared with control BAL cells (Tables 4 and 5). However, the BAL fluid of *C. parvum*–treated rats both at 8 and 16 h exhibited marked anti-trypsin and anti-elastase activity compared with control BAL fluid (Tables 4 and 5).

The BAL cells from *C. parvum*–treated rats 8 and 16 h after instillation exhibited limited but significantly enhanced ability to induce fibronectin lysis (Table 6). It was clear that the protease activity was mainly due to PMN, whereas the macrophages were more active than PMN in inducing epithelial permeability (Figure 4).

Role of Tumor Necrosis Factor

TNF activity in BAL fluid was elevated both 8 and 16 h after instillation of *C. parvum* compared with almost undetectable TNF levels in control BAL fluid (0.05 ± 0.04 U/ml). The levels of TNF were more dramatically increased 8 h (4,054 ±

TABLE 4

Effect of BAL cells or fluid from rats 8 h after C. parvum *instillation on protease-induced fibronectin degradation**

	Trypsin (*cpm*)	Elastase (*cpm*)
Protease	5,531 (188)	3,989 (220)
+ Control BAL cells	5,600 (369)	3,410 (120)†
+ Cp BAL cells	6,150 (218)†	4,946 (251)‡
+ Control BAL fluid	4,293 (353)‡	2,613 (688)†
+ Cp BAL fluid	1,003 (322)§	0 (420)§

* The results are presented as mean (SEM) of free ^{125}I cpm of three to five experiments in triplicate. + Cp BAL cells (or fluid) = protease + BAL cells (or fluid) from rats 8 h after *C. parvum* instillation. The concentration of both trypsin and elastase was 5 μg/ml. Significant differences from protease alone: † $P < 0.05$, ‡ $P < 0.01$, and § $P < 0.001$.

TABLE 6

Fibronectin lysis induced, and TNF released, by BAL leukocytes from rats 8 or 16 h after C. parvum *instillation**

Population	Fibronectin Lysis (*cpm*)	TNF Activity (*U/ml*)
Control	361 (64)	35 (5)
8h	440 (66)†	225 (60)‡
8hPMN	623 (110)§	20 (6)
8hMØ	383 (40)	2,702 (858)‡
16h	781 (77)‡	80 (11)‡
16hPMN	946 (51)‡	17 (4)§
16hMØ	422 (84)	266 (47)‡

* The results of fibronectin lysis assay are presented as mean (SEM) free ^{125}I cpm of three experiments in triplicate. The concentration of leukocytes was 2×10^6/ml. TNF results are presented as mean and SEM of 14 to 20 experiments. See Table 1 for definitions of abbreviations. Significant differences from control values: † $P < 0.01$, ‡ $P < 0.001$, and § $P < 0.005$.

804 U/ml) compared with 16 h (667 ± 114 U/ml; $P < 0.001$, $n = 14$ to 22 experiments) after *C. parvum* instillation. Leukocytes from *C. parvum*–treated rats produced TNF in greater amounts than did leukocytes from control animals. However, leukocytes from animals 8 h after *C. parvum* instillation produced greater amounts of TNF ($P = 0.01$) than did 16 h leukocytes (Table 6). Separation of the BAL leukocytes into neutrophil- or macrophage-enriched populations showed that macrophages produced significantly greater amounts of TNF, especially the enriched macrophage population from BAL cells 8 h after *C. parvum* instillation (Table 6). The specificity of TNF detected in BAL fluid and in the supernatant of BAL leukocytes was confirmed by neutralization with rabbit anti-TNF serum (data not shown).

In a preliminary experiment, we found that 20 μl of anti-TNF serum was not enough to switch off TNF-induced increased epithelial permeability (data not shown). However, simultaneous instillation of 100 μl of anti-TNF antibody abolished the increase in epithelial permeability caused by *C. parvum* instillation (Figure 5). This effect was associated with a limited reduction in the numbers of neutrophils and macrophages in BAL (Figure 5). The effect of TNF on the permeability of A549 type II epithelial cells was also assessed *in vitro*. Concentrations of TNF-α ≥ 10 U/ml induced increased epithelial permeability of A549 cells, without cell detachment or lysis (Figure 6).

TABLE 5

Effect of BAL cells or fluid from rats 16 h after C. parvum *instillation on protease-induced fibronectin degradation**

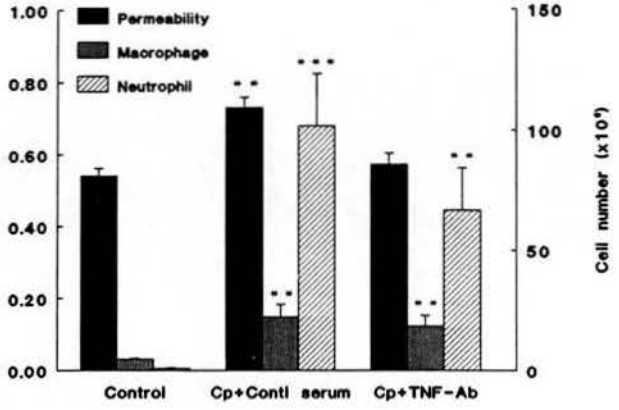
	Trypsin (*cpm*)	Elastase (*cpm*)
Protease	2,449 (142)	1,674 (115)
+ Control BAL cells	2,820 (140)†	1,853 (58)
+ Cp BAL cells	2,697 (66)	1,508 (75)
+ Control BAL fluid	1,697 (169)‡	1,857 (123)
+ Cp BAL fluid	204 (45)§	83 (72)§

* The results are presented as mean (SEM) free ^{125}I cpm of three to six experiments in triplicate. + Cp BAL cells (or fluid) = protease + BAL cells (or fluid) from rats 16 h after *C. parvum* instillation. The concentration of both trypsin and elastase was 0.625 μg/ml. Significant differences from protease alone: † $P < 0.05$, ‡ $P < 0.01$, and § $P < 0.001$.

Discussion

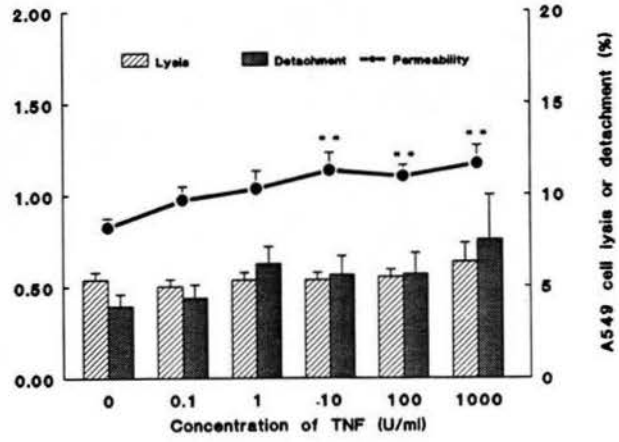
This study has focused on the mechanisms of increased alveolar epithelial permeability during acute lung inflammation. In a rat model, we induced an acute alveolitis characterized by a transient but marked inflammatory cell influx consisting mainly of neutrophils, which was associated with an increase in airspace epithelial permeability, measured as the passage of ^{125}I-BSA from the airspace to the blood. This technique has been validated by a number of workers. Bhalla and co-workers have shown that similar changes in epithelial permeability occur in response to O_3 when ^{125}I-BSA or ^{99m}Tc-DTPA was used as the tracer (22–24). Furthermore, the passage of ^{125}I-BSA across the airspace epithelium has been confirmed by autoradiographic techniques and transmission electron microscopy in the rat (23). In addition, the increased permeability of ^{125}I-BSA from airspace to blood was similar to the increased permeability from blood to airspace shown in a later study when ^{125}I-BSA was injected intravenously (25). In our studies, we showed similar increases in ^{125}I-BSA permeability after intratracheal instillation of LPS and in total protein in BAL fluid as an independent measure of alveolar/capillary permeability.

The peak increase in alveolar epithelial permeability occurred 8 h after intratracheal instillation of *C. parvum*, whereas the maximal neutrophil and macrophage recruitment into the airspaces appeared at 16 h. In a study of O_3 exposure in the rat, Bhalla and co-workers (10) showed that the maximal increase in airway permeability occurred 8 to 16 h after exposure, which corresponded with the maximum increase in the number of neutrophils in BAL. In another similar study of acute O_3 exposure in mice, lavagable neutrophils reached a peak 6 h after exposure; however, increased lung epithelial permeability was maximal 24 h after O_3 exposure (22). These variations in the time courses of epithelial permeability and corresponding neutrophil influx in these studies may result from species difference in the re-



ure 5. Effect of anti-TNF antibody on *C. parvum*–induced leuyte influx into BAL and epithelial permeability (as expressed in ure 3). Each histogram/point represents mean and the bars the M of five to six rats. For clarity, the data for lymphocytes have n omitted. Cp+Contl serum = *C. parvum* plus rabbit con- serum; Cp+TNF-Ab = *C. parvum* plus rabbit anti-TNF se-. Significant differences from control values: ** $P < 0.01$ and $P < 0.001$.

nse to an inhaled oxidant. However, in our model of acute ammation, the increase in epithelial permeability occurred ore the maximal increase in neutrophil numbers in BAL. To test the hypothesis that leukocytes were involved in increase in airspace permeability after treatment with *parvum*, we depleted rat peripheral blood neutrophils by intravenous injection of neutrophil antibody. This treatnt dramatically reduced both neutrophil and macrophage ux into the airspaces in the *C. parvum*–treated animals l reduced epithelial permeability to control levels, conning a central role for leukocytes in increasing epithelial meability in this model of acute lung inflammation. Sim-



ıre 6. Effect of TNF-α on A549 epithelial cell monolayer perbility. See text for calculation of epithelial permeability. Each nt represents mean and the bars the SEM of nine to 12 experits. Each histogram represents mean and the bars the SEM of to 10 experiments. Significant differences from control values: $P < 0.01$.

ilar results were reported by Bhalla and co-workers (10) in rats made leukopenic with cyclophosphamide, which prevented the increase in tracheal mucosal permeability to ^{99m}Tc-DTPA produced by O_3 exposure. Their studies also showed that pretreatment with indomethacin, to inhibit cyclooxygenase products, also prevented the increase in epithelial permeability produced by O_3. By contrast, studies by Kleeberger and Hudak (26) showed that pretreatment with indomethacin or cyclophosphamide in the mouse reduced neutrophil influx after O_3 exposure but failed to significantly attenuate airway epithelial permeability. They concluded that the influx of neutrophils did not play a major role in O_3-induced changes in permeability in the murine lung. O_3 is a powerful oxidant that produces only a modest neutrophil influx into the airspaces. Thus, studies with O_3 are not directly comparable with our studies of *C. parvum*–induced inflammation. Moreover, the separate effects of neutrophils and macrophages were not assessed in these studies.

We addressed the question why alveolar epithelial permeability was significantly elevated at 8 h but not at 16 h after *C. parvum* instillation, despite the greater leukocyte number in BAL at 16 h. We investigated the ability of inflammatory BAL leukocytes from rats 8 and 16 h after *C. parvum* treatment to alter epithelial permeability when instilled into control animals. Our results show that although the differential cell counts of both cell populations were similar, upon reinstillation into the lungs of normal rats, in the same numbers, only BAL mixed leukocytes from rats 8 h after *C. parvum* instillation induced a significant increase in epithelial permeability. However, the magnitude of the increased epithelial permeability produced by the re-instillation of inflammatory cells was not as great as that occurring in *C. parvum*–treated animals *in vivo.* Removal of the macrophages and re-instillation of neutrophil-enriched BAL cell populations from animals 8 h after *C. parvum* produced similar increases in epithelial permeability as the mixed leukocyte populations. This was not the case with 16 h neutrophil-enriched populations. However, enriched macrophage populations produced the greatest increase in epithelial permeability upon re-instillation. The effects of re-instillation of inflammatory leukocytes into control animals cannot exactly mimic the inflammatory conditions that follow *C. parvum* instillation. In particular, some of the results produced by reinstillation of separated cells may be due to the separation procedures. Despite their limitations, these data suggest that the presence of macrophages rather than neutrophils in the distal airspaces induces increased epithelial permeability in this model of lung inflammation. However, a contribution from neutrophils cannot be ruled out.

Several mechanisms could account for the increased permeability produced by airspace leukocytes 8 h after *C. parvum.* Robbins and associates (27) found that stimulation of neutrophils and mononuclear cells with PMA increased the adherence of these cells to epithelial cell monolayers, which may be a necessary interaction leading to an alteration in epithelial permeability. Using an *in vitro* culture system of rat alveolar type II cells, Sugahara and colleagues (11) demonstrated that neutrophils that phagocytosed opsonized zymosan increased the permeability of the epithelial monolayers compared with resting neutrophils. These observations indi-

te that neutrophil activation may be an important step en-ling neutrophils to alter epithelial permeability. However, ukocytes from BAL 8 and 16 h after *C. parvum* had similar ility to release O_2^- and to degrade fibronectin, the latter a measure of proteolytic activity *in vitro*, and were thus esumably equally active but induced different levels of epi-elial permeability. However, PMA-stimulated mixed and parated leukocyte populations from BAL 8 h after *C. par-m* released more O_2^- than did the corresponding 16 h cell pulations. Thus, it is possible that leukocyte stimulation *vivo*, releasing more reactive oxygen species, could ac-unt for the increased epithelial permeability 8 h after *parvum* instillation. Because the 16 h BAL leukocyte pop-ations also released more O_2^- upon stimulation and the mber of leukocytes, particularly neutrophils, was much eater in BAL 16 h after *C. parvum* at a time when epithe-l permeability was not increased, this explanation is not nvincing.

GSH is an important antioxidant in the lungs that main-ns the reduced state of the sulfhydryl groups of a number sulfhydryl-dependent enzymes. Endogenously generated droperoxides and other oxygen intermediates are inacti-ted via the enzyme GSH peroxidase, which utilizes GSH a proton donor (28). To investigate whether increased epi-elial permeability resulted from a disturbance of the GSH tioxidant system in the rat lung, we depleted lung GSH ntent by an intraperitoneal injection of BSO, a GSH syn-sis inhibitor. In a previous study, we have shown that a luction in intracellular GSH in alveolar epithelial cells by O is associated with increased permeability of epithelial l monolayers *in vitro*, which potentiates the increased per-ability produced by oxidants (18). However, only when GSH content in rat lungs was reduced by > 90% (to simi-levels to those that can be achieved *in vitro*) by repeated ections of BSO, was there a modest increase in epithelial meability. Coursin and Cihla (29) suggested that BSO atment alone did not induce lung injury, despite biochemi-alterations that were apparent in lung tissue after BSO atment, such as a marked reduction in GSH peroxidase ivity. Presumably, although BSO treatment alone may not e caused lung injury, it may have altered the resistance ung tissue to damage due to the loss of the reducing prop-es of GSH (30).

Although there was a dramatic increase in alveolar epi-lial permeability after intratracheal instillation of *C. par-n*, the reduction of GSH in these animals was trivial. reover, the combination of *C. parvum* instillation and O treatment had an additive effect in reducing GSH con-t in the lungs but not in increasing alveolar epithelial per-ability. We assume that the lack of an additive effect of *parvum* instillation and BSO on alveolar epithelial per-ability suggests different mechanisms of *C. parvum*– and O-induced increased epithelial permeability, whereas the ergetic interaction between GSH reduction and O_3 ex-ure (31) could result from the fact that both treatments ck the GSH antioxidant system. The data in this study in-te that although leukocytes from BAL of *C. parvum*–ted animals demonstrate enhanced release of oxygen rad-s, this does not result in a sufficient reduction in lung I to induce epithelial permeability. Nor was there any difference in the GSH in lungs 8 and 16 h after *C. parvum* instillation, as a measure of oxidant stress, to account for the difference in epithelial permeability at these two time points (data not shown). Although a marked reduction in this important antioxidant in the lungs does lead to increased epithelial permeability, as shown by the experiments with BSO, this is not the explanation of the increased epithelial permeability in *C. parvum*–treated animals. We did not study other antioxidant systems in the lungs; however, our results do not suggest that a disturbance in the oxidant-antioxidant balance in *C. parvum*–treated lungs plays a major role in inducing increased airspace epithelial permeability.

It has been well documented in our laboratory that inflammatory BAL cells derived from *C. parvum*–treated animals are able to induce detachment of cultured human type II epithelial cells and degrade extracellular matrix by protease- and oxidant-mediated mechanisms (16, 17, 32). To determine if differences in leukocyte proteolytic activity could explain the difference in epithelial permeability found in *C. parvum*–instilled rats, we therefore studied the ability of inflammatory BAL cells from animals 8 and 16 h after *C. parvum* treatment to damage the connective tissue component fibronectin, which is normally present in lung extracellular matrix and basement membrane. Both populations of BAL cells, whether mixed leukocytes or pure neutrophils, from rats 8 and 16 h after *C. parvum* instillation, induced similar degrees of fibronectin lysis, consistent with our previous findings (32). However, BAL fluid from *C. parvum*–treated rats showed considerably higher inhibitory activity on both trypsin- and elastase-induced fibronectin lysis than did control BAL. These data indicate the presence of high antiprotease activity, probably α_1-proteinase inhibitor (33), in the airspaces of *C. parvum*–treated animals. Thus, it is unlikely that the increased epithelial permeability in *C. parvum*–treated lungs was due to unchecked enhanced proteolytic activity from BAL leukocytes, since this would be suppressed by the antiprotease activity found in BAL fluid. Furthermore, differences in proteolytic activity between 8 and 16 h populations or anti-proteolytic activity in BAL cannot account for the differences in airspace epithelial permeability at these two time points in *C. parvum*–treated animals.

Epithelial cells produce a number of cytokines, such as interleukin-8, interleukin-6, granulocyte/macrophage colony-stimulating factor (34), as well as TNF (35). These cytokines induce neutrophil migration through endothelial (13) and human type II epithelial cell monolayers in a dose-related fashion (36). In addition, the production of these cytokines may be increased by exposure of epithelial cells to stimuli, such as O_3 (37) and other cytokines (34). Therefore, the increased epithelial permeability that occurs in lung inflammation could result from the secretion and release of products such as cytokines from epithelial cells. We have shown in preliminary studies that rat type II epithelial cells are capable of producing TNF in response to LPS stimulation (data not shown). However, the present study indicates that activated alveolar macrophages are the major source of TNF in the airspaces.

Although there are no reports showing the relationship between TNF and epithelial cell permeability, a number of studies have revealed that TNF increases the permeability of

endothelial cells (14, 15). The release of TNF from macrophages and its presence in BAL in this study indicate that TNF may be the factor that induces epithelial permeability in this model. This hypothesis is supported by the induction of increased A549 epithelial cell permeability by TNF *in vitro* and also by the inhibition of *C. parvum*–induced epithelial permeability by anti-TNF antibody. Although the A549 cell line may differ in many respects from primary culture cells, we have previously shown in this *in vitro* model that these cells respond in a similar way to primary culture rat type II epithelial cells in terms of changes in epithelial permeability and detachment (18, 38). The higher the levels of TNF in BAL and the greater TNF released from 8 h BAL leukocytes compared with BAL 16 h after *C. parvum* instillation could therefore account for the increased airspace permeability 8 h compared with 16 h after this treatment. The greater amount of TNF in the supernatant from macrophage-enriched populations compared with the population of mixed inflammatory leukocytes from rats 8 h after *C. parvum* instillation may be explained by the removal of neutrophils. The presence of neutrophils with TNF receptors may reduce the levels of TNF by binding with their receptors (39) or by the release of a TNF inhibitor (40).

Thus, the changes in the epithelium that result in increased epithelial permeability in *C. parvum*–treated rats may be due, at least in part, to TNF. Proposed mechanisms by which TNF affects the epithelium include alterations in the microfilamentous and microtubular cytoskeletal components, disruption of epithelial tight junctions, transport of macromolecules through paracellular pathways, or elevated endocytic transport across the alveolar epithelium, without disturbing the integrity of the epithelium. Further studies will be required to clarify these mechanisms.

In summary, intratracheal instillation of *C. parvum* in rats causes a transient alveolitis characterized by neutrophil and macrophage influx and increased epithelial permeability. Enhanced proteolytic activity from inflammatory cells, alterations in antiproteases, or a disturbance of the lung's oxidant/antioxidant balance cannot explain this increased alveolar epithelial permeability. However, the data in this study suggest that TNF, released from alveolar macrophages, may be the major factor that produces increased epithelial permeability and may at least be one factor that induces neutrophil influx.

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Supercoiled plasmid DNA as a model target for assessing the generation of free radicals at the surface of fibres*

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With 3 figures

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Key words: DNA, plasmid; Plasmid DNA; Supercoiled plasmid DNA; Free radicals; Radicals, free; Fibres, surface; Asbestos; Ceramic fibres; Vitreous fibres; Crocidolite asbestos; Amosite asbestos; Amphibole asbestos; Man made fibres

Summary

The ability of respirable amosite and crocidolite asbestos, refractory ceramic fibres (RCFs) and man made vitreous fibres (MMVFs) to cause free radical injury to plasmid, φX174 RFI DNA was assessed. The amosite and crocidolite asbestos caused substantial damage to the DNA and, in the main, the free radicals responsible for the asbestos-mediated DNA damage were hydroxyl radicals as determined by inhibition with mannitol. Asbestos fibre-mediated damage to the DNA was completely ameliorated by the chelation of fibre-associated iron by pre-treatment of fibres with desferrioxamine-B, confirming the importance of iron in the production of free radicals. MMVFs and RCFs produced modest free radical damage to the DNA, which was prevented by mannitol but not by iron chelation.

Introduction

The physical/chemical properties of asbestos and man-made fibres have been postulated to be responsible for their carcinogenicity, with particular reference to the shape and durability of fibres within the lung (DONALDSON et al. 1992). The role of active oxygen species in asbestos-induced disease has been extensively documented and fibre surface-derived free radicals have been implicated as a factor in toxicity (reviewed in KAMP et al. 1992). Iron is present in large amounts in some forms of asbestos and asbestos catalyses many of the same reactions as iron, such as lipid peroxidation and DNA damage (KAMP et al. 1992.

The purpose of the present experiments was to identify and quantify the potential of a range of respirable industrial fibres to cause free radical damage, using a model biomacromolecule, φX174 RF1 plasmid DNA.

Material and methods

Fibres: The amosite asbestos fibres were as used previously (DAVIS et al. 1986). Crocidolite, asbestos, refactory ceramic fibres (RCF) 1, 2, 3 and 4 and man-made vitreous fibres (MMVF) 10, 11, 21 and 22 were obtained from the TIMA Fibre Repository and were as used in the RCC studies.

Induction and measurement of fibre-mediated free radical damage to DNA: Two hundred and ninety ng φ X174 RF plasmid DNA were incubated with the different fibres at an equal fibre number of either 616,600 (WHO fibres > 5 μm long, < 3 μm diameter, aspect ratio > 3 : 1) in 20 μl of solution for all experiments. Two hundred and ninety ng of DNA were also used for untreated DNA. Each treatment or control sample was incubated at 37 °C in a water bath for 8 hours. Four μl Tracking Dye were added to the control and treated DNA samples and the three plasmid forms (super-coiled, relaxed coil and linearised plasmid) were separated by electrophoresis for 16 hours at 30 volts on a 0.8 % agarose gel (Sigma, Poole, UK). A positive/negative photograph was taken of each ethidium bromide (Sigma, Poole, UK) -stained agarose gel under UV light. The proportion of the plasmid forms, which provide an indication of the free radical damage to the plasmid, was quantified by scanning laser densitometry (LKB Bromma, Appsala, Sweden) providing each plasmid form as a percentage of the total DNA present in each sample.

The use of Desferrioxamine-B to determine the role of iron in the fibre-mediated free radical damage to plasmid DNA was carried out by pre-treating the fibres for 24 hours at room temperature with 10 mM DSF-B prior to addition to the DNA for treatment incubation. The fibres were taken

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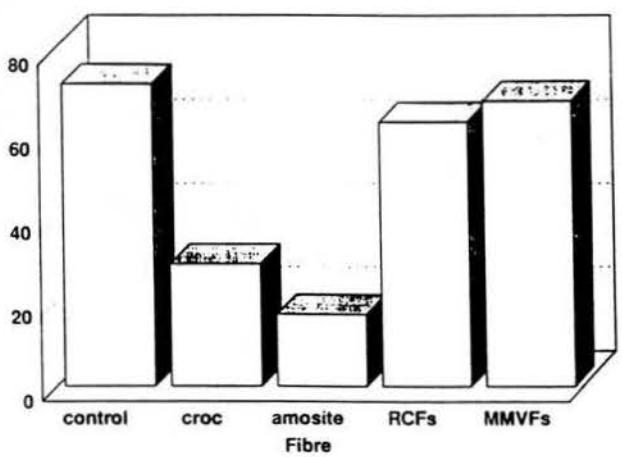
om the pre-incubation mixture, including DSF-B, and dded to the plasmid treatment solution. Mannitol (BDH hemicals, Poole, UK) was prepared as a stock solution at) mM in filter-sterilised distilled water. The mannitol was dded to the DNA treatment mixtures during experiments to final concentration of 4 mM.

esults

Fibre-mediated free radical damage to plasmid NA: Free radical damage to the DNA plasmid was dectable as a depletion of supercoiled plasmid DNA and ncrease in the proportion of relaxed coil and linearised lasmid. The extent of DNA damage caused by the diffe-nt fibres is shown in figure 1. The greatest damage was aused by the asbestos fibres whilst the man-made fibres roduced marginal damage.

Effect of hydroxyl radical scavenger and iron che-tion on fibre-mediated DNA damage: The addition of mM mannitol alone to the plasmid did not produce a gnificant change in the profile of plasmid DNA forms ompared to control but did result in an amelioration of e long fibre amosite and crocidolite-mediated damage igure 2). It had a protective effect on the small amount f injury that was caused by the man made fibres (data ot shown).

DSF-B alone did not affect the amount of supercoiled NA. However DSF-B did ameliorate the damage cau-d by both crocidolite and amosite asbestos (figure 3) hilst having no protective effect on the man made fibre-ediated damage.



ig. 1. Free radical activity of asbestos and man-made fibs as measured by decrease in % supercoiled DNA. Croc = ocidolite; RCFs = average of the TIMA Refractory ceraic fibres (see text); MMVFs average of the TIMA manade vitreous fibres (see text). Data is the mean of 3–6 exeriments.

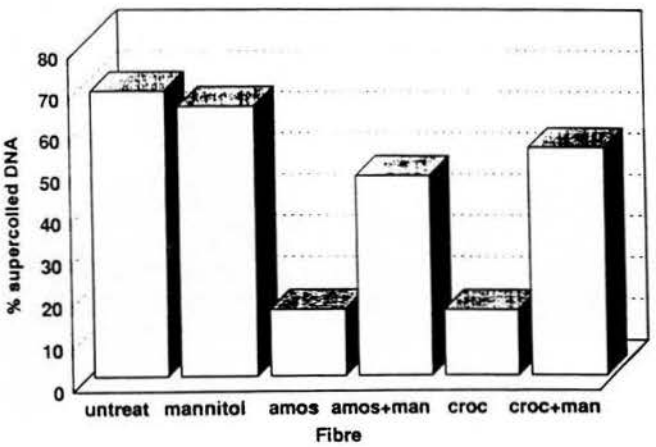


Fig. 2. Protective effect of mannitol on the free radical activity of asbestos fibres as measured by decrease in % supercoiled DNA. Amos = amosite; man = mannitol; croc = crocidolite. Data is the mean of 3–6 experiments.

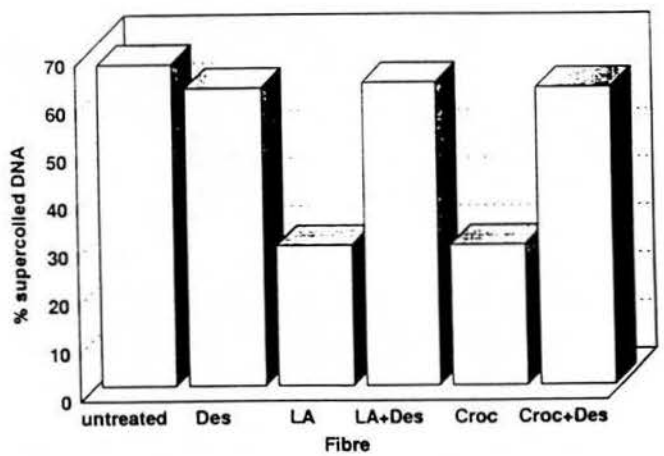


Fig. 3. Protective effect of the iron chelator Desferal on free radical activity of asbestos fibres as measured by decrease in % supercoiled DNA. LA = amosite; Des = desferal; Croc = crocidolite. Data is the mean of 3–6 experiments.

Discussion

The fact that two samples of amphibole asbestos, long amosite and crocidolite fibres, both cause considerable damage to the plasmid DNA is in agreement with the previous work of LUND and AUST (1993). The significant free radical damage that these asbestos fibres cause to the DNA in this model system does indeed reflect the general pathogenicity of these particular types of fibre as detected in previous studies. The results we describe here also indicate that the significant pathogenic activity of these asbestos fibres may be related to their ability to cause hydroxyl radical-mediated damage to macromolecules such as lipid, protein and particularly DNA, *via* iron.

The use of mannitol, a specific hydroxyl radical scavenger, ameliorated the fibre-derived damage to the plasmid DNA in the present study, clearly implicating the hydroxyl radical. It may be assumed that the residual damage that was not prevented by mannitol may be due to the action of O_2-, H_2O_2 or other oxidant species not scavenged by mannitol. Iron present on asbestos fibre surface has previously been implicated as the source of radical production, via the Fenton reaction (KAMP et al. 1992).

The man-made fibres showed much less free radical activity than asbestos fibres, but did have a detectable effect on plasmid DNA which was consistent but small enough to not attain statistical significance in the case of MMVFs but attained significance in the case of RCFs. The amelioration by mannitol of the small amount of damage to the plasmid DNA that was caused by man-made fibre treatment implicated hydroxyl radicals but chelation of iron had no effect.

The finding that RCF fibres generated significant levels of free radicals, may underlie the greater carcinogenicity of RCF fibres. As the DSF-B treatments of the RCFs and MMVFs did not produce any amelioration of the DNA damage, it can be concluded that iron does not play a role in the free radical-generating potential of RCFs and MMVFs in the model used here.

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etection of surface free radical activity of respirable industrial bres using supercoiled φX174 RF1 plasmid DNA

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he ability of a number of respirable industrial fibres, nosite and crocidolite asbestos, refractory ceramic fibres CFs) and man-made vitreous fibres (MMVFs), to cause ee radical injury to plasmid φX174 RFI DNA was assessed. he oxidative DNA damage was observed as depletion of percoiled DNA after fibre treatment and was quantified scanning laser densitometry. The mechanism of fibre-ediated damage was determined by the use of the specific droxyl radical scavenger mannitol and the iron chelator sferrioxamine-B. The amosite and crocidolite asbestos used substantial damage to DNA that was dose-related. ie free radicals responsible for the asbestos-mediated NA damage were hydroxyl radicals, as determined by hibition with mannitol. Asbestos fibre-mediated damage DNA was completely ameliorated by the chelation of re-associated iron with desferrioxamine-B. The amount Fe(II) and Fe(III) released by equal numbers of the fferent fibre types at equal fibre number was determined. ie fibres released very small amounts of Fe(II) and there re no significant differences between the fibre types. The res released substantial amounts of Fe(III); MMVF 21 leased significantly more Fe(III) than any of the other res and short fibre amosite also released more Fe(III) an three of the MMVFs and two of the RCFs. When ility to release Fe(II) and Fe(III) was compared with ility to cause DNA damage there was not a good correla-n, because only the long amosite and crocidolite caused bstantial free radical injury to DNA; this contrasts with MVF 21 and short amosite being the two fibres that eased the greatest amounts of iron. The loss of ability damage DNA in DSF-B-treated asbestos fibres shows at iron at the surface of asbestos fibres definitely has a e in generating hydroxyl radicals. However, it is clear at some fibres, such as short amosite and MMVF 21, ease large quantities of iron without causing free radical mage, whilst neither long amosite nor crocidolite released re iron than the other fibres. The exact role of iron in re reactivity therefore remains unresolved, but fibre-und iron not released from the surface of asbestos could important. Further research is under way to investigate s possibility.

roduction

e fact that exposure to asbestos fibres causes an increased k of pleural mesothelioma and bronchogenic carcinoma of the lungs has been extensively documented (1,2). Initial studies on the mechanisms of asbestos fibre-induced pathology have centred on the importance of physical properties, such as fibre length (3,4), fibre dimensions and composition (5,6), surface area (7) and solubility (8). Recent studies on man-made vitreous fibres (MMVFs*) and refractory ceramic fibres (RCFs) have shown that these fibre types also cause pathology (9). The physical properties of these man-made fibres have been postulated to be responsible for their carcinogenicity, with particular reference to the durability and residence time of fibres within the lung (10,11). Hesterberg and Barrett (12) and others have suggested that the inability of the lung to remove longer, thinner fibres results in their interaction with the mitotic apparatus of the cell, leading to chromosomal aberrations.

The role of active oxygen species in asbestos-induced disease has been extensively documented and fibre surface-derived free radicals have been implicated as a factor in toxicity (13–15). Iron is present in large amounts in some forms of asbestos and asbestos catalyses many of the same reactions as iron, such as lipid peroxidation, DNA damage (16) and hydroxyl radical formation (17). This suggests that iron available at the fibre surface or leaching from it plays a role in damaging biomolecules. Many studies have identified asbestos-complexed iron as a mediator of free radical damage (13,18–20) and iron mobilization from fibres is important in the level of toxicity of some fibres (21,22).

The purpose of the present experiments was to identify and quantify the potential of a range of respirable industrial fibres to cause free radical damage using a model biomacromolecule, φX174 RF1 plasmid DNA. Surface free radical activity of asbestos has been identified as a possible mechanism of pathogenicity and so the quantification of fibre free radical-mediated damage may provide an insight into the differential toxicity of asbestos and non-asbestos fibres. To confirm the role of iron in free radical production fibres were pretreated with desferrioxamine-B (DSF-B) to chelate iron and inhibit redox reactions of iron, prior to incubation with the plasmid DNA. The release of iron from the surfaces of the fibres was also determined. The use of the hydroxy radical (·OH) scavenger mannitol allowed us to investigate the role of ·OH in DNA damage. We also assessed the surface free radical activity of a long and a short fibre amosite asbestos that we have utilized in previous studies (3,4).

Materials and methods

Fibres and reagents

Three asbestos types and a range of man-made fibres were used in this study. The long and short amosite asbestos fibres were as used previously (23). Crocidolite asbestos, RCF 1, 2, 3 and 4 and MMVFs 10, 11, 21 and 22 were obtained from the TIMA Fibre Repository and were as used in the RCC studies (9) and will be referred to by the designations used in this study, because these are commonly used and the data here can be compared with the original study and others using these materials. The nature of the different samples are shown in Table I. All fibres were used at equal fibre number.

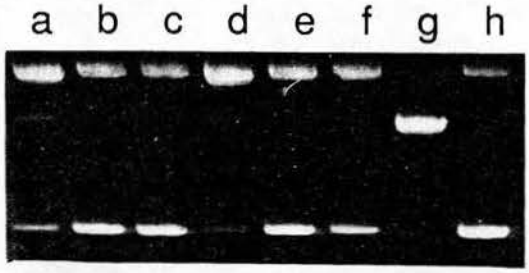
φX174 RF closed circular, supercoiled plasmid DNA was obtained from Gibco Europe (Paisley, UK) and was used for all control and DNA treatment

breviations: MMVFs, man-made vitreous fibres; RCFs, refractory ceramic es; DSF-B, desferrioxamine-B.

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. Fibre composition

	Composition
	Kaolin-based refractory ceramic fibre
	High purity refractory ceramic fibre
	Zirconium-containing refractory ceramic fibre
	High temperature-treated sample of RCF 1
: 10	Fibre glass
: 11	Fibre glass
: 21	Insulation wool
: 22	Insulation wool



Effect of asbestos and asbestos + mannitol treatments on the t forms of plasmid DNA. Photograph of a DNA gel following ion of plasmid DNA with long fibre amosite asbestos (LFA) or lite asbestos with or without mannitol. (a) LFA; (b) LFA + 2 mM ol; (c) LFA + 4 mM mannitol; (d) crocidolite; (e) crocidolite M mannitol; (f) crocidolite + 4 mM mannitol; (g) linearized DNA ; (h) DNA control.

ients. The restriction endonuclease *Pst*I was also obtained from Gibco and was used to linearize the X174 RF DNA plasmid as a control in eriments. The hydroxyl radical scavenger D-mannitol and the iron DSF-B were purchased from Sigma (Poole, UK).

on and measurement of fibre-mediated free radical damage to DNA

-RF plasmid DNA (240 ng) was incubated with the different fibres at l fibre number of either 616 600, 924 900 or 1 233 200 fibres (WHO ·5 μm long, <3 μm diameter, aspect ratio >3:1) in 20 μl of solution experiments, achieved by the addition of filtered ultra-pure distilled Two hundred and ninety nanograms of DNA were also used for d DNA and *Pst*I-linearized DNA control samples included in each ent. Each treatment or control sample was incubated at 37°C in a ath for 8 h.

microlitres of tracking dye were added to the control and treated DNA and the three plasmid forms (supercoiled, relaxed coil and linearized) were separated by electrophoresis for 16 h at 30 V on a 0.8% agarose ma, Poole, UK). See Figure 1 for a typical gel and explanation. A negative photograph was taken of each ethidium bromide (Sigma, JK) stained agarose gel under UV light. The proportion of the plasmid which provide an indication of the free radical damage to the plasmid, antified by scanning laser densitometry (LKB Bromma, Uppsala,), providing each plasmid form as a percentage of the total DNA in each sample.

on of iron

of DSF-B (Sigma, Poole, UK) to indicate the role of iron in the fibre- d free radical damage to plasmid DNA was carried out by pretreating s for 24 h at room temperature with 10 mM DSF-B prior to addition NA for treatment incubation. The fibres were taken from the pre- on mixture, including DSF-B, and added to the plasmid treatment

of mannitol as a hydroxyl radical scavenger

l (BDH Chemicals, Poole, UK) was prepared as a stock solution at n filter-sterilized distilled water. The mannitol was added to the DNA t mixtures during experiments to a final concentration of 4 mM.

earance of a typical stained gel is shown in Figure 1; the treatments led in the legend. Scanning laser densitometry of the negative of the photograph was used to determine the per cent of each DNA form. Plasmid DNA damage is expressed in the figures and tables in the present study as the change in per cent supercoiled DNA form. This was 70–80% in the control plasmid and decreased with free radical damage as the DNA was 'nicked' to become first circular and then linearized.

Measurement of Fe(II) and Fe(III)

Ferrozine and DSF-B (both Sigma, Poole, UK), specific chelators of Fe(II) and Fe(III) respectively, were utilized to detect the release of each iron type from the panel of fibres over an 8 h incubation period. Fibres were made 8.24×10^8 fibres/ml in citric acid buffer, pH 7.2. The mixtures were incubated in a rotating incubator at 37°C for 8 h. Following incubation the fibres were removed from solution by centrifugation at 4000 r.p.m. for 10 min and the supernatant decanted. The concentrations of Fe(II) and Fe(III) were determined by adding ferrozine or DSF-B to a final concentration of 1 mM. The amount of each iron type present in the samples was determined by comparison with standard curves of $FeSO_4$ [Fe(II)] and 1 mM ferrozine and $FeCl_3$ [Fe(III)] with 1 mM DSF-B read at 562 and 430 nm respectively. The amount of each iron type released from fibres was therefore expressed as μM Fe(II) or Fe(III)/ 8.24×10^8 fibres/ml.

Statistical analysis

One- or two-way analysis of variance was utilized to determine the significance of treatment effects utilizing the Tukey correction when comparisons between multiple treatment groups were required.

Results

Asbestos-mediated free radical damage to plasmid DNA

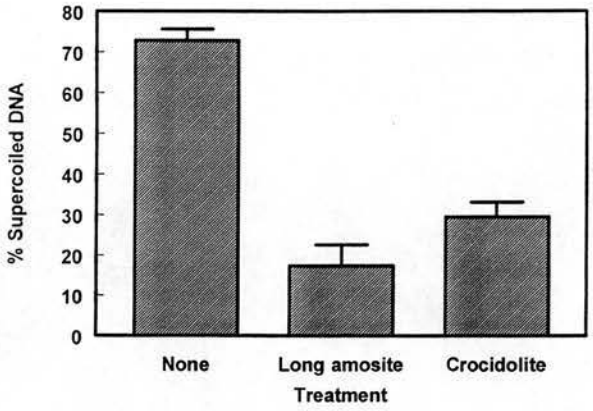
The amosite and crocidolite asbestos samples produced considerable free radical damage to the DNA plasmid, detectable as a significant depletion of supercoiled plasmid DNA and increase in the proportion of relaxed coil and linearized plasmid (Figure 2). The amosite asbestos damaged the DNA with a mean reduction of supercoiled DNA of 55.5% (significant difference from untreated DNA, $P < 0.001$) compared with the crocidolite-mediated depletion of supercoiled DNA of 43.0% (significant difference from untreated DNA, $P < 0.001$).

The free radical effect on the plasmid was related to the dose of fibres (Table II), with a reduction of supercoiled DNA corresponding to an increase in fibre number.

The long amosite fibres caused a reduction in supercoiled plasmid DNA which was significantly greater than that caused by short amosite ($P < 0.01$) (Figure 3).

Effect of a hydroxyl radical scavenger on asbestos-mediated DNA damage

In order to determine the nature of the free radical damage to the plasmid from the amosite and crocidolite asbestos treatments the hydroxyl radical scavenger mannitol was included in the asbestos treatments. The addition of 4 mM mannitol alone to the plasmid did not produce a significant change in the profile of plasmid DNA forms compared with the control, with only a 3.7% loss of supercoiled DNA (Figure 4). The addition of mannitol did, however, result in an amelioration of the long fibre amosite-mediated damage to the supercoiled form of the plasmid, with 15.7% supercoiled DNA in plasmid treated with long fibre amosite, increasing to 47.9% when 4 mM mannitol was present with the long fibre amosite (Figure 4). In the analysis of variance of the data in Figure 4 there was a significant treatment effect of $F = 50.63$, $P < 0.001$. A similar amelioration of the DNA-damaging effect of crocidolite was observed when mannitol was added to the crocidolite treatment. The supercoiled plasmid form increased from 15.64% with crocidolite alone to 54.8% with the addition of mannitol to the crocidolite treatment (Figure 5). Once again, analysis of variance of the data in Figure 5 revealed a highly significant treatment effect ($F = 28.25$, $P < 0.01$).

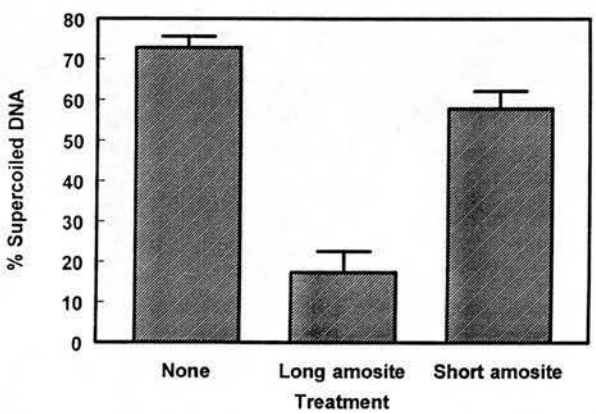


g. 2. Percentage supercoiled DNA following treatment with crocidolite d amosite asbestos compared with untreated control. Each bar represents e mean and ± SEM of values from nine separate experiments.

able II. Effect of fibre number on free radical-induced plasmid damage

reatments ibre no.×10^5)	Percent supercoiled DNA	
	Experiment 1	Experiment 2
ntreated DNA	94.6[a]	72.5
rocidolite 6.2	43.3	32.7
9.2	30.5	31.6
12.3	33.4	22.0
ong amosite 6.2	27.3	33.6
9.2	16.9	10.1
12.3	0.0	19.5

Each data point is the result of a single experiment.



g. 3. Percentage supercoiled DNA following treatment with long and ort amosite compared with untreated control. Each bar represents the ean and ± SEM of values from at least five separate experiments.

ffect of iron chelation on asbestos-mediated DNA damage

on present on the fibres has been implicated in damage to acromolecules by asbestos, with hydroxyl radicals as the fector radical. To confirm this fibres were pretreated with SF-B to chelate the iron at the surface. DSF-B alone did not fect the amount of supercoiled DNA. However, DSF-B did neliorate the damage caused by both crocidolite and amosite bestos (Figure 6). Analysis of variance revealed a significant atment effect ($F = 13.25$, $P < 0.001$) and a t-test of fibres one versus fibres + DSF-B yielded a significant difference < 0.05).

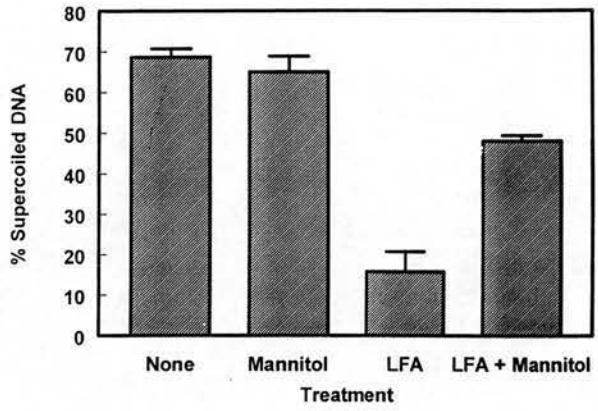


Fig. 4. Percentage supercoiled DNA following treatment with mannitol, long amosite and amosite + mannitol compared with untreated control. Each bar represents the mean and ± SEM of values from four separate experiments.

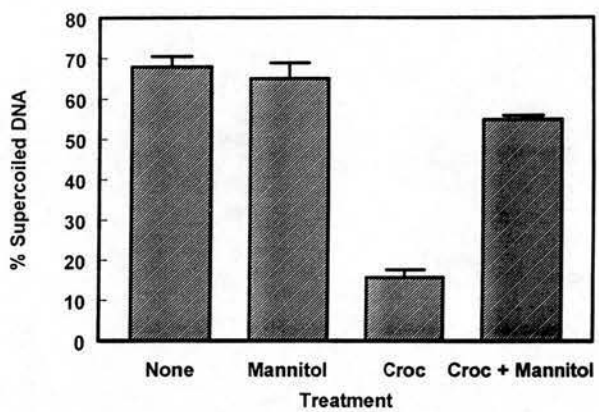


Fig. 5. Percentage supercoiled DNA following treatment with mannitol, crocidolite and crocidolite + mannitol compared with untreated control. Each bar represents the mean and ± SEM of values from four separate experiments.

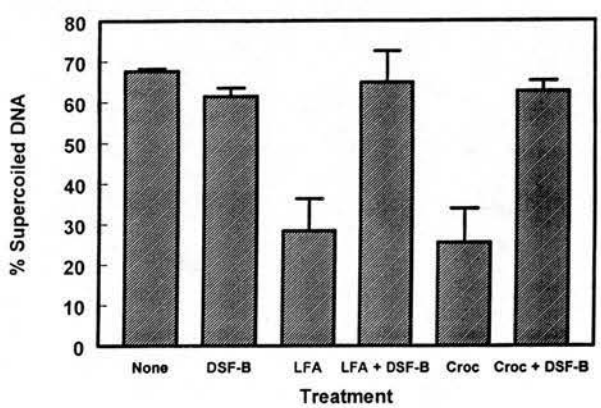


Fig. 6. Effect of DSF-B on DNA breakage caused by long fibre amostie and crocidolite. Each bar represents the mean and ± SEM of values from three separate experiments.

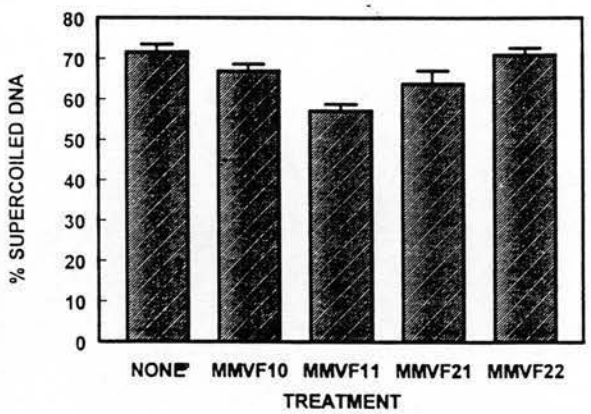
Effect of MMVFs and RCFs on plasmid DNA

Analysis of variance of experiments using the four different MMVFs showed no global treatment effect (Table III) ($F = 2.02$, $P < 0.05$), despite an average 6.2% depletion of supercoiled DNA (Figure 7). Even when MMVF 22, which had virtually no ability to cause DNA breakage, was excluded

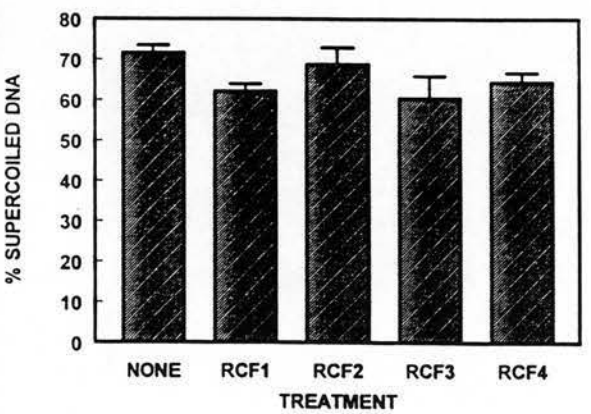
ble III. Effect of mannitol or desferrioxamine-B on MMVF-mediated mage of supercoiled DNA

eatment	Fibres only[a]	+Mannitol[b]	+DSF-B[b]
ntreated DNA	71.6 ± 0.3	71.3 ± 0.3	67.5 ± 1.1
MVF 10	66.9 ± 1.7	71.9 ± 1.9	64.7 ± 2.7
MVF 11	67.1 ± 1.6	71.6 ± 4.1	68.6 ± 2.8
MVF 21	63.8 ± 3.2	71.4 ± 0.5	70.1 ± 3.1
MVF 22	70.9 ± 1.7	72.9 ± 1.6	64.4 ± 4.3

e Table 1 for an explanation of the abbreviations.
esults presented as the mean ± SE, a single measurement in six separate periments.
esults presented as the mean ± SE, a single measurement in two separate periments.



. 7. Percentage supercoiled DNA following treatment with MMVFs 10, 21 and 22 compared with untreated control. Each bar represents the an and ± SEM of values from six separate experiments.



8. Percentage supercoiled DNA following treatment with RCFs 1–4 pared with untreated control. Each bar represents the mean and ± SEM alues from six separate experiments.

m the ANOVA, there was still no significant treatment ect. A *t*-test comparing all control DNA (71.2 ± 2.4, mean SEM) with all treatments again gave no significant difference m MMVF (67.6 ± 1.1). Analysis of variance of the five F experiments revealed no significant global treatment ect ($F = 2.51$, $P < 0.05$) (Figure 8). However, when all of RCF treatments were taken together (62.9 ± 1.7) and npared with all of the relevant untreated controls (71.2 ±) in a *t*-test there was a significant difference ($P < 0.05$). hese results suggest that the RCF fibres have greater ability

Table IV. Effect of mannitol or desferrioxamine-B on RCF-mediated damage to supercoiled DNA

Treatment	Fibres only[a]	+Mannitol[b]	+DSF-B[b]
Untreated DNA	71.6 ± 1.9	71.3 ± 0.3	67.5 ± 1.1
RCF 1	62.1 ± 1.8	65.4 ± 2.3	55.2 ± 10.8
RCF 2	68.8 ± 4.1	69.1 ± 6.2	54.4 ± 7.6
RCF 3	60.4 ± 5.5	68.5 ± 5.3	41.9 ± 25.5
RCF 4	64.4 ± 2.3	69.9 ± 1.04	52.3 ± 9.9

See Table 1 for an explanation of the abbreviations.
[a]Results presented as the mean ± SE, a single measurement in six separate experiments.
[b]Results presented as the mean ± SE, a single measurement in two separate experiments.

to directly damage DNA than MMVFs. As shown in Tables III and IV, the small amount of DNA damage caused by MMVFs and RCFs was consistently ameliorated by mannitol, demonstrating a role for hydroxyl radicals. The data in Tables III and IV, however, preclude iron in hydroxyl radical production by RCFs and MMVFs as, in contrast to the results found with asbestos, DSF-B did not protect from fibre-mediated DNA damage.

Release of iron from fibre samples

In order to further analyse the role of iron in free radical injury caused to plasmid DNA by fibres we determined the release of Fe(II) and Fe(III) from the different fibre types. This was taken as an indication of the ability of fibres to give up iron to the local environment, where they could undergo Fenton chemistry leading to the generation of free radicals. Under these conditions none of the fibre types released substantial amounts of Fe(II) (Figure 9) and there was no statistically significant difference between the different fibres. However, Fe(III) was released by all of the fibres (Figure 10). MMVF 21 released the greatest amount of Fe(III) into solution, significantly more than any of the other fibres. Amongst the other fibres there were no significant differences in their ability to release Fe(III) when compared to equal fibre number. When ability to release Fe(II) and Fe(III) was compared with ability to cause DNA damage, at equal fibre number, there was not a good corelation, since only MMVF 21 released significant iron, whilst long amosite and crocidolite caused free radical injury to DNA.

Short fibre amosite was compared with long fibre amosite at equal mass, since this is the most relevant comparison and because of the low proportion of WHO fibres short amosite contains (Table V). Although short amosite had little ability to cause DNA damage it released more Fe(III) than long amosite.

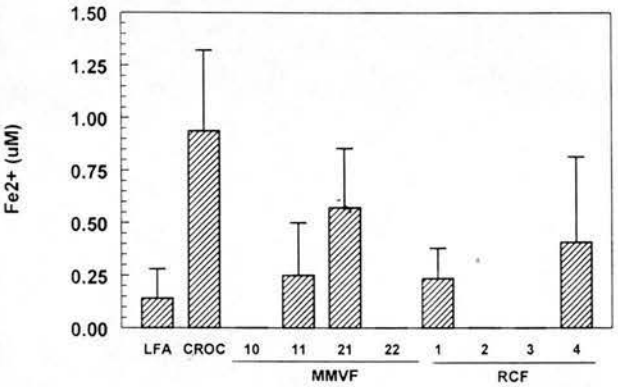
Discussion

The fact that two samples of amphibole asbestos, long amosite and crocidolite asbestos fibres, both caused considerable damage to plasmid DNA is in agreement with the previous work of Lund and Aust (21). The significant free radical damage that these asbestos fibres cause to the DNA in this model system does indeed reflect the general pathogenicity of these particular types of fibre as detected in previous studies (1,2). Lund and Aust (21) also reported that crocidolite asbestos induced a significant degree of DNA plasmid strand breakage *in vitro*. Turver and Brown (16) observed that UICC crocidolite asbestos caused DNA strand breakage when incubated with C3H10T½ cells in culture and that asbestos also initiated lipid peroxidation. Johnston and Maples (24) also demonstrated that

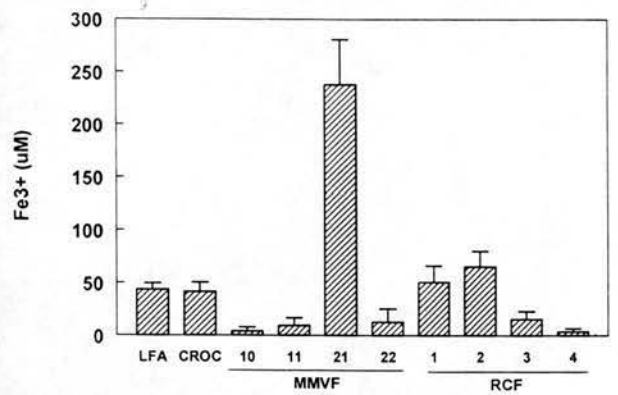
Table V. Release of iron from equal mass of short or long amosite

ibre type	Fe(II) mean (SEM)	Fe(III) mean (SEM)
hort fibre amosite	0.29 (0.21)	107.4 (0.54)
ong fibre amosite	0.14 (0.14)	43.2 (6.08)

ee Table I for an explanation of the abbreviations.



g. 9. Release of Fe(II) from 8.4×10^8 fibres of the various fibre types.



g. 10. Release of Fe(III) from 8.4×10^8 fibres of the various fibre types.

ocidolite can induce p53 protein, which can be mediated by droxyl radicals generated via Fenton chemistry (25). The ility of crocidolite asbestos to initiate DNA damage *in vitro* s been related to ability to cause mesothelioma *in vivo* by lachi *et al.* (20). Long amosite was found to have substantial ological activity by inhalation and intraperitoneal injection 23). Vallyathan has also demonstrated the oxygen radical-nerating potential of amosite and crocidolite fibres compared th other respirable industrial fibres (26). Lund *et al.* (19) ve suggested, from work with asbestos and asbestos bodies rieved from human lung, that amosite asbestos causes nificant oxidative damage to biomacromolecules, including NA. The results we describe here also indicate that the nificant pathogenic activity of these asbestos fibres may be ated to their ability to cause hydroxyl radical-mediated mage to macromolecules, such as lipid, protein and possibly NA, via iron (15).

Many studies have identified fibre dimension as being critical fibre toxicity. Hesterberg and Barrett (5) identified fibre gth as being directly related to the clastogenic activity of lustrial fibres and, similarly, Hart *et al.* (6) suggested that re length is directly proportional to *in vitro* toxicity. We scribe here the contrasting abilities of long and short amosite asbestos fibre samples that we have studied previously (3,4,23) to cause free radical damage to plasmid DNA. Because molecules of plasmid DNA interact with fibres in this assay we do not believe that fibre length is a factor, rather it is the chemical activity of the fibre surface that is being measured in this assay.

MMVFs are generally considered to be less harmful than asbestos fibres (9), but may not be without hazard (29). The results presented here reveal that the MMVFs have much less free radical activity than asbestos fibres, but do have a detectable harmful effect on plasmid DNA which is consistent, but small enough to not attain statistical significance in this series of experiments. The amelioration by mannitol of the small amount of damage to plasmid DNA caused by MMVF treatment implicated hydroxyl radicals, but chelation of iron had no effect.

In contrast to the activity of long amosite described extensively above, short amosite had no significant free radical activity, when compared to equal mass. Previously we have implicated only the difference in fibre length as the arbiter of the different pathogenic potential of these two samples (3,4,23). One possible explanation for the difference in free radical activity of the long and short amosite samples is that milling of the long amosite to produce the short sample has altered the surface chemistry and this was borne out by the data on Fe(III) release, which showed a difference between long fibre amosite and short fibre amosite. Indeed, the relative massive increase in the surface area of short amosite compared with long amosite (increased number of ends plus shards) suggests that the 'specific activity' of the short fibre surface is substantially less than that of the long amosite surface. This is an important finding in relation to the interpretation of previous studies that have used these two samples (4,23).

The products of asbestos fibre-derived oxidation reactions have been identified as hydroxyl radicals (26,27), superoxide anions and H_2O_2 (21). The use of mannitol, a specific hydroxyl radical scavenger, ameliorated fibre-derived damage to plasmid DNA in the present study, clearly implicating the hydroxyl radical. It may be assumed that the residual damage which was not prevented by mannitol may be due to the action of O_2^-, H_2O_2 or other oxidant species not scavenged by mannitol.

When the amounts of releasable Fe(II) and Fe(III) were determined from equal numbers of each fibre type it was clear that MMVF 21 insulation wool released the highest levels of Fe(III), whilst there were no differences in the small amounts of Fe(II) released by the fibres. The release of iron seen with MMVF 21 was despite the fact that MMVF 21 had negligible ability to cause DNA damage in the plasmid assay.

Mobilizable or released iron from the asbestos fibre surfaces has previously been implicated as the source of radical production, via the Fenton reaction (13,16,18–21). It has been proposed by Lund and Aust (21) that Fe(II) reacts with O_2 to produce Fe(III) and a superoxide anion. The superoxide anion would provide a mechanism by which the Fe(III) could be recycled to Fe(II). Continued renewal of the source of O_2^- and some electron donating capacity on the fibre surface may lead directly to the production of further hydroxyl radicals by reaction with H_2O_2. The results presented here, using direct DSF-B iron chelation of long amosite and crocidolite fibres and a concomitant lowering of the free radical activity, confirmed that iron was a key factor leading to DNA damage. These results confirm the work of Lund and co-workers (18,19,21) and Faux *et al.* (28) using crocidolite asbestos. Although the data generally support the contention that iron

important, the data on the release of iron by fibres did not 'eal any tendency for the fibres with ability to cause DNA nage, the long amosite and crocidolite, to release more iron n the fibres that did not cause DNA damage.

The data produced here are therefore conflicting as to the e of iron in the harmful effects of fibres in general. It ɔears that the two amphibole asbestos types used here do nage DNA via iron that can be chelated on their surface. wever, it is also clear that MMVF 21 releases the greatest ounts of iron, but causes no DNA damage. Similarly, all of fibres released comparably small amounts of Fe(II). Thus n that remains associated with the surface of fibres may be most important, not the released iron. Additionally, it may that in biological systems the presence of physiological ɔlators causes iron to be mobilized from the surfaces of ferent fibre types with qualitative or quantitiative differences m the release seen under the buffer-alone conditions used e.

When compared with long amosite at equal mass, short osite released more iron, but had little effect on plasmid lA.

t is necessary to add two caveats regarding the data obtained e. The first is that there may be differences in the surface a between the different fibre samples used. Although all nparisons were done at equal fibre number and the RCFs l the MMVFs were size-selected to have similar dimensions, re is a tendency for the RCFs to be slightly longer and so y would have more surface area (Bunn *et al.*, 1993). In lition, there may be some particles that are too small to nply with the WHO criterion (>5 μm long, <3 μm wide, ect ratio >3:1) but could contribute to the surface area; se could contribute to the results obtained here. The second eat relates to the findings of the low activity of short fibre ɔsite. Short amosite would have a large number of 'ends', sibly with different chemistry from the 'normal' surface, t these might not bind or could even repel DNA, although would appear to be unlikely and we would predict the face area of short amosite to be relatively high compared h long amosite.

The finding that RCF fibres generated significant levels of ɔ radicals may underlie the greater carcinogenicity of RCF es (9). These results would also appear to be in agreement h Miller *et al.* (30), where both MMVF and RCF fibres duced cytokine induction *in vitro*, whilst only RCF fibres ılted in any *in vivo* stimulation. However, the data here do show any marked difference between RCF and MMVF it might be reasonable to consider them as both having ilar activities, both of which are substantially less than estos. A separate study on the mechanism of the marginal ɔ radical activity displayed by MMVFs and RCFs is ranted.

ımosite and crocidolite asbestos have iron contents of as ɔh as 36% of the total weight (31). The MMVFs used in study have a range of iron content from 0.07 (MMVF 10) '.0% (MMVF 21) and the RCF fibres had a range of iron tent from 0.05 (RCF 2) to 0.97% (RCF 1 and 4) (from the IA Repository Data Sheets). As DSF-B treatment of the Fs and MMVFs did not produce any amelioration of the A damage, this further supports the contention that iron s not play a role in the free radical-generating potential of Fs and MMVFs.

ıll of the RCFs and MMVFs released some iron, although ıtly Fe(III), providing the possibility that in the complex milieu of the lung, in the presence of other reductants such as H_2O_2, vitamin C, glutathione and proteins, there could be redox cycling with production of hydroxyl radicals. Further studies are in progress to clarify whether hydrogen peroxide and the other reductants mentioned above, which can be presented in fibre-exposed lung, may increase the levels of harmful hydroxyl radicals at the surface of MMVFs or RCFs, as previously reported for asbestos (32).

In conclusion, the results presented here suggest that the free radical activity of asbestos is much greater than that of RCFs and MMVFs. Furthermore, surface iron was involved in the generation of hydroxyl radicals in the case of amosite and crocidolite asbestos. RCFs and MMVFs did, however, cause detectable, though minimal, free radical damage to DNA, particularly the RCF fibres. This was hydroxyl radical-mediated, but did not involve iron and, in general, there were no differences in the ability of different fibres to release iron, with the exception of MMVF 21. MMVF 21 released relatively large amounts of iron, but did not cause DNA damage. The data presented here generally support a role for iron in the generation of free radicals at the surface of amphibole asbestos, but confirm that the release of iron alone is not sufficient for free radical-mediated DNA damage in the case of short amosite and MMVF 21.

The data do, however confirm that man-made fibres, via the generation of injurious hydroxyl radicals, do have the potential to cause cellular damage and, by implication, such fibres may cause pathological change in the same way as asbestos, albeit at a much lower level (14,15). The mechanism that leads to the formation of these free radicals remains obscure, but is the focus of further research.

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Section 7

Section 7 Reviews, development and methodology papers

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AN IMPROVED MACROPHAGE SPREADING ASSAY - A SIMPLE AND EFFECTIVE MEASURE OF ACTIVATION

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Abstract

The development of a quantitative spreading assay of macrophage activation is described. The assay involved incubation of macrophages on glass coverslips for 1 hour and assessment of cell size using a microscope attached to a microcomputer-assisted digitising system which allowed the diameter of 200 cells to be assessed within 10 minutes. Mouse peritoneal macrophages were used in the development of the assay. Internal consistency of the assay was shown by minimal inter-observer, intra-observer and inter-animal variation. Validation of the assay as a measure of macrophage activation was confirmed by the use of *in vivo* and *in vitro* activating agents. Once validated the assay was used to detect activation in alveolar macrophages from rats exposed to airborne asbestos. The macrophage spreading assay described here is quick, reliable, consistent and easy to perform and has a potentially wide application in studies of macrophage function and dysfunction.

Introduction

Macrophage activational state has been examined by utilising various well documented biochemical or functional parameters (for example 1,2). Of these, increased spreading *in vitro* was one of the earliest reported properties of activated macrophages (3) and is apparently one of the earliest expressions of the activated phenotype (4). Spreading therefore provides a potentially reliable measure of cell function applicable to the assessment of macrophage activation.

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As part of a series of investigations into the pathogenic and immunomodulatory effects of industrial dust we have been examining the activational state of alveolar macrophages obtained, by broncho-pulmonary lavage, from rats exposed to airborne dust. During this work it was noted that the observed spreading of macrophages on glass seemed to correlate well with certain other indices of macrophage activation. In view of the potential simplicity of an assay of spreading we set out to explore this relationship and to develop a quantitative method for assessment of spreading. For this purpose the mouse peritoneal cavity was chosen as a convenient source of macrophages for the preliminary development work, during which the influence of a number of *in vitro* and *in vivo* conditions were examined.

Materials and Methods

During the course of preliminary development work a number of methods of determining the degree of macrophage spreading were considered. The measurement of the maximum diameter of the spread cells was subsequently selected for detailed examination, since this was a simple and convenient indicator of the ability of a cell to spread. The assay, as described, is therefore based upon the measurement of maximum cell diameter of a 200 cell sample taken as representative of the population.

Tables I(a) and (b) summarise the experimental approaches taken to assess the potential sources of variation in the application of the assay and to examine the assumption that the macrophage spreading assay was a measure of macrophage activation.

The Macrophage Spreading Assay

Preparation of macrophages for assay

Macrophages were obtained from mice and rats as described below. Clean, 6 x 22 mm coverslips (Chance/Propper) were set out on flat racks in triplicate groups. 100 µl of suspensions of macrophages (10^6/ml in Hams F_{10} + 10% foetal calf serum (Gibco)) were pipetted onto triplicate coverslips which were then placed in a humidified incubator at 37°C in 5% CO_2 for 60 minutes. Coverslips were then rinsed to remove non-adherent cells by moving them gently back and forth 10 times in a standard manner in a 250 ml beaker of saline within an overall time of 10 seconds. Coverslips were then replaced on racks and 100 µl of methanol added to fix the cells.

TABLE I

Summary of experiments designed (a) to determine potential sources of variation in the macrophage spreading assay; (b) to examine the relationship between macrophage spreading and activation.

(a)

POTENTIAL SOURCE OF VARIATION	DESIGN
Inter observer	The same preparation was assessed by three different operators
Intra observer	One operator assessed the same preparation on three separate occasions
Inter animal	Animals within the same treatment group were assessed and compared

(b)

EXPERIMENT	DESIGN
Effects of activating agents *in vivo*	Spreading was assessed in *C. parvum* and saline elicited peritoneal macrophages
Effects of activating agents *in vitro*	Spreading was assessed in control macrophages cultured in medium alone or medium + PMA† or MDP‡
Effect of activation by culture *in vitro*	Spreading was assessed in macrophages cultured *in vitro* for up to 48 hours
Comparison of spreading with other assays of macrophage activation	Control and *C. parvum* activated macrophages were compared with regard to spreading, tumour cell killing, Fc receptor function
Alveolar macrophages from animals inhaling chrysotile asbestos	Spreading was assessed in alveolar macrophages from control rats and rats exposed to airborne asbestos

† PMA - Phorbol myristate acetate; ‡ Muramyl dipeptide.

After 5 minutes the coverslips were washed and stained with Giemsa stain (Gurr) (1:10 Giemsa buffer (pH 6.8)) for 10 minutes, washed in two changes of tap water, and left to dry. Wher dry the coverslips were inverted on a microscope slide on a drop of DPX (Gurr) to render the preparation permanent.

Instrumentation

The measurements were made on a digitising board with an electronic cursor connected to a microcomputer (GDS1. Graphic Information Systems Limited, Scotland). With this system a pinpoint of light (from an LED) on the cursor is superimposed on the image of the stained macrophage preparation using a camera lucida attachment to a Nikon FE optical microscope. At the beginning of the assay the system was calibrated using a graduated graticule such that the cell measurements could be expressed directly in μm.

Quantitation of spreading

The stained macrophage preparations were viewed at a magnification of X1500 using an oil immersion X100 objective lens. The maximum diameters of 200 macrophages were measured by scanning up to 40 fields across the centre of the coverslip, and storing the size data on tape for subsequent analysis. Occasional adherent neutrophils were not assessed. The assays were undertaken without pre-knowledge of treatment conditions.

Analysis and presentation of data

Preliminary examination of the data revealed that the measurement of cell spreading produced a population distribution that could be described as positively skewed. In view of this, the non-parametric Kolmogorov-Smirnov statistical test (5) was used on cumulative size distributions to compare cell populations. Further, in order to convey the maximum amount of information about the cell populations, the data has been presented in the Figures and Tables as the median, quartile, and range for each distribution. In some cases the data from several preparations were pooled prior to analysis.

Conventional assays of activation

To ensure that increased spreading correlated with other markers of activation two methods of detecting activation were applied, in parallel with the spreading assay, to test preparations of macrophages:

(i) Tumoricidal function was assessed by incubating fibrosarcoma target cells, pre-labelled with ^{3}H thymidine, with saline- or C. parvum-elicited macrophages at a macrophage to target cell ratio of 10:1. After 48 hours in culture the ^{3}H thymidine counts remaining in the tumour cells was assessed by liquid scintillation counting; (ii) Fc receptor avidity was assessed by incubating saline-, or C. parvum-elicited adherent macrophages with conventional antibody-sensitised sheep red blood cells and estimating the percentage of rosette-forming cells; details of both these methods are given elsewhere (6).

Sources of Variation

Observer variation

Table I(a) summarises the experiments undertaken to examine the major sources of variation in the macrophage spreading assay.

Factors that influence cell spreading

Table I(b) summarises the experiments undertaken to determine whether macrophage activating stimuli caused macrophage spreading.

Effects of maintenance in vitro on macrophage spreading and activation

Peritoneal exudate cells were obtained from unstimulated mouse peritoneal cavities by washing with 6 x 2 ml aliquots of cold Dulbecco Phosphate buffered saline A (Dul. A) containing 10 U/ml of heparin (Pularin). Coverslip cultures were prepared by placing cleaned coverslips in 60mm plastic Petri dishes (Sterilin) prior to the addition of cells suspended in culture medium (Hams F_{10} + 10% Foetal calf serum + antibiotics). After allowing 60 minutes for adherence of the cells the coverslips were washed in the standard manner and medium added to the Petri dishes. The cells were maintained in medium for up to 48 hours. At 4, 24 and 48 hours, coverslips were removed, fixed and stained for assay as described above.

Effects of activating agents added in vitro

Coverslip cultures of mouse peritoneal adherent cells were prepared as described above with the exception that the following agents were added to the medium prior to culture: phorbol myristate acetate (PMA) (Sigma) was added at a final concentration of 1 $\mu g/ml$; muramyl dipeptide (MDP) (Sigma) was added into separate cultures at a final concentration of

10 μg/ml. After 4 hours incubation the coverslips were removed, fixed and stained for assay as described above.

Effects of activating agents in vivo

CF1 mice aged 6-10 weeks old were injected intraperitoneally with 0.2 ml (1.4 mg) Corynebacterium parvum (Wellcome), or with 0.5 ml of Dul. A as a control. Five days later the peritoneal exudate cells were harvested with 3 x 2 ml washes of cold Dul. A containing 10 U/ml of heparin (Pularin). Cells were washed, counted, and maintained at 10^6/ml in Hams F_{10} medium + 10% FCS (Gibco) on ice until used in the spreading assay.

Assessment of the effect of dust on alveolar macrophages in vivo

Male PVG inbred spf rats were exposed for up to 3 months to an airborne cloud of UICC chrysotile A asbestos (7) at a target concentration of 10 mg/m^3 of respirable dust for 7 hours per day, 5 days per week. Details of the exposure chamber design and procedures for dust generation and monitoring have been described previously (8). Untreated rats were maintained under spf stock conditions in the animal house. All animals received ad libitum access to pelleted laboratory diet and tap water.

Alveolar macrophages were obtained from the rats by bronchoalveolar lavage using 4 x 10 ml washes of phosphate buffered saline, according to the method of Myrvik, Leake and Fariss (9). Subsequent cell treatment was as described for the mouse peritoneal cells.

Results

The results of typical size distributions from spread and unspread populations are presented in Figure 1. It can be seen that both populations are positively skewed, with the spread populations having a greater range of cell sizes, and a greater proportion of larger cells. It is also noticeable that not all the cells respond similarly, since at least some of those cells in the population exposed to an activating stimulus (in this case C. parvum adminstered in vivo) are of a similar size to those in the unstimulated population.

Sources of Variation

The results of the comparison between three different operators are presented in Table II and it can be seen that the values obtained were all very similar, no significance being attached to the differences at conventional levels ($P > 0.05$). Table III shows the results of the

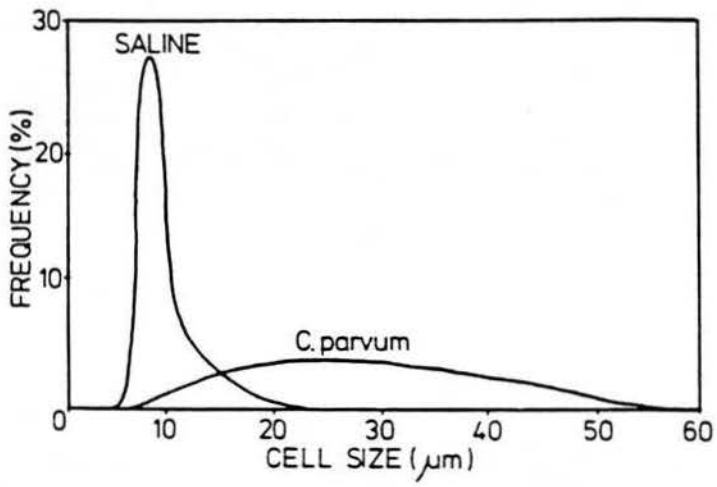


FIGURE 1

Typical size distibutions of resting (saline elicited) and activated (C. parvum elicited) peritoneal macrophages to show positive skew in both cases.

TABLE II

Summarised size distributions from macrophage spreading assays carried out by 3 different operators on the same preparation

Operator	Cell Size μm				
	Percentiles			Range	
	25	50	75	Min	Max
1	12.6	16.1	21.0	5.9	40.8
2	13.0	17.5	21.2	8.0	45.3
3	12.9	16.4	20.6	8.1	51.1

No significant difference ($P > 0.05$) between any of the operators

TABLE III

Summarised size distributions of macrophage spreading assays carried out by one operator on one preparation on 3 separate occasions

Assay	Cell size (μm)				
	Percentile			Range	
	25	50	75	Min	Max
1	13.0	17.5	21.6	8.0	45.3
2	13.0	16.0	20.5	8.0	41.1
3	14.3	16.4	19.2	10.5	34.0

No significant difference ($P > 0.05$) between any of the assessments

comparisons of the same slide assessed by one operator on 3 separate occasions; once again there was no statistically discernible difference ($P > 0.05$).

In order to test the variation in the response of individual animals to the same macrophage activating challenge, the spreading assays from several animals in an experimental group were compared and it was found (see Table IV) in all cases that the animals responded similarly, with no detectable difference ($P > 0.05$) between any animals in an experimental group.

Factors that Influence Cell Spreading

Figure 2a shows that maintenance of macrophages on coverslip preparations in culture was associated with a consistent increase in the degree of spreading throughout the 48 hour period of observation, but that there was relatively little increase during the first 4 hours in culture.

Figure 2b shows that the culture of macrophages with the well known activating agents MDP or PMA was found to be associated in each case with a degree of cell spreading that was significantly different from controls ($P > 0.001$) at 4 hours. Spreading continued to increase over the subsequent 24 hours (data not shown).

TABLE IV

Comparison of macrophage spreading assay results from separate animals given the same treatments

Treatment	Median cell size (μm)			
	Animal			
	1	2	3	4
Peritoneal				
5d Saline	8.1	9.3	8.8	-
5d C. parvum	27.4	27.8	27.6	-
Alveolar				
Control	9.8	10.0	9.7	10.0
16 days asbestos exposure	15.3	14.9	15.8	14.4

No significant differences ($P > 0.05$) between the animals in any treatment group

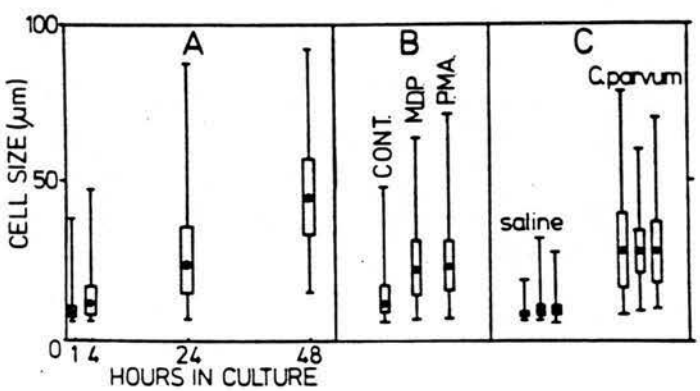


FIGURE 2

Summarised size distributions‡ (median, percentiles, and range) of (a) control peritoneal macrophages maintained in culture on coverslips from 1-48 hours; (b) control peritoneal macrophages maintained for 4 hours in medium alone, medium + muramyl dipeptide (10 μg/ml) or medium + phorbol myristate acetate (1 μg/ml); (c) peritoneal macrophages from mice injected with saline or C. parvum.

(b)* denotes significant difference ($P < 0.001$) from control macrophages maintained in medium.

(c)* denotes significant difference ($P < 0.001$) between saline and C. parvum elicited macrophages.

‡ circle = median; box = 25th to 75th percentiles; bars = range.

The use of a conventional in vivo macrophage activating agent (intraperitoneal injection of C. parvum 5 days previously) was found to result in a cell population that exhibited a significantly ($P < 0.001$) greater rapid spreading in vitro than control populations from animals given intraperitoneal saline (Figure 2c). Using conventional assays of macrophage activation, it was found that the C. parvum-elicited macrophages were activated, and Table V shows that the increased spreading described above was accompanied by significant changes in tumoricidal activity and Fc receptor avidity.

Effects of Chrysotile Asbestos on Alveolar Macrophage Activation

Having established that the assessment of macrophage spreading in vitro could be used as an assay of activation, the technique was applied to examine the effects of exposure of rats to airborne chrysotile asbestos. The results are presented in Figure 3, and it can be seen that the alveolar macrophages derived from dusted animals were

TABLE V

Comparison of the macrophage spreading assay with two other assays of macrophage activation on control (saline elicited) and activated (C. parvum elicited) macrophages

		Macrophages elicited with	
		Saline	C. parvum
Assay system	Spreading *	8.1 μm	27.4 μm
	Tumour cell cytoxicity †	1407 ± 130 cpm	513 ± 103 cpm
	Fc receptor avidity ‡	16.3 ± 4.6%	43.3 ± 3.6%

* Median cell size - see Figure 2c for further details of size distribution of C. parvum activated macrophages.

† Expressed as ^{3}H Thymidine cpm in viable prelabelled fibrosarcoma target cells co-cultured for 48 hours at a macrophage: tumour cell ratio of 10:1.

‡ Expressed as percentage rosettes formed following 1 hour incubation of adherent macrophages with sensitised sheep red blood cells.

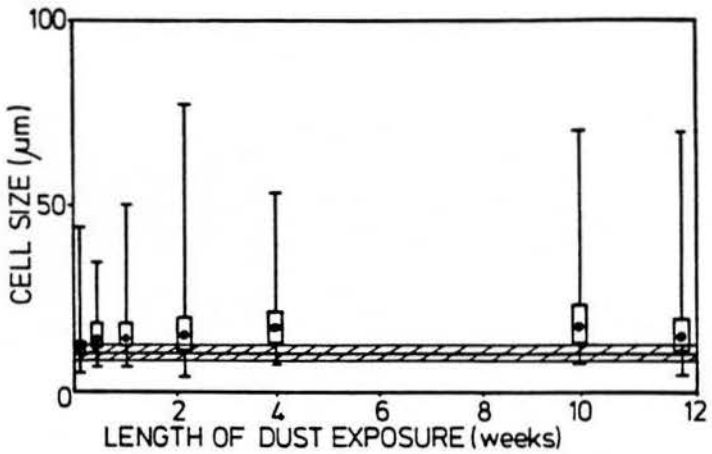


FIGURE 3

Summarised size distribution (median, percentiles and range - see below‡ of alveolar macrophages from rats exposed to airborne chrysotile asbestos for up to 3 months. Each time point contains pooled data from 4 animals. Hatched area shows control data (median and quartiles) from a total of 14 untreated animals over the period of the study. Significant differences ($P < 0.001$) between treated and controls at all time points.

‡ circle = median; box = 25th to 75th percentiles; bars = range.

significantly more spread than matched controls at all the times examined after the commencement of exposure.

Discussion

The present work has confirmed that measurement of the ability of macrophages to spread in vitro can provide a useful index of activation. The assay as described has shown that the degree of spreading correlated well with the activational state as assessed by other, widely used, methods of estimation. Using maximum cell dimension as the basic parameter, the simple standardised protocol produced a permanent record of the cell population from which it was possible to assess both the degree and extent of activation. The procedure was greatly assisted by the use of a microcomputer-linked digitising system that provided a rapid method of storage and analysis of data derived from microscopic evaluation of the stained cell preparations. Using this system an estimate of macrophage spreading can be obtained from a slide preparation in less than 10 minutes, with the added advantage that the permanent preparations can be assessed when convenient, thus enabling more complex, labile, assays to be undertaken on the day of an experiment.

It is worth noting that, although a microcomputer is helpful, it is not a prerequisite for the assay, since oil immersion optical microscopy with a calibrated eye-piece graticule could be used to manually produce a cell size distribution.

Several studies have used spreading as a marker of macrophage activation, and several methods of assessment have been described. Thus some have used semi-quantitative measures such as presence of pseudopods, aprons or veils or percentage of cells with double the diameter of unspread controls (10-15) or scoring in subjective morphological categories (16-20). Other studies have provided more objective measures of spreading such as photography, tracing, and weighing of cell images (21,22), photography and quantitation of macrophage area (23,24), and macrophage diameter by micrometry (25). Our approach is based upon micrometry of the largest cell dimension (notionally called "diameter") since this was considered to provide a valid estimate of spreading and required only one measurement per cell. We chose to estimate 200 cells as a representative sample of a cell population that could be easily and quickly measured.

Development work (Fig. 2) indicated that in vitro culture caused some degree of spreading, even in control macrophages, if culture was prolonged (24-48 hours). However relatively little of this spreading occurred in the first 4 hours and so the arbitrary 1 hour time point adopted in the routine assay can be considered to be representative of the metabolic (activational) state of the cell in vivo.

Preliminary examination of the data revealed that the measurement of cell spreading produced a population distribution that was positively skewed (see Figure 1). For the purposes of comparison of cell populations. it was therefore necessary to avoid those statistical tests which assume a normal distribution, and we chose the Kolmogorov-Smirnov test, a non-parametric test of cumulative size distributions. Further, in order to convey the maximum information about the populations in this first description of the assay, the data has been presented in the figures using median, quartiles, and range, with the median cell size being a useful simple summary statistic of a population.

Using the Kolmogorov-Smirnov test it was possible to examine the sources of variation in the assay and it was shown that the assay was internally consistent. There was thus no significant difference (at the

5% level) between three operators assessing the same preparation (Table II), or between one individual assessing the same preparation on three separate occasions (Table III). It was also found (Table IV) that there was no significant difference between cell populations derived from separate animals.

It was shown that incubation of control macrophages with PMA or MDP *in vitro* also induced a marked increase in spreading, detectable within 4 hours, and continuing up to 48 hours. In addition when the macrophages were derived from the mice pre-treated by intraperitoneal *C. parvum* 5 days earlier they were found to have an increased ability to spread *in vitro*. These findings are particularly important since the above treatments *in vivo* and *in vitro* are known to be potent inducers of macrophage activation as defined by conventional criteria (26-28). Increased Fc receptor avidity and tumour cell killing are standard measures of macrophage activation and were found to parallel the increased rapid spreading noted in *C. parvum* elicited macrophages.

Once it had been validated, the macrophage spreading assay was applied to experiments on the consequences of inhalation of chrysotile asbestos dust. Thus it was demonstrated that alveolar macrophages, derived by bronchopulmonary lavage from rats, were capable of producing a spreading response *in vitro*. It was also shown that the inhalation of chrysotile asbestos was associated with an elevation in the macrophage activational state *in vivo* that was first detected within 2 days of initial exposure and was sustained throughout the duration of a 3 month period of dusting. It is interesting to note that other workers have described, using various criteria, alveolar macrophage activation *in vivo* following exposure to fibrogenic dusts (29-31). Further, Morgan *et al* (32) recently found that rats given a pulmonary burden of dust produced an alveolar macrophage response detected as an increase in mean cell size when compared with macrophages from untreated rats. It should be noted, however, that macrophage size was measured from cytospin preparations and was not therefore an assay of spreading although increased size could reasonably be assumed to be indicative of increased metabolic state.

The present work has established the value of a quantitative assay of macrophage spreading as an index of activation that is simple to obtain and interpret. This has been confirmed by the use of macrophage

activating agents in vivo and in vitro and by examination of the effects of exposure of animals to the inhalation of mineral dusts. The assay seems to have a potentially wide application in studies of macrophage function and dysfunction.

Acknowledgements

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Concanavalin A receptors and capping in control and activated macrophages

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Summary

Macrophages activated *in vivo* with *Corynebacterium parvum* and asbestos showed an increased sub-population of cells which capped spontaneously on incubation with fluoresceinated Concanavalin A compared to saline-induced control macrophages. This capping was unaffected by colchicine but was inhibited by cytochalasin B. The spontaneous capping of activated macrophages did not appear to be directly related to the total number of Concanavalin A receptors as measured by specific ^{125}I-labelled Concanavalin A binding to activated and control macrophages. Capping also did not appear to be related to the position of Concanavalin A receptors as judged by trypsin accessibility of bound ^{125}I-labelled Concanavalin A.

Introduction

Macrophages occupy a central role in the inflammatory (Allison *et al.*, 1978) and immune systems (see papers in Unanue & Rosenthal, 1980). The plasma membrane of the macrophage is seen as the primary site of the sensory and effector roles of this cell including endocytosis (Walters & Papadimitriou, 1978), macrophage–lymphocyte interactions in antigen recognition (Rosenthal *et al.*, 1975; Lipscomb *et al.*, 1981), handling of immune complexes via specific receptors for immunoglobulin and complement (Mantovani, 1981) and functional responses to lymphocyte products via specific lymphokine receptors (David *et al.*, 1980).

Macrophages can undergo profound changes in their metabolic, cytochemical, morphological and secretory status in response to a variety of agents (Ogmundsdottir & Weir, 1980) and, while there is some disagreement over terminology, these changes can be collectively called activation (Cohn, 1978; Morahan, 1980). Macrophage activation results in changes in many surface properties such as ectoenzyme activity, pinocytosis (Cohn, 1978), Fc receptor activity (Rhodes, 1975) and surface morphology (Nabarra *et al.*, 1978).

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Macrophages can specifically bind a number of lectins to their plasma membrane including Concanavalin A (Oliver & Berlin, 1975), *Lotus tetragonolobus* lectin (Homma *et al.*, 1981), wheat germ agglutinin (Hadley *et al.*, 1977), phytohaemagglutinin and pokeweed mitogen (Loor & Roelants, 1974). Concanavalin A (Con A) specifically binds to D-mannosyl and D-glucosyl residues at the cell surface and the macrophage Con A receptor has been studied with regard to the effect of spreading and phagocytosis (Lutton, 1973), ligand-induced capping (Williams *et al.*, 1979) and transmembrane organization (Adachi *et al.*, 1980).

Intraperitoneal asbestos injection in mice induces an activated macrophage population (Donaldson *et al.*, 1982) and activated macrophages may play a role in the aetiology of asbestos-related disease (Miller, 1978); *Corynebacterium parvum*-activated macrophages are well known for their growth regulating effect on tumour cells (see papers in James *et al.*, 1977).

In the present paper, we report on the mobility and number of Con A receptors on macrophages activated *in vivo* with asbestos and *C. parvum*. These experiments form part of a wider study into the effects of asbestos on macrophage membranes.

Materials and methods

Mice

Male CBA/Ca mice 12–16 weeks old at the time of injection, were used throughout. Mice were housed under conventional conditions and were killed by ether inhalation when required.

Macrophages

Control macrophages were obtained three days after intraperitoneal injection of 0.5 ml sterile Dulbecco's phosphate-buffered saline 'A' (Dul A). Activated macrophages were obtained five days after the intraperitoneal injection of 0.2 ml (1.4 mg) of *Corynebacterium parvum* (*C. parvum*) (heat killed; Wellcome) or three days after intraperitoneal injection of 2.5 mg chrysotile asbestos (UICC sample) in 0.5 ml sterile Dul A. In some experiments macrophages were obtained three days after intraperitoneal injection of 1% latex spheres (Bacto latex 8.1 μm diameter; Difco) in Dul A or 10% Proteose peptone (Difco) in Dul A.

Macrophage cultures were prepared by washing out the peritoneal cavity with 3 × 2 ml of RPMI 1640 medium (Gibco) containing 10 U/ml heparin (Pularin). In ^{125}I-labelled Con A binding experiments, the proportion of adherent cells was ascertained and found to be 74.2% for saline, 49.3% for chrysotile and 65.6% for *C. parvum*. Activated peritoneal exudate cells were adjusted to yield the same concentration of adherent cells as saline peritoneal exudate cells – 4.5×10^6/ml in RPMI 1640 plus antibiotics and 10% foetal calf serum (Gibco). One hundred microlitres of exudate cell suspension were pipetted onto washed 6 × 22 mm glass coverslips and incubated for 1 h at 37° C. These coverslips were then vigorously washed in phosphate-buffered saline (PBS) to remove non-adherent cells; the resulting adherent cell cultures are referred to as macrophage cultures.

Concanavalin A

Con A was obtained from Pharmacia (London), fluoresceinated Con A (FITC-Con A) from Sigma (London), and Na^{125}I from Amersham International (Amersham).

FITC-Con A-induced capping

Two hundred microlitres of FITC-Con A (20 μg/ml Dul A) were pipetted onto macrophage cultures which were then incubated for 20 min at 37° C. Coverslip cultures were washed, fixed in ethanol and mounted on slides in PBS–glycerol (1:1 v/v). Coverslips were examined in a fluorescence microscope and the percentage of capped macrophages in 200 cells was assessed for each coverslip.

In experiments on pharmacological modification of capping, colchicine (0.1 mM) and cytochalasin B (0.1 mM) (Sigma, London) were prepared in PBS and 100 μl of each drug added to macrophage cultures; cultures were then incubated at 37° C for up to 1 h prior to treatment with FITC-Con A at 37° C for 20 min.

The specificity of the FITC-Con A binding was demonstrated by a reduction to barely detectable fluorescence by pre-incubation either with 20 μg/ml unlabelled Con A or in the presence of 50 mM methyl mannoside, the specific ligand for Con A.

^{125}Iodination of Con A

Con A was iodinated by the chloramine T method of Hunter (1973) and passed down a Sephadex G25 column to remove unbound ^{125}I. In the pooled ^{125}I-labelled Con A-containing fractions eluted from the G25 column, 90–95% of ^{125}I counts were trichloracetic acid-precipitable and the specific activity was, on average, 90 μCi/mg Con A.

^{125}I-labelled Concanavalin A binding assay

One hundred microlitres of ^{125}I-labelled Con A (200 μg/ml) were pipetted onto macrophage coverslip cultures, or coverslip only controls for non-specific binding, and left for 15 min at room temperature. Coverslips were then extensively washed in PBS and the coverslip associated counts determined in a gamma counter. The average background counts due to non-specific binding to coverslips ranged from 10–20% of the binding to macrophage coverslip cultures. A minimum of eight replicate coverslip cultures was used for each condition.

The specificity of the ^{125}I-labelled Con A binding to macrophages was demonstrated by a reduction to background binding in the presence of 50 mM methyl mannoside, the specific ligand for Con A. Any confusing effects due to endocytic uptake of ^{125}I-labelled Con A was ruled out by demonstrating no effect on binding following pre-treatment with the endocytosis inhibitors iodoacetate (1 mM) and sodium azide (2 mM).

For phagocytosis experiments, 100 μl 1% Latex spheres (Bacto latex 0.81 μm diameter; Difco) were added to macrophage cultures. Cultures were then incubated for 1 h at 37° C followed by vigorous and extensive washing to remove unphagocytosed Latex before assaying for ^{125}I-labelled Con A binding.

Trypsin accessibility of Con A binding sites

Following binding of ^{125}I-labelled Con A, coverslips were placed in 0.1 mg/ml trypsin (Difco) in PBS and allowed to incubate for 15 min at room temperature. Coverslips were then agitated in the trypsin solution and removed with forceps; trypsin-associated and coverslip-associated counts were then determined. The trypsin-released counts were expressed as a percentage of the total counts.

Statistics

The significance of differences was analysed using Student's *t*-test.

Results

FITC-Con A-induced capping

Saline-induced control macrophages had a small population of cells which formed caps spontaneously on incubation with FITC-Con A at 37° C (Table 1). Both *C. parvum*- and asbestos-activated macrophages had populations of spontaneously capping macrophages approximately eight times greater than the saline controls (Table 1). Both Latex- and Proteose peptone-induced macrophages had proportions of spontaneously capping macrophages similar to the saline-induced macrophages (Table 1).

Time course of capping

The time course of FITC-Con A-induced capping was similar for both *C. parvum*- and asbestos-activated macrophages (Fig. 1) reaching a plateau by 20 min and increasing little thereafter.

Table 1. Proportion of macrophages from various sources capping on incubation with FITC-Con A (20 μg/ml) at 37° C or room temperature (20° C). 200 cells counted.

Macrophage source	*Conditions of FITC-Con A treatment*	*Mean % (± S.D.) capped macrophages*	*Number of experiments*
3 day saline	20 min, 37° C	4.1 ± 2.3	8
3 day asbestos	20 min, 37° C	32.2 ± 4.2	5
5 day *C. parvum*	20 min, 37° C	32.4 ± 5.0	6
5 day *C. parvum*	20 min, room temp.	1.8 ± 0.8	3
3 day Proteose peptone	20 min, 37° C	1.2 ± 1.1	2
3 day Latex	20 min, 37° C	3.2 ± 0.9	1

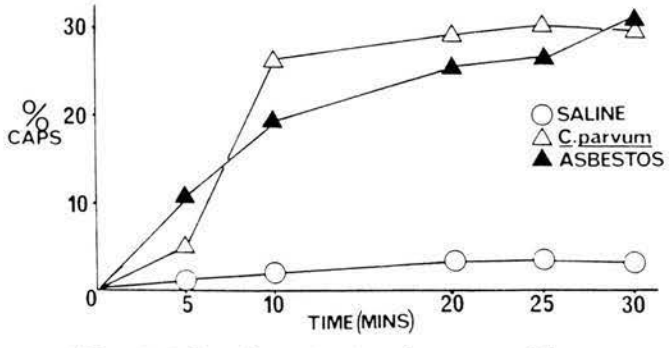


Fig. 1. Time course of Con A-induced capping in asbestos- and *C. parvum*-activated macrophages and in saline control macrophages incubated with FITC-Con A (20 μg/ml) at 37° C.

Pharmacological modification of capping

Colchicine and cytochalasin pre-treatment did not significantly affect the incidence of capping in control macrophages. Colchicine pre-treatment caused a slight insignificant increase in the proportion of capping in activated macrophages whereas cytochalasin pre-treatment reduced capping of activated macrophages to control levels (Table 2).

Table 2. Effect of pre-treatment with the microtubule-disrupting drug colchicine, and the microfilament-disrupting drug cytochalasin B, on FITC-Con A-induced capping in control and activated macrophages. 200 cells counted.

Macrophage source	*Pre-treatment before FITC-Con A* (20 min, 37° C)	*Mean % (± S.D.) capped macrophages*	*Number of experiments*
3 day saline	Saline 1 h, 37° C	4.2 ± 1.6	3
	0.1 mM colchicine (1 h, 37° C)	5.6 ± 4.8	4
	0.1 mM cytochalasin 'B' (1 h, 37° C)	3.0 ± 5.2	4
3 day asbestos	Saline 1 h, 37° C	32.7 ± 4.1	3
	0.1 mM colchicine (1 h, 37° C)	39.5 ± 10.5	3
	0.1 mM cytochalasin 'B' (1 h, 37° C)	6.0 ± 2.9	3

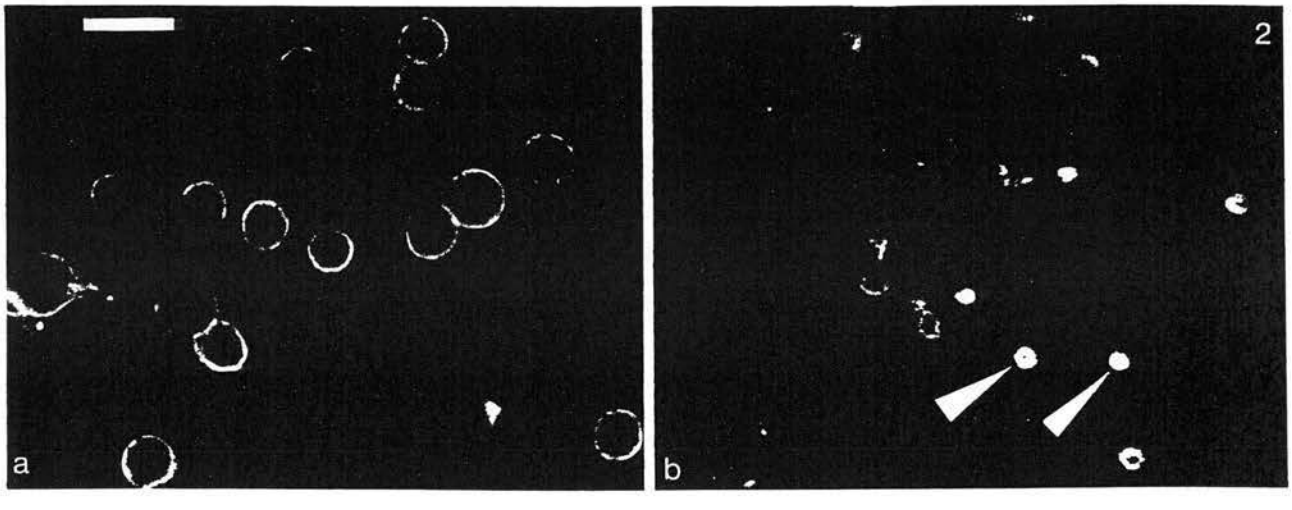


Fig. 2. (a) Three day saline-induced macrophages incubated with FITC-Con A (20 μg/ml) at 37° C for 1 h; no capping sub-population. (b) Three day asbestos-induced macrophages treated as above; capping cells arrowed. Scale bar: 10 μm.

Table 3. (a) Effect of Latex phagocytosis on ^{125}I-labelled Concanavalin A binding; non-specific binding to control coverslips = 3359 ± 909 counts per minute. (b) Binding of ^{125}I-labelled Con A to equal numbers of control and activated macrophages; non-specific binding to control-coverslips = 10 957 ± 367 counts per minute.

Macrophage source	[^{125}I] *Counts/coverslip* (mean ± S.D.)
(a)	
Control macrophages*	28 046 ± 3945
Control macrophages after Latex phagocytosis	16 227 ± 2515
(b)	
Control macrophages	38 960 ± 2616
3 day asbestos	36 361 ± 2084
5 day *C. parvum*	52 207 ± 4855

*Control macrophages = 3 day saline induced.

Appearance of caps

The caps produced in activated macrophages following incubation with FITC-Con A for 20 min at 37° C are shown in Fig. 2 along with saline-induced non-capping macrophages for comparison. Some evidence for pinocytic uptake of FITC-Con A was evident as pale speckled fluorescence, most noticeably in the activated populations.

Binding of ^{125}I-labelled Con A; effect of phagocytosis

Phagocytosis of Latex particles by control macrophages resulted in a significant (42.4%; $P < 0.001$) reduction in ^{125}I-labelled Con A binding compared to appropriate controls (Table 3).

Binding of ^{125}I-labelled Con A to activated macrophages

^{125}I-labelled Con A bound to asbestos-activated macrophages to the same extent as to control macrophages (Table 3); *C. parvum*-activated macrophages bound significantly more ^{125}I-labelled Con A (27.2%; $P < 0.001$) than control macrophages.

Table 4. Percentage of ^{125}I-labelled Con A bound to control and activated macrophages released by trypsin (0.1 mg/ml, 15 min, room temp.).

Macrophage source	*Mean % (± S.D.) trypsin-released counts*
3 day saline	31.9 ± 2.9
3 day asbestos	33.2 ± 1.3
5 day *C. parvum*	30.7 ± 2.4

Trypsin accessibility of ^{125}I-labelled Con A binding sites

The proportion of ^{125}I-labelled Con A binding sites which were accessible to trypsinization was the same for control and both types of activated macrophage (Table 4).

Discussion

Experiments to determine the proportion of macrophages which formed caps on incubation with FITC-Con A revealed that both *C. parvum*- and asbestos-activated macrophages had capping sub-populations of 32%. This figure contrasts with 4% capping macrophages in the control, saline-induced macrophages. Since Latex-induced macrophages had control levels of capping, it is clear that phagocytosis alone is not always a sufficient stimulus for inducing the capping sub-population. Since Proteose peptone-induced macrophages also had low levels of spontaneously Con A capping cells, not all macrophage-activating agents induce an increased capping sub-population.

Evidence that macrophage activation may play a role in Con A-induced capping exists in previously published work. Thus Pick & Wilner (1979) found that resting peritoneal macrophages had no capping cells while Williams *et al.* (1979), using peritoneal macrophages induced with sodium caseinate, a procedure likely to induce some activation, found approximately 10% spontaneously capping macrophages. Similarly, the finding of approximately 30% spontaneously Con A capping guinea-pig alveolar macrophages is in keeping with the suggestion that resting alveolar macrophages, as measured by several parameters, are in a consistently higher activational state than resting peritoneal macrophages (Hopper *et al.*, 1979).

Pharmacological modification of capping showed that, in our hands, colchicine had virtually no effect in promoting capping although previous studies have demonstrated that with colchicine treatment the level of spontaneously induced Con A caps in macrophages can be increased 2–30-fold (Williams *et al.*, 1977, 1979; Pick & Wilner, 1979). It is clear, however, that in all these studies, the majority of macrophages were not colchicine-sensitive, being either spontaneously capping or non-capping despite colchicine treatment. In our assay system with CBA mouse peritoneal macrophages, the mobility of Con A receptors is clearly not inhibited by colchicine-sensitive microtubules. The abolition of capping in activated macrophages following cytochalasin B pre-treatment confirms that microfilaments are necessary for Con A-induced capping in peritoneal macrophages (Williams *et al.*, 1979) and spleen cells, presumed to be lymphocytes (De Petris, 1974).

Nathan & Root (1977) have reported that macrophages activated by a range of agents *in vivo* responded to Con A and other membrane perturbing agents by releasing hydrogen peroxide, the important endogenous macrophage microbicidal agent. Oliver *et al.* (1977), from their study, suggested that an increase in hydrogen peroxide content, in polymorphonuclear cells at least, could have a direct role in tubulin oxidation and therefore inhibition of microtubule assembly, leading to an increase in capping. This explanation of capping in activated macrophages, reflecting an increase in oxidative

metabolism, is an attractive one despite our failure to find an obvious role for microtubules using colchicine and the reported difference in the cytoskeletal requirement for capping between macrophages and polymorphonuclear leucocytes (Williams *et al.*, 1979).

In order to ascertain whether the increased ability of activated macrophages to cap spontaneously on incubation with FITC-Con A was related to the total number of Con A receptors on the surface of control and activated macrophages, the binding of ^{125}I-labelled Con A to such macrophage populations was compared. In initial experiments, the finding of a significant reduction in ^{125}I-labelled Con A binding following Latex phagocytosis was in agreement with the original experiments of Lutton (1973); this suggested that our assay system, which was based on Lutton's, was comparable.

The total ^{125}I-labelled Con A binding to asbestos-activated macrophages was the same as for control macrophages. Previous work concerning the effect of environmental pollutants on lectin binding to alveolar macrophages has shown that ozone and nitrogen dioxide do not cause an increase in the binding of ^{3}H-labelled Con A to alveolar macrophages compared to control macrophages (Goldstein *et al.*, 1977). Hadley *et al.* (1977), however, found increased binding of the lectin wheatgerm agglutinin to ozone-treated macrophages.

C. parvum-activated macrophages bound approximately 30% more ^{125}I-labelled Con A than control and asbestos-activated macrophages. Since activation can occur via several different pathways or combinations of pathways (Rabinowitz & Hamburg, 1980) and since activation is not an all or none process but is rather a sequential adoption of properties obtained through differentiation signals, it is not surprising that there is considerable variation in activational phenotypes (Cohn, 1978; Hopper *et al.*, 1979). Due to the very different natures of asbestos and *C. parvum*, it is likely that they activate macrophages via different pathways. Asbestos- and *C. parvum*-activated macrophages possess different activational phenotypes, *C. parvum* macrophages being tumouricidal while asbestos-activated macrophages are not (Donaldson *et al.*, 1982). The increased binding of ^{125}I-labelled Con A to *C. parvum*-activated macrophages could, therefore, reflect a functionally related difference in the membrane of the tumouricidally activated macrophage. Increased binding could also be due to increased cell surface polysaccharide as reported for macrophages involved in cell-mediated immunity (Hammond & Dvorak, 1972).

The foregoing discussion, on the heterogeneity of expression of the activated macrophage phenotype, emphasizes the danger of regarding the Con A capping described above as a universal member of macrophage activation until it has been shown to occur with a range of activating agents *in vivo* and *in vitro*.

While trypsin accessibility of Con A binding sites is reported to be altered between fibroblasts and their transformed counterparts (Marques, 1978), the change from the unactivated to the activated state in peritoneal macrophages did not result in a change in the proportion of membrane-bound ^{125}I-labelled Con A which was trypsin releasable.

Thus, although the total number of Con A binding sites was increased in the *C. parvum*-induced macrophages compared to both saline- and asbestos-induced macrophages, there was no difference in the trypsin accessibility of these additional binding sites.

Conclusion

These experiments have demonstrated that interaction between the macrophage membrane Con A receptor glycoprotein and the cytoskeleton is altered in a sub-population comprising approximately one-third of activated macrophages, as shown by the ability of this sub-population to cap spontaneously on incubation with FITC-Con A. The capping was found to be microfilament-mediated. The ability to cap in the activated macrophage sub-population did not appear to be due simply to an alteration in the number of Con A receptor sites on activation, since two different activating agents were used to induce capping but only one caused a significant increase in Con A receptor sites. Trypsin treatment released similar amounts of bound Con A from the surfaces of control and activated macrophages suggesting that, by the criterion of trypsin accessibility, the position of the Con A receptor in the complex milieu of the macrophage surface was the same in both control and activated macrophages. These results support previous findings that the macrophage Con A receptor glycoprotein is a transmembrane molecule and can be linked to the cytoskeleton as shown by its ability to cap under various conditions. We suggest that macrophage activation may be a factor in the contradictory reports on the proportion of peritoneal macrophages which can cap spontaneously with Con A treatment; differences in activation could also explain the difference in spontaneous Con A capping reported between alveolar and peritoneal macrophages. Such an alteration in membrane glycoprotein–cytoskeletal association can be envisaged as a further manifestation of the well-documented membrane related changes which accompany macrophage activation and could also reflect their increased oxidative metabolism.

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Fluoresceinated α_2-Macroglobulin as a Probe for Studying Macrophages

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The potential value of fluorescein-conjugated human α_2-macroglobulin as a probe for studying macrophages in murine peritoneal exudate and spleen cell suspensions has been investigated using a fluorescence-activated cell sorter. These studies revealed that the number of α_2-macroglobulin-positive cells in the mixtures examined correlated closely with their macrophage content as determined morphologically. Furthermore cells separated on the FACS on the basis of their strong α_2-macroglobulin binding exhibited macrophage morphology and expressed Fc receptors on their surface. Conversely the α_2-macroglobulin-negative population contained few macrophages or Fc rosette-forming cells. Extensive blocking and comparative binding studies with a number of purified human α_2-macroglobulin preparations and derivatives thereof, and a variety of purified proteins, confirmed that the binding of fluorescein-conjugated α_2-macroglobulin to peritoneal exudate cells was specific. Furthermore the binding occurs in a highly reproducible manner. These observations suggest that fluorescein-conjugated α_2-macroglobulin in conjugation with flow cytometry is a sensitive and reliable method for both identifying and isolating subpopulations of macrophages.

Key words: *α_2-macroglobulin – macrophage probe*

Introduction

During the past few years it has been firmly established that macrophages function as major regulatory and effector cells in both immune and inflammatory responses (see Nelson, 1981). As a consequence procedures for identifying these cells in heterogeneous mixtures and for enriching or depleting cell suspensions of the same are of considerable importance.

Classically cells have been identified as macrophages on the basis of a variety of criteria including their morphology, cell surface receptor expression, intracellular enzyme content and their capacity to phagocytose inert or opsonized particles. Their enrichment has generally been achieved by exploiting their adherence properties although other techniques widely used include unit gravity and discontinuous gradient sedimentation and countercurrent centrifugal elutriation. The depletion of the macrophage content of cell suspensions has also been achieved by a variety of

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methods including adherence, the magnetic separation of carbonyl iron-treated cells and the use of specific and non-specific cytotoxic reagents (for review see Herscowitz et al., 1981)

While all the above techniques have contributed greatly to our understanding of the central role of macrophages in immune and inflammatory responses and are widely used in assessing immune status, there is a continuing need for additional procedures for identifying and separating macrophages. The recent observations on the clearance of α_2-macrogobulin/protease complexes by macrophages in vivo (Debanne et al., 1975, 1976) and the demonstration of α_2-macroglobulin receptors on macrophage cell surfaces (Kaplan and Nielsen, 1979a, b), together with the successful exploitation of fluorescein-tagged α_2-macroglobulin as a probe for similar receptors on fibroblasts (Maxfield et al., 1978) suggested to us an alternative approach to these problems. In this paper we set out to establish whether fluorescein-labelled human α_2-macroglobulin in conjunction with flow cytometry could be used as a sensitive, specific and reliable probe to identify and enrich macrophages in heterogeneous cell mixtures. From the outset this approach seemed particularly appealing in view of the possible role of α_2-macroglobulin in immune and inflammatory responses (Harpel and Rosenberg, 1976; Hubbard, 1978; James, 1980; Ganea et al., 1983) and the observation that monocytes synthesise small amounts of this protein (Hovi et al., 1977).

Materials and Methods

The preparation of α_2-macroglobulin and its derivatives

The α_2-macroglobulin preparations for use in these studies were obtained from fresh frozen normal human plasma by a 2-stage procedure. The initial step involved metal chelate affinity chromatography (Porath et al., 1975) of 20 ml of plasma on a 10×1 cm Zn affinity absorbent column (Boehringer Corporation (London), Lewes, East Sussex) which had been pre-equilibrated with 0.1 M phosphate buffer pH 6.5. After extensive washing with equilibrating buffer to remove unbound serum components, the α_2-macroglobulin fraction was recovered by elution with 0.1 M acetate buffer pH 4.5 containing 0.8 M NaCl. In order to remove any trace amounts of contaminating immunoglobulin the α_2-macroglobulin-rich eluate was concentrated by ultrafiltration, dialysed against phosphate-buffered saline (pH 7.2, 0.06 M containing 0.15 M NaCl) and subsequently applied to an anti-human immunoglobulin CnBr Sepharose column.

The purity of the final products was routinely determined by immunoelectrophoresis employing antisera to both whole human serum and α_2-macroglobulin or by PAGE-gel electrophoresis under non-denaturing conditions (Laemmli, 1970). The trypsin-binding capacity of selected preparations was also determined using chromatographically purified bovine pancreas trypsin and the chromogenic substrate benzyl arginyl paranitroanilide (Sigma Chemical Co. Poole, Dorset).

For the sake of brevity these preparations are hereafter referred to as normal α_2-macroglobulin or α_2-macroglobulin. This is to distinguish them from the α_2-mac-

roglobulin trypsin complex preparations used as probes in some of the binding studies and the purified slow and fast α_2-macroglobulin preparations (Barrett et al., 1979) used in some of the blocking studies (see later).

Fluorescein-conjugated derivatives of normal and trypsin-treated α_2-macroglobulin were prepared essentially as follows. After equilibration against 0.25 M carbonate-bicarbonate pH 9.0, 0.05 mg of fluorescein isothiocyanate per mg of protein was added and the mixture gently stirred overnight at 4°C. Free fluorescein isothiocyanate was removed by gel filtration on a Sephadex G-25 column equilibrated in phosphate-buffered saline.

In certain instances the fluorescein-tagged α_2-macroglobulin was subjected to further processing (see below). This involved either separation on Sephadex G-200, with the aim of isolating possible low molecular weight protein contaminants, or ultracentrifugation (90,600 $\times$ g for 30 min) to remove aggregates.

The preparation and staining of cells with fluorescein-conjugated α_2-macroglobulin preparations

For convenience sake most of the studies reported were performed with pooled *C. parvum*-elicited peritoneal exudate cell preparations from adult CBA mice; these were normally obtained as follows. Mice were injected (i.p.) with 0.4 mg of heat killed *C. parvum* (Wellcome Biotechnology, Beckenham, Kent) in 0.5 ml of sterile Dulbecco A. Five days later peritoneal exudate cells were harvested by washing out the peritoneal cavity with 3 $\times$ 2 ml of Dulbecco A containing 10 U/ml of heparin. Control cell preparations were obtained from the peritoneum of mice injected with Dulbecco A alone. On occasions in order to obtain neutrophil-rich exudates, the peritoneal cells were harvested 2 days following *C. parvum* injection. Spleen cell suspensions were obtained from untreated mice by conventional techniques. In certain instances adherent cell depleted and enriched peritoneal exudate and spleen cell suspensions were prepared as follows. Following washing and resuspension in RPMI 1640 containing 10% foetal calf serum 5 ml of cell suspension (10^7 cells/ml) was applied to a plastic petri dish. The dishes were incubated at 37°C for 1 h after which the non-adherent cells were washed off with RPMI 1640. The adherent cells were recovered by 5 min incubation at 37°C in 5 ml of 10 mM Lignocaine in RPMI 1640 followed by scraping with a rubber policeman.

All cell preparations were spin washed and suspended (5 $\times$ 10^6 cells/ml) in RPMI 1640 and kept on ice before use.

The flow cytometric investigation of cells stained with fluorescein-conjugated α_2-macroglobulin

For routine FACS analysis 200 μl of suspension containing 10^6 cells was added to replicate wells of a round bottom microtitre plate. The plate was spun gently to sediment the cells. Following decantation of the supernatant the cells were spun washed in cold medium. The cells were gently resuspended in 100 μl of a 1 mg/ml solution of fluorescein-conjugated α_2-macroglobulin and incubated on ice for 30 min. The cells were then spun washed (3 times) in 200 μl of cold RPMI in plastic tubes. These preparations were kept on ice until analysed in the FACS.

In order to establish the specificity of the cell binding of fluorescein-conjugated α_2-macroglobulin, blocking studies were performed with a variety of protein preparations. These included 'normal' α_2-macroglobulin, its slow and fast forms, α_1-protease inhibitor, anti-thrombin III, human IgG, heat-aggregated human IgG (56°C for 30 min), human IgM and IgA of myeloma origin, bovine serum albumin, trypsin and human plasma which had been depleted of α_2-macroglobulin on a zinc affinity column (see above). Blocking studies were performed by simultaneously incubating peritoneal exudate cell preparations with fluorescein conjugated α_2-macroglobulin and any one of the above proteins after which the cells were processed as above and examined in the FACS.

The binding of fluorescein-conjugated α_2-macroglobulin was routinely assessed in a FACS IV fluorescence-activated cell sorter (Becton Dickinson, FACS Systems, Sunnyvale, CA). The instrument was preset so that unstained preparations gave less than 10% fluorescence-positive cells and 10,000 cells were counted on each analysis. The results are presented as the relative cell number versus log fluorescence intensity.

In the sorting studies the fluorescence-positive and -negative cell populations were collected in cold RPMI 1640. Differential counts were performed on May-Grünwald Giemsa stained cytospin preparations while Fc receptor expression was determined as previously described (Donaldson et al., 1982). The percentage of cells forming rosettes was assessed for 200 counted cells. It should be stressed that unless otherwise stated serum-free buffers were used throughout these studies.

Where relevant the data was treated by regression analysis and differences in group means tested for significance using the Minitab computer program obtained from Minitab Project, 215 Pond Laboratory, University Park, PA 16801 (copyright 1982, Pennsylvania State University).

Results

Physicochemical and biochemical properties of α_2-macroglobulin preparations

Immunoelectrophoretic analysis indicated that α_2-macroglobulin preparations isolated as above were of extremely high purity. In addition polyacrylamide gel electrophoresis of the preparations under non-denaturing, non-reducing conditions indicated that they were predominantly of the slow (that is the native) electrophoretic form (Barrett et al., 1979). This was confirmed in a number of preparations by trypsin binding studies which revealed that 100 μg of α_2-macroglobulin could preserve the esterolytic activity of 8–10 μg trypsin from inhibition by soya bean trypsin inhibitor. Furthermore following interaction with trypsin and methylamine, the α_2-macroglobulin was converted into the characteristic fast form (Barrett et al., 1979). The conjugation procedure referred to above usually resulted in conjugates with a fluorescein/protein ratio of 8 : 1.

Identification of α_2-macroglobulin-binding cells in murine peritoneal exudate and spleen

All the fluorescein-α_2-macroglobulin preparations used to date have exhibited a

remarkably consistent binding pattern with *C. parvum*-elicited peritoneal exudate cells. For example the first 3 conjugates bound to 68.5, 60.4 and 62.0% of these cells.

Having established that fluorescein-labelled α_2-macroglobulin bound to cells in murine peritoneal exudate a series of experiments were performed to identify the cell population responsible for binding. In the initial series of experiments binding to peritoneal exudate cells obtained at various times following *C. parvum* injection, and

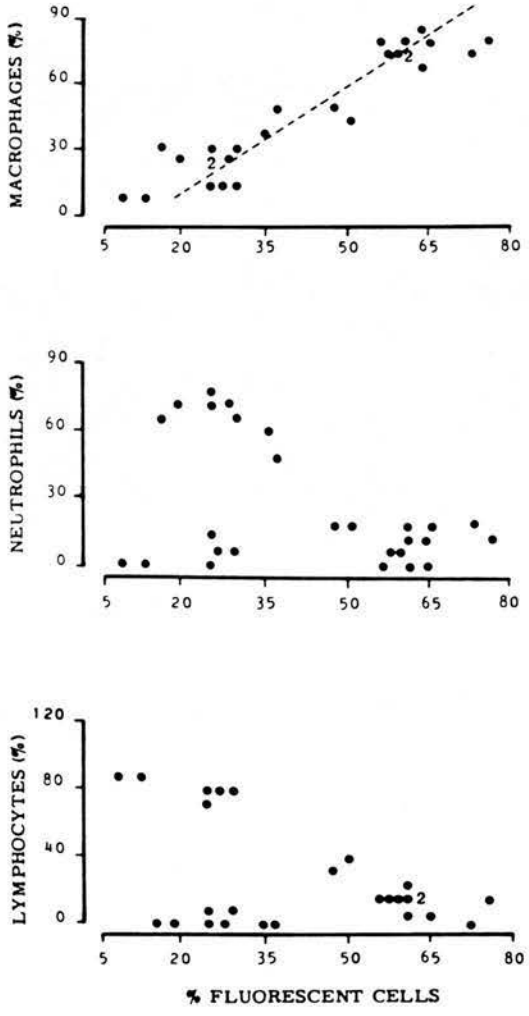


Fig. 1. The relationship between the α_2-macroglobulin-binding cell number and the macrophage, neutrophil and lymphocyte content of various murine cell preparations. In these studies the α_2-macroglobulin-binding cell content was assessed by FACS analysis while the identity of the cells was determined by morphological analysis of stained cytospin preparations. The results presented are the pooled data from 3 separate experiments. The numbers adjacent to certain data points indicate superimposed values.

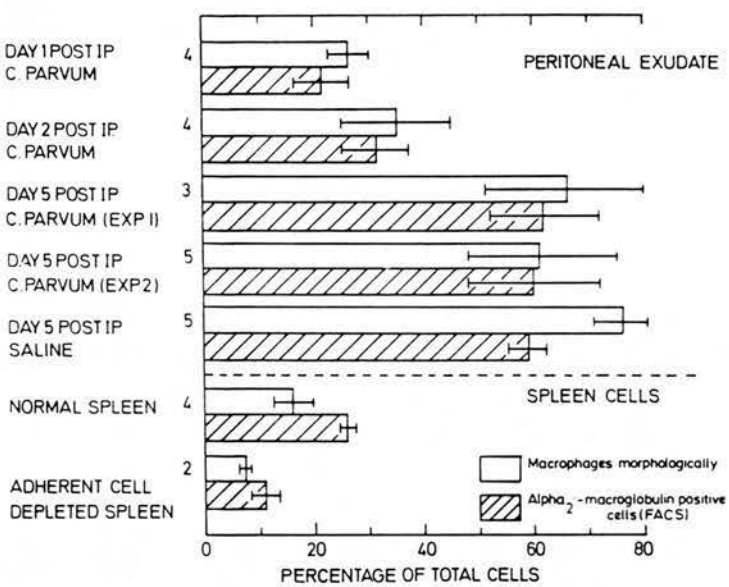


Fig. 2. A comparison of the macrophage and α_2-macroglobulin-positive cell content of various murine cell preparations. The values presented are mean values $\pm$ standard deviation of counts on 3–8 mice.

to normal mouse splenocytes was examined. The cellular composition of these preparations was also determined by differential counting of stained cytospin preparations. These studies (see Fig. 1) revealed that there was a close correlation between the macrophage content and α_2-macroglobulin-positive cell numbers of the cell suspensions examined ($R^2 = 89.1\%$; $P < 0.001$). The lymphocytes and neutrophils on the other hand were negatively correlated ($R^2 = 17.4\%$ and 16.2% respectively). When the complete data was separated into each treatment group and displayed as the mean $\pm$ standard deviation of the percentage of cells which are macrophages or α_2-macroglobulin-positive, the close correlation is still evident in the *C. parvum*-elicited peritoneal exudate cell preparations (Fig. 2). It should be pointed out however that such a close correlation was not observed with saline-elicited peritoneal exudate or normal spleen cells.

Additional experiments revealed that the α_2-macroglobulin binding cells resided in the adherent cell fraction (see Fig. 3). These observations, which were highly reproducible, also indicated that there was considerable heterogeneity in the adherent cell population with respect to α_2-macroglobulin binding. This heterogeneity was also confirmed by Fc rosette analysis and morphological examination.

As the data presented above indicated that fluorescein-conjugated α_2-macroglobulin bound to an adherent peritoneal exudate cells population, presumably macrophages, it seemed reasonable to determine whether cells separated with this probe, showed the phenotypic characteristics of such cells. Table I contains morphological and Fc receptor data on cells separated in the FACS on the basis of fluorescein-conjugated α_2-macroglobulin binding. It is readily apparent that cells

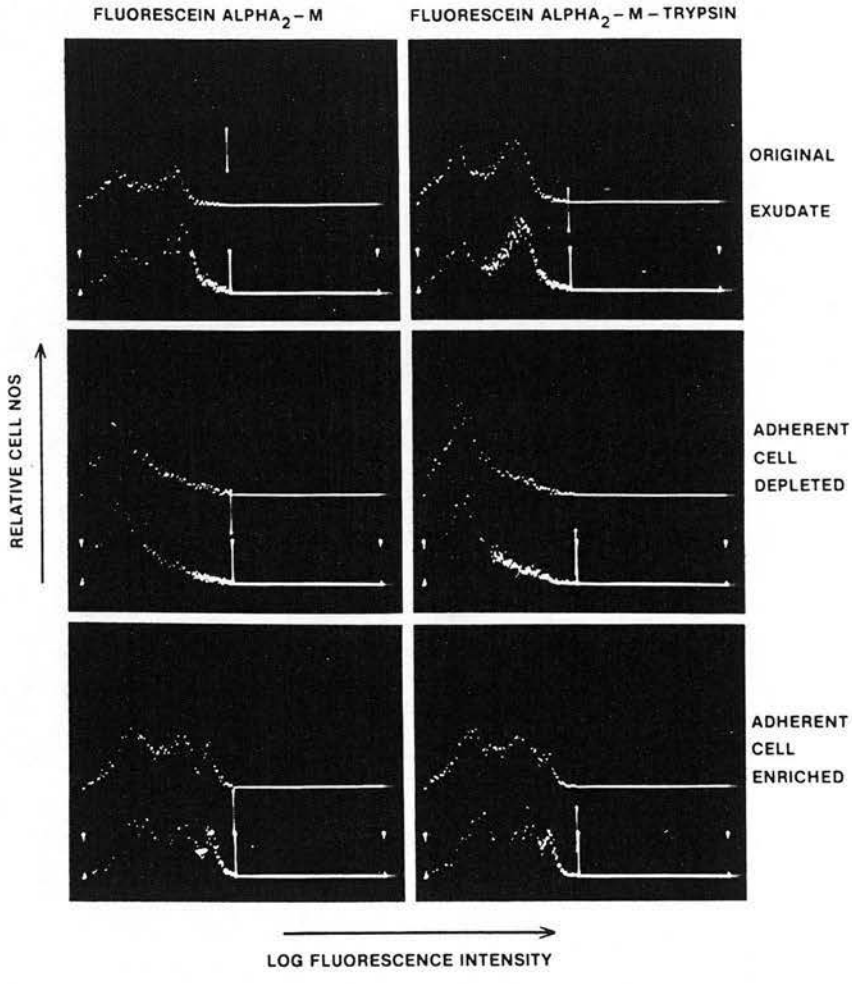


Fig. 3. A comparison of the binding abilities of fluorescein-conjugated α_2-macroglobulin and fluorescein α_2-macroglobulin trypsin complex to various murine peritoneal exudate cell preparations. In this figure the results of duplicate analysis are presented.

binding this probe exhibit macrophage morphology and express Fc receptors on their surface. Conversely the α_2-macroglobulin-negative populations contain few macrophages or Fc rosette-forming cells.

The specificity of binding of fluorescein-conjugated α_2-macroglobulin to murine peritoneal exudate cells

The studies performed to establish the specificity of the interaction of fluorescein conjugated α_2-macroglobulin with cells were essentially of 2 kinds. The first series were designed to determine if the binding was dependent upon any modification which may have occurred in the α_2-macroglobulin during isolation or labelling. Such

TABLE I

THE CHARACTERISTICS OF MURINE PERITONEAL EXUDATE CELLS SEPARATED IN THE FACS ON THE BASIS OF THE BINDING OF FLUORESCEIN-CONJUGATED HUMAN α_2-MACROGLOBULIN

Note the high macrophage and Fc receptor-positive cell content of the α_2-macroglobulin-binding fraction.

Experiment no.	Separated population	% Macrophages by morphology	% Fc rosette-forming cells
1	α_2-Macroglobulin-positive	97.0 ± 1.7 [a]	76.6 ± 2.3
	α_2-Macroglobulin-negative	8.3 ± 1.6	1.0 ± 1.0
2	α_2-Macroglobulin-positive	95.7 ± 0.9	81.7 ± 5.5
	α_2-Macroglobulin-negative	7.5 ± 0.7	6.0 ± 3.0

[a] Mean ± standard deviation of 3 separate analyses.

modifications might include protease-mediated conversion from the slow to fast form of the molecule. (Barrett et al., 1979), the release of low molecular weight peptides which accompanies this conversion (Pan et al., 1980: Hubbard et al., 1983) or the formation of aggregates, all of which might bind more avidly than the original α_2-macroglobulin. The other experiments were undertaken to exclude the possibility that the binding was due to trace amounts of contaminating proteins which might bind to other receptors known to be present on macrophage membranes.

Comparative studies revealed that at the concentrations tested the standard fluorescein α_2-macroglobulin preparation (which is predominantly electrophoretically slow) bound as effectively to murine peritoneal exudate cells as did the electrophoretically fast fluorescein-conjugated α_2-macroglobulin trypsin complex (see Fig. 3). Furthermore the effect was highly reproducible. Indirect evidence that the slow form of α_2-macroglobulin was capable of binding was obtained in blocking studies (see below). It is also interesting to note that the trypsinized form of the molecule exhibits a similar pattern of binding to adherent and non-adherent subpopulations as does the normal α_2-macroglobulin.

Studies with Sephadex G-200 fractions of fluorescein-conjugated α_2-macroglobulin indicated that both high and low molecular weight constituents bound to peritoneal exudate cells. However on a mole to mole basis the binding of the high molecular weight fraction, which contains both free and enzyme-complexed α_2-macroglobulin, was superior to that of the low molecular weight fraction (Fig. 4). The latter would contain the immunoreactive peptides reputed to be cleaved from α_2-macroglobulin following protease binding (see earlier).

Overall, the blocking studies with various purified proteins confirmed the specificity of the interaction and also revealed additional information. Thus unlabelled α_2-macroglobulin and its slow and fast derivatives readily blocked the binding of fluorescein-conjugated α_2-macroglobulin to peritoneal exudate cells while similar amounts of trypsin, bovine serum albumin, anti-thrombin III, IgG and IgM were without significant effect. The results of one of these blocking experiments are portrayed in Fig. 5. α_2-Macroglobulin depleted serum also had little, if any, effect on

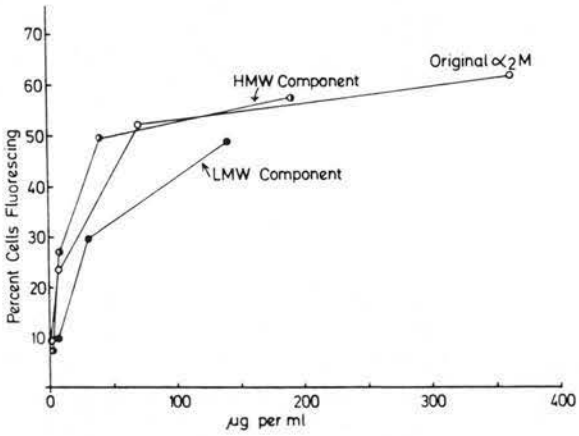


Fig. 4. A comparison of the binding to murine peritoneal exudate cells of fluorescein-conjugated α_2-macroglobulin and sephadex G-200 fractions therefrom.

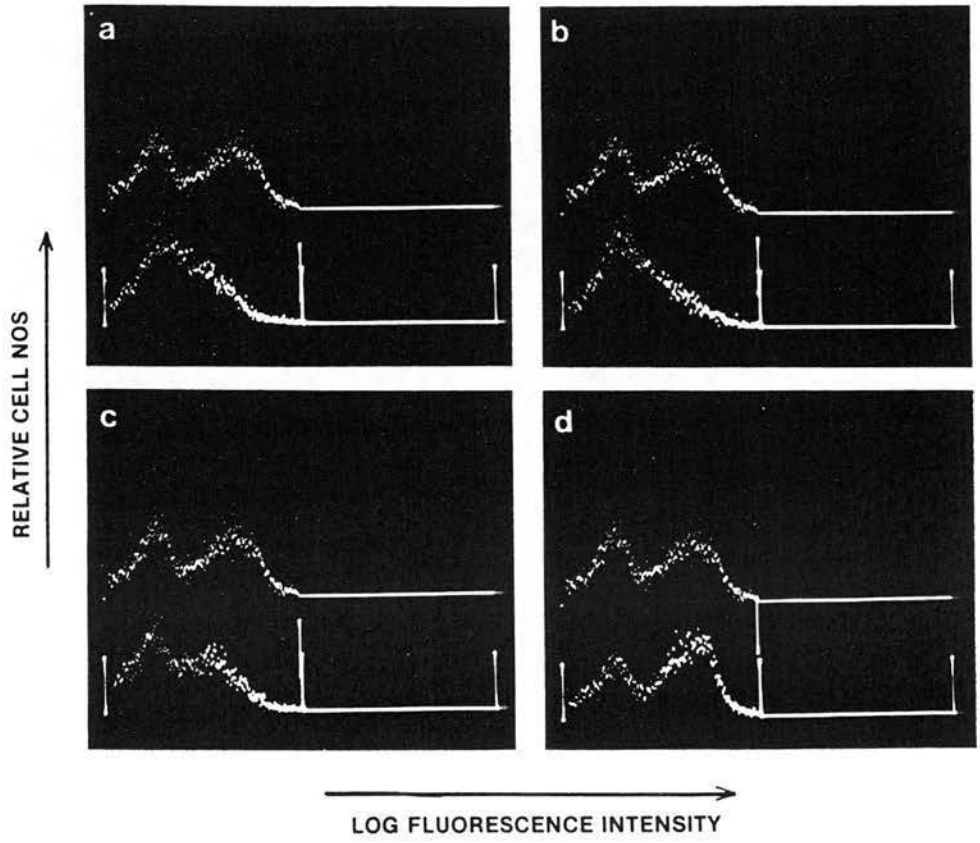


Fig. 5. The effect of various proteins on the binding of fluorescein-conjugated α_2-macroglobulin by murine peritoneal exudate cells. In these studies *C. parvum*-elicited peritoneal exudate cells were simultaneously incubated with fluorescein-conjugated α_2-macroglobulin (100 µg) and 100 µg of the following unlabelled proteins: (a) α_2-macroglobulin, (b) fast form of α_2-macroglobulin, (c) α_2-protease inhibitor, and (d) anti-thrombin III. The binding pattern observed with fluorescein-conjugated α_2-macroglobulin is displayed on the top of each photograph.

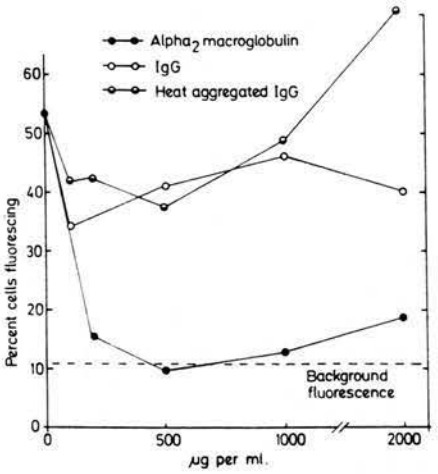


Fig. 6. The effect of heat-aggregated IgG on the binding of fluorescein-conjugated α_2-macroglobulin by murine peritoneal exudate cells. In these studies *C. parvum*-elicited peritoneal exudate cells were simultaneously incubated with fluorescein-conjugated α_2-macroglobulin (100 μg) and a range of concentrations of unlabelled α_2-macroglobulin, normal human IgG or heat-aggregated IgG.

the binding of the isolated protein. Of particular interest, however, were the observations that aggregated IgG treatment could enhance the binding of the α_2-macroglobulin probe while α_1-protease inhibitor had some blocking effect (see Figs. 5 and 6). It should also be noted that the ultracentrifugation of fluorescein α_2-macroglobulin immediately before use did not significantly afffect its binding. Finally it should be stressed that when blocking occurred in these studies there was a reduction in both the total number of fluorescent cells and in the intensity of fluorescence as determined by channel analysis.

Discussion

The presence of receptors for α_2-macroglobulin on both macrophages and fibroblasts has been known for some time (see for example Kaplan and Nielsen, 1979a, b: Van Leuven et al., 1979; Dickson et al., 1981a, b; Frey and Afting, 1983; Marynen et al., 1984). Such receptors have usually been demonstrated by the uptake of ^{125}I- or fluorescein-labelled α_2-macroglobulin by cell cultures in vitro followed by gamma scintillometry or fluorescence microscopy. We have experienced difficulties with both these approaches. In the first place in our hands products labelled with ^{125}I by the chloramine-T techniques exhibited low binding, a problem also noted by others with Ficoll-separated rat spleen cells (Miyanga et al., 1982). Furthermore while the fluoresceinated derivates bound to cells, the intensity of fluorescence was always low, rendering accurate identification and counting of α_2-macroglobulin-binding cells extremely difficult. It was in the light of the above problems that we adopted the approach described in the present paper. From our results it is clear that

fluorescein-conjugated α_2-macroglobulin, in conjunction with flow cytometry, provides a sensitive and highly reproducible means of quantifying and isolating the α_2-macroglobulin receptor-positive cell component of heterogenous cell mixtures, and that the bulk of such cells are macrophages. While most of the results presented were obtained using 100 μg of conjugated α_2-macroglobulin per test it should be stressed that similar results can be obtained with as little as 5–10 μg of this material per test (see Fig. 4).

We believe that the use of α_2-macroglobulin as a macrophage probe could be of considerable practical and theoretical importance. In the first place it provides a method for enumerating and sorting macrophages which is based on the expression of a cell surface receptor of considerable theoretical interest which is already widely used to study membrane receptor-ligand interactions and turnover on macrophages (Kaplan and Nielsen, 1979b; Kaplan, 1980) and fibroblasts (see for example Van Leuven et al., 1979; Dickson et al., 1981a, b; Via et al., 1982; Van Leuven, 1984). In this connection it is important to note that a number of biologically important macromolecules (in addition to endoproteases) appear to be simultaneously endocytosed with α_2-macroglobulin. These include insulin and epidermal growth factor (Maxfield et al., 1978) and presumably platelet-derived growth factor, some of which is known to bind to α_2-macroglobulin (Huang et al., 1984).

Of additional interest is the apparent heterogeneity of α_2-macroglobulin receptor expression which is particularly obvious within the adherent cell-enriched preparations. Thus within this population there are some cells which strongly bind α_2-macroglobulin while others have little if any affinity for this probe. Such heterogeneity is not surprising considering the known variability in size, density, cell surface and secretory phenotype, and functional activity of macrophage-enriched populations-including adherent peritoneal exudate cell preparations (Walker, 1974; Hopper et al., 1979; Lee, 1980; Werb and Chin, 1981). The source of this heterogeneity remains to be determined although it should be noted that the binding of α_2-macroglobulin trypsin complexes by murine peritoneal exudate cells is not influenced by their method of elicitation (Imber et al., 1982). Finally in the light of the experimental evidence already available (Kaplan and Nielsen, 1979a) and our own preliminary binding studies, it is apparent that the application of α_2-macroglobulin probes will probably not be species restriced in mammals.

The current studies also revealed a number of other points worthy of comment. Of particular interest were the observations that aggregated IgG treatment of peritoneal exudate cells at 0°C appeared to augment the binding of fluorescein-conjugated α_2-macroglobulin suggesting that the cross-linking of Fc receptors results in an increased exposure of α_2-macroglobulin receptors. While the bulk of these exposed receptors are probably on macrophages, the possibility that other Fc receptor bearing cells contribute, e.g., B cells, cannot be overlooked. Also worthy of note is the observation that under the conditions used the fluoresceinated α_2-macroglobulin appeared to bind as effectively to peritoneal exudate cells as its trypsinized derivative. These observations are of interest in light of the preferential binding of α_2-macroglobulin trypsin complexes to high affinity receptors on fibroblasts (Dickson et al., 1981b) and macrophages (Kaplan, 1980) and their more rapid clearance in

vivo (Debanne et al., 1975). Possible explanations include the presence in the 'normal' α_2-macroglobulin preparation of sufficient complexed material to saturate all available sites (Van Leuven et al., 1979) or the preferential binding in our system to the more abundant low affinity receptors known to be present on fibroblasts (Dickson et al., 1981b) and presumably on macrophages.

It is clear that further investigations will be necessary before the true value of fluorochrome-labelled α_2-macroglobulin as a macrophage probe will be established. Nevertheless it is believed that flow cytometry studies with labelled α_2-macroglobulin and other important ligands, such as transferrin, epidermal growth factor and platelet-derived growth factor, will greatly improve our understanding of basic cell biology in general and the immune response in particular.

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Comparisons of the Biological Effects of Mineral Fibre Samples Using in Vitro and in Vivo Assay Systems

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Introduction

At the Institute of Occupational Medicine in Edinburgh, we have studied the in vitro effects of a series of mineral fibre samples using three different permanent cell lines. The results of this work have been summarised in the companion paper by Gormley et al.. Relatively consistent grading of the dusts was obtained by the different test systems with preparations of chrysotile asbestos consistently being found to be more active than varieties of amphibole or other mineral fibres. In addition there was an overall relationship between toxicity and fibre length. Most of the dust preparations used in these in vitro studies have also been tested in rats using either long term dust inhalation or intraperitoneal injection (Davis et al. 1978, 1980, 1984; Bolton et al. 1982). This paper reports how the results of the animal studies compare with the in vitro work.

Material and Method

Of the 19 fibrous dusts tested for their in vitro pathogenicity by Gormley et al., 15 have been used in intraperitoneal injection studies with rats and 12 for long term inhalation studies. By definition, all inhalation studies were conducted with 'elutriated' dusts and 13 of the 15 samples used for injection had also been collected from the inhalation chambers and correspond to the elutriated (E) dusts examined in vitro. The two exceptions were heat cleaned wet dispersed chrysotile and unextracted wet dispersed chrysotile which were not available in elutriated form. As reported by Gormley et al. size distribution data obtained using scanning electron microscopy was produced for many of the dust preparations used for the in vitro studies and these figures were used for comparisons with disease production in both the inhalation and injection studies.

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samples since most of them produced mesotheliomas in almost 100% of animals. However, the mean tumour induction period did vary considerably between the dusts and provided a useful system for grading the results of these studies (Table 2).

Comparisons of Tables 1 and 2 show that the dust samples were relatively consistent in their ability to produce three different types of pathological change. Many dusts occupy similar positions in both tables and in other cases, where table positions are different, the data are so close together that the change has no pathological significance. In general, chrysotile samples, particularly those produced by the wet dispersion process (WDC) proved to be the most harmful dusts with only tremolite of the amphibole samples showing similar activity. Samples of crocidolite and amosite were only moderately fibrogenic and carcinogenic, with a specially prepared long fibre amosite sample being the most harmful of these. A sample of amosite prepared so that it contained extremely few fibres > 5 μm in length, produced very little pathology.

The in vivo findings agree in general with the results from in vitro studies since both systems identify chrysotile and particularly wet dispersed chrysotile, as being more harmful than most amphibole preparations and both agree that the short fibre amosite preparations were almost completely without harmful effects. Three exceptions to the general trend are of interest. Both tremolite and ceramic fibre appear more harmful in the inhalation studies than the in vitro results suggested. However, for these two dusts, the findings from injection studies agree much more closely with in vitro results. In addition the unextracted wet dispersed chrysotile proved more harmful in the in vivo injection tests than in vitro although this was probably due to the fact that it could not be as well dispersed for the in vitro studies as the other dusts.

The calculation of Spearman's rank correlation co-efficients (Table 3) showed that the similarities between in vivo and in vitro gradings was significant in most cases. Similar comparisons confirmed the relationship between in vivo biological activity and fibre length (Table 4). The production of pulmonary disease was most closely correlated with the presence of relatively long fibres in the dust samples and these correlations were statistically significant for lengths > 10 μm. The speed of mesothelioma production following intraperitoneal injection, however, was less closely associated with fibre length. As with the in vitro studies there appeared to be little relationship between in vivo pathogenicity and fibre diameter.

The inhalation chambers and dust generators used were described by Beckett et al. (1975). All the inhalation studies used groups of 48 rats of the AF/Han strain. These were mainly treated with dust clouds with a respirable mass concentration of 10 mg/m^3 of air for a period of 1 year although four groups were dosed at a level of 4 mg/m^3. These were the three samples of wet dispersed chrysotile and milled chrysotile. After the end of dusting, animals were allowed to live out their full life span. During the histological examination of lung tissue, the number of pulmonary tumours produced by each dust was recorded, as well as the percentages of lung tissue affected by interstitial fibrosis. These were obtained using an electronic image analyser (Graphic Information Systems Ltd., GDS1), by methods reported by Davis et al. (1984).

For the injection studies most dusts were tested using groups of 32 AF/Han rats although for a few dust samples the group size was 24. Each animal received a dose of 25 mg of dust administered as a single intraperitoneal injection suspended in 2 ml of phosphate buffered saline.

Information from the in vitro studies (described in detail by Gormley et al.) has been used to rank the reactivity of dust samples from the three cell lines in ascending order. In each case the dust with the highest reactivity is ranked number 1.

Statistical comparisons between the in vivo and in vitro results, as well as comparisons between the animal data and the fibre length and diameter distributions, were undertaken using the Spearman Rank Correlation test. Fibre size information was expressed as the number greater than a stated size per unit mass of dust.

Results

For comparisons to compare the results of in vitro studies undertaken with the same dust samples, the results from animal experiments have been graded in three ways. The results from inhalation studies have been graded by the number of malignant pulmonary tumours produced in each experiment although data on the total number of pulmonary tumours produced as well as levels of pulmonary interstitial fibrosis are also presented for comparison (Table 1).

It was found that the 25 mg dose used in the injection studies was too high for detailed comparisons of tumour numbers to be made between the different dust

Discussion

This comparison of results of in vitro and in vivo studies has shown quite good agreement and suggests that the specific in vitro tests that were used may provide an indication of the potential pathogenicity of fibrous mineral dusts towards laboratory rats.

With fibrous dusts it is important to discriminate between fibrogenic and carcinogenic potential and some cell lines respond in both ways. Chamberlain and Brown (1978) suggested that non fibrous dusts which can be fibrogenic but not carcinogenic show in vitro toxicity to phagocytic cells only. Non phagocytic cell lines such as A549 and V79/4 appeared to react only with carcinogenic fibrous dusts. Our in vitro studies confirm these suggestions to the extent that the positive control dust, quartz (DQ_{12}) was toxic to the phagocytic cell line, $P388D_1$, but relatively inactive towards the A549 and V79/4 cell lines (Wright et al. 1984; Brown et al. 1984). However, in our in vivo studies only fibrous dusts were used and the most reactive ones were both fibrogenic and carcinogenic to lung tissue. For this reason, although we were able to confirm that the in vitro tests provided a good indication of pathogenicity, it was not possible to examine the suggested discrimination between fibrogenicity and carcinogenicity. In the in vivo studies reported here and in the in vitro studies of Gormley et al. it has been found that the activity of the dust samples tested shows some correlation with the proportion of long fibres present. This agrees with the prediction of Stanton et al. (1972, 1977) who used information from studies involving intrapleural implantation. However, in the present study the relationship between disease and fibre length appeared to be more marked within the lung tissue following inhalation than in the peritoneal cavity following injection. Stanton also suggested that a low fibre diameter as well as length was important in carcinogenicity and this does not show so clearly from the present study. However, Stanton calculated that the most dangerous fibres had diameters below 1.5 µm and the majority of fibres in our dust samples fell into this category.

The results of both the in vivo and in vitro assays compared in the present study highlighted the major problem in the interpretation of experiments conducted with asbestos dusts. Virtually all assays of biological activity show that chrysotile samples are at least as harmful as the amphiboles, and in many cases the chrysotiles have been the most dangerous materials tested. This is in contrast to human epidemiology, which clearly indicates that for mesothelioma production at least, the amphiboles and particularly crocidolite have been

the most harmful asbestos types in industrial use. Part of the explanation of these findings might be that the amphiboles are naturally much more dusty materials than chrysotile and past industrial exposures may have been correspondingly higher. An additional factor may also be the relative durability of the different asbestos types in lung tissue. This suggestion is supported by findings from human lung tissue at autopsy where amphibole dust often predominates even when chrysotile is known to have made up the majority of dust exposure (Gylseth 1983). It is likely, therefore, that while chrysotile is the most biologically active type of asbestos, much of it is removed from human lung tissues before it is able to produce disease.

Acknowledgements. These studies were undertaken as part of the research programme funded by the Asbestosis Research Council.

Table 1. Comparison of in vivo findings with in vitro ranking for 12 samples of fibrous dust. Inhalation studies

Type of dust	Malignant pulmonary tumours	Total pulmonary tumours	Interstitial fibrosis*	$P388D_1$**	V79/4	A549
Tremolite	18	20	14.5	7	6	7
Wet dispersed chrysotile (WDC)	14	21	9.6	3	10	1
Milled chrysotile	14	16	8.8	4	4	4
Milled WDC	13	18	12.8	1	1	2
Factory WDC	10	21	12.1	2	2	3
Long amosite	10	13	10.0	8	5	10
UICC chrysotile A	8	15	9.1	6	3	5
Ceramic aluminium silicate	3	4	5.0	12	12	8
Brucite	2	5	2.9	5	7	6
Factory amosite	0	2	8.5	9	9	9
UICC crocidolite	0	1	1.4	8	8	11
Short fibre amosite	0	0	0.1	11	11	12

Table 2. Comparison of in vivo findings with in vitro ranking for 15 samples of fibrous dust[a]. Injection studies

Type of dust	Percentage of animals with mesothelioma	Mean mesothelioma induction period in days	In vitro grading[b]		
			$P388D_1$	V79/4	A549
Wet dispersed chrysotile	100	312	4	12	1
Milled WDC	92	327	1	1	2
Factory WDC	100	338	3	2	3
Tremolite	93	365	8	6	9
Unextracted WDC	97	397	10	14	12
UICC chrysotile A	97	400	7	3	6
Milled chrysotile	95	400	5	4	5
Heat cleaned WDC	100	407	2	10	4
UICC crocidolite	90	416	13	9	14
Brucite	96	418	6	8	8
Long fibre amosite	95	520	11	5	13
Factory amosite	94	566	12	11	11
Anthophyllite	84	613	9	7	7
Short fibre amosite	4	837	14	13	15
Ceramic aluminium silicate	9	913	15	15	10

[a] Data expressed as a percentage of lung tissue area.

[b] In this ranking the figure 1 indicates the highest level of activity.

Table 3. Spearman's correlation co-efficients for the comparisons of the in vivo and in vitro pathogenicity of mineral fibre samples

	$P388D_1$	V79/4	A549	Combination
Mean mesothelioma induction period	75[a]	47[a]	64[a]	52[a]
Pulmonary malignant tumours	61[a]	49	68[a]	65[a]
Pulmonary interstitial fibrosis	56[a]	63[a]	62[a]	62[a]

[a] Significant at the 5% level.

Table 4. Spearman's correlation co-efficients for the comparison of fibre lengths and in vivo pathogenicity

	>0	>1	>2	>3	>4	>5	>6	>7	>8	>9	>10	>15
Mean mesothelioma induction period	27	43	58	72[a]	78[a]	53	53	47	61[a]	46	46	34
Malignant pulmonary tumours	-46	-29	-15	27	44	32	19	27	54	38	81[a]	78[a]
Pulmonary interstitial fibrosis	14	14	14	71	77	60	54	60	67	60	94[a]	94[a]

[a] Significant at the 5% level.

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The effect of products from bronchoalveolar-derived neutrophils on oxidant production and phagocytic activity of alveolar macrophages

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SUMMARY

Neutrophils and alveolar macrophages are found together in the alveolar region during pulmonary inflammation where neutrophil products could influence important macrophage defensive functions. We set out therefore to investigate the ability of neutrophil products to modulate alveolar macrophage phagocytosis and oxidant production. Neutrophils derived from acutely inflamed rat lung were incubated along with a range of potential triggers of neutrophil secretion and supernatants collected. Using two quantitative assays of rat alveolar macrophage phagocytosis, the supernatants were found to have no effect except for the quartz supernatant, which slightly enhanced phagocytosis via non-specific receptors and the PMA supernatant, which caused reduction in phagocytosis via non-specific and Fc receptors; this reduction could however be mimicked by PMA alone. None of the supernatants affected macrophage production of superoxide anion or hydrogen peroxide except for the PMA supernatant and once again the inhibitory effect of the PMA supernatant could be mimicked with PMA alone. It is concluded that products of inflammatory neutrophils do not affect phagocytosis or oxidative metabolism of alveolar macrophages, although in quartz-exposed lung neutrophils may exert a small enhancing effect on macrophage phagocytosis.

Keywords neutrophil macrophage oxidant phagocytosis

INTRODUCTION

During alveolar defence the alveolar macrophage performs a range of functions protective to the alveolar surface, with phagocytosis and killing of microbes by oxygen dependent processes as central mechanisms in the fulfilment of this role (Hunninghake *et al.*, 1979). During alveolar inflammation neutrophils are recruited to the alveolar region and both neutrophils and alveolar macrophages are found together in close proximity in the alveolar spaces as revealed by bronchoalveolar lavage profiles (Gee & Fick, 1980; Crystal *et al.*, 1981).

Neutrophils produce a range of enzymes and cytokines which have been shown to affect other cell types and this modulation could take the form of enhancement of cellular functions and responses, including those of macrophages, which would aid host defence (Nakamura, Goto & Yoshinaga, 1982; El Hag & Clark 1984; Bird, Sheng & Giroud, 1984). However it is possible that, as in other inflammatory/immunologic situations (Fantone & Ward 1984; Martin 1984), neutrophils may exert a deleterious influence on macrophage function. To investigate this, and as part of a programme of work on responses of the lung to inhaled, inflammation-generating dusts, we set out to assess *in vitro* the effect of alveolar-derived neutrophils on two key macrophage functions—phagocytosis and oxidant production.

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MATERIALS AND METHODS

Animals

SPF/syngeneic PVG rats, obtained from the Institute of Occupational Medicine Animal Unit, and more than twelve weeks of age, were used throughout. To obtain a neutrophil-rich bronchoalveolar lavage population, rats were injected intratracheally with 1·4 mg of heat-killed *Corynebacterium parvum* (Wellcome, Beckenham) 16 h prior to lavage.

Lavage

The rats were killed by Nembutal (Ceva Limited, Watford) overdose, the lungs were removed and lavaged with 4×10 ml volumes of saline at 37°C, with massaging to increase the yield.

Preparation of supernatants

The neutrophil-rich (70–90%) 16 h *C. parvum*-elicited bronchoalveolar cell population was placed in 75 cm^2 flasks (Sterilin, Feltham) at 10^6 cells/ml (15×10^6 cells total) in MEM plus 10%

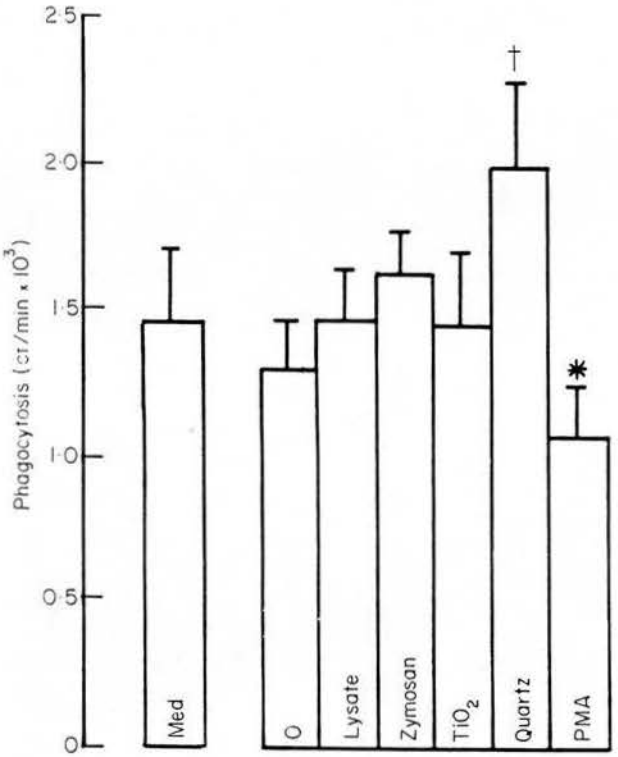


Fig. 1. The effect of various supernatants prepared from bronchoalveolar neutrophils, on phagocytosis of zymosan by control alveolar macrophages. Medium alone (med); unstimulated supernatant (0); soluble fraction of freeze-thawed neutrophils (lysate); the remaining bars are supernatants stimulated with the substances indicated (TiO_2 = titanium dioxide; PMA = phorbol myristate acetate). Data derived as mean ± s.e.m. of four replicates in three separate experiments. * $P < 0{\cdot}05$; † $P < 0{\cdot}001$ (significant differences from medium alone).

heat-inactivated fetal calf serum (Gibco, Paisley) or F10 medium (Gibco, Paisley) plus 2% bovine serum albumin (BSA). The following stimulating agents were added to separate flasks: 0, titanium dioxide—rutile form (500 μg/ml; Tioxide Limited, Stockton-on-Tees, UK), zymosan (500 μg/ml; Sigma, Poole, UK), DQ_{12} standard quartz (500 μg/ml), phorbol myristate acetate (PMA) (1 μg/ml; Sigma, Poole, UK). The cells were incubated for 24 h and the supernatants collected, centrifuged to clarity and stored at −70°C. In addition, a lysate was prepared from an equal aliquot of cells by disrupting the cells with three cycles of freeze/thawing (−70°C to 37°C) followed by centrifugation and then storing at −70°C. In some experiments PMA was incubated overnight in complete medium in the absence of neutrophils to assess the effect of PMA alone.

Assays of macrophage phagocytosis

Two different assays of phagocytosis were utilized in this study specifically to allow examination of the role of non-specific and receptor-mediated mechanisms in phagocytosis. The ^{125}-I zymosan assay reflects the ability of macrophages to phagocytose via non-specific receptors while the ^{51}Cr SRBC assay relies on Fc receptor-mediated phagocytosis.

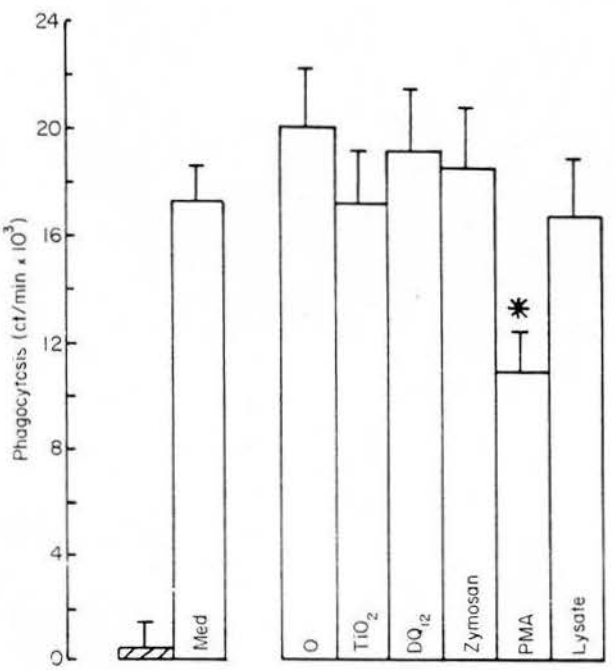


Fig. 2. The effect of various neutrophil supernatants on phagocytosis of ^{51}Cr-labelled sheep red blood cells. The hatched bar represents phagocytosis of SRBC coated with irrelevant antibody. The remaining bars represent phagocytosis of ^{51}Cr-labelled sheep red blood cells coated with specific anti-sheep red blood cell antibody. Abbreviations as for Fig. 1. Results represent mean ± s.e.m. of five replicates in two separate experiments. * $P < 0{\cdot}01$ (significant difference from medium alone).

^{125}I-zymosan phagocytosis assay

This assay was carried out essentially according to the method of Ezekowitz *et al.* (1985). Zymosan was iodinated using the chloramine T method. Briefly, 100 mg of zymosan (Sigma, Poole) in 2 ml of PBS were iodinated with 60 μl of chloramine T solution (1 mg/ml) and 18·5 MBq of ^{125}I. The reaction was stopped after 20 min by adding 10 μl of sodium metabisulphite (8 mg/ml). The ^{125}I-zymosan was spin/washed five times, brought to a volume of 5 ml in PBS and dialysed overnight against three changes of PBS containig 10 mM NaI. The zymosan was washed once more with PBS and resuspended at 40 mg/ml. Alveolar macrophages at 10^6/ml in complete medium were added in 200 μl (2×10^5 cells) to quadruplicate microtitre plate wells (Sterilin, Feltham) and allowed to adhere for 2 h at 37°C. These were then washed and 200 μl of the various supernatants were added followed by a 10 μl volume of ^{125}I zymosan yielding a final concentration of 2 mg/ml; the plates were incubated at 37°C for 1 h. The wells were then washed five times with PBS to remove free zymosan and then with 200 μl of trypsin/EDTA (Gibco, Paisley) for 5 min at 37°C to remove cell surface-bound zymosan. The trypsin/EDTA was then removed and the wells washed once with PBS before adding 200 μl of 5% Triton (Sigma). Solublized cells and phagocytosed ^{125}I-zymosan were then counted in a gamma counter.

^{51}Cr-labelled sheep red blood cell phagocytosis assay

^{51}Cr-labelled sheep red blood cells (^{51}Cr-SRBC) were prepared by incubating 10^7 washed SRBC with 370 kBq of ^{51}Cr (Amersham International, Amersham, UK) for 1 h at 37°C then dividing the ^{51}Cr-SRBC into two parts. These were centrifuged, supernatants discarded and irrelevant antibody (mouse IgG, 2

mg/ml; Sigma, Poole) or monoclonal anti-SRBC antibody (MAS 013C, 2 mg/ml; Sera-Lab, Crawley Down) added to the resuspended pellet at 10 μg/10^7 ^{51}Cr-SRBCs and incubated at 37°C for 15 min. The cells were then spin/washed three times and resuspended in medium at $2{\cdot}5 \times 10^7$ ^{51}Cr-SRBC/ml in complete medium. Alveolar macrophage cultures were prepared as described for the ^{125}I-zymosan phagocytosis assay except that a total of $2{\cdot}5 \times 10^5$ cells were set up in individual removawells (Dynatech, Billinghurst) and allowed to adhere for 1 h. Supernatants were then added for 1 h before being washed. To quintuplicate wells were added 100 μl ($2{\cdot}5 \times 10^6$ ^{51}Cr-SRBC) of irrelevant antibody-coated ^{51}Cr-SRBC or anti-SRBC-coated ^{51}Cr-SRBC and they were incubated at 37°C for 90 min. The medium was then removed and 100 μl of red cell lytic buffer were added for 5 mins to disrupt the non-phagocytosed ^{51}Cr SRBC. The cells were washed twice with PBS and individual wells were counted in a gamma counter.

Treatment with supernatants

In some experiments ^{125}I-zymosan was pre-treated by incubating in supernatant at 10 mg/ml at 37°C for 1 h, and then washing three times. In another sequence of experiments the alveolar macrophages were pre-treated with supernatant for 2 h at 37°C before chilling to 4°C. Binding of ^{125}I-zymosan to the alveolar macrophages was then assessed at 4°C for 1 h by excluding the trypsin/EDTA step, so that only attached ^{125}I-zymosan was assessed.

Measurement of superoxide anion production

Superoxide anion was measured according to the method of Johnstone (1981). Bronchoalveolar macrophages from control rats were prepared and $0{\cdot}25 \times 10^6$ cells adhered to 300 mm plastic petri dishes (Sterilin, Feltham) for 2 h and then pre-treated with neutrophil supernatant or control medium for 2 or 24 h. This medium was then removed, the wells washed, and 1·5 ml of reaction buffer (phosphate-buffered saline plus 1 mg/ml cytochrome C and 2 mg/ml dextrose) added to each plate. Freshly prepared PMA (1 μg/ml) or zymosan (100 μg/ml) were immediately added to some plates and replicate plates also received superoxide dismutase (38 μg/ml; Sigma, Poole). Plates were incubated for 2 h at 37°C and the supernatants harvested and centrifuged to clarity before reading against a reagent blank at 550 nm in a scanning spectrophotometer. The superoxide dismutase-inhibitable cytochrome C reduction was converted to superoxide anion concentration according to Johnstone (1981) and expressed as nanomoles superoxide anion/$0{\cdot}25 \times 10^6$ cells/2 h. In some experiments macrophages were pre-treated with PMA (1 μg/ml).

Measurement of hydrogen peroxide

Hydrogen peroxide was assayed according to the method of Pick & Keisari (1980). Cultures of alveolar macrophages were set up as for superoxide anion and pre-treated with supernatant. One millilitre of reaction mixture (10 mM phosphate buffer plus 1 mg/ml dextrose; 8·4 mg/ml NaCl and 50 μg/ml horseradish peroxidase (Sigma, Poole)) was added to each dish and these were incubated for 2 h. The supernatants were then harvested, centrifuged to clarity and alkalized with 30 μl of 1M NaOH prior to reading at 610 nm in a spectrophotometer against a reagent blank. Standards were prepared from reagent hydrogen peroxide (Sigma, Poole) and a linear regression carried out using a Casio FX3600P calculator from which unknowns were obtained and expressed as nanomoles/$0{\cdot}25 \times 10^6$ cells/2 h.

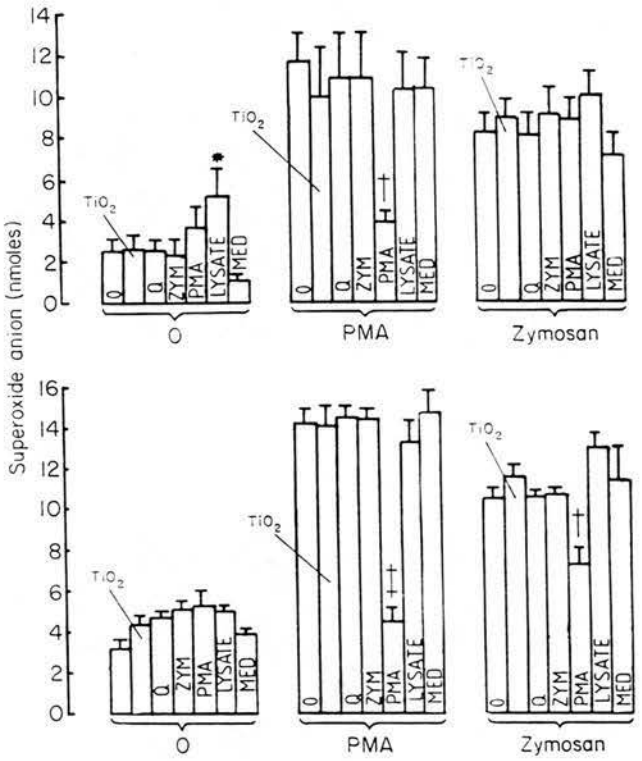


Fig. 3. The production of superoxide anion by rat alveolar macrophages triggered with 0, PMA or zymosan. Each column represents pre-treatment of the alveolar macrophages for 2 h with supernatant from neutrophils stimulated *in vitro* with the indicated substance: untreated (0); titanium dioxide (TiO_2) quartz (Q); zymosan (zym); phorbol myristate acetate (PMA); soluble component of lysed neutrophils (lysate); medium alone (med). Upper graph: the neutrophils were prepared in medium containing fetal calf serum. Lower graph: Supernatants were prepared in medium containing BSA. Mean ± s.e.m. of triplicate plates in three separate experiments. Significant differences from medium alone * $P < 0{\cdot}05$; † $P < 0{\cdot}01$; ‡ $P < 0{\cdot}001$.

RESULTS

The effect of neutrophil supernatants on alveolar macrophage phagocytosis

General. Time courses of phagtocytosis in both of the phagocytosis assay systems revealed a clear time-dependent uptake of both ^{125}I-zymosan and ^{51}Cr-SRBC, plateauing at around 1 h in each case. In the ^{51}Cr-SRBC assay the Fc receptor dependence of the phagocytosis was evident from the extremely low levels of phagocytosis of ^{51}Cr-SRBC coated with irrelevant antibody. In the case of the ^{51}Cr-SRBC assay there was no question that phagocytosis was occurring since all non-internalized SRBCs were lysed. In the ^{125}I-zymosan assay however, removal of adherent, non-phagocytosed zymosan depended on detachment with protease and so in some experiments one set of replicates was kept at 4°C, a temperature at which phagocytosis, but not binding, is inhibited. This showed less than 20% of the cell association of ^{125}I-zymosan found with identically treated macrophages kept at 37°C, confirming that phagocytosis was indeed being measured.

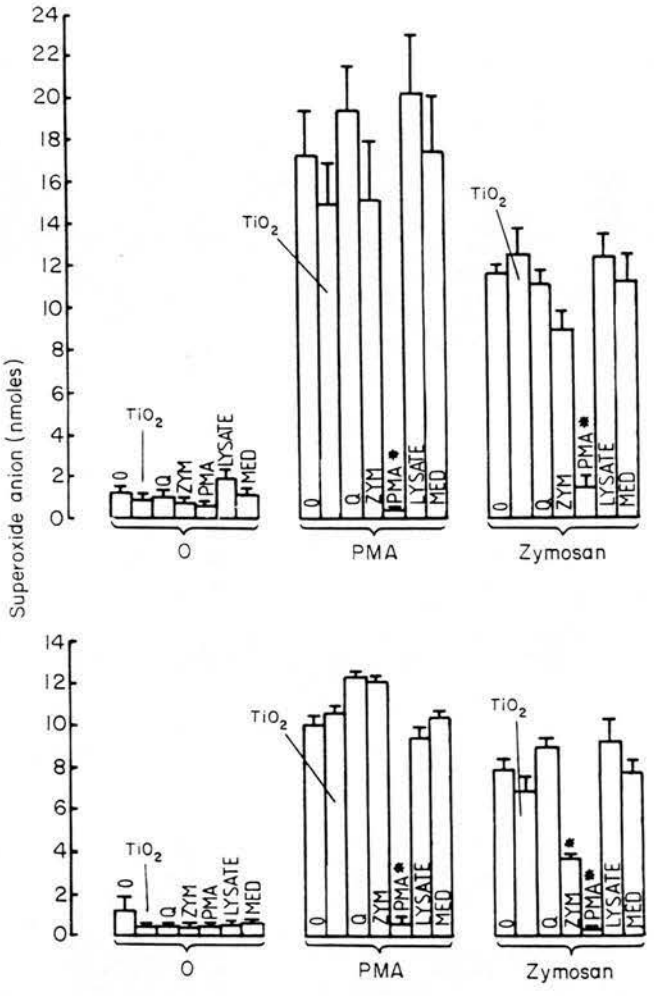


Fig. 4. Production of superoxide anion by rat alveolar macrophages triggered with 0, PMA or zymosan. Abbreviations and interpretation are as for Fig. 3 except that pre-treatment with neutrophil supernatant was for 24 h. * $P < 0{\cdot}001$.

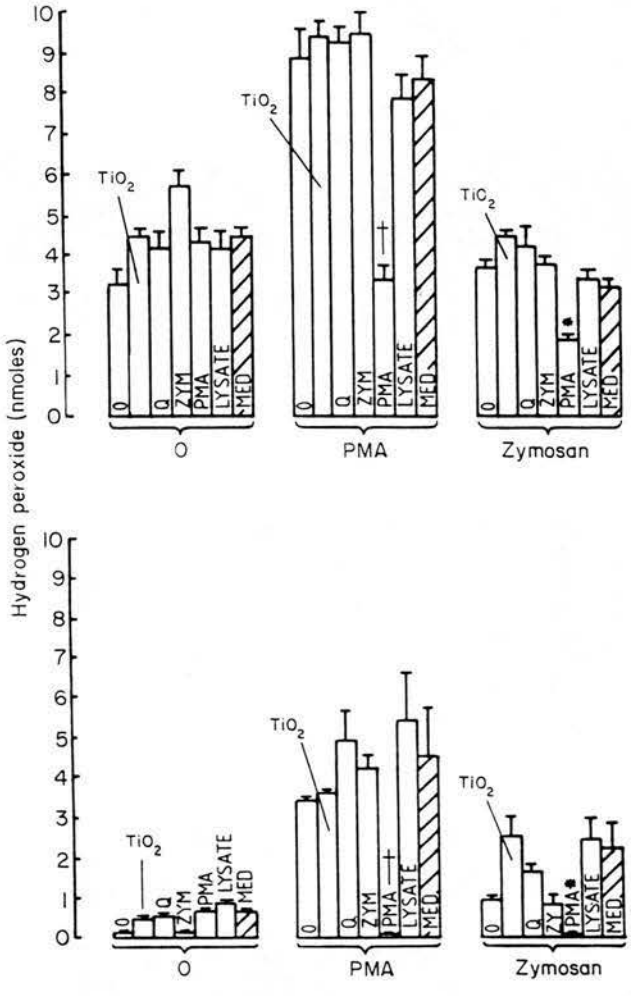


Fig. 5. Production of hydrogen peroxide by rat alveolar macrophages triggered with 0, PMA or zymosan. Pre-treatment with supernatant for 2h (upper) or 24 h (lower). Supernatant was prepared in medium containing BSA. Abbreviations and interpretation as for Figs. 3 and 4. * $P < 0{\cdot}05$; † $P < 0{\cdot}001$.

The effect of neutrophil supernatants on phagocytosis of ^{125}I-zymosan

Figure 1 shows that only supernatants from quartz- and PMA-treated neutrophils significantly modulated phagocytosis of ^{125}I-zymosan by control alveolar macrophages and that none of the other treatments of the neutrophils, or the lysate, affected phagocytosis. In the case of quartz supernatants there was a significant ($P < 0{\cdot}001$) increase in phagocytosis while with PMA-treated neutrophil supernatant, there was significant ($P < 0{\cdot}05$) inhibition of phagocytosis. Figure 2 shows the effect of the supernatant on phagocytosis of ^{51}Cr-SRBC by alveolar macrophages. Only the PMA-stimulated neutrophil supernatant caused inhibition of phagocytosis of ^{51}Cr-SRBC and quartz supernatants had no effect.

The effect of the quartz and PMA supernatant in the zymosan assay was further examined in a series of experiments which revealed that with pre-incubation of ^{125}I-zymosan in the different supernatants, there was no preferential retention or loss of counts from the ^{125}I-zymosan, which might account for the differences in phagocytosis (data not shown). However, pre-incubation of ^{125}I-zymosan in the PMA supernatant caused less ^{125}I-zymosan to be phagocytosed subsequently and accounted for the reduced phagocytosis found with this supernatant. In contrast however, the quartz supernatant did not affect the ^{125}I-zymosan particles in a way which influenced subsequent phagocytosis (data not shown). The PMA-stimulated neutrophil supernatant therefore inhibited ^{125}I-zymosan phagocytosis by acting at the level of the particle and not on the macrophage, whilst the quartz supernatant (effective only in the ^{125}I-zymosan assay) operated at the level of the macrophage to increase phagocytosis.

Further studies on the PMA supernatant effect in the ^{51}Cr-SRBC assay were concentrated on examining whether it was due to the PMA in the supernatant or to a PMA-stimulated neutrophil product. By incubating PMA alone in medium the effect of PMA could be assessed and it was found to cause inhibition of phagocytosis to the same extent as PMA-stimulated neutrophil supernatants: medium 4913 ± 489 ct/min; supernatant from PMA-stimulated neutrophils 4015 ± 385 ct/min; PMA alone 3446 ± 407 ct/min (all data given as mean $\pm$ s.d. of 5 replicates in 2 separate experiments). The effect of the PMA supernatant was therefore an artefact associated with the PMA present. This effect was not a toxic one since trypan blue

Table 1. Effect of pre-treatment with PMA on triggered release of superoxide anion by alveolar macrophages

Pre-treatment		Trigger: 0	PMA	Zymosan
0	2 h	2·7±0·3*	12·3±0·6	10·4±0·4
	24 h	0·0±0·0	8·3±0·5	7·4±0·2
PMA	2 h	4·3±0·3	3·1±0·2	11·2±0·3
	24 h	0·0±0·0	0·0±0·0	0·2±0·1

* Mean ± s.e.m. (nanomoles superoxide anion; $n = 3$)
PMA trigger, 2 and 24 h, 0 pre-treatment v PMA pre-treatment $P < 0.001$. Zymosan trigger, 24 h, 0 pre-treatment v PMA pre-treatment $P < 0.001$.

exclusion studies on macrophages incubated for 24 h in 1 μg/ml PMA showed viability of >90% in both the PMA-treated and control macrophages.

Effects of neutrophil supernatants on alveolar macrophage oxidant production

Triggering of untreated alveolar macrophages with PMA or zymosan caused substantial production of both superoxide anion and hydrogen peroxide. Following 2 h of pre-treatment in neutrophil supernatant based on MEM plus FCS, the most marked effect was a significant decrease ($P < 0.001$; all comparisons with medium alone) in production of superoxide anion by alveolar macrophages pre-treated with PMA-stimulated supernatants and subsequently triggered with PMA (Fig. 3). There was also a small but significant ($P < 0.05$) increase in untriggered production of superoxide anion by alveolar macrophages pre-treated with neutrophil lysate. Two hours of pre-treatment with BSA-based supernatant medium from PMA-stimulated neutrophils also caused alveolar macrophages to release decreased amounts of oxidant on treatment with both PMA ($P < 0.001$) and zymosan ($P < 0.01$).

Increasing the pre-treatment time with neutrophil supernatants to 24 h emphasized the inhibitory effect of PMA-stimulated neutrophil supernatants on the ability of alveolar macrophages to be triggered with both PMA and zymosan ($P < 0.001$) (Fig. 4). Pre-treatment with the BSA-based, zymosan-stimulated, supernatant for 24 h also produced a significant decrease in alveolar macrophage oxidative response to zymosan ($P < 0.001$) (Fig. 4).

A clear increase of 70–100% in total superoxide anion release was evident with supernatants based on FCS compared to BSA, at the 24 h time point.

Figure 5 shows the levels of hydrogen peroxide produced in experiments when alveolar macrophages were cultured with supernatants based on BSA medium for 24 hours then triggered, that is similar to those shown in the lower half of Figs 3 and 4. The similarities are striking with marked inhibition of the ability of PMA to trigger macrophages pre-treated with PMA-stimulated supernatants ($P < 0.001$) and a similar loss of responsiveness to triggering with zymosan in the PMA supernatant-treated macrophages. The ability of zymosan to trigger macrophages treated with zymosan supernatant was also reduced but this did not attain statistical significance.

In order to determine whether it was a PMA-stimulated neutrophil product, or PMA, which was mediating the inhibitory effect in PMA-stimulated supernatants, macrophages were pre-treated with PMA alone for 2 or 24 h. These macrophages were then washed and their ability to be triggered with PMA or zymosan was assessed. As shown in Table 1, 2 h of pre-treatment with PMA caused significant inhibition of ability to be triggered with PMA, but not by zymosan. By 24 h of PMA pre-treatment, however, the response to both PMA and zymosan was significantly ($P < 0.001$) reduced.

DISCUSSION

The first part of the study showed clearly that, with two key exceptions, alveolar macrophage phagocytosis was not affected by supernatants prepared from bronchoalveolar-derived inflammatory neutrophils. Initial findings revealed that quartz supernatants caused stimulation of ^{125}I-zymosan phagocytosis while PMA supernatants caused inhibition of phagocytosis in both the ^{125}I-zymosan and ^{51}Cr-SRBC assays. Experiments revealed that PMA supernatant-mediated inhibition in the ^{125}I-zymosan assay was due to an effect of the supernatant at the level of the particle to make ^{125}I-zymosan apparently bind less to the macrophage membrane and to be phagocytosed less. Experiments with PMA alone, in the ^{51}Cr-SRBC assay, revealed that the inhibitory effect of PMA-stimulated neutrophil supernatant could be mimicked with PMA alone. Supernatants from PMA-stimulated neutrophils therefore inhibit phagocytosis by different mechanisms in two different assay systems. However, of particular interest to the aims of this study was that neither of these was attributable to the effect of a neutrophil product on the macrophages, being due to an effect on the particles and an effect of the PMA trigger. The increased ^{125}I-zymosan phagocytosis found with quartz supernatant was, however, apparently not at the level of the particle, since there was no increased binding or enhanced phagocytosis of quartz ^{125}I-zymosan pre-treated with the quartz supernatant. The effect of the quartz supernatant was therefore a direct one on the macrophage to stimulate phagocytosis of ^{125}I-zymosan to a small but significant extent.

The second part of the study revealed that the oxidative metabolism of alveolar macrophages is not substantially modulated by treatment with products obtained by treating bronchoalveolar-derived neutrophils with titanium dioxide, quartz, zymosan or with products from lysed neutrophils. Supernatants obtained by treating neutrophils for 2 h with PMA did, however, cause inhibition of the macrophage oxidative burst in response to PMA; treatment for 24 h resulted in virtual complete inhibition of the response to both PMA and zymosan.

Studies on alveolar macrophages pre-treated with PMA alone for 2 or 24 h revealed the same effect, with specific loss of PMA responsiveness at 2 h and loss of responsiveness to both PMA and zymosan present after 24 h. This confirmed that the inhibitory effects produced by supernatants obtained from PMA stimulation of neutrophils, were artefactual, due to PMA, and were not due to neutrophil products. The inhibitory effect of PMA on peritoneal macrophage response to further PMA stimulation has been described in detail by Berton & Gordon (1983) and loss of PMA membrane receptors from macrophages accounts for this refractory state. PMA-mediated loss of

responsiveness to zymosan, as found in the present study with longer-term exposure to PMA, was also described by these workers but its mechanism remained obscure.

The generally increased production of oxidants by alveolar macrophages incubated for 24 h in serum compared to BSA is in agreement with the conditioning effect of serum on rabbit alveolar macrophage oxidative metabolism described by Gerberick, Willoughby & Willoughby (1985).

Neutrophils are normally found in the circulation but are recruited into tissue at sites of inflammation where they are found to be different to circulating neutrophils, being in an activated state with increased secretions (Wandall, 1982; Baggiolini & Dewald, 1985). During inflammation in the alveolar region of the lung variable numbers of neutrophils are recruited (Keogh & Crystal 1982). Several studies have demonstrated that neutrophils and macrophages can affect each other. Thus neutrophil products cause peritoneal macrophages to take up thymidine and show increased chemiluminescence (Bird, *et al.*, 1984), release a substance which specifically inhibits interleukin-1 (Tiku *et al.*, 1986) and also may be involved in macrophage activation to the tumoricidal state (Chapes & Haskill, 1984). Neutrophils have also been reported to modulate the functions of lymphocytes (El Hag *et al.*, 1986) and natural killer cells (El Hag & Clark 1984) and to injure lung cells (Martin, 1984).

In the present study products from bronchoalveolar-derived, unstimulated neutrophils or neutrophils stimulated *in vitro* with zymosan, titanium dioxide or PMA, or products from lysed neutrophils, generally failed to affect oxidant release or phagocytosis by control alveolar macrophages. However, in the ^{125}I-zymosan phagocytosis assay only, supernatants from quartz-treated neutrophils enhanced alveolar macrophage phagocytosis.

The accumulation of neutrophils in the alveoli of experimental animals exposed to quartz by inhalation (Donaldson *et al.*, 1988) or intratracheal instillation (Morgan *et al.*, 1980; Bowden & Adamson 1984) suggests that conditions certainly exist whereby neutrophils could influence alveolar macrophages following deposition of quartz. Enhanced uptake of a toxic particle like quartz by alveolar macrophages could have some adaptive value to the host since the particles would be more quickly cleared from the lung. One study from our own laboratory has revealed that quartz can stimulate neutrophil metabolism *in vitro* (Gormley, Kowolik & Cullen, 1985) so possibly stimulating release of the enhancing factor. These were, however, peripheral blood neutrophils as opposed to the inflammatory bronchoalveolar neutrophils used here.

Work is in progress to further characterize release of the stimulatory factor generated by quartz-treated neutrophils and to examine other macrophage functions which could be modulated by neutrophil products.

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In vitro assays for detecting carcinogenic mineral fibres: a comparison of two assays and the role of fibre size

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The activities of 13 carcinogenic fibrous dust samples were compared in two *in vitro* test systems using V79/4 and A549 cells. Eleven of the dusts had similar ranks in both assays but two samples had disparate results. The relationship between the fibre length and diameter distributions of 12 of the samples and their activity in each assay was examined. A significant association between fibre length and biological activity occurred in both assays. This relationship showed increasing strength of association with increasing fibre length. The only significant association between fibre diameter and activity *in vitro* was demonstrated with fibres >0.2 μm in the A549 assay.

Introduction

It has long been established that the inhalation of mineral fibres can cause serious health problems in man. Of particular importance for the present study is the well established association between exposure to fibrous minerals and development of malignant disease (1). The mechanism of fibre carcinogenesis is not understood but, experimentally, fibre dimensions have been shown to be a major factor. In a series of detailed *in vivo* studies, Stanton *et al.* (2–4), examined the relationship between fibre length and diameter and the potential to cause mesotheliomas following implantation into the pleural cavity of rats. The chemical, crystallographic and morphological structures of the samples varied widely but nevertheless a significant correlation was demonstrated between carcinogenicity and the number of fibres present with lengths >8 μm and diameters <1.5 μm. Similarly Wright and Kuschner assessed the effect of fibre length on the induction of fibrosis by asbestos and glass fibre samples. They concluded that fibre length, rather than fibre type, was important in determining fibrogenicity and ultimately carcinogenicity. Pott (6) reported that fibres of 20 μm length and <0.25 μm diameter have high carcinogenic potency and this decreases with decreasing length and increasing diameter. Several *in vitro* studies have also demonstrated a relationship between fibre length and biological activity. Ririe *et al.* (7) and Hesterberg *et al.* (8) compared milled and unmilled glass fibres and showed that the long fibres were more active. Brown *et al.* (9,10) demonstrated that the correlation between fibre length and biological activity was similar to the *in vivo* correlations reported by Stanton (4) and reaffirmed his suggestion that there may be a threshold length below which fibres may be inactive. Chamberlain *et al.* (11) and Wade *et al.* (12) reported similar findings.

In the present study a series of fibrous materials are compared in two *in vitro* assay systems. The relationship between activ *in vitro* and the dimensional characteristics of the dusts is amined and is compared with previously published *in vivo* formation on tumour production and fibrosis, obtained us many of the same dust samples.

Materials and methods

Dust samples

Thirteen fibrous dust samples were tested in this study. Two isometric dusts v included as controls in each experiment and these were the rutile form of titar dioxide (TiO_2) and quartz (DQ_{12}) (13). Some of the fibrous dusts had been for *in vivo* inhalation experiments and the samples for *in vitro* studies had collected from the inhalation chambers by an elutriation process (14). These d. therefore, consisted mainly of respirable particles and they have been indic by the prefix E in the script. Chrysotile samples: UICC chrysotile A; E. U chrysotile A and superfine chrysotile (SFA chrysotile). Amphibole samples: U crocidolite; E. UICC crocidolite; E. UICC anthophyllite; UICC amosite; E. fibre amosite (E.L.F. amosite) (dust sample prepared from raw amosite); fibre amosite (S.F. amosite) which was prepared by a grinding and sedim tion process; E. factory amosite which was obtained from the air filtration sy of an asbestos factory; and a sample of a commercially available tremolite Korea (E. tremolite). Two other fibrous materials were tested and these E. ceramic (a man-made, fibrous aluminium silicate) and E. brucite. Fibre le and diameter distributions were available for 12 of the dusts which had bee amined previously by scanning electron microscopy (SEM*), using the me of Schneider (15). The only test sample for which no fibre size data were avai was E. factory amosite. The total number of fibres in 10^{-10} g of each sa was calculated and this value, together with the SEM size data was used to calc the actual number of fibres present in each 10^{-10} g of each fibrous sample, v was greater than the stated length (0–20 μm in 1 μm increments) or diar (0.1–1.0 μm in 0.1 μm increments).

V79/4 colony inhibition assay

This assay was performed as described by Chamberlain and Brown (16) e that the concentrations of dust and cells were changed because of the very cyt nature of some of the samples. In brief, V79/4 Chinese hamster lung fibrol (obtained from the MRC Pneumoconiosis Unit, Llandough Hospital, Pen South Glamorgan) were routinely passaged and also assayed in the presen dust using a growth medium composed of minimum essential medium (M — Gibco-Europe, Paisley, Scotland) containing 10% foetal calf serum (G and 100 μg/ml kanamycin (Winthrop Ltd, Surbiton-upon-Thames, Surrey). C inhibition estimations were performed by plating out 5 ml aliquots of me containing 75 cells/ml and 0, 5, 10, 25, 75 or 100 μg dust/ml, onto 4 × 5(Petri dishes (Sterilin, UK). These were incubated for 6 days at 37°C, in a mosphere of 5% CO_2 in air. The colonies were then fixed in 10% formal s and stained with 1% methylene blue. The number of colonies containing cells were counted and the activity calculated as a percentage of a control c count from dishes of cells which had been incubated in the absence of dust concentration of dust causing a 50% reduction in the cloning efficiency of V cells (ED_{50}) was measured in three independent experiments.

A549 assay

The A549 assay determines the ability of dusts to induce an increase in the dia of A549 (human alveolar type II-like) cells. The assay was performed usin technique described by Chamberlain and Brown (16) but with an increas oculum of cells per flask because some of the samples tested proved to be toxic.

A549 cells (obtained from MRC Pneumoconiosis Unit, Llandough Hos Penarth, South Glamorgan) were routinely cultured and assays of dust ac were performed using Dulbecco's modified Eagles medium (DMEM—Gi containing 10% heat-inactivated foetal calf serum and 100 μg/ml kanamycin activity of dusts in this assay was measured by plating out 5 ml aliquots of m containing 2 × 10^5 cells/ml and 25 or 50 μg dust/ml, into 25 cm^2 tissue c flasks (Sterilin). Five ml of pure CO_2 were injected into each flask and were incubated at 37°C for 4 days. The cells were then detached from the

*Abbreviations: SEM, scanning electron microscopy; MEM minimal essential medium; DMEM, Dulbecco's modified Eagle's medium.

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using trypsin/EDTA (Gibco) and the resulting cell suspensions were pipetted onto Neubauer haemocytometer chambers. Each Neubauer slide preparation was photographed and the cell size distribution was obtained by measuring the cell diameters in relation to the Neubauer grid lines. The diameter of 100 cells per test sample was obtained from the photographs using a Graphic Instruments GDS1 image analyser, and the results were expressed as either the median volume diameter of the cell population or as the percentage of cells >25 μm in diameter.

Statistical analysis

In order to facilitate statistical comparisons of the results obtained in the V79/4 and A549 assays, the data from each was examined and dusts were ranked according to their activity in each assay. For comparisons between the two assays and all other analyses in which two sets of ranks were compared, Spearman's rank correlation co-efficient was used (17). In the A549 assay, where the dusts were ranked according to their cytotoxicity (on the basis of the mean of at least three experiments) at 25 and 50 μg/ml, a comparison of the dust rankings at the two concentrations revealed a significant correlation ($r = 0.921$), and thus a composite rank was calculated.

The ranking of samples in the V79/4 assay was based on the mean of the ED_{50} values obtained in three separate experiments. This figure was calculated from the colony counts using a Genstat linear interpolation programme (18). Data were available on the number of fibres in each length and diameter category per 10^{-10} g of dust and this information was used to rank the dusts according to the number of fibres at each length and diameter. The fibre length and diameter ranks for each size category were then compared with both the A549 and V79/4 ranks.

Table I. Effect of dusts in the V79/4 assay

Dust	ED_{50} ± SD
E. ceramic	>100
TiO_2	85 ± 26.0
DQ_{12}	78.3 ± 37.5
SF amosite	41.1 ± 27.3
UICC crocidolite	33.8 ± 7.1
E. factory amosite	31.2 ± 9.3
E. UICC crocidolite	28.4 ± 22.4
E. brucite	26.4 ± 11.2
UICC amosite	25.4 ± 10.8
SFA chrysotile	24.4 ± 7.0
E. UICC anthophyllite	21.1 ± 6.5
UICC chrysotile A	17.3 ± 3.3
E. tremolite	17.3 ± 4.5
E. LF amosite	11.2 ± 3.4
E. UICC chrysotile A	11.0 ± 1.3

The ED_{50} is the concentration of dust causing a 50% reduction in the cloning efficiency of the V79/4 cells and is the mean of three independent experiments.

Results

V79/4 assay

The results of the V79/4 assay are presented in Table I where the dusts are listed according to their cytotoxicity as expressed by the ED_{50}. The dust concentrations at which the dusts were tested in this assay were between 5 and 100 μg/ml. Since there could be no accurate extrapolation beyond these points, those dusts whose ED_{50}s were ≥100 were taken as = 100.

The two non-fibrous controls were virtually inactive in this assay. In general, the amphiboles showed a wide range of cytotoxicity, but were less cytotoxic overall than the chrysotiles. Of the amphiboles, tremolite and anthophyllite proved to have the greatest biological effect, with SF amosite proving to be the least active. The most active sample, E. UICC chrysotile was considerably more active than the non-elutriated UICC chrysotile. The E. ceramic sample was completely inert and showed less activity than the two controls.

A549 assay

The results of the A549 assay are presented in Table II where the dusts are listed according to their activity at 25 and 50 μg/ml. In general, the increase in both the mean cell diameter and percentage of cells >25 μm was greater at the higher dust concentration. However, the relative effect of each dust, when compared with the others, was similar at both concentrations. The TiO_2 and DQ12 controls both had a negligible effect in this assay. As in the V79/4 assay, the amphiboles again showed a wide spread of activity and were, in general, less active than the chrysotiles. At 50 μg/ml in particular the chrysotiles showed a marked increase in percentage of cells >25 μm when compared with the other dusts. The E. ceramic sample, which was inert in the V79/4 assay, proved to be active in this system.

Statistical comparison of the V79/4 and A549 assays

Dusts were ranked from 1 to 13 according to their activity in each assay, with the figure 1 indicating the least active and the

Table II. Effects of dusts in the A549 assay

Sample	25 μg/ml			Sample	50 μg/ml		
	Size	SD	% >25 μm		Size	SD	% >25 μm
Controls	17.15	± 1.33	1.10				
DQ_{12}	16.95	± 0.30	1.25	TiO_2	16.83	± 0.41	0
TiO_2	17.45	± 1.4	1.50	DQ_{12}	17.42	± 0.66	0
E. UICC crocidolite	17.46	± 0.7	1.67	SF amosite	17.71	± 1.39	1.25
SF amosite	17.52	± 1.4	2.25	E. UICC crocidolite	17.80	± 0.51	3.67
UICC crocidolite	17.75	± 1.1	3.00	E. LF amosite	18.73	± 0.11	6.00
UICC amosite	18.18	± 0.5	4.25	E. factory amosite	18.81	± 0.29	4.00
E. UICC anthophyllite	18.45	± 1.6	2.33	E. tremolite	19.00	± 1.38	5.67
E. LF amosite	18.57	± 0.0	4.67	E. ceramic	19.28	± 2.03	4.33
E. factory amosite	18.70	± 1.0	3.33	UICC crocidolite	19.40	± 1.78	10.75
E. ceramic	18.72	± 1.6	3.00	UICC amosite	20.05	± 1.55	10.75
E. tremolite	19.73	± 2.0	9.00	E. UICC anthophyllite	20.27	± 0.55	9.67
E. UICC chrysotile A	19.74	± 0	13.00	E. brucite	20.47	± 1.67	7.67
E. brucite	20.20	± 0.9	7.00	UICC chrysotile A	22.68	± 1.33	30.25
UICC chrysotile A	21.32	± 1.7	22.50	E. UICC chrysotile A[a]	22.98	± –	32.00
SFA chrysotile	21.91	± 2.0	25.75	SFA chrysotile	23.18	± 1.41	31.75

100 cells per dust were measured in each assay and dusts were usually tested at least three times.
The cell diameters quoted are the mean of the results obtained in three experiments.
[a]Tested once only.

Table III. Comparison of ranking of V79/4 and A549 assays

Dust	V79/4 rank	A549 rank
E. ceramic[a]	1	8
S.F. amosite	2	1.5
UICC crocidolite	3	4
E. factory amosite	4	5.5
E. UICC crocidolite	5	1.5
E. brucite	6	10
UICC amosite	7	5.5
SFA chrysotile	8	13
E. UICC anthophyllite	9	8
UICC chrysotile A	10	12
E. tremolite	11	8
E. LF amosite[a]	12	3
E. UICC chrysotile A	13	11

Spearman's correlation coefficient for all 13 dusts; $r = 0.431$, $P > 0.05$.
Spearman's correlation coefficient for 11 dusts (excluding the two anomalous dusts marked [a]; $r = 0.743$, $P > 0.05$.
[a]Dusts which are considered to be anomalous because of their differential activity in the two assays.

figure 13 indicating the most active. Where two dusts or more showed the same activity a rank was allocated which is the mean of their combined rank values. Eleven of the dusts had similar ranks in both assays but the two remaining samples E. ceramic and E.L.F. amosite showed disparate results. E. ceramic was virtually inactive in the V79/4 assay but ranked in the mid-range in the A549 assay. Conversely, although E.L.F. amosite had low activity in the A549 assay, it was very active in the V79/4 assay.

The ranking of dusts in the V79/4 and A549 assays was compared using Spearman's rank correlation coefficient (see Table III). No significant association between the assays could be demonstrated when all 13 dusts were included in the analysis ($r = 0.431$, $P > 0.05$) but the relationship became significant when the two anomalous dusts were removed ($r = 0.743$, $P < 0.05$). The experimental figures in each assay for dusts with adjacent ranks were often very close and overlaps might be expected to occur. However, the correlation between the two assays indicates that the overall ranking of the dusts is correct.

Effect of fibre length

The fibre length and diameter data which were available for 12 of the dusts were compared with the cytotoxicity ranks in each assay. In Table IV the correlation coefficients are presented for fibre length versus activity in each assay. For this set of data, where 12 different dusts were used, a correlation coefficient of ≥ 51 indicates a significant association between the two measures ($P \leq 0.05$).

The strength of association between the V79/4 assay and the fibre length data can be seen to increase with fibre length, reaching a significant association at number of fibres >3 μm. In the A549 assay, significance of association is present at number of fibres >4 μm. The strength of association increases with fibre length but reaches a peak value at >8 μm. Less confidence can be attached to correlations produced at lengths >15 μm due to the small numbers of fibres available for the analysis.

Effect of fibre diameter

Table V details the association between fibre diameter and the A549 and V79/4 assays. No association could be found between fibre diameter and the V79/4 assay. The only significant association which could be demonstrated in the A549 assay occurred at number of fibres >0.2 μm ($P \leq 0.05$).

Table IV. Spearman rank correlation coefficients for the relation between fibre length and cytotoxicity in the two assays

Assay	Fibre length											
	>0	>1	>2	>3	>4	>5	>6	>7	>8	>9	>10	>
V79/4	6	22	33	54	53	70	70	81	80	81	87	8
A549	19	30	39	50	55	62	59	57	70	62	53	2

For this set of data, with 12 different dusts, a correlation coefficient of >51 indicates a significant association between the two measures ($P > 0.05$).

Table V. Spearman rank correlation coefficients for the relationship betw fibre diameter and cytotoxicity in the two assays

Assay	Fibre diameter									
	≥0.2	≥0.3	≥0.4	≥0.5	≥0.6	≥0.7	≥0.8	≥0.9	≥1.0	>
V79/4	12	−1	10	8	11	5	−12	−12	−14	-
A549	−47	−37	−24	−20	−6	−3	13	13	19	

For this set of data, where 12 different dusts were used, a correlation coefficient of ≤ -47 is significant ($P \leq 0.05$).

Discussion

Many experimental studies have examined the carcinoge potential of mineral fibres. In our laboratory we have use *vivo* exposure to examine characteristics of mineral fibre cinogenesis (14) and *in vitro* exposure, using various target c to assess fibre toxicity (19). The aim of the *in vitro* studies been 2-fold — to elucidate the cellular mechanisms of f pathogenicity and to develop *in vitro* assay systems, wh response correlates with the carcinogenic potential of fibre assessed by more expensive and time-consuming *in vivo* ass the experiments reported here are concerned with the latter proach. A panel of size-characterized fibrous minerals, wi had already been tested in rats for their carcinogenic poten were tested in the A549 and V79/4 *in vitro* assays which h been reported to respond preferentially to carcinogenic fib Detailed comparison of the *in vitro* activity and *in vivo* cinogenic responses to this panel of mineral samples have b published elsewhere (20). In the present paper we report or comparison of the two *in vitro* assay systems and the effe fibre dimensions on the *in vitro* activity of fibrous miner

Statistical comparisons of the A549 and V79/4 assays sho a significant correlation between responses in the two a systems for 11 of the 13 dusts tested, but the two remaining were completely dissimilar in their activities in each assay ceramic was inactive in the V79/4 assay but active in the A assay. Conversely, E.L.F. amosite was active in the V79/4 a but showed little activity in the A549 assay. The effect of t two samples in each *in vitro* assay was compared with their cinogenicity *in vivo* (20) but no obvious relationship eme which could explain the anomalous behaviour of the two d

Chamberlain *et al.* (11) compared the cytotoxic effect of on mouse peritoneal macrophages, V79/4 and A549 cells demonstrated a 'very high level of concordance' between the assays. The fibrogenic mineral quartz was inactive in the non-macrophage assays whilst a range of fibrous materia known *in vivo* carcinogenicity had a range of activities in all *in vitro* systems which correlated well with their *in vivo* eff This led to the suggestion that a combination of all three a might form the basis of a screening system to differentiate

ween the fibrogenic and carcinogenic potentials of dust samples. The present study confirms and extends the suggestion that the A549 and V79/4 assays response to carcinogenic fibres. However, it is clear that certain dusts act differently in each of the non-macrophage assays and this evidence for the differential activity of dusts in separate assay systems highlights the risk of applying a single test system where false positive or negative results may arise. In the present study the discrimination between fibrous (carcinogenic) and toxic, non-fibrous (fibrogenic) minerals by the two non-macrophage assays is reaffirmed. Quartz, a non-fibrous cytotoxic dust is inactive in both the V79/4 and A549 assays; in contrast, the fibrous dusts, with the exception of E. ceramic in the V79/4 assay, all showed some activity in the two test systems.

We have also demonstrated an association between activity in the V79/4 and A549 assays and fibre length. In both assays, the association strengthens with increasing length and these results suggest that although shorter fibres are less active than long fibres they may not be completely inactive. Very little relationship was, however, observed between fibre diameter and activity *in vitro*; only in the A549 assay with fibres >0.2 μm did the association reach significance. These results do not preclude a correlation between toxicity and fibre diameter but suggest that below the diameter of ~ 1 μm there is no clear increase in response with further diameter reductions.

In vivo studies in our laboratory showed that all of the fibrous dusts used in this study had some carcinogenic potential (20). Those studies also revealed that the relationship between *in vivo* carcinogenicity and fibre length strengthened with increasing fibre length but very little relationship was observed between fibre diameter and *in vivo* carcinogenicity. These results are in close accord with the relationships between fibre length and fibre diameter and activity in the V79/4 and A549 assays described in the present paper.

We conclude that each of the *in vitro* tests utilized in the present study can be a useful screen for detecting carcinogenicity of mineral fibres. However, disparate responses may arise between the two assay systems and so the use of both assays is desirable. The increasing response in the two tests with increasing fibre length, is similar to that reported in many *in vivo* studies of fibre carcinogenesis (reviewed by Davis, 21). The results presented in this study therefore add support to the suggestion (22) that the A549 and V79/4 short-term *in vitro* assays may be used as an aid in predicting the carcinogenicity of mineral fibres.

Acknowledgements

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SHORT COMMUNICATION

Assessment of Mineral Dust Cytotoxicity toward Rat Alveolar Macrophages Using a ^{51}Cr Release Assay

Assessment of Mineral Dust Cytotoxicity toward Rat Alveolar Macrophages Using a ^{51}Cr Release Assay. DONALDSON K., AND BROWN, G. M., (1988). *Fundam. Appl. Toxicol.* **10**, 365–366. An assay was developed to assess the cytotoxicity of mineral dust by measuring release of ^{51}Cr from prelabeled rat alveolar macrophages. Optimal conditions for the assay are described, the most notable being use of 2% albumin instead of fetal calf serum. The assay demonstrated loss of label into the supernatant when prelabeled macrophages were cultured with the two pathogenic mineral dusts, quartz and chrysotile asbestos. In contrast the inert mineral dust titanium dioxide had very little effect on ^{51}Cr release by rat alveolar macrophages. © 1988 Society of Toxicology.

The demonstration that, *in vitro*, some pathogenic mineral dusts can be toxic to alveolar macrophages has been suggested as a likely factor in the etiology of pneumoconiosis (Harington *et al.*, 1975).

The assessment of alveolar macrophage cytotoxicity following treatment with dusts *in vitro* has been confined, in the main, to three methods: (i) assessment of ability to exclude vital dyes; (ii) measurement of release of the cytoplasmic enzyme lactate dehydrogenase; and (iii) measurement of release of lysosomal enzymes.

Despite the increased use of miniaturized radioactive-label-and-release assays in inflammation and immunology research, we know of no such assay to specifically assess mineral dust-induced injury to macrophages. In view of the potential benefits, we developed a miniaturized ^{51}Cr label-and-release assay using rat alveolar macrophages as the target cells.

MATERIALS AND METHODS

General. We chose to validate the assay by studying quartz, a compact cytotoxic dust, chrysotile asbestos, a cytotoxic fibrous dust, and titanium dioxide (TiO_2), a particulate found to be nontoxic in many previous studies.

Animals. Syngeneic PVG rats obtained from our own animal unit and 12–16 weeks of age were utilized throughout.

Cells. Rat alveolar macrophages were obtained by bronchoalveolar lavage of resected lungs with 4 × 10 ml lavages of saline (37°C); approximately 7 million cells were obtained from each rat. Cells were washed, counted, and diluted to 10^5 cells/ml in minimum essential medium (MEM; GIBCO, Paisley) containing 2% bovine serum albumin (BSA; Sigma, Poole).

Dusts. Three dusts were tested in this assay: (i) an inert particulate—the rutile form of TiO_2 (Tioxide Ltd, Tyne & Wear); density 4.25 g/cm^3, median volume diameter 2.4 μm; (ii) a toxic, nonfibrous crystalline dust—quartz (DQ_{12} sample); density 2.65 g/cm^3, median volume diameter 1.35 μm; (iii) a toxic, fibrous dust—chrysotile asbestos (UICC reference sample A); density 2.55 g/cm^3, median diameter 0.25 μm and median length 2.6 μm.

^{51}Chromium. ^{51}Cr was obtained as sodium chromate in sterile sodium chloride solution at 1.3 GBq/mg Cr (370 MBq/mmol) from Amersham International, Aylesbury.

Cytotoxic assay. Cells were set up in 96-well microtiter plates (Sterilin, Feltham) at 5 × 10^4 cells/well then labeled overnight with 74 kBq ^{51}Cr/well at 37°C in 5% CO_2 resulting in uptake of 5–9 × 10^3 counts per minute routinely. The cells were washed once with sterile phosphate buffered saline (PBS) and dusts were added at the appropriate dilution (10 and 25 μg/well) in 200 μl MEM + 2% BSA. After 24 hr incubation at 37°C, the supernatant medium was harvested and counted in a gamma counter (Rackgamma, LKB; South Croydon) to assess counts released from damaged cells. In early experiments the cell layer was then lysed with 0.1% Triton X-100 (Sigma; Poole) and counted as above to estimate the counts present in intact cells.

Statistical analysis. Repeat experiments were analyzed by analysis of variance using the Minitab computer program. Differences between means were tested for significance using the *t* test.

RESULTS AND DISCUSSION

Studies on optimal culture conditions indicated that 2% bovine serum albumin was a

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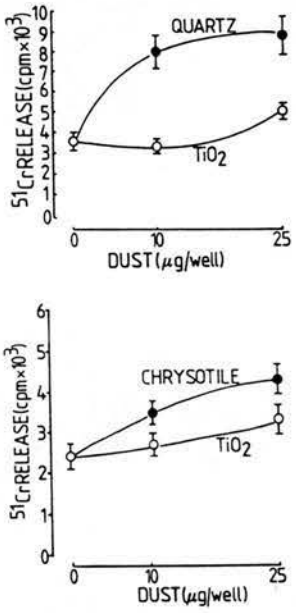


FIG. 1. Upper. ^{51}Cr counts released into the medium from rat alveolar macrophages cultured with 10 or 25 μg TiO_2 or quartz. Lower. Similar results obtained with TiO_2 or chrysotile asbestos. Results represent means ± SEM from triplicate wells in seven separate experiments. There was significantly more ^{51}Cr release with quartz and chrysotile at both doses compared to TiO_2 ($p < 0.001$).

better medium supplement than 2 or 10% fetal calf serum as measured by ^{51}Cr retention. In preliminary experiments the released and cell-bound ^{51}Cr were measured and found to vary conversely with dose following injury due to quartz or chrysotile asbestos. In multiple subsequent experiments only release of ^{51}Cr was measured showing this to be a sufficiently reliable index, on its own, for detecting mineral dust-mediated macrophage damage (Fig. 1). The assay described here has demonstrated the toxicity, at low dose, of both quartz and chrysotile asbestos, dusts shown to be cytotoxic in numerous previous studies; in addition it showed little response to TiO_2, a dust of known low toxicity. The assay is quick and simple to set up and uses many fewer cells than the conventional assays of macrophage cytotoxicity used previously. The doses of dust used show the ^{51}Cr release assay to be as sensitive, or more sensitive, than the conventional assays. The small amounts of dust used make the assay ideal for studying environmental or special experimental samples of dusts available only in limited quantity.

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Early cellular responses to calcium phosphate ceramics

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The early cellular response to calcium phosphate powders and sintered ceramics was quantified using two test systems. Cytotoxicity was assessed by a Cr^{51} release assay, using human fibroblasts and mouse macrophages. Inflammatory cell recruitment was measured in the mouse peritoneal cavity by total and differential cell counts. The results for the two test systems gave different rankings for the materials. Dense hydroxyapatite ceramic was the only material to manifest cytotoxicity, while tricalcium phosphate powder was the material with the greatest inflammatory potential in the mouse peritoneal cavity. The cell recruitment ranking of the materials indicated that calcium phosphate powders were most active, while calcium phosphate sintered ceramics showed much less activity.

Introduction

In recent years, bioceramics of calcium phosphate have been introduced into dentistry, for the restoration of hard tissue defects caused by periodontal disease, local tooth loss, or post extraction ridge resorption.[1–4] These materials, which are chemically similar to the inorganic phase of bone or dentine, have been shown to elicit neither undesirable biological response nor gross local or systemic toxicity.[5] However, the mechanisms of the host tissue response to these materials are still unclear, although recent work suggests that a combination of physical and chemical factors is involved.[6] Furthermore, surface form appears to influence the bioreactivity of these materials.[7] In consequence, a broad based study of cellular and tissue reactions to calcium phosphate materials has been initiated in an attempt to describe more precisely the nature of the host response to these biomaterials. The initial phase of this study was designed to quantify the inflammatory potential, differential cellular response and bioreactivity of these materials.

Tissue culture assessment of biocompatibility and biotoxicity has been used in medicine for many years, but has not yet gained widespread use in dentistry, although Kawahara *et al.*[8] recommended the use of such techniques for the testing of dental materials.

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In 1973, Spangberg[9] introduced the Cr^{51} release assay as a means of cytotoxicity testing for dental materials using fibroblast cell lines, the amount of radioactivity released being directly proportional to the degree of call lysis. This type of assay has recently been modified to allow the use of primary macrophages for the measurement of cytotoxicity of mineral dust samples.[10]

Peritoneal inflammation following injection of foreign materials is a well known phenomenon and the cellular response to injection of mineral dust samples has been shown recently to be highly repeatable,[10,11] providing quantitative data on the ability of materials to recruit leukocytes as part of this local reaction. Neutrophils form a characteristic part of acute inflammatory responses, as do macrophages. However macrophages are part of the mononuclear phagocytic system which is also implicated in the generation of hard tissue resorbing cells. The peritoneal cavity test was used in this study to provide quantitative information on inflammatory potential of the calcium phosphate materials and their ability to recruit the different leukocyte populations.

This paper presents the responses to three calcium phosphate bioceramics and calcium phosphate powders when tested in these two assay systems. The results were then discussed in the light of the known clinical response to these materials, especially their local toxicity, inflammatory potential and long-term stability in the tissues.

Materials and methods

Peritoneal cavity inflammation test

Materials, as supplied, were suspended in sterile phosphate buffered saline (PBS) at a concentration of 5mg/ml and 0.5ml (2.5mg) was injected into the peritoneal cavity of groups of three male syngeneic C57B16 mice, 10–12 weeks old. (The Calcitite and Interpore specimens were ground in pestle and mortar to achieve a particle size equivalent to the other test materials.) Two days later the mice were killed by ether overdose and the peritoneal cavity lavaged with 3×2ml volumes of PBS + 10U/ml of Heparin. Cells were stored on ice prior to counting and preparation of cytocentrifuge smears, which were stained with Diff-Quik (Merz-Dade, Dudingen, Switzerland) for differential counting. The total numbers of macrophages, neutrophils, lymphocytes and other granulocytes were calculated from the total and differential counts for each animal[10] and then averaged for each material. The data was tested for significance using a 'T' test which allows for

Table 1 Materials and suppliers

Test materials	Physical form	Chemical form
Calcitite (C)	Dense sintered ceramic	Hydroxyapatite
Interpore (I)	Porous sintered ceramic	Hydroxyapatite
Synthograft (S)	Porous sintered ceramic	Tricalcium phosphate
Dense hydroxyapatite (H*)	Powder	Hydroxyapatite
HA 130 (H)	Powder	Hydroxyapatite
TCP118 (T)	Powder	Tricalcium phosphate
Control materials	**Physical form**	
Titanium oxide	Rutile form powder	
ST×1	Montmorillonite clay powder	
Test materials suppliers		
Calcitite	Calcitec Inc., California, USA	
Interpore	Interpore, International, Irvine CA, USA	
Synthograft	Johnson & Johnson, East Windsor NJ, USA	
H* (Dense hydroxyapatite)	British Charcoal and MacDonald, Greenock, UK	
HA130 and TCP118	Albright and Wilson Ltd, Oldbury, UK	
Control material suppliers		
Titanium oxide	Tioxide Ltd, Stockton-on-Tees, UK	
ST×1	Source Clays Repository, University of Missouri, USA	

differences in variance (Welch test) between the samples compared.

Chromium^{51} release cytotoxicity test

Four materials were tested (TCP118 and H* were not included owing to their non availability at this time) against two different cell types:

1) Human embryonic fibroblasts (HEF cells) which were supplied by the Virology Department, Edinburgh University. Following explant these cells had been passaged seven to eight times in Eagle's medium containing 5% foetal calf serum and penicillin/streptomycin. They were maintained in the same medium during testing.
2) Mouse peritoneal macrophages were obtained by peritoneal lavage of C57B16 mice with 3 × 2ml volumes of sterile PBS. The cells were stored on ice until counted and then resuspended in RPMI medium with 10% FCS, and maintained in this medium for the course of the experiment.

Suspensions of each cell type were adjusted to 5×10^6/ml and dispensed in 100μl (5×10^4 cells) aliquots into plastic 96-well tissue culture plates. The plates were incubated at 37°C (5% carbon dioxide) for four hours, and then 7.4KBq of Cr^{51} labelled sodium chromate (Amersham International, Amersham, UK) in 100μl of the relevant medium was added to each well. The plates were incubated for 24 hours and the wells washed with three changes of PBS to remove free activity in the supernatant. The wells were each then filled with 200μl of medium containing 10μg of the test powder, control wells were filled with 200μl of medium, and incubated for 24 hours. Twenty-four wells were used for each material and 24 for each set of controls. At the end of this period the supernatant in the wells was harvested using the Skatron harvesting system. The cells in the positive control wells were lysed with phenol to release the total activity into the supernatant prior to harvesting.

The activity in the supernatant from each well was measured in a Wallac GTL 300–500 Gamma counter and the mean for each group calculated. Significance levels were calculated using a Student's t-test separate variance estimate.

Results

Cell recruitment test

Leukocyte recruitment to the peritoneal cavity in response to the titanium dioxide negative control exhibited a very low level response, whereas the response to the toxic positive control showed a high level of macrophage and neutrophil recruitment. The macrophage and neutrophil results for the test materials showed significant differences (see Table 2 for '*P*' values) from the negative control for tricalcium phosphate (TCP) and hydroxyapatite (HA) powder materials, but not for the sintered ceramic forms. The increase for TCP powder compared to TiO_2 was substantial and highly significant, indicating that this material has considerable inflammatory potential. The response to the sintered ceramic materials suggested that TCP had a marginally lower activity than HA, but these differences were not significant. The lymphocyte response to all the materials was, with one exception, similar to that of the negative control. The exception, HA Dense (H*) powder, produced a lymphocyte response significantly greater than that of the negative control and

Table 2 Leukocyte recruitment test: mean cell counts $\times 10^6$ (SE)

Material	Macrophages	Neutrophils	Lymphyocytes
TCP 118 Powder (T)	11.30 (1.28)*	4.00 (0.18)***	0.54 (0.18)
HA 130 Powder (H)	8.95 (0.38)**	2.99 (0.63)*	0.33 (0.17)
HA Dense Powder (H*)	8.47 (1.56)	2.99 (0.42)*	1.26 (0.18)*
Interpore (I)	7.11 (1.09)	1.67 (0.28)	0.81 (0.36)
Calcitite (C)	6.90 (1.11)	0.93 (0.21)	0.18 (0.06)
Synthograft (S)	5.66 (0.51)	1.79 (0.05)	0.48 (0.03)
Control −ive (TiO_2	5.59 (0.44)	0.94 (0.25)	0.30 (0.06)
Control +ive (ST×1)	15.12 (3.00)	13.14 (4.42)	0.29 (0.06)

Sig. *$p< 0.05$, **$p<0.01$, ***$p<0.001$ v Control −ive

the other HA powder. The numbers of basophils and eosinophils recruited by each of the test materials and controls were below 0.2×10^6. The data are displayed in Table 2, and Figures 1–3.

Chromium^{51} release cytotoxicity assay

The results for the two cell types were similar, suggesting the same ranking for the test materials. The only significant difference was between dense HA ceramic (calcitite) and the negative control using macrophages test cells. The results suggest that porous forms of sintered ceramics, particularly HA, reduce isotope release in this test below that seen for negative controls, but this effect was not significant. The difference between isotape release for dense HA ceramic and porous HA ceramics was significant at the 5% level. All the materials tested showed low levels of toxicity compared to the positive control. The data are displayed in Table 3, and Figures 4 and 5.

Discussion

The cell types used in the cytotoxicity tests, macrophages and fibroblasts were chosen because of their importance in early post implantation wound healing.[12] The low levels of toxicity found for the sintered ceramic test materials with both cell types corresponds well with clinical findings[5,6] that bioceramics of calcium phosphate are well tolerated when used as implant materials.

The negative control used in the cell recruitment test was titanium oxide a material widely used in the food and cosmetic industries, with a known low cell and tissue toxicity.[10] Indeed titanium metal or its alloys are now widely accepted as ideal metallic implant materials, because their surfaces are covered with a tenacious film of this oxide which has excellent tissue compatability. The reaction seen to this material in the mouse peritoneal cavity is considered to represent the baseline response. The positive control is a clay mineral (Montmorrilonite) of known high toxicity.[13]

The speed and quality of the early tissue healing around an implant material will not only be influenced negatively by the degree of surgical trauma and the toxicity of the material, but also, particularly with materials of low toxicity, by any positive ability the material may have to recruit cell types which are active in wound healing. The peritoneal cavity assay provides an indication of the ability of materials to recruit leukocyte populations to their site of implantation, to take part in the early postimplantation tissue response. The sintered ceramic materials tested all provoked moderate numbers of macrophages and much

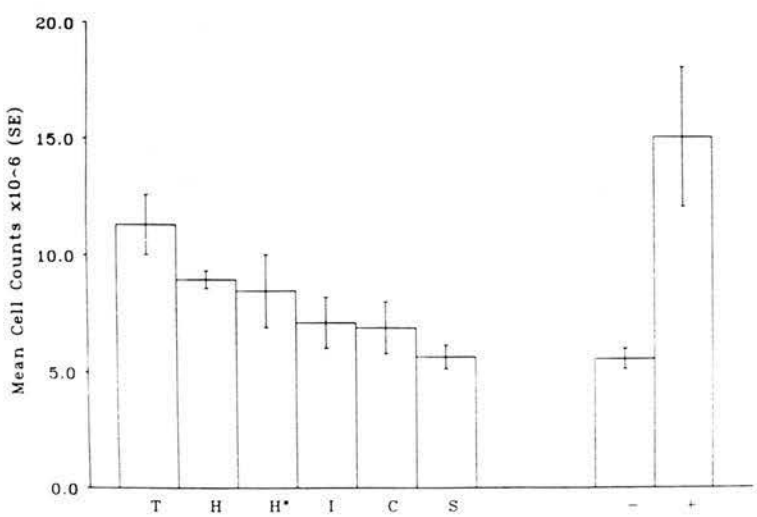


Figure 1

lower numbers of neutrophils. This suggests that these materials generally have a small inflammatory action. However, the potential of a material to recruit macrophages may be of further significance when considering bioceramics, since both macrophages and osteoclasts, the cells responsible for hard tissue resorption, are derived from a common precursor – the circulating monocyte.

The chemistry, surface topography and particle size of these materials as supplied was not identi-

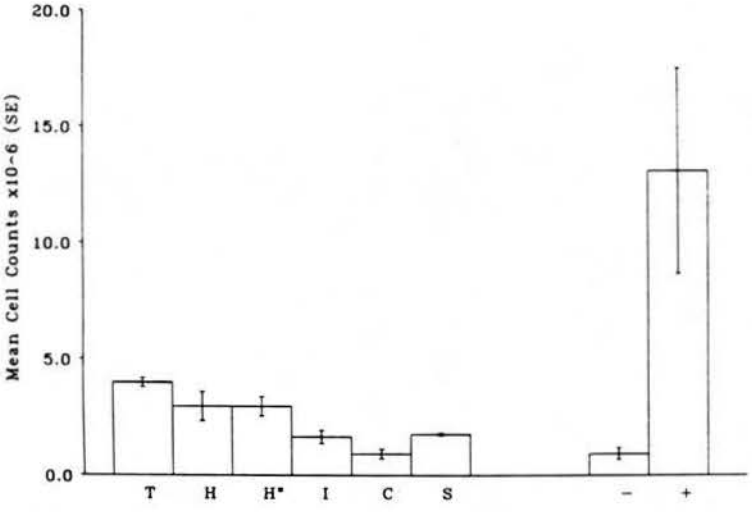


Figure 2

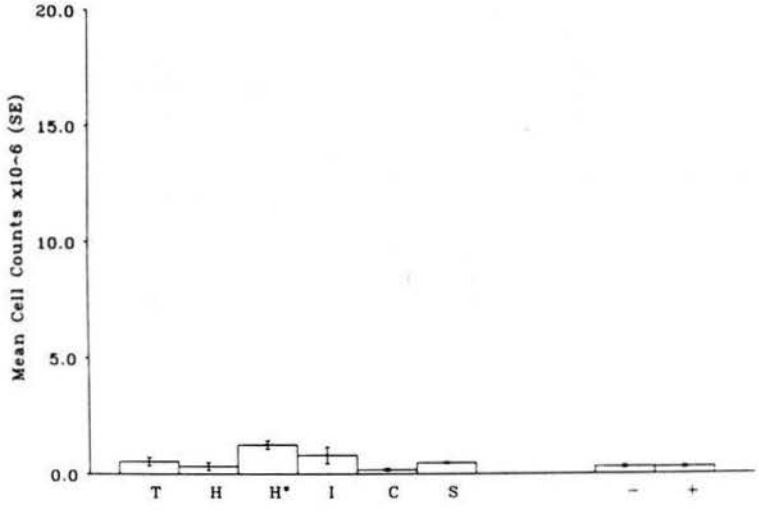


Figure 3

Figures 1–3 Bar charts showing mean (±SE) differential cell counts 2 days following injection into peritoneal cavity. T = TCP118 Powder; H = HA130 Powder; H* = HAst powder; I = Interpore; C = Calcitite; S = Synthograft; – = Control –ive (TiO_2); + = Control +ve (ST×1).

cal. Our aim in this study, however, was to examine the biological response to the materials in the form in which they are supplied for clinical use. Future work will be aimed at finding which of the aforementioned characteristics is the major determinant of biological activity. We therefore confine ourselves, at present, to describing the biological response to the materials in the forms commonly provided for in clinical dentistry.

No clear correlation was seen between the known resorbability of the materials tested and their ability to recruit macrophages. This may

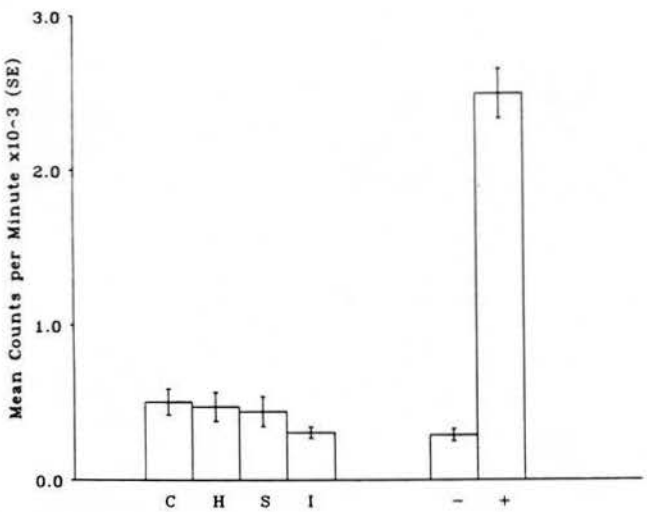


Figure 4

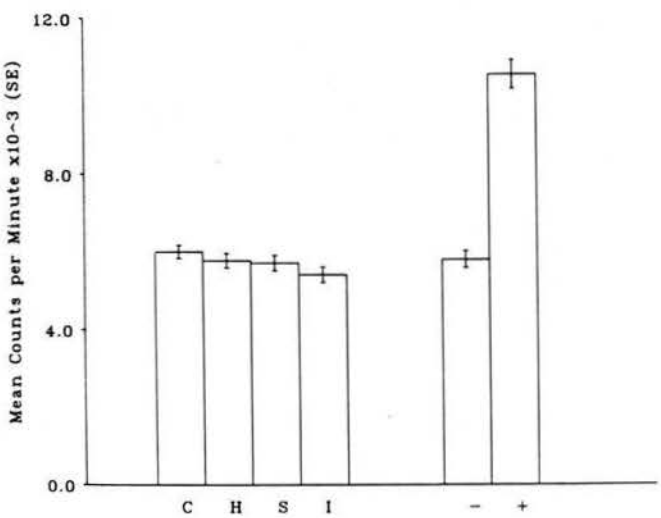


Figure 5

Figures 4 and 5 Bar charts showing mean (+SE) Cr^{51} release after 24 hours exposure to the tested materials. C = Calcitite; H = HA130 Powder; S = Synthograft; I = Interpore; – = Control –ive; + = Control +ve (Phenol).

well be due to the short time period used (two days); if a longer time period had been used the early inflammatory response could have waned, allowing any potentially resorptive monocytic response to become more apparent. Work is currently in progress to explore this possibility.

Table 3 Cr^{51} release assay: mean counts per min × 10^3 (SE)

Material	Fibroblasts	Macrophages
Calcitite (C)	6.01 (0.17)	0.508 (0.085)*
HA130 Powder (H)	5.78 (0.19)	0.477 (0.094)
Synthograft (S)	5.72 (0.20)	0.446 (0.097)
Interpore (I)	5.42 (0.20)	0.309 (0.037)
Control −ive (no mat.)	5.82 (0.22)	0.292 (0.040)
Control +ive (phenol)	10.60 (0.37)	2.500 (0.161)

*$p<0.05$ v Control −ive

The results from the cytotoxicity assay for both macrophages and fibroblasts show a similar ranking for the tested materials. The results suggest, however, that the macrophage is the more sensitive cell type. The only test material established as being in any way cytotoxic to macrophages in this study was dense HA ceramic and this material of all those tested is the least resorbable when used clinically. Cytotoxicity to cells of the monocytic series in areas of hard tissue genesis could be expected to manifest itself as suppression of osteoclast activity locally around the implanted material. This could provide part of the explanation for the known resistance to resorption of dense HA sintered ceramic.

The lack of any significant cytotoxicity to fibroblasts correlates well with the known osteoconductive properties of these ceramic materials, since fibroblasts and osteoblasts have a similar ontogeny. The results for porous HA ceramic hint that this may even enhance fibroblast survival in the test system. Any similar effect following implantation would enhance wound healing and encourage early bone formation.

When the results from the two test systems are compared, the low cytotoxicity established in the Cr^{51} release assay indicates that the cell recruitment observed in the mouse peritoneal cavity, by the same materials, is unlikely to be solely due to material toxicity and may therefore indicate a weak, noninflammatory macrophage recruitment response. Neither test system was able to detect any marked difference in the response to porous ceramics when compared with dense ceramics; the pore sizes in these materials are very large on a cellular scale and so may not be significant to the effects detected in cell cultures.

The inflammatory response to the powder materials tested was greater than that seen for the ceramics; this suggests that the sintering process by which the powders such as these are converted into ceramics effectively reduces their inflammatory potential. The difference must be due to the change in physical form as a result of sintering, since the materials are chemically similar. These results give biological credence to the histological evidence[5,7] that the physical form as calcium phosphate can alter the nature of the host response.

The lymphocyte response in the peritoneal cavity test was very small, with the exception of HA*, which suggests that this particular preparation contains a weakly antigenic contaminant. This finding emphasizes the importance of only using materials for clinical implantation which have been thoroughly tested and found to be free of undesirable side effects.

Further work is currently in progress to characterize the response of a third cell type: osteoblasts to different forms of calcium phosphate. These three cell types macrophage, fibroblast and osteoblast, are those sequentially involved in post-implantation wound healing in bone.[12] It is therefore important to assess the response of these three cell types to extend our understanding of the host reaction to calcium phosphate, particularly the influence of physical form.

Conclusions

1) Calcium phosphate materials were not cytotoxic in this study with the exception of dense HA ceramic (Calcitite) which was mildly cytotoxic to macrophages.
2) The inflammatory stimulus produced by all the sintered ceramic materials tested was low; in contrast all of the powder materials produced more inflammation in the mouse peritoneal cavity than the sintered materials.
3) Both of the test systems used showed that sintered ceramics of calcium phosphate have high biocompatability; this finding correlates well with clinical experience.

Acknowledgements

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EFFECTS OF PRODUCTS FROM INFLAMMATORY PULMONARY NEUTROPHILS ON ALVEOLAR MACROPHAGE CHEMOTAXIS, SPREADING, AND THYMIDINE INCORPORATION

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Abstract—Since macrophages and neutrophils are found together in the alveolar region of the lung during alveolar inflammation, we assessed whether neutrophil products could influence three key functions of alveolar macrophages: chemotaxis, spreading, and thymidine incorporation. Neutrophils were obtained from the lungs of rats treated by intratracheal instillation of heat-killed *Corynebacterium parvum* and cultured overnight, alone or in the presence of zymosan, PMA, an inert particulate (titanium dioxide), or a toxic dust, (quartz). Supernatants were collected from these cells and a lysate, obtained by freeze-thawing neutrophils, was also used. Neutrophil supernatants caused a slight reduction in chemotaxis and a significant loss of ability to spread on glass which varied depending on the in vitro treatment of the neutrophils. In addition neutrophil supernatants also had a substantial effect in stimulating uptake of thymidine which was, once again, very dependent on the treatment of the neutrophils during preparation of the supernatants, with unstimulated and TiO_2-treated neutrophils producing maximum stimulation. The increases in thymidine uptake were not matched by increased proliferation, suggesting that another signal may be necessary for expansion of alveolar macrophage numbers during alveolar inflammation.

INTRODUCTION

Neutrophils are found in the alveolar spaces during inflammatory lung disease (1, 2) and in smokers (3). Neutrophils may be present in variable proportions ranging from a modest increase in smokers (3), to more than 10% in some asbestos workers (4), and exceeding 50% in adult respiratory distress syndrome (ARDS) (5). Neutrophils possess powerful effector functions that can influence other cells (6–11), and it is possible that neutrophils could affect the normal

defensive functions of alveolar macrophages (12) in ways that could exacerbate the alveolitis or enhance macrophage defensive functions. We therefore set out to assess the effect of secretory products from neutrophils, derived from inflamed rat lung, on normal rat alveolar macrophage functions. In a previous paper (13) we described the failure of neutrophil supernatants, generally, to significantly modulate two important functions of alveolar macrophages: phagocytosis and oxidant production. We report here on the effect of neutrophil supernatants on alveolar macrophage chemotaxis, spreading, and uptake of thymidine. Supernatants were prepared by incubating pulmonary neutrophils alone or with a number of potential "triggers." This neutrophil-enriched population can be triggered to undergo an increased metabolic burst as we have previously described (14), which is likely to generate important modulatory molecules.

MATERIALS AND METHODS

Animals. Syngeneic, SPF/PVG rats obtained from the Institute of Occupational Medicine animal unit and more than 12 weeks old were used throughout. To obtain a neutrophil-rich bronchoalveolar lavage population, rats were injected intratracheally with 1.4 mg of heat-killed *Corynebacterium parvum* (Wellcome, Beckenham) 16 h prior to lavage.

Bronchoalveolar Lavage. The rats were killed by Nembutal (Ceva, Watford) overdose and the lungs removed prior to lavage with 4 × 10 ml volumes of saline at 37°C with massage to increase the yield.

Preparation of Supernatants. The neutrophil-rich (70–90%), 16-h *C.parvum*-elicited bronchoalveolar leucocyte population was placed in 75-cm^2 flasks (Sterilin, Feltham) at 10^6 cells/ml (15×10^6 cells total) in MEM + 10% heat-inactivated fetal calf serum (Gibco, Paisley, U.K.) or F10 medium (Gibco) + 2% bovine serum albumin (BSA) (Sigma, Poole, U.K.). The following stimulating agents were added to separate flasks: 0, titanium dioxide, rutile form (500 μg/ml, Tioxide Limited, Stockton-on-Tees, U.K.), zymosan (500 μg/ml, Sigma), DQ_{12} standard quartz (500 μg/ml), and phorbol myristate acetate (PMA, 1 μg/ml, Sigma). The cells were incubated for 24 h and supernatants collected, centrifuged to clarity to remove particles, and stored at −70°C. In addition a lysate was prepared from an equal aliquot of cells by disrupting the cells with three cycles of freeze–thawing (−70°C to 37°C) followed by centrifugation and then storing at −70°C. In some experiments PMA was incubated overnight in complete medium in the absence of neutrophils to assess the effect of PMA alone.

Assay of Chemotaxis. Chemotaxis was assessed using Blindwell chambers. Two hundred microliters of the chemotaxin solution, normally zymosan-activated serum (1 mg/ml of serum; 37°C for 1 h, zymosan centrifuged out) were placed in the lower compartment of the chamber and a 5-μm nuclepore (Pleasanton, California) filter placed on top. The top part of the chamber was then screwed down, and 6×10^5 control alveolar macrophages in 400 μl of F10 + 2% BSA or neutrophil supernatant prepared in F10 + 2% BSA were placed in the top compartment. The chambers were then incubated for 3.5 h at 37°C in 5% CO_2. Cells in the upper compartment were then pipetted gently up and down before decanting. The filter was removed, washed with two dips in phosphate-buffered saline (PBS), and stained with Diff-Quik (Merz-Dade, Dudingen, Switzerland). The filters were allowed to dry before mounting in plastic mountant under a cover slip. The number of migrated cells in five high power fields (×1000) was assessed for each filter, and two chambers were set up for each experimental condition.

To check that true chemotaxis was occurring and to assess random movement (chemokinesis), a modified "checkerboard" analysis was performed with 5% ZAS in upper, lower, or both compartments.

Spreading Assay. Bronchoalveolar cells were suspended at 10^6/ml in F10 + 2% BSA or neutrophil supernatant that had been prepared in F10 + 2% BSA, and 100 μl of cells placed on 6 × 22-mm alcohol-clean, cover slips on racks. Cover slip cultures were incubated for 1 h at 37°C in 5% CO_2. The cover slips were then washed vigorously in saline to remove nonadherent cells, fixed in methanol, and stained in Diff-Quik. After allowing them to dry in air, the cover slips were inverted on a drop of mountant to obtain a permanent preparation. The maximum diameter of 200 spread macrophages was determined using a microscope interfaced with a digitizing board and microcomputer as described by Donaldson et al. (13).

Uptake of [^{3}H]Thymidine. Alveolar macrophages were incubated at 5 × 10^5/well in quadruplicate microtiter plates for 1 h to allow adherence. The nonadherent cells were removed by washing and F10 + 2% BSA or neutrophil supernatant prepared in F10 + 2% BSA was added in 100 μl, to triplicate wells. After four days, [^{3}H]thymidine (0.25 MBq, 10 μl) was added to each well and the cells harvested with a cell harvester 24 h later onto glass fiber paper. These were allowed to dry, and the incorporation of [^{3}H]thymidine measured by liquid scintillometry in a β counter.

In a number of experiments, counts of cells were carried out in parallel with the experiments measuring uptake of [^{3}H]thymidine. Alveolar macrophages (5 × 10^5) were added to 30-mm petri dishes (Sterilin, Feltham) and allowed to adhere for 1 h before washing to remove nonadherent cells. Medium was then replaced with F10 + 2% BSA or neutrophil supernatant prepared in F10 + 2% BSA. After five days of incubation, the cells were removed with a plastic cell scraper and the total cells counted.

Statistical Analysis. Data from repeat experiments were examined by analysis of variance, and suspected differences in the means of treatment groups were analyzed by *t* test.

RESULTS

Chemotaxis. Before testing for the ability of neutrophil supernatants to modulate macrophage chemotaxis, we tested whether any of the neutrophil supernatants contained chemotactic activity. This was necessary because, since the assay was performed with alveolar macrophages in these supernatants in the upper compartment and the chemotaxin (zymosan-activated serum, ZAS) in the lower compartment, any chemotactic activity in the upper chamber would tend to reduce the chemotactic gradient. As shown in Table 1, the level of chemotaxis demonstrable in the neutrophil supernatants was negligible; the response to the ZAS was, however, very large, proving that the assay system was working effectively. Both zymosan and PMA, however, caused some stimulation of chemotaxin release by neutrophils, but this was substantially less than that produced by ZAS and was considered unlikely to interfere with the chemotaxis assay. The effect of neutrophil supernatants on the ability of the alveolar macrophages to chemotact is shown in Figure 1. This reveals that all of the supernatants caused some inhibition of chemotaxis by alveolar macrophages compared to medium alone. This only attained statistical significance, however, with

TABLE 1. Chemotactic activity in the neutrophil supernatants both unstimulated and stimulated in vitro with the stipulated triggers. Results $\bar{X}$ (SEM) in 10 high power fields.

Trigger present	Chemotaxis
O	0.7 (0.2)
Zymosan	2.6 (0.4)
PMA	5.5 (0.6)
TiO_2	0.8 (0.2)
Quartz	1.1 (0.3)
ZAS	86.3 (3.7)
Medium alone	0.1 (0.1)

zymosan-stimulated supernatant, which caused a 32% reduction ($P < 0.05$) and with the PMA-stimulated supernatant, which caused a 69% reduction ($P < 0.001$).

Modified "checkerboard" analysis revealed the vast majority of the migration to be due to chemotaxis and only a small proportion was chemokinetic movement.

Since we had previously described the inhibitory effect of PMA alone on several macrophage functions, we suspected that the inhibitory effect of the PMA supernatant could have, in fact, been due to PMA carried over. We there-

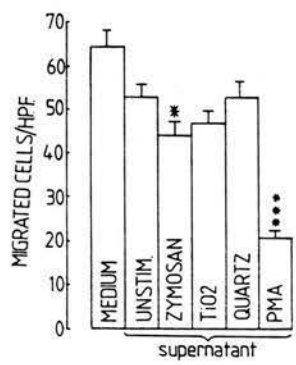


Fig. 1. Migration of alveolar macrophages towards zymosan activated serum; alveolar macrophages in medium alone or supernatants from neutrophils, untreated or treated with the agents indicated. Results represent mean ± SD of 10 HPF in 2 separate chambers for each condition in 3 separate experiments. Significant reductions from medium alone—*$P < 0.05$; ***$P < 0.001$.

TABLE 2. Chemotaxis of alveolar macrophages in medium or medium + 1 μg/ml PMA. Results × (SEM) of 10 high power fields in 2 separate experiments.

Treatment	Chemotaxis
Medium	53.2 (5.0)
Medium + PMA	32.0 (4.0)

fore tested for a direct effect of PMA on alveolar macrophage chemotaxis. Table 2 demonstrates that there was indeed a marked (40%) reduction in chemotaxis caused by the presence of PMA alone. It is likely therefore that the reduction caused by the PMA supernatant was mediated to a large extent by the PMA carried over in the supernatant and was not attributable to a neutrophil product produced by PMA-stimulated neutrophils.

Macrophage Spreading. When alveolar macrophages were allowed to spread on glass for 1 h in the presence of neutrophil supernatants, there were significant effects with some of these supernatants in inhibiting spreading (Figure 2). While unstimulated supernatant caused a slight decrease in spreading, this was not significant; among the supernatants from neutrophils stimulated with the various agents, zymosan, quartz, and PMA treatment all caused inhibition of the ability of macrophages to spread. In the case of PMA stimulation, however, we suspected that the PMA itself could be responsible for the inhibition and so PMA alone was tested for its ability to affect spreading. This revealed that PMA in medium caused significant reduction ($P < 0.001$) in

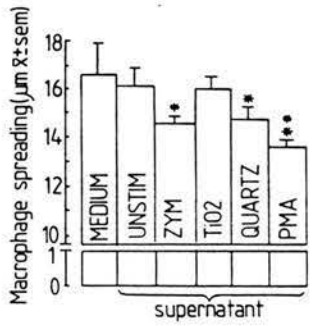


Fig. 2. Spreading of alveolar macrophages on glass for 1 hour in the presence of medium or neutrophil supernatants obtained by treatment with the indicated agents. Results represent mean ± SD of 3 separate experiments. Significant reductions compared to medium alone:—*$P < 0.05$; **$P < 0.01$.

spreading of alveolar macrophage compared to medium alone: medium, 14.70 ± 0.3; medium + PMA, 13.30 ± 0.2 (results expressed as mean ± *SEM* of 200 cells counted).

Figure 3A shows the size distribution of macrophages allowed to spread on glass following treatment with medium alone or supernatant from neutrophils treated with quartz; Figure 3B shows the effect, on the size distribution, of treating the alveolar macrophages with supernatant from PMA-treated neutrophils. In both cases the effect of neutrophil supernatants was to substantially increase the proportion of cells in the smaller size categories and reduce the number in the higher size categories.

Uptake of [^{3}H]Thymidine. Figure 4 illustrates the uptake of [^{3}H]thymidine by alveolar macrophages treated with the various neutrophil supernatants for five days. It is clear that the addition of supernatant from unstimulated neutrophils (marked UNSTIM 0 in Figure 4) caused a large-scale significant increase in [^{3}H]thymidine uptake by the alveolar macrophages. Furthermore, release of this stimulatory product by neutrophils was significantly and substantially decreased by the presence, during the preparation of the supernatant, of zymosan, quartz, or PMA but was unaffected by presence of TiO_2. Figure 5 shows the uptake of [^{3}H]thymidine by alveolar macrophages treated for five days with varying dilutions of control neturophil supernatant. There is clear evidence of a dose-dependent stimulation of [^{3}H]thymidine uptake. Lysate caused a stimulation of thymidine uptake by alveolar macrophages which was less than that

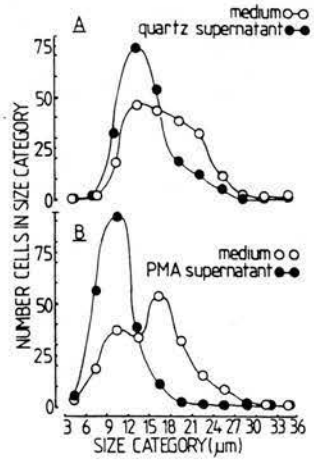


Fig. 3. Size data from the macrophage spreading assay showing macrophages in different size categories. (A) comparison between medium alone and supernatant from PMN treated with quartz. (B) comparison between medium and supernatant from PMN treated with PMA. Total of 200 cells counted in each case.

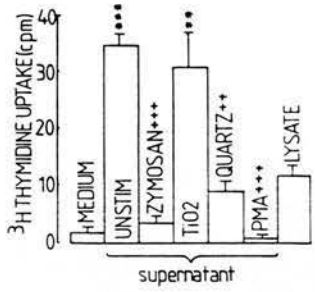


Fig. 4. Uptake of ^{3}H thymidine by alveolar macrophages treated with supernatants from neutrophils treated with the indicated agents. Significant increase compared to medium alone $^{**}P < 0.01$; $^{***}P < 0.001$. Significant reduction compared to treatment with supernatant from unstimulated neutrophils. $++P < 0.01$; $+++P < 0.001$. Results represent mean ± SEM of quadruplicate wells in 3 separate experiments.

produced by unstimulated neutrophils (Figure 4). In two experiments supernatants were added to macrophages and, along with uptake of [^{3}H]thymidine, total cell numbers were assessed after five days of incubation. These experiments revealed that while the uptake of thymidine increased by up to 3.5-fold in these experiments (190 ± 13 cpm to 857 ± 60 cpm, mean ± *SEM* of five replicate wells in two separate experiments), the cell numbers remained exactly the same (control 3.8 ± 1.8 × 10^5/plate; supernatant-treated 3.8 ± 1.9 × 10^5/plate).

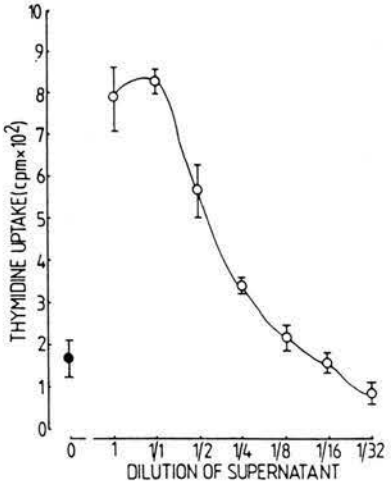


Fig. 5. Uptake of ^{3}H thymidine by alveolar macrophages treated with various dilutions of supernatant from unstimulated neutrophils. Results represent mean ± SEM of quadruplicate wells.

TABLE 3. Cell numbers ($\times 10^4$) after addition of PMN supernatant; results given as mean (SD) of duplicate plates in 2 separate experiments.

Dilution of Supernatants							
1	1/1	1/2	1.4	1.8	1/16	1/32	MEDIUM
5.2 (0.4)	4.0 (1.5)	3.8 (1.3)	3.2 (0.4)	3.4 (0.8)	3.6 (0.8)	3.5 (1.0)	3.8 (1.8)

Furthermore, in experiments analogous to those shown in Figure 5, with dilution of supernatant, there was no concommitant increase in cell numbers (Table 3).

DISCUSSION

During different diseases, and in smokers, neutrophils are found in the jlveolar space where they could influence the defensive functions of alveolar macrophages; such effects could be to enhance macrophage functions or inhibit them. The results described here form part of a study to examine the effects, using a rat model, of secretory products produced by neutrophils derived from inflamed lung, on the functions of normal alveolar macrophages. In a companion paper (14), we described the secretory products from pulmonary neutrophils failed to affect alveolar macrophage oxidative metabolism and had no substantial effects on phagocytosis.

In the present paper we report on the effects of pulmonary-derived neutrophil secretory products on alveolar macrophage chemotaxis, spreading, and [^{3}H]thymidine uptake in vitro. All of the neutrophil supernatants caused slight inhibition of chemotaxis. In the case of the PMA-stimulated neutrophil supernatant, however, the reduction in chemotaxis was substantial but, as we have reported for other macrophage functions (14), experiments with PMA alone revealed the inhibition to be caused by PMA itself and not by a PMA-stimulated neutrophil product. It is not possible to say whether the small decrease in macrophage chemotactic activity produced by neutrophil supernatants is biologically significant. In pulmonary infection, a slight decrement in the ability of macrophages to chemotact caused by neutrophil products may be minor in comparison to the overall benefits gained from the presence of neutrophils in the alveolar region with consequent increase in microbicidal activity. One previous study (9) reported failure of a neutrophil product to affect macrophage chemotaxis, although no data were supplied. In that study acute peritoneal exudate

guinea pig neutrophils also produced a factor that stimulated macrophage phagocytosis at 10 min but not by 30 min. We consider this to be unlikely to have substantial biological significance in host defense.

Loss of ability to chemotact may be important, however, in inflammation due to mineral dusts, where we have described that macrophages from pathogenic dust-exposed lung have decreased ability to chemotact (15). This loss of chemotactic activity was greatest where inflammation was greatest and consequently where greatest numbers of neutrophils were present in the alveolar region (15).

Spreading on glass is another surface-related function of macrophages that is a measure of the activity (13). Once again all the supernatants caused inhibition of spreading; this was only significant in the case of zymosan, quartz, and PMA, which reduced spreading by 2–3 μm. It seems likely that the reduction in spreading is related to the reduction in chemotaxis, since both require membrane mobility. In the case of PMA, the inhibition of spreading was not substantially less than that produced by zymosan or quartz although it was anticipated, from the results of PMA alone on other parameters of macrophage activity, that PMA might substantially inhibit spreading. It was notable that PMA supernatant did not cause spreading of alveolar macrophages nor did PMA alone although PMA is known to cause spreading of peritoneal macrophages *in vitro* (13).

Neutrophils have been reported to secrete products that cause a range of target cells to take up increased levels of thymidine (7, 8, 10, 11). We report here that the supernatants from resting neutrophils cause substantial enhancement of uptake of [^{3}H]thymidine by alveolar macrophages. However, we noted that the production of the stimulating factor(s) was completely inhibited by the presence of zymosan, quartz, or PMA during the preparation of the supernatants; it was not, however, effected by the presence of TiO_2, the inert dust. Most strikingly, however, the uptake of thymidine was not correlated with increases in cell number. Thus the neutrophil secretory product caused thymidine incorporation, but did not result in cells entering the M phase of the cycle. Furthermore, phagocytosis of zymosan or quartz prevented production of the signal while phagocytosis of TiO_2 did not. The significance of these effects, if any, for normal defense in the inflamed alveolus is not obvious. However, signals that initiate DNA synthesis in macrophages have been previously reported in inflammatory exudates, which would very likely contain secretory products of neutrophils (16, 17). In addition, other workers have described that two different stimuli are required for resting macrophages to commence uptake of thymidine (18, 19). We consider it is likely that the uptake of [^{3}H]thymidine by macrophages shown by Bird et al. (11) in the presence of neutrophil supernatants may not have been accompanied by cell division, and certainly no counts of cell number were included in the paper. In another report, using lung-derived

epithelial cells, increased uptake of [^{3}H]thymidine in the absence of increases of total DNA suggests that thymidine uptake in the absence of DNA synthesis can occur (20).

Overall these results have revealed that products secreted by neutrophils in inflamed alveoli could influence macrophages by causing slight slowing of chemotactic responses, probably by affecting the cell membrane and its associated structures, as revealed by the decreased spreading caused by the supernatants. The relevance of these effects, and the likely importance in vivo, is questionable since the effects were small, even at equivalents of high neutrophil–macrophage ratios. The ability of neutrophil supernatants to stimulate thymidine uptake was of particular interest, possibly reflecting some stimulation of macrophage activity. The relationship of this to the expansion of macrophage numbers in inflamed alveoli is unclear, but a second signal to the macrophage, arising in the inflammatory milieu of the alveolitis, could be sufficient to cause the macrophages to divide and thus contribute to the increased numbers found in the alveolar region during inflammation.

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CHAPTER

6

Bronchoalveolar Lavage in the Assessment of the Cellular Response to Fiber Exposure

KENNETH DONALDSON AND GERALDINE M. BROWN

I. Bronchoalveolar Lavage

The intention here is to review the use of bronchoalveolar lavage (BAL) in assessing and understanding the cellular response of the lung to fiber exposure. Most of the toxicology of the early response to fibers in the lung can be studied only in animals and so this chapter will concentrate in large part on animal studies. Human studies will, however, be described where they are available. It is not appropriate here to describe, in detail, the technique of BAL as the method has been described fully in recent reviews of its application in humans (Crystal *et al.*, 1986; European Task Group on BAL, 1989; American Thoracic Society, 1990) and experimental animals (Henderson, 1984; Subcommittee on Pulmonary Toxicology, 1989). The lavage fluid contains a sample of the cells and other substances lining the airspaces and these can be assessed using standard procedures. In humans the technique can be modified to sample either airway or alveolar cells (Robinson *et al.*, 1988), but in small laboratory rodents the entire bronchoalveolar space is sampled.

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II. Sources of Variability in the Use of Bronchoalveolar Lavage

In humans, the technique of bronchoalveolar lavage is subject to sampling problems due to limitations imposed by the selection of only small areas of the lung. The total surface area lavaged is not accurately known since the dilution of epithelial lining fluid by instilled saline is variable. The procedure is therefore standardized by normalizing the constituents of the lavage fluid to a single constituent such as albumin (Gee and Fick, 1980) or, more recently, urea (Rennard *et al.*, 1986). No method of expressing human lavage constituents can be ideal because of the possible confounding effects of focal alterations in permeability, which could alter the concentration of the denominator. The use of control subjects in human studies can serve to standardize the method to some extent but does not completely circumvent the problem of knowing whether there has been adequate sampling of the lung periphery. Nevertheless, the use of BAL was instrumental in the initial findings on the role of inflammation in the pathogenesis of interstitial lung disease (Crystal *et al.*, 1981) and the technique is still of considerable value as a research tool, although its role in diagnosis has been questioned.

Use of experimental animals has the advantage that the entire lung can be removed and lavaged, so reducing sampling variability and eliminating some of the problems encountered in human studies; control animals can be used to obtain standard levels of BAL constituents. All BAL studies are, however, limited by the assumption that a lung exposed to toxic substances or one that is diseased may be different in lavageability from a normal lung. The benefits that accrue from the use of experimental animals are offset by the problems of species differences and the relevance of the findings to human disease processes.

In practice, other sources of variability are inevitably present and these can influence the yield and proportions of cellular and biochemical BAL constituents. The yield and activity of leukocytes obtained by bronchoalveolar lavage have been shown to be related to the microbial status of the animals. Starling and Balish (1981) showed that germ-free rats had significantly fewer macrophages, with lower amounts of lysosomal enzyme activity, than conventionally housed rats. Furthermore, when the germ-free rats were exposed to bacteria either in their food or by being housed under conventional conditions for 2 weeks, the numbers and activities of alveolar macrophages increased. The anatomy of the lung differs between species and this can alter the site and deposition of inhaled dust (Warheit and Hartsky, 1990); this, in turn, is likely to influence the cellular response. The numbers of cells recovered in the

BAL are related to age of the animal (Brain and Frank, 1968) and so this factor must be taken into account in any long-term studies. Maximum recovery of bronchoalveolar leukocytes can be obtained by using lavage solutions warmed to 37°C, which are free of divalent cations such as calcium and magnesium (Brain and Frank, 1973). Gentle massage of the resected rodent lung during the lavage process can also dramatically increase the yield of cells (Brain, 1970). With increasing numbers of lavage washes, the recovery of cells is greater but the number of cells obtained with each wash is progressively less. It might be assumed that the macrophages obtained early or late during the lavage process might have different characteristics, but this appears not to be the case (Lehnert and Morrow, 1984). Care must be taken in the quantification of BAL leukocyte populations since there may be selective loss of certain cell types during the preparation procedure (Saltini *et al.*, 1984). In most cases these problems of variability can be overcome by adhering to a standard protocol and taking account of the limitations of the technique.

III. Relevance of Bronchoalveolar Lavage Leukocytes to the Leukocyte Population of the Interstitium and to Pathological Processes

In addressing the use of BAL in fiber-related lung toxicity, one important limitation of the technique should be highlighted since it qualifies all interpretations of the results obtained. That caveat is that the constituents of the BAL do not necessarily reflect the events occurring within the interstitium, where most of the pathological lesions/changes occur (Haslam *et al.*, 1980). However, in some studies, the bronchoalveolar lavage cell population has been shown to reflect the interstitial population (Semenzato *et al.*, 1985; Weissler *et al.*, 1986) and to correlate with the stage of the disease process (Crystal *et al.*, 1981).

Macrophages from the bronchoalveolar space and from the interstitium of untreated rats do differ in superoxide anion production, enzyme activity, and phagocytic activity (Drath *et al.*, 1982). Differences in macrophage function also exist in human lung between the alveolar and the interstitial populations (Weissler *et al.*, 1986). In rats exposed by inhalation to the nonfibrous pathogenic dust, quartz, clear differences were evident between alveolar and interstitial macrophages with regard to growth factor production and enzyme secretion (Sjostrand *et al.*, 1991). The same is likely to pertain in fiber-exposed lung.

In fiber-related lung disease, changes in the alveolar leukocyte popu-

lation are likely to be of relevance to the disease process, since the initial insult arises from the deposition of fibers in the alveolar space. Our own data suggest that fiber-exposed alveolar leukocytes initiate tissue damage, possibly by mediating epithelial injury (Donaldson *et al.*, 1988b), thus permitting passage of fibers, alveolar macrophage-derived injurious products (Brown and Donaldson, 1988), and growth factors (Bauman *et al.*, 1988) into the interstitium, leading to inflammation and fibrosis. Once interstitial inflammation is instituted, then the interstitial leukocyte population may become an additional source of injurious mediators.

IV. The Use of Bronchoalveolar Lavage in Evaluating the Toxic Effects of Fibers in the Lung

There are two main uses of BAL in the toxicological assessment of mineral fibers. The first relates to its use in detecting a toxic effect; the second is concerned with using BAL data to understand the mechanism of the toxic effect. These two aims are obviously interdependent, since an understanding of mechanism allows a rational choice of the most appropriate marker of toxicity. In the field of mineral fiber toxicology, considerable progress has been made in using BAL for both purposes. The vast majority of this work has been focused on asbestos and the early work confirmed the ability of asbestos fibers to cause an inflammatory response in the lung in both occupationally exposed humans and experimentally exposed animals. This was followed by a period in which BAL yielded important information on the kinetics of response, on the mechanisms underlying the accumulation of leukocytes in the lung following asbestos fiber exposure, and also on the role of these cells in mediating tissue damage and, ultimately, the disease processes that lead to fibrosis and cancer. Hopefully, the technique will find increasing use in the toxicology of man-made fibers in the future.

V. Bronchoalveolar Lavage Leukocytes from Fiber-Exposed Lung

A. Numbers and Proportions of Leukocytes

The use of BAL to obtain cells from the airspaces of fiber-exposed animals has allowed a substantial understanding of the effects and results of such exposure. Many studies have demonstrated changes in the cellular

profile of the BAL. In humans the comparisons tend to be among controls, asbestos-exposed individuals, and patients with asbestosis. Accurate data on the type of fiber to which the workers have been exposed and the extent of the exposure are seldom available. The general finding is that patients with asbestosis have more severe indices of inflammation, such as increased total cells or the presence of granulocytes, than asbestos-exposed individuals and that both groups have more inflammation than controls. The effect of smoking, which causes an increase in both alveolar macrophages and neutrophils, has to be taken into consideration but the effects of fiber exposure are, generally, evident in both smokers and nonsmokers, and so the effects of cigarette smoke will not be dealt with here. Within the expanded leukocyte population present in the bronchoalveolar space, the increase in macrophages is a constant finding and may be due to monocyte migration and local cell division (Spurzem *et al.*, 1987). Increased representation of lymphocytes is also a common occurrence (Spurzem et al., 1987; Costabel *et al.*, 1986; Garcia *et al.*, 1989; Gellert *et al.*, 1985), but the most dramatic effect is to be seen in the neutrophil population. A substantial increase in neutrophils has been described in many (Jaurand *et al.*, 1980; Hayes *et al.*, 1988; Garcia *et al.*, 1989; Delclos *et al.*, 1989; Gellert *et al.*, 1985) but not all (Bignon *et al.*, 1978) human studies and there is more of an increase in patients with asbestosis than in asbestos-exposed workers (Hayes *et al.*, 1988; Garcia *et al.*, 1989). In one BAL study of man-made mineral fiber (MMMF)-exposed workers, 3 out of 12 of the workers showed an increase in the total lavageable cells. The authors, however, described no "inflammatory cells" (Forni *et al.*, 1989). Increased levels of eosinophils have been described in the BAL of asbestos-exposed and asbestotic individuals (Xaubet *et al.*, 1986; Robinson *et al.*, 1988a; Delclos *et al.*, 1989), but this is not a finding in all studies.

In animal models of asbestos fiber exposure the increases in BAL leukocytes have, in general, been confirmed under more strictly controlled conditions of exposure with single, well-defined fiber populations. Exposure to asbestos has resulted in increased numbers of retrievable leukocytes in Begin's sheep model, in which between 100 mg and more than 1 g of asbestos were instilled into sheep lungs via a bronchoscope (Begin *et al.*, 1983a). Begin has also utilized the model to demonstrate that other fibrous minerals, such as carborundum (Begin *et al.*, 1989) and attapulgite (Begin *et al.*, 1987), are capable of causing a sustained increase in the inflammatory cell profile of the BAL over months following bolus instillations. Although the drawbacks of the instillation approach are discussed elsewhere in this book, the use of good controls, such as latex and graphite, which show low activity, helps to strengthen this work.

The mouse has also been used to study the BAL response to inhaled fibers. Bozelka *et al.* (1983) described an increase in the total cell number

and in the neutrophils in mice inhaling 10 mg/m^3 of standard UICC chrysotile. Adamson and Bowden (1987a,b) extended work on the mouse using the instillation approach and demonstrated sustained neutrophil alveolitis following the instillation of up to 0.5 mg of a long fiber sample of amosite. However, the use of a short sample of the same mineral was associated with a transient, resolving inflammatory response in the BAL, although macrophage numbers remained elevated. This supported previous inhalation (Davis *et al.*, 1986) and instillation (Lemaire, 1985) studies in rats and intraperitoneal inoculation studies in mice (Donaldson *et al.*, 1989), correlating increased fiber length with increased inflammogenicity and pathogenicity.

The mouse has also been used to study the response to glass fiber (Johns Manville Code 100) and showed an inflammatory response following intratracheal instillation (Lombard and Hubbard, 1990). Although the inflammation was transient as judged by the proportion of PMN, the macrophages still appeared activated by Day 30, as judged by visual scoring of cells stained for acid phosphatase.

The rat has been most widely used in studies of the effect of fibers on the lung probably because it is the standard animal in toxicology work. Donaldson *et al.* (1988a) exposed rats to 10 mg/m^3 of UICC chrysotile and amosite for 7 hr a day, 5 days a week, for up to 75 days. As shown in Fig. 1 the cells obtained by BAL were loaded with fibers readily identifiable by light microscopy. The effect of this deposition of fibers was a late, modest increase in the number of macrophages (Fig. 2), but this was accompanied by a substantial early recruitment of neutrophils (Fig. 3); the inflammation was sustained over a further 2 months when the rats were breathing normal air. Short-term exposure to a high airborne mass concentration of fibers and the pattern of response that follows cessation of exposure, as just described, may provide an insight into the potential pathogenicity of a dust. The ability of fiber to continue to deliver "injury" to the lung could be an important factor in explaining (or predicting) its potential to cause disease (Vincent and Donaldson, 1990). Using this approach Warheit *et al.* (1991) have demonstrated transient airspace inflammation in response to wollastonite inhalation but prolonged lung damage with a similar length exposure to asbestos.

Donaldson *et al.* (1987) studied the effect of instillation of 2.5 mg of a ceramic fiber, aramid fiber, and UICC chrysotile on the short-term inflammatory response in the rat. The proportion of neutrophils was approximately the same for all three fiber types at 2 days, being three to four times more than that produced by the inert, respirable-sized dust titanium dioxide (TiO2). Lemaire (1985) instilled a short and a long sample of chrysotile and found a resolving neutrophil alveolitis with the short sample while the long produced a neutrophilia in the lavage that continued for up to 21 days.

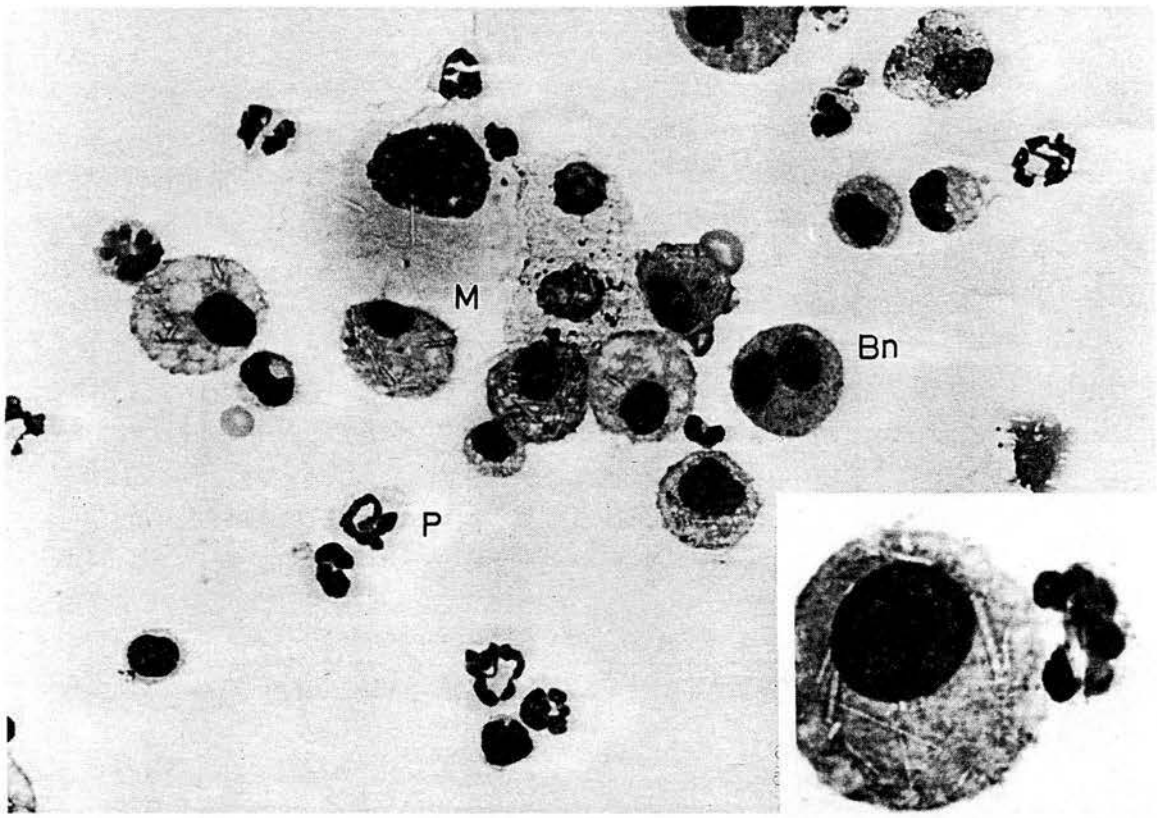


Fig. 1 Light microscope appearance of cells lavaged from the lungs or rats that had been inhaling UICC amosite asbestos (10 mg/m^3) 7 hr per day, 5 days per week, for 2 months. A group of large and vacuolated macrophages (M) can be seen with phagocytosed fibers present inside them (see inset). Polymorphonuclear neutrophils (P) can be seen, indicating inflammation, as well as a binucleate macrophage (Bn). Original magnification ×40, inset ×100.

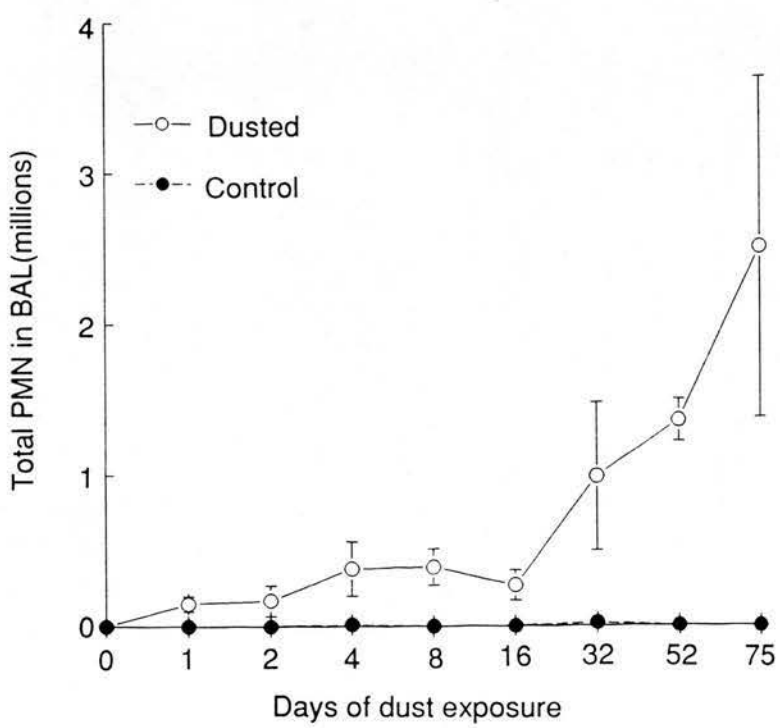


Fig. 2 Total numbers of macrophages in bronchoalveolar lavage fluid (BAL) during inhalation exposure to 50 mg/m^3 of amosite asbestos. A modest increase in macrophage numbers recovered by lavage is measured over time of exposure. Results show mean of four rats; bars indicate ± 1 SD.

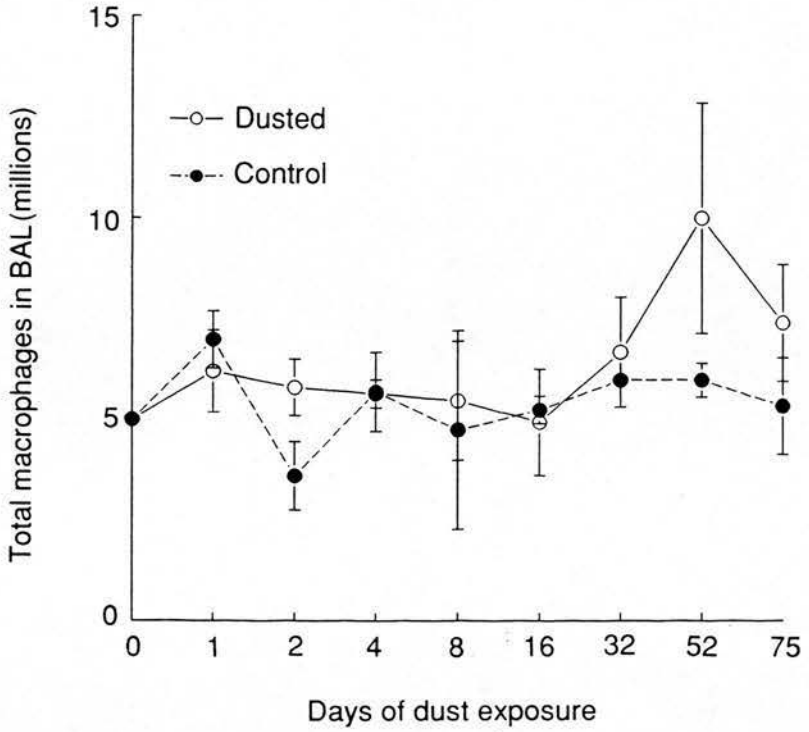


Fig. 3 Total numbers of PMNs (neutrophils) in bronchoalveolar lavage fluids (BAL) during inhalation exposure to 50 mg/m^3 of amosite asbestos. A substantial recruitment of neutrophils into the lung is observed after 16 days of exposure. Results show mean of four rats; bars denote ± 1 SD.

Kagan *et al.* (1983) exposed rats to airborne UICC crocidolite and chrysotile for up to 18 months and described a reduction in the total number of lavageable leukocytes, although there were increases in the proportion of PMN, suggesting an inflammatory response. The latter study contrasts, in general, with the majority of studies described earlier, which demonstrated recruitment of inflammatory cells to fiber-exposed lung.

B. Mechanisms of Recruitment

The recruitment of inflammatory leukocytes to sites of inflammation is largely influenced by chemotactic gradients. Leukocytes migrate from the vascular space, following the gradient, and accumulate at the inflammatory focus. Several BAL studies in animals and humans have demonstrated sources of chemotactic activity in the bronchoalveolar region of fiber-exposed lung. In sheep exposed to chrysotile asbestos, the release of chemotactic activity for PMN by BAL leukocytes was increased in a dose-dependent manner (Rola-Plezczynski *et al.*, 1984). Increased release of chemotactic activity for PMN by BAL macrophages was also evident in hamsters exposed to chrysotile asbestos (Glassroth *et al.*, 1984) and

by Hayes *et al.* (1988) in a clinical study of asbestotics. This activity may be partly due to release of LTB_4 (Garcia *et al.*, 1989) or fibronectin (Rom *et al.*, 1987; Begin *et al.*, 1986a).Kagan *et al.* (1983b) showed that alveolar macrophages from rats exposed to UICC chrysotile and crocidolite asbestos released increased chemotactic activity for macrophages and suggested that such activity might account for the accumulation of macrophages at sites of asbestos deposition in the lungs. A further mechanism governing the accumulation of macrophages and PMN at sites of asbestos fiber deposition is likely to be direct activation, by fibers, of complement components in the lung lining fluid, with the production of the chemotaxin C5a (Warheit *et al.*, 1985). The demonstration of increased protein in BAL fluid at time points at which increased chemotaxin was also demonstrated suggests a mechanism by which sufficient levels of complement components may be sustained in fiber-damaged lung (Warheit, 1986). Corroborative evidence of the importance of C5a in leukocyte recruitment to fiber-exposed lungs was provided by the reduced recruitment of leukocytes in rats treated with cobra venom factor to deplete circulating complement (Warheit, 1985). Increased levels of chemoattractant activity in BAL fluid have been demonstrated in BAL of chrysotile asbestos-exposed sheep (Rola-Plezczynski *et al.*, 1984) and in asbestos workers (Garcia *et al.*, 1989). Evidence that leukocyte recruitment is important in the inflammatory response to fibers comes from the work of Oberdorster *et al.* (1984), who showed an influx of monocyte-like cells following intratracheal instillation of UICC amosite asbestos. Besides recruitment of leukocytes from the pulmonary circulation, total numbers of macrophages in the BAL in fiber-exposed lung may be augmented by local cell proliferation. In asbestos-exposed subjects both newly recruited monocytes and proliferating macrophages were demonstrated in the BAL (Spurzem *et al.*, 1987), indicating that both mechanisms are involved in the accumulation of macrophages in asbestos-exposed lung. In rats instilled with UICC amosite asbestos the increase in BAL macrophages was also due, in part, to an influx of monocyte-like cells (Oberdorster *et al.*, 1984). Paradoxically, while chemotactic gradients are present in fiber-exposed lung, the alveolar macrophages from fiber-exposed lung may be impaired in their ability to respond to the gradient (Donaldson *et al.*, 1990).

C. Immunological Functions

The important role of leukocytes in immune responses, and the role of immunopathological processes in disease have led to the use of BAL to study the effect of fibers on immune responses and associated leukocyte activities in the lung. Mineral fibers are not antigenic by normal criteria

so there can be no classical immune responses to the fibers. However, foreign surfaces can activate complement so there could, in theory at least, be opsonization of fibers with C3b and recruitment of immune cells by C5a. The inflammatory response that ensues may then cause nonspecific activation of immune cells through release of cytokines leading to T-cell-mediated inflammation. Cytokine-mediated increased expression of MHC Class 2 molecules at the surface of immunocompetent cells may also provide the conditions for enhanced immunological reactivity and autoimmune phenomena. Many studies have described a quantitative increase in BAL lymphocytes following exposure to fibers in humans (Spurzem *et al.*, 1987; Wallace *et al.*, 1989; Garcia *et al.*, 1989; Costabel *et al.*, 1986; Gellert *et al.*, 1985), mice (Bozelka *et al.*, 1987), rats (Donaldson *et al.*, 1988a), and sheep (Begin *et al.*, 1981). This alone does not prove that immunological phenomena are occurring since secondary infection following dust-mediated injury could produce this effect.

Bronchoalveolar leukocytes are generally suppressive to immune responses (Kusaka *et al.*, 1990); this is understandable in view of the undesirability of immune response occurring in the fragile tissues of the alveolar region. Immune response in the lung is generally confined, therefore, to the bronchus-associated lymphoid tissue and the mediastinal nodes. Lymphocytes are, however, present in the BAL and these are predominantly T cells. Following human asbestos exposure, analysis of the T cell subsets has revealed an increase in the T4 : T8 (helper : suppressor) ratio in three studies (Costabel *et al.*, 1987; Delclos *et al.*, 1989; Wallace *et al.*, 1989). An increase in this ratio produces an environment in which immunological activity will tend to be increased and where a T4 cell product, lymphokine, is likely to be produced in increased amounts, favoring inflammation and fibroplasia. Other studies have tended to confirm immunostimulation in asbestos-exposed workers as measured by increased soluble IL2 receptors in the BAL (Delclos *et al.*, 1989) and increased production of interferon-γ by cultured BAL cells (Robinson *et al.*, 1988). One human study, however, reported that exposure to asbestos for more than 5 years was associated with a reduced T4 : T8 ratio, suggestive of a generalized suppressive environment in the lung of asbestos workers (Gellert *et al.*, 1985).

In the rat model, asbestos inhalation is associated with increased expression of MHC Class 2 (Ia) antigen on the surface of alveolar macrophages (Miller, 1979; Kagan *et al.*, 1989). Since incubation of alveolar macrophages with asbestos *in vitro* failed to cause enhanced expression of Ia in the latter study, then it must be the environment of a fiber-exposed lung that is responsible for the increased expression of Ia. Functional support for this hypothesis has come from studies in the rat (Miller, 1979) and sheep (Stankova *et al.*, 1989), where the normally immunosup-

pressive activities of BAL are reversed, becoming immunostimulatory following fiber exposure.

A role for the immune response in restraining the fibrogenic response in fiber-exposed sheep was suggested by Begin *et al.* (1988). In asbestos-exposed sheep receiving the immunosuppressive drug cyclophosphamide, the inflammatory response in the BAL was increased compared with sheep receiving asbestos alone. While the BAL profile showed clear worsening of the inflammation with cyclophosphamide treatment, the authors described accompanying bronchopneumonia. This infection could have had the effect of enhancing the inflammatory/fibrotic process in the drug-treated group and so raises a question as to the validity of the conclusion that immunosuppression favors fiber-induced fibrosis.

D. Production of Growth Factors and Cytokines

Asbestosis is characterized by the derangement of lung structure and in particular an overgrowth of connective tissue, leading to diffuse interstitial fibrosis; in addition small airways fibrosis is also found. Other fibers would be expected to produce the same patterns of pathological change. The mechanisms governing the increase in connective tissue molecules in fibrotic lesions and fibrosed airways are not elucidated but interstitial fibroblasts are likely to play a major part in the disease process through secretion of collagen and other connective tissue molecules. The turnover of connective tissue that occurs in normal lung is thought to be mediated largely by fibroblasts, which secrete both connective tissue molecules and the proteinases that can degrade connective tissue. The development of fibrotic lesions in inflamed lungs may therefore arise from alterations in fibroblast function, leading to a derangement in this delicate balance between extracellular matrix production and degradation.

A mechanism whereby stimulation of fibroblasts could occur in fiber-exposed lung is the increased secretion of the cytokines, tumor necrosis factor (TNF), and interleukin 1 (IL-1) by BAL leukocytes. In rats instilled intratracheally with amosite asbestos there was increased secretion of both IL-1 and TNF by BAL macrophages (Brown *et al.*, 1991b). Secretion of TNF was greater with a long fiber sample than with a short fiber sample of amosite asbestos, which is consistent with the well-documented relationship between fiber length and pathogenic potential. Increased secretion of TNF has also been reported for BAL leukocytes from mice exposed to chrysotile asbestos (Bissonette *et al.*, 1989).

Further evidence that altered fibroblast function may contribute to the pathogenesis of fiber-associated lung disease has been provided by studies showing modulation of fibroblast function *in vitro* by BAL

leukocytes. Lemaire *et al.* (1986) demonstrated that BAL macrophages from rats dosed intratracheally with chrysotile asbestos released a factor that stimulated fibroblast proliferation and that these animals went on to develop lung fibrosis. BAL macrophages from sheep exposed, by intratracheal instillation to UICC chrysotile asbestos, were also shown to release fibroblast-stimulating activity (Lemaire *et al.*, 1983). Fibronectin is one of the secretory products of macrophages that acts as a competence factor in the cell cycle and enhances the activity of growth factors. BAL macrophages from sheep exposed to asbestos consistently secrete enhanced levels of fibronectin compared with controls (Begin *et al.*, 1986b) and so, in these animals, the capacity of the lung fibroblasts to respond to growth factors may be enhanced. BAL macrophages from patients with asbestosis also showed increased secretion of fibronectin but, in asbestos workers with no disease, the levels were similar to controls.

The importance of BAL macrophage-derived growth factors in the pathogenesis of fiber-associated lung disease is, however, equivocal. Adamson and Bowden (1990) evaluated the secretion of fibroblast stimulating activity by BAL macrophages from rats exposed intratracheally to long fiber and short fiber crocidolite asbestos. BAL macrophages from rats exposed to short fiber asbestos secreted factors that enhanced both proliferation and secretion of collagen by fibroblasts in culture but there was no evidence of a fibrotic response in these animals. Conversely, exposure to long fiber crocidolite caused no increase in the secretion of fibroblast growth factors by BAL macrophages, but did induce pulmonary fibrosis. The authors contended, from these results, that growth factors released into the airspaces of the lung may not reach the interstitium to interact with fibroblasts, but may be excluded by the epithelial barrier or may be inactivated by blocking factors in the lung-lining fluid. They conclude that stimulation of fibroblast growth factor release by interstitial macrophages might be far more important than the release of growth factor by BAL macrophages. The ability of fibers to cross the epithelium may thus be an important descriptor of their pathogenicity.

E. Injurious Effects of Bronchoalveolar Lavage Leukocytes

The association between fiber deposition in the lung and the accumulation of inflammatory leukocytes has been well documented, as described previously. The beneficial effect of leukocyte recruitment is obvious in the clearance of potentially harmful particles. However, there is now a substantial body of work in support of the hypothesis that inflammatory leukocytes themselves play a major role in disease processes. The evolved function of the phagocytes is a host defense against microbial agents but, although their bactericidal properties are beneficial to the

host, they can also prove to be harmful, through the extracellular release of toxic products such as oxidants and proteinases. Damage to epithelial cells could be particularly important in leading to prolonged inflammation (Donaldson *et al.*, 1988b).

The evidence for proteinase-mediated tissue damage in fiber-exposed lung is derived from studies showing increased levels of proteinases in BAL fluid and BAL leukocytes following fiber exposure. Lysosomal acid proteinases can degrade connective tissue molecules (Brown *et al.*, 1991a) and have been implicated in tissue damage in the lung. The acid proteinases are inactive at the pH of normal lung tissue but degranulation within aggregations of inflammatory leukocytes may provide a localized acid milieu. Acid lysosomal proteinases are increased in BAL leukocytes from human asbestos-exposed subjects (Jaurand *et al.*, 1980) and in rats exposed to chrysotile asbestos (Tetley *et al.*, 1976). While Jaurand's study showed no increase in the levels of lysosomal proteinases in human BAL fluid, increased levels of acid proteinase activity in the BAL fluid were found accompanying the inflammatory cell infiltrate into the lungs of rats inhaling UICC chrysotile (Donaldson *et al.*, 1988a). The connective tissue-degrading activity of leukocyte neutral proteinases is also established (Brown and Donaldson, 1988), but little work has been done to investigate the role of the neutral proteinases in fiber-mediated lung damage. Plasminogen activator has, however, received some attention in view of its important roles in the clotting and fibrinolysis cascades. Plasminogen activator was shown to be increased in both BAL fluid and leukocytes from human asbestos-exposed subjects and in sheep exposed to chrysotile asbestos (Cantin *et al.*, 1989).

The potential of inflammatory leukocytes to induce oxidant-mediated tissue damage in fiber-exposed lung tissue was demonstrated by Rom *et al.* (1987) in a study that showed increased spontaneous production of superoxide anion and hydrogen peroxide in asbestos-exposed individuals. However, in rats inhaling chrysotile or crocidolite asbestos, the BAL leukocytes exhibited reductions in both spontaneous and triggered oxidant production at early time points (Petruska *et al.*, 1990). It is not known whether oxidant production by the rat leukocytes would be restored with continued exposure. Further studies into the role of oxidant-mediated injury in fiber-exposed lung are clearly warranted, particularly in view of the possible role of oxygen radicals in carcinogenesis.

VI. Fiber Content of Bronchoalveolar Lavage

BAL may be used to gain information on the fiber burden or asbestos-body burden in the bronchoalveolar space. This information may be of

value in assessing exposure, to some extent, although it does not include the interstitial component of the dust, which could be the most important. Typical of these studies is the presence of a great degree of variability in the number of asbestos bodies within asbestos-exposed groups, ranging from 0 to 6000 asbestos bodies per million lavaged cells (Roggli *et al.*, 1986). In general, however, there are more asbestos bodies in occupationally exposed individuals than in nonexposed groups and more again in those with asbestosis (De Vuyst *et al.*, 1987); more asbestos bodies were found in patients with asbestosis than in those with other types of interstitial fibrosis (Roggli *et al.*, 1986).

Begin (1986c), in his sheep model, has related the prevalence of airways disease alone, or airways disease plus alveolitis, with the amount of asbestos fibers present in the BAL following instillation. Using BAL fiber burden as an index of lung fiber retention there was approximately four times more fiber retained in the BAL of sheep that developed alveolitis plus airways disease than sheep that had airways disease only. This could mean that individuals who can more efficiently clear the fibers from their lungs are less likely to develop alveolitis while small airways fibrosis is the first response to deposition of asbestos. These workers also showed that an increased accumulation of fibers in the BAL occurred in sheep that went on to develop interstitial lung disease compared with those that did not (Begin and Sebastien, 1989). The relationship between fibers in the BAL and retention in the interstitium was not considered in this study but has been addressed in humans (Sebastien *et al.*, 1988), where a reasonable correlation between BAL asbestos body count and lung parenchymal asbestos bodies has been demonstrated.

Wright *et al.* (1986) also addressed the relationship between BAL fiber burden and pathophysiological change in guinea pigs exposed to UICC amosite by instillation. These workers also concluded from their results that airway deposition and inflammatory response precedes the interstitial inflammation, the latter occurring only if a sufficient dose penetrates through the airway walls and enters the interstitium. Of particular interest was the finding that the length and aspect ratio of fibers in the BAL were less than those of the fibers retrieved from the lung tissue itself. This suggestion, that there is a selective retention of long fibers in the pulmonary interstitium with preferential clearance of shorter fibers, has been supported in rats (Kauffer *et al.*, 1987). Such a selective retention of long fibers could, in part at least, explain the relative potency of long fibers, which has been alluded to throughout this report.

A potential mechanism governing the retention of fibers in the lung may be the impaired chemotactic activity of leukocytes following fiber exposure *in vivo* (Warheit *et al.*, 1984; Donaldson *et al.*, 1990). This im-

portant finding has implications for the persistence of both fibers and inflammatory leukocytes in the bronchoalveolar region and could contribute to increased tissue injury by prolonging the inflammatory response to the initial fiber deposition.

VII. The Relevance of Bronchoalveolar Lavage to the Assessment of Fiber-Related Lung Disease

It is important to relate BAL parameters to other measures of the disease process such as lung function, gallium scan, and X ray. Xaubet *et al.* (1986) reported that crackles on auscultation, arterial oxygen pressure, and alveolar–arterial oxygen pressure difference in humans correlated with extent of PMN alveolitis in asbestos-exposed subjects. Begin described reduced total lung capacity, vital capacity, compliance, diffusion capacity, and arterial oxygen pressure in sheep exposed to asbestos by instillation and noted that BAL markers of inflammation correlated highly with decreasing lung function parameters (Begin *et al.*, 1983).

Robinson *et al.* (1986) compared the lavage cellular profile with radiological scores in 29 controls and 27 patients with asbestosis and found no relationship between percentage PMN or eosinophils and radiological evidence of asbestosis, or between BAL profile and abnormal lung function.

In a unique study Begin *et al.* (1986b) assessed the relationship among BAL profile, gallium scan, lung function, and radiography. They concluded that gallium scan could be used as an early marker of macrophage accumulation in the lungs of asbestos-exposed sheep, which preceded detectable radiographic or physiologic changes. A significant increase in BAL fibronectin was the only other index of lung injury that could be detected as early as the gallium change, suggesting that the retention of gallium in the lungs may be related to macrophage activation and fibronectin secretion.

Delclos *et al.* (1989) found increased positivity of the gallium scan among asbestos workers and patients with asbestosis, but there was no relationship between the gallium scan and BAL cell profile, radiology, or pulmonary function tests. Thus it is evident that the BAL profile, while indicating that there is inflammation in the bronchoalveolar region, is probably too nonspecific to correlate well with the extent of the disease. Careful animal studies, such as those of Begin, may be more revealing of the relationship between the BAL profile and the pathological/physiological change. However, the caveat alluded to earlier, concerning the relevance of BAL to the events occurring on the other side of the epithe-

lium, means that the relationship between the BAL profile and the actual pathological processes that cause radiographic or lung function change may never be clear.

VIII. Conclusions on the Use of Bronchoalveolar Lavage to Study the Cellular Response to the Deposition of Fibers

BAL has been used extensively to unravel the complex series of events that occur following the deposition of fibers in the lung, and the study of both human subjects and animal models has contributed to substantial progress in understanding these events. There is general agreement that, if deposition of fibers is sufficient, there is an excess accumulation of leukocytes that can be lavaged from the bronchoalveolar space containing increased proportions of neutrophils; a role for other granulocytes in the subsequent events is by no means yet precluded. Much progress has been made in understanding the mechanisms whereby this leukocyte recruitment occurs. These new leukocyte populations are "activated" as judged by many criteria and have the potential to injure lung cells and extracellular matrix components, stimulate mesenchymal cells, and induce immunological phenomena, which could contribute to disease processes. Although much has been achieved, there remain areas where further work is required. In particular the relevance of the huge data base on the effects of asbestos to the man-made mineral fibers needs to be addressed by focusing research on these asbestos substitutes. Also, the problematic relationship between the BAL changes and the events occurring in the interstitium is in need of further investigation. This relates to the thorny question of the meaning of changes in BAL indices in relation to the type and degree of lung disease, which also need to be further clarified.

Following an initial period of quantification and identification of BAL components from fiber-exposed lung, it is now time for more use of BAL cells and soluble components to study cellular interactions in the alveolar region, however difficult these postlavage *in vitro* studies are. As well as revealing new information on the mechanisms of fiber-induced lung disease, the huge amount of information obtained so far should be used to choose markers for use in testing for pathogenicity of new fibers and in testing hypotheses on the nature of fiber pathogenicity, which entail exposure of animals to special test dust clouds. Such short-term assays, if properly designed and conceived, can provide invaluable information, greatly needed, on the nature of fiber toxicity.

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Hypothesis

Particulate air pollution and acute health effects

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Summary

Epidemiological studies have consistently shown an association between particulate air pollution and not only exacerbations of illness in people with respiratory disease but also rises in the numbers of deaths from cardiovascular and respiratory disease among older people.

Meta-analyses of these studies indicate that the associations are unlikely to be explained by any confounder, and suggest that they represent cause and effect. We propose that the explanation lies in the nature of the urban particulate cloud, which may contain up to 100000 nanometer-sized particles per mL, in what may be a gravimetric concentration of only 100–200 µg/m3 of pollutant. We suggest that such ultra-fine particles are able to provoke alveolar inflammation, with release of mediators capable, in susceptible individuals, of causing exacerbations of lung disease and of increasing blood coagulability, thus also explaining the observed increases in cardiovascular deaths associated with urban pollution episodes.

This hypothesis is testable both experimentally and epidemiologically.

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In the 1940s and 1950s, coal was the major fuel for industrial and domestic use, and cities were frequently affected by winter smogs, when temperature inversions trapped smoke close to ground level and the air contained high concentrations of soot, sulphur dioxide, and other pollutants. One such episode, in London in 1952, was associated with excess deaths from respiratory and cardiovascular causes.[1] Research at that time, recently reanalysed,[2] showed an association between particle and sulphur dioxide concentrations in the air and risks of death and acute respiratory disease. The UK Clean Air Act in 1956 led to reduction in urban pollution and elimination of winter smogs. The causes of the excess deaths were never explained, but it was believed that the problem had been eliminated.

Two facts make it necessary to question this assumption: the increase in motor vehicles on roads world-wide with the consequent rise in exhaust emissions, and evidence of continuing associations between air pollution and ill-health. The former has resulted in a change in the type of pollution in cities, and the latter has shown effects on health at levels of pollution previously considered harmless. Associations have been shown between overall mortality and episodes of respiratory disease, and concentrations of small particles measured in the microgram range. These findings have encountered some scepticism, partly because the concentrations of particles at which effects seem to occur are low by comparison with those to which many people are exposed in industrial workplaces without apparent harm; and partly because no plausible hypothesis has yet been advanced to explain the associations. We propose such an hypothesis.

Studies in fourteen different locations have shown that overall daily mortality increases as the concentration of small particles in the air rises.[3,4] The relationship is present across a wide range of climatic conditions and meta-analysis has shown that atmospheric temperature or other pollutants are unlikely to explain the association.[3] Where specific causes have been examined, a significant excess of cardiovascular as well as respiratory deaths related to particulate pollution has been found.[5,6] The strongest associations are in older individuals and in smokers. There is also evidence linking particulate air pollution with acute attacks of asthma[7] and other respiratory diseases.[8–11]

Particles in the air

Particulate air pollution has been traditionally measured by drawing air through a filter paper and measuring the density of the black stain, the "black smoke method". However, most urban pollution is now derived from vehicle exhausts and is less black. Increasingly, therefore,

is being measured by the mass of particles that pass ough a size-selective orifice with a 50% collection iciency cut-off at 10 μm aerodynamic diameter M10), chosen to represent those particles most likely to ch the lung acinus. Measurements of black smoke or M10 are both expressed in μg/m³, but do not measure ite the same thing. Nevertheless, the epidemiological ation discussed above in general hold true whichever thod is used.

Studies of earlier London urban pollution showed rticles to be predominantly very small; whilst half the ss comprised particles less than 1 μm in diameter, half number were less than 0·1 μm.[12] As many as 150 000 rticles were found per mL. They were predominantly dic, the pH of smog during episodes sometimes being low as 2. There are also large numbers of small rticles in the rural atmosphere, averaging 5000–10 000 r mL and rising to 25 000–30 000 per mL when luenced by traffic 1 km distant.[13] Studies in the UK nes M, Harrison RM, Dept of Environmental ealth, University of Birmingham, UK, personal mmunication), with a condensation-nucleus counter, ve shown particle counts in Birmingham of 00–50 000 per mL, sometimes rising to 100 000 per L. Taking the latter figure, we calculate that during a llution episode each lung acinus could receive on erage some 30 million particles and each alveolus about 00 particles every 24 hours, about 50% being posited. Since ventilation is not evenly distributed, pecially in chronic lung disease, some alveoli would ceive much larger doses.

The chemical composition of urban particulate clouds ries considerably,[14] but the fine fraction, below about 5 μm diameter, to which PM10 approximates, is about lf carbon and half salts, mainly ammonium sulphate d ammonium nitrate. In urban air, the particles are rived mostly from combustion, especially by diesel hicle engines, and the carbon has chemicals adsorbed to its surface. Very small particles, below about 1 μm in ameter, may remain suspended for weeks, whereas ose greater than 2·5 μm are removed by settling and by in in a matter of hours. A cloud of the finest particles ll thus drift for many miles and may cause pollution ross national boundaries. Moreover, very fine particles adily penetrate into buildings, and most indoor vironments are unlikely to provide substantial otection from the risk of inhaling them. Indeed, it has en shown that indoor personal monitoring samples may ntain as much chemical particulate matter as is found outdoor fixed-point sampling in the same general ea.[15]

At present, PM10 is measured in nine UK cities. oncentrations recorded to date indicate average values er a day of around 15–35 μg/m³ with daily maxima of to 70 μg/m³. Meta-analysis of epidemiological data om the USA has suggested that rises of 10 μg/m³ are companied by an increase in relative risk of mortality of out 1% in the exposed population,[3,16] including elevated sks from both respiratory (around 3·4%) and cardiac round 1·4%) causes. The lower percentage increase in rdiovascular deaths represents a higher absolute total, nce these deaths are much more frequent. Other studies ve shown rises in emergency-room visits for asthma and creases in hospital admissions for respiratory diseases hen PM10 rises.[9,16,17] These findings imply an important fect on public health.[11] Much higher concentrations of particles occur in some of the world's largest cities, and here the effects on the health of populations already having a high prevalence of respiratory disease may be particularly important.

In general, studies of industrial cohorts have not shown increases in overall mortality rates even in workers exposed to high gravimetric concentrations of dust.[18,19] Any hypothesis explaining the observed associations between air pollution, morbidity, and mortality must take account of this fact. It must also explain the association of inhaled particles with cardiovascular and respiratory disease.

If epidemiological association between particulate pollution and disease is causal, it is likely that part of the explanation lies in the fact that the general population includes a much higher proportion of people who, by reason of arteriosclerosis or chronic airway disease, are at increased risk of death or exacerbation of their illness when subjected to environmental change than do industrial populations. The other part of the explanation must lie in the nature of the particles. A key point is that the urban pollution cloud comprises predominantly very small acidic particles, many of them in the nanometer range which readily penetrate indoors and persist for long periods in air, whereas industrial dust clouds consist mainly of much larger particles usually formed by the abrasion of rocks.

How particles may cause harm

About 50% of nanometer particles are deposited by brownian movement. Soluble particles will increase in diameter by absorption of water as they pass down the airway, but on reaching the alveoli they make contact with a water-repellant surface and will be handled by the lung's defences like other particles of similar size. Insoluble particles are predominantly carbon, of complex shape and high surface-to-volume ratio, and will be handled similarly. There is evidence that particles below about 100 nm in diameter behave differently from larger respirable ones and a particle which is non-toxic in the micrometer size range may be toxic in the nanometer range. Thus, rats exposed to equal weights of titanium dioxide in two aerosol size ranges, fine and ultra-fine (around 0·25 and 0·02 μm, respectively), retain more ultra-fine particles in the interstitial tissue of the lung, developing a marked airspace inflammatory response.[20,21] This interstitial retention probably arises from the failure of alveolar macrophages to phagocytose the large numbers of small particles, together with the action of surfactant in impelling them into the alveolar space.[22] These studies were carried out at a high airborne-mass concentration, but teflon fume particles, 30 nm in diameter at only 200 μg/m³, have also been shown to cause acute pulmonary toxicity in rats.[23] The teflon-generating system produces more ultra-fine particles than does the titanium dioxide system. It is part of our hypothesis that very small but chemically reactive particles in urban air pollution produce such a reaction in human beings. That transport of chemicals on the surface of particles may be important in the cause of lung inflammation has been shown in recent studies in which iron complexed on the surface of fly-ash particles promoted oxidative lung injury, and this effect was reduced after removal of surface iron by washing.[24]

Whatever the precise mechanism of induction, we propose that alveolar inflammation provoked by ultra-fine

particles, in addition to promoting exacerbations of lung disease, has an additional effect on the coagulability of blood, increasing the susceptibility of individuals to acute episodes of cardiovascular disease. Several haematological factors, including plasma viscosity, fibrinogen, factor VII, and plasminogen activator inhibitor, are not only known to be predictive of cardiovascular disease[25] but also rise as a consequence of inflammatory reactions. Such low-grade inflammation may in part be responsible for the otherwise unexplained variations in fibrinogen and white-cell counts seen in the general population.[26] Low-grade inflammation may be particularly important in altering the coagulability of blood as a result of activation of mononuclear cells in the lung.[27] Activated white cells have been shown to initiate and promote coagulation[28] by releasing tissue factor, which initiates the conversion of factor X to factor Xa, the first reaction of the final common clotting pathway.[29] Alveolar inflammation may also cause the release of interleukin-6 from macrophages and thus stimulate hepatocytes to secrete fibrinogen.[30]

The hypothesis

We propose that acidic ultra-fine particles characteristic of air pollution provoke alveolar inflammation which causes both acute changes in blood coagulability and release of mediators able to provoke attacks of acute respiratory illness in susceptible individuals. The blood changes result in an increase in the exposed population's susceptibility to acute episodes of cardiovascular disease; the most susceptible suffer the most.

This hypothesis, being based on the number, composition, and size—rather than on the mass—of particles, accounts for the observed epidemiological relations, and explains excess deaths occurring in people who may be indoors, and why similar serious effects are not seen in industrial workers exposed to much higher concentrations of dust measured by weight. By drawing attention to the possible effects of alveolar inflammation on blood coagulability, we propose an explanation of the observed and hitherto unexplained association of pollution episodes with cardiovascular deaths.

The hypothesis is testable epidemiologically, by investigating relations between blood coagulability and changes in air-particulate concentrations; and experimentally in rats by studying the airspace inflammation induced by ultra-fine particles and its effects on mediator release and coagulation factors.

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RESPIRABLE INDUSTRIAL FIBRES: PATHOLOGY IN ANIMAL MODELS

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Abstract—This paper discusses the general efficacy of the rat as a model for studying the pathogenicity of respirable fibres in humans. Rats show differences in lung structure from man and these could influence the pulmonary response to some degree, but they do develop interstitial fibrosis, carcinoma and mesothelioma when exposed to respirable fibres and these same types of lesions are found in fibre-exposed humans. Because of the importance of long fibres in causing pathological change, a substantial number of long respirable fibres must be used in inhalation studies if the relative pathogenicity of different fibre types is to be assessed. From data on the fibre burden in human disease and information on build-up of fibres in rat studies in a range of different fibres, a 1 year exposure to a cloud of 200 fibres ml^{-1} minimum, preferably more, is recommended. The rate of dissolution of the fibres within the lung milieu will be an an important factor in determining the pathogenicity.

Many questions remain unanswered as to the mechanism of lung pathogenicity caused by respirable fibres particularly in the areas of durability, fibre shape, fibre surface chemistry and the exact mechanism, at the cellular level whereby fibres can cause pathological change. The use of rat inhalation studies, properly conceived and designed, remain a key approach whereby these questions can be answered.

INTRODUCTION

THIS paper addresses the efficacy of the rat as a model to study the diseases which fibres provoke in humans. This involves a review of the species difference in lung structure and pathological response. It also examines the important size ranges and airborne fibre concentrations necessary for the development of pathology.

SPECIES DIFFERENCES IN LUNG STRUCTURE

The aspects of lung structure that vary between species and which could influence the outcome of animal studies and, in particular, their relevance to the human situation, have been extensively studied and reviewed by Warheit recently (WARHEIT *et al.*, 1988, 1993; WARHEIT, 1989; WARHEIT and HARTSKY, 1990). These studies have emphasized the differences between species with regard to the sequence of events following exposure to airborne particles. The present review will consider principally the comparison between the rat, the animal of choice for inhalation studies with fibres, and the human.

A primary consideration, addressed elsewhere, is the obligatory nose breathing of rats which differs from the mouth and nose breathing found in humans; the relationship this has to deposition is addressed elsewhere in this review. The next important consideration is the actual pattern of branching that is found in the airways. In humans this pattern is 'dichotomous' with the bifurcation in a central position with regard to the flow and the two branches coming off at similar angles (bipodal branching). In laboratory rodents, however, the branching is assymmetrical with a major daughter from each bifurcation that continues along a roughly central path with minor daughters that

branch off at regular intervals (monopodal branching). The number of branches from the trachea to the terminal bronchiole is variable but similar in the rat and the human, being 10–20 (WARHEIT, 1989). If fibres deposit in the bronchioles, they encounter cell types which are similar in the rat and human but which differ in proportion (WARHEIT, 1989). The pattern of the branching appears to influence the regional deposition within the rat lung suggesting that there may be important differences in the detailed sites of deposition between the rat and the human, where the branching pattern is quite different.

In the centriacinar region the most striking difference between rats and man is the absence of a respiratory bronchiole in the case of rats. Unlike the human, the rat terminal bronchiole opens out into alveolar ducts (WARHEIT and HARTSKY, 1990). These then divide to form the alveolar duct bifurcations where the majority of particles deposit and where the early lesions occur (WARHEIT *et al.*, 1984). In contrast, in the human, fibres are likely to deposit in respiratory bronchioles and their alveoli. Because of this difference in anatomy, the major site of fibre deposition differs between the rat and the human. The impact that this has on the course and development of fibre-related disease between rat and human is unknown.

Once deposited in the alveolar region, the fibres may be phagocytosed by the alveolar macrophages. Species differences in alveolar macrophage function have been noted with regard to morphology, phagocytic activity and migration (WARHEIT *et al.*, 1988). It would not therefore be surprising if there were differences between humans and rats with regard to important macrophage functions.

With reference to mesothelioma development it seems likely that fibre must be transported to the pleura. Nothing is known about the relative abilities of different species to transport fibres to the pleural tissue.

In conclusion, this data on comparative anatomy suggests that there may be differences in the extent, pattern and site of fibre deposition in rats compared to humans. In the upper airways there may be more deposition in the rat than is seen in the human. This is shown by the decreased penetration efficiency to the deep lung in rats as compared to humans (JONES *et al.*, 1993). In the lung periphery, deposition is likely to be centriacinar in both species although the fibre is likely to deposit on different types of bronchiole. No conclusions can yet be drawn about the rat and human with regard to anatomical–physiological differences in the transport of fibres to the pleura.

SPECIES DIFFERENCES IN LUNG PATHOLOGY

Most of the animal data relating fibre inhalation and pathology that have been described here has been obtained from experiments using rats and from all considerations the rat does appear to be the best available animal model. Theoretically primates should provide the best available model for human disease and both baboons and monkeys have been used although cost considerations dictate that only very small numbers of animals can be tested in each experiment. Both monkeys and baboons develop asbestosis following the inhalation of high dust levels and mesotheliomas have also been recorded for both in inhalation studies (WAGNER, 1963; GOLDSTEIN *et al.*, 1983; DAVIS, 1989). Pulmonary carcinomas have not been reported following dust inhalation in primates which may be due to the small number of animals so far reported but also may reflect a naturally low incidence of these tumours in primates when the synergistic effect of tobacco smoke is excluded. Rats certainly develop asbestosis,

pulmonary carcinomas and mesotheliomas following asbestos inhalation (WAGNER *et al.*, 1974; DAVIS *et al.*, 1978) and so do hamsters (HESTERBERG *et al.*, 1992). Mice develop pulmonary asbestosis but their ability to develop pulmonary tumours in response to asbestos inhalation has been difficult to substantiate because many strains have a very high spontaneous incidence of these tumours (LYNCH *et al.*, 1957; REEVES *et al.*, 1974). Mesotheliomas have not been recorded in mice following asbestos inhalation. Guinea pigs, gerbils and rabbits have all developed pulmonary asbestosis in inhalation studies (WAGNER, 1963; REEVES *et al.*, 1974) but asbestos-related lung tumours have not been reported.

Whether or not all recorded differences between animal species regarding asbestos-related disease reflect real variations in biological results or merely the fact that many studies in the past had technical flaws in dust cloud generation and experimental design is uncertain. Recent evidence, however, has demonstrated one set of interspecies differences that may truely reflect a different response to fibres that have been deposited in the pulmonary parenchyma. A large series of inhalation experiments have been undertaken in Switzerland using carefully standardized fibre clouds of asbestos and man-made vitreous fibres (MMVF). Both rats and hamsters were exposed. The rats have responded in a way that appears similar to many previously published studies in that they readily developed pulmonary asbestosis and pulmonary carcinomas but produced only a few mesotheliomas. The hamsters developed less asbestosis and fewer pulmonary tumours but many more mesotheliomas (up to 35% with one ceramic fibre) (HESTERBERG *et al.*, 1992). The most likely explanation of this difference is that hamsters clear fibres from the pulmonary parenchyma more readily than rats but transport a larger proportion to the pleural surfaces. This process needs to be explored in more detail. It cannot be stated that the hamster is a better experimental model of human mesothelioma development than the rat until it is known in which species dust transport comes closest to the human pattern.

One apparent difference is known to occur between the production of asbestos-related pulmonary carcinomas in rats and humans. In rats these tumours develop in peripheral sites within the lung parenchyma and are most frequently adenocarcinomas with the tumour cells exhibiting close structural relationships to type II pneumocytes. In humans, pulmonary carcinomas in asbestos workers occur mainly in the larger bronchial tubes and are usually of squamous type. A possible explanation of this discrepancy may be that most of the human tumours to be considered result from a combination of exposure to asbestos and cigarette smoke with the tumour site corresponding to that for cigarette exposure alone for reasons that are not understood. Peripheral adenocarcinomas of the lung do occur in humans and some pathologists suggest that in non-smoking asbestos workers the tumours are generally of this type (WEHNER, 1989). There is no concensus on this matter, however, perhaps because pulmonary carcinomas are infrequent in asbestos workers who do not smoke.

FIBRE SIZE AND PATHOGENICITY

While fibre dimensions are of great importance in determining which fibres penetrate to the pulmonary parenchyma, not all sizes of fibre deposited in this region are equally pathogenic.

Considerable evidence has been accumulated from experimental studies that the

pathogenicity of fibres vary with their length, the long fibres being the most dangerous. The first work on fibre dimensions was included in the original programme of Gardner with unexpected results. Because it was known that quartz-related pathology increased with a reduction in particle size, Gardner's group included finely ground (short fibre) chrysotile asbestos in their experiments. The shorter fibre length did not merely fail to increase pulmonary fibrosis compared to long fibre material but actually significantly reduced the level of fibrosis in the treated animals (VORWALD *et al.*, 1951). Further substantiation of these findings are found in the work of KING *et al.* (1946), when asbestos cut to different lengths on a special microtome was administered to rabbits by intratracheal injection. Far more pulmonary fibrosis was produced by long fibres (15 μm) than short (2.5 μm). Similar findings were subsequently reported by other groups of workers (KLOSTERKÖTTER, 1973; SZYMCZYKIEWICZ and WIECEK, 1961). That pulmonary fibrosis, resulting from glass fibre administration, depended on fibre length was demonstrated by WRIGHT and KUSCHNER (1977), who used intratracheal injection into rats of specially sized glass fibres.

The importance of fibre length in relation to neoplasia was demonstrated in two laboratories by STANTON *et al.* (1977, 1978), STANTON and WRENCH (1972), POTT (1978) and POTT and FRIEDRICHS (1972). These workers demonstrated that following the intrapleural or intraperitoneal implantation of asbestos and other mineral fibres the development of mesotheliomas was most closely related to the number of fibres in any dust preparation that were >8 μm in length and <0.25 μm in diameter. The type of mineral appeared unimportant in these studies with fine glass fibre being almost as carcinogenic as asbestos and no minerals containing long thin fibres produced negative results. Some studies have, however, suggested that short fibre asbestos may be carcinogenic when injected (KOLEV, 1982; LE BOUFFANT *et al.*, 1985). These results are difficult to interpret since short fibres are usually only reported as a proportion of the total. With the ability of asbestos to split longitudinally as well as transversely it is quite possible to reduce the mean fibre length of a sample greatly while actually increasing the number of long fibres/unit mass. WAGNER *et al.* (1984b) demonstrated that following the injection of finely ground short crocidolite the small proportion of long fibres present were selectively retained in the pleural granulomas produced by this dust.

Since injection techniques exaggerate tissue response to fibres by bypassing the elutriation mechanisms of the upper airways, it is important to determine whether or not fibrosis and neoplasia are related to fibre length when dust is inhaled. The difficulties in preparing suitable dusts in large quantities have inhibited this work, and only a very few studies with satisfactory dusts have been possible. DAVIS *et al.* (1986a) reported long-term inhalation and injection studies in rats in which a sample of amosite with almost all fibres <5 μm in length was compared to a normal amosite dust preparation containing many long fibres. The short fibre material produced neither fibrosis nor neoplasia, apart from a single mesothelioma in the highest dose injected, while the long fibres were highly pathogenic. An attempt was made to duplicate this study using chrysotile (DAVIS and JONES, 1988) but the intended short fibre preparation contained a small but perhaps significant proportion of long fibres and some fibrosis and pulmonary tumours were produced in rats although the response was much less than with long fibre chrysotile. When injected, the short chrysotile produced fewer tumours at any dose than the long. Perhaps the most dramatic demonstration of the importance of fibre length was reported by WAGNER *et al.* (1985) and WAGNER (1990).

The mesothelioma incidence of almost 100% following the inhalation of erionite in rats was reduced to zero when short fibre preparations of this material were used. Data from experimental inhalation studies published by DAVIS *et al.* (1978, 1986a) indicate that different fibre lengths may be needed to produce disease in the pulmonary parenchyma than to cause mesothelioma. Of the three amosite samples tested in these studies, both a very short fibre sample and the medium length fibre, UICC reference sample produced minimal pulmonary fibrosis and almost no pulmonary tumours. In contrast a long fibre sample produced large amounts of fibrosis and significant numbers of pulmonary tumours. When the three dusts were injected at the same dose, both the long fibre sample and the UICC material produced mesotheliomas in almost all animals while the short-fibre material produced none. The implications of these results are that very short fibres produce no disease at any site, medium length fibres (8–10 μm) can cause mesotheliomas but longer fibres (perhaps 15–25 μm) are needed to produce disease in the pulmonary parenchyma.

AIRBORNE FIBRE CONCENTRATIONS REQUIRED TO PRODUCE PATHOGENIC RESPONSES

From the above considerations it appears that for dust clouds of mineral fibres to damage the pulmonary parenchyma of rats they must contain significant numbers of fibres >20 μm in length and <1 μm in diameter. Fibres >10 μm in length cause mesotheliomas although the proportion of these tumours occurring in rat inhalation studies is quite small. For humans, it may be assumed that similar dimensions are important in disease production. Even with fibres of the most critical dimensions, however, the production of recognizable disease requires a sufficient pulmonary fibre burden to be accumulated and maintained.

Examination of the lungs of asbestos workers at autopsy has given good indication that this required burden is high, for example, it is generally accepted that pulmonary asbestosis will not occur until the lung fibre burden exceeds 5×10^5 fibres g^{-1} of dried lung [fibres >5 μm in length by phase contrast optical microscopy (PCOM)] although in severe cases of asbestosis fibre counts can be as high as 1000×10^6 fibres g^{-1} of dried lung (DAVIS, 1984). As would be expected from the association of asbestosis and pulmonary carcinoma, the lung fibre burdens from the carcinoma cases in asbestos workers occur over the same fibre range. For mesothelioma production the situation is much more complicated. Firstly no data are available for the fibre burden at the pleura itself in mesothelioma cases and this is what will be most important. Measurement of the fibre burden in the pulmonary parenchyma in mesothelioma cases range from the highest burdens found in asbestosis down to the background level for the rural population since approximately 30% of mesotheliomas are not associated with known exposure to asbestos and are either due to other causes or are spontaneous. Human evidence shows that mesotheliomas can develop without asbestosis being present and therefore at lower pulmonary fibre burdens than are required for this fibrosis. At present, however, it is not known whether there is a minimum figure for lung burdens of asbestos fibre to cause mesothelioma.

With the short lifespan of laboratory rodents available to build up significant fibre burdens in the pulmonary parenchyma, most experimental studies have been undertaken at extremely high fibre levels (1000–3000 fibres ml^{-1} >5 μm by PCOM for

asbestos). This type of exposure for 1 year does produce pulmonary fibre burdens similar to those recorded for humans. Thus DAVIS *et al.* (1986a) reported that in experiments with amosite, a dust cloud of 2060 fibres ml^{-1} of air (> 5 μm in length by PCOM) administered for 1 year produced a mean pulmonary dust burden of 3570 μg of dust. Since dried rat lungs weighed between 1 and 2 g depending on the individual and degree of pulmonary damage, this would produce a fibre burden of 300×10^6–700×10^6 fibres g^{-1} of dried lung. This calculation, based on the approximation that the number of fibres > 5 μm in length/unit mass of fibre in the lung tissue is similar to that in the original dust cloud, shows that the number of fibres in the rat lungs was in the same range as the 1×10^6–1000×10^6 fibres g^{-1} of dried lung reported from cases of human asbestosis. Direct extrapolation from these figures might suggest that the critical 1×10^6 fibres g^{-1} of dried lung required to produce the earliest signs of asbestosis (in humans) could be reached following a 1 year exposure in rats to a dust cloud as low as 3–5 fibres ml^{-1}. However, this calculation makes no allowance for the effects of the reduced clearance demonstrated in rats following periods of exposure at very high fibre levels. In addition, since only the earliest signs of asbestosis are produced in rats exposed at this fibre level to amosite asbestos, a material known to be very dangerous, similar dose levels will be of little use to examine fibres of lower although not insignificant pathogenicity. As we have stated most 12-month inhalation studies with asbestos in rats have used exposure concentrations of 500–3000 fibres ml^{-1} and have usually produced large amounts of pulmonary disease. One example of a lower exposure which still produced marked disease was reported by DAVIS *et al.* (1986b). In this study a special chrysotile sample was examined at only 100 fibres ml^{-1}. However, this material was an example of wet dispersed chrysotile which had been treated during preparation in a manner that facilitated its rapid separation to individual fibrils in tissues so that the tissue burden of fibre units was much higher than indicated by the original dust cloud. Exposure of rats to ceramic aluminium silicate fibre at 100 fibres ml^{-1} (> 5 μm in length by PCOM) has produced small amounts of fibrosis and a few pulmonary tumours (DAVIS *et al.*, 1984). Similar studies with this type of ceramic fibre at 200 fibres ml^{-1} (> 20 μm in length by PCOM) have produced much more serious pulmonary disease including many mesotheliomas in hamsters (HESTERBERG *et al.*, 1992). Experimental studies involving the use of very fine glass fibre at fibre levels of approximately 1000 fibres ml^{-1} produced almost no response (WAGNER *et al.*, 1984a; MCCONNELL *et al.*, 1984) which at this exposure level appears to be definite evidence of low pathogenicity for this fibre type. Studies with glass wool and rock wool were negative at dust levels of 323 and 257 fibres ml^{-1}, respectively (> 5 μm in length), which could be due either to low durability for these fibre types or to the fact that only about 10% of these fibres were greater than 20 μm in length and less than 1 μm in diameter. Other negative studies with glass and rock wool at fibre levels of 94 and 41 fibres ml^{-1}, respectively (> 5 μm in length), have the same problem of fibre size distribution but the lower of these two exposure levels might produce little or no detectable disease even if the fibres had harmful potential similar to that of asbestos (LE BOUFFANT *et al.*, 1987).

From these considerations it appears that inhalation studies aimed at examining the pulmonary pathogenicity of fibres should be undertaken at dust cloud concentrations of at least 100 fibres ml^{-1} (fibres > 20 μm in length by PCOM) and preferably at 200 fibres ml^{-1}.

This recommendation, however, creates practical problems for undertaking inhalation studies to examine the potential pathogenicity of fibrous materials with a relatively large mean fibre diameter but with a small proportion of respirable fibres. To produce clouds of more than 100 respirable fibres ml^{-1}, large masses of dust would need to be generated (mostly non-respirable) and this would produce an overwhelming 'sandstorm' effect in the inhalation chambers unless large quantities of aerosol are generated and then separated aerodynamically so that only the fine fraction is delivered to the inhalation chamber. One ideal approach to long-term inhalation studies with this type of product would be the use of differential sedimentation techniques to produce a dust greatly enriched in respirable fibres, but to do this on a scale large enough to produce enough material (for dust clouds) is not practicable. However, smaller amounts (5 g) can be produced and while these do not allow an examination of inhalation-induced gross pathology they enable cellular, inflammatory and other responses to be studied and enable one of the essential features in fibre pathogenicity, namely fibre durability, to be examined. Thus fibre research should proceed by carefully designed studies of the carcinogenicity and fibrogenicity of materials for which adequate dust clouds can be produced, together with comparisons of the characteristics of fibres and the tissue responses (to innoculated doses of fibres) at the cellular and biochemical levels. The fibre characteristics to be examined should include dimensions, composition, durability and—in some cases—surface molecular structure.

It is expected that the inhalation studies of carcinogenicity and fibrogenicity will identify characteristics of potentially dangerous fibres. The presence of such fibres can then be assessed for a wider range of bulk materials (which may not be suitable for generation of high concentration aerosols) from the other types of test.

FIBRE PERSISTENCE AND PATHOLOGY

One further essential factor in disease production, however, is fibre durability in the lung tissue. The cumulative harmful effect of fibres in the lung will depend on both how much fibre is deposited and how long it is retained. Fibres that dissolve fast enough to prevent significant accumulation in lung tissue are likely to be less dangerous. The interpretation of any pathology study has to take into account the durability of the fibre in lung tissue. Exposure to high airborne fibre concentrations might not produce any disease if the fibre dissolves fast. The extrapolation from animal studies to humans requires an examination of the relative persistence of the fibres over the life time of each species. To achieve this, there is a need to develop reliable assays to determine the durability of fibres in the biochemical conditions of the lung.

KEY QUESTIONS IN EXPERIMENTAL RESEARCH INTO THE EFFECTS OF INDUSTRIAL FIBRES ON THE LUNG

The main questions that remain to be addressed, or are currently being addressed in the field of research into the health effects of industrial fibres, are in the following broad areas.

(a) *Durability*

Do some fibres undergo dissolution in the lung following deposition such that they become less harmful to the lung? If so, then what are the parameters that are important

in determining durability so that it can be reliably assessed in a predictive test? If durability could be measured *in vivo* and *in vitro* then 'safe' non-durable fibres could become the goal of the fibre manufacturers.

(b) *Fibre shape*

It is known that the fibrous shape, to a large extent, determines pathogenic potency. However, the key lengths, diameters and aspect ratios have not yet been identified. Optimal characterization of the fibre sample populations used in any experiments should be a key aim; relating key size parameters to biological activity should then follow.

(c) *Fibre chemistry*

Although shape is an important determinant of fibre pathogenicity, fibre chemistry and in particular the chemistry of the fibre surface, affects toxicity. This is true from the point of view of solubility alone, but may also be an important factor in the interaction of the fibre with cells and so there could be a more fundamental role for differences in fibre chemistry.

(d) *Mechanisms of fibre-related pathology*

Despite years of intense research into asbestos, there has been little progress in understanding why fibres of certain length and diameter have the ability to cause fibrosis and cancer. A key aim of research into the new generation of industrial fibres should therefore be to fully investigate the cellular and sub-cellular events that occur when cells of the respiratory tract interact with fibres. The techniques and approaches used in pathobiological research, in all its disciplines, should be brought to bear on this important aspect of respiratory disease.

CONCLUSIONS

This paper examines the extent to which animal studies can reproduce the pathological consequences which arise in humans, and reviews the role of these experiments in establishing the relative hazard of different types of fibre.

Although the mechanisms of disease causation by fibres are not fully understood, it has been clearly demonstrated that certain sizes of fibre are necessary for the production of most of these diseases. If fibre size were a complete and sufficient definition of what constitutes hazardous fibres, then similar pathological results should be produced by similar levels of exposure as defined simply by the number concentration of fibres (in the critical size range) irrespective of which type of fibre is used. If this were shown to be the case, then safe working conditions would be defined simply by controlling exposure to fibres in this size range. Of course, achieving these safe working conditions (for the critical size of fibre) may be much easier for some materials than for others.

The biological response to fibres is probably affected by the surface properties, chemical and mineralogical, and therefore a series of controlled experiments is likely to reveal that some types of material are inherently much safer or more dangerous than others. The purpose of the present programme of work is to identify the characteristics (morphological, chemical or mineral) which affect the development of pathology and thus enable appropriate safety precautions to be defined.

Fibres are an important and valuable resource for industry and society. However, some fibres have been associated with diseases which may arise a long time (decades) after exposure. It is therefore very important that we obtain as clear an understanding of the mechanisms and relevant factors leading to these diseases so that proper, sufficient and appropriate safety procedures can be used in order to ensure that these commercially valuable materials are exploited fully but safely and without the stain or fear of disease.

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Respirable industrial fibres: mechanisms of pathogenicity

K Donaldson, R C Brown, G M Brown

Background

Fibrous materials have much to commend them in industrial applications, where they can offer, in varying degree, reinforcement, thermal and electrical insulation, flexibility, and strength. In the first part of this century the fibrous material that was available, cheaply and in bulk, that could provide these properties to industry was the asbestos group of silicate minerals (table 1). The fibrous, crystalline, asbestiform habit, however, also means that during mining and industrial use fibres are released into the air. Some of these are in the respirable size range and may be deposited in the bronchiolar-alveolar region. Subsequently we have come to realise that asbestos inhalation is associated with several different lung diseases—namely, asbestosis (interstitial fibrosis), carcinoma of the lung, mesothelioma, and small airways disease—and the association is now well documented. This information on the harmful effects of asbestos has led to bans in some countries and considerable litigation and claims for damages throughout the developed world. Regulatory and legal pressures on asbestos have contributed to an increase in the use of alternative materials, both natural and manmade, including some fibrous materials. More importantly, advances in materials science have identified a ever widening range of applications in which the fibrous nature of a material offers positive advantages. Thus in addition to their traditional use in thermal insulation new fibre composites have found new uses, such as metal reinforcement, to which asbestos was never suited (table 2).

The finding that in at least some biological assays some non-asbestos fibres could cause the same types of effect as asbestos has raised the question of whether all fibres have the same pathogenic potential as asbestos. A major approach in present research on fibres is therefore to compare any fibre under study with asbestos and to gauge the potential for causing lung disease in the light of experience with asbestos. Thus the spectre of asbestos permeates all discussion of research into respirable fibres, of whatever origin, leading to a complex interplay of science, politics, and commercial pressure.

Table 1 The asbestos minerals

Serpentine group	*Amphibole group*
Chrysotile	Crocidolite
	Amosite
	Anthophyllite
	Tremolite
	Actinolite

Table 2 Industrial fibres other than asbestos

Natural	*Manmade*
Erionite	Slag wool
Wollastonite	Rock wool
Attapulgite	Glass wool
Sepiolite	Continuous filament glass
Halloysite	Ceramics
	Alumina
	Zirconia
	Silicon carbide
	Graphite
	Boron
	Aromatic amide

This review aims to address only the scientific ideas that dominate research into the mechanisms underlying the development of diseases caused by respirable industrial fibres. The constraints of space and the breadth of the published research prevent this review from being comprehensive. Further details can be obtained from recent reviews on mechanisms of fibre pathology[1] and on non-asbestos fibres and their health effects in general.[2-4]

Role of fibre size, shape, and chemistry

DEPOSITION

A fibre, as defined by the World Health Organisation for measurement of the exposure of workers to airborne fibres in factories and mines, is a structure longer than 5 μm, less than 3 μm in diameter, and with an aspect ratio (length:diameter) greater than 3:1. The deposition of a particle in the lung is a function of the "aerodynamic diameter" (particle size expressed in terms of settling speed). For fibres this is governed predominantly by the actual diameter, density and length being of subsidiary importance. Only fibres with a physical diameter of 3 μm or less may be deposited in the alveolar region, the efficiency of deposition increasing with decreasing diameter to a physical diameter of about 1 μm; beyond this the efficiency of deposition may begin to decrease again. Subsequently thin fibres will be deposited in the alveolar region of the lung even if they are

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very long (up to 30 or 40 μm). In contrast, for compact particles density is more important, and particles with diameters greater than about 5 μm are unlikely to reach the alveolar region. The site of the deposition of fibres in the lungs of rats was elucidated by the elegant studies of Brody *et al*[5] and Warheit *et al*[6] who used microdissection and scanning electron microscopy to follow the fate of chrysotile asbestos fibres delivered to the rat lung during a single exposure. These fibres were found to be deposited almost exclusively around the terminal bronchioles and the first alveolar duct bifurcations, where the fibres could first be seen lying free and later phagocytosed by macrophages. Our own studies, using amosite asbestos and glass fibres, have confirmed this sequence of events and fibres up to 35 μm long can be seen lying on the epithelium of the terminal bronchioles and the alveolar ducts and inside macrophages (fig 1).

FIBRE LENGTH AND PATHOGENIC POTENTIAL

There has been some progress towards understanding the attributes of fibres that imbue them with the potential for causing pathogenic change. Pioneering studies, such as those of Wright and Kuschner,[7] and Stanton and Layard,[8] showed that short fibres (below 5 μm) are low in pathogenicity and that increasing pathogenicity accompanies increasing fibre length beyond 8–10 μm. In particular, the impressive studies of Stanton and co-workers[8] using implantation of fibres into the pleural space in the rat, showed that a range of quite different fibrous materials could all cause pleural mesotheliomas provided that there were fibres of the appropriate sizes. The optimum size range for the production of mesotheliomas in this system was over 8 μm long and below 0·25 μm in diameter.[8]

In the early 1980s the Institute of Occupational Medicine in Edinburgh obtained two samples of amosite asbestos, one containing a substantial proportion of long fibres and the other composed exclusively of short fibres. The short fibres were separated from the long by milling and sedimentation. The two samples were very similar in all other respects, such as diameter, elemental composition, and crystallographic features. Milling reduced a substantial proportion (63%) of the particles in the short fibre sample to an aspect ratio of less than 3:1—that is, they could not be defined as fibres. The remaining 37% were fibres by this criterion with an average length of 2·7 μm. These two samples have now been extensively studied by inhalation and instillation and some of the results are summarised in table 3. We believe this to be some of the most compelling evidence showing the importance of fibre size in determining the biological activity of respirable fibres.

One important factor that contributes to the pathogenic potential of long fibres is the evidence from several studies that long fibres are cleared from the deep regions of the lung less effectively than are short fibres.[11 12] This may be a consequence of failure of macrophages to ingest long fibres completely or of difficulty in moving once they have phagocytosed a long fibre. Long fibres may even interact with several macrophages, which may not then be able to act in concert. Such a reaction might mark the onset of a granulomatous response. With regard to the latter possibility, macrophages from the lungs of rats exposed to asbestos by inhalation have been found to have decreased ability to migrate towards a chemotaxin.[13] Thus long fibres could be more harmful to the lung because they are retained, whereas the short fibres are cleared more effectively. But although this presumably contributes to the toxicity of long fibres the evidence from studies using some in vitro assay systems that do not rely on clearance (see, for example, Brown *et al*[14] and Donaldson *et al*[15]) confirms that longer fibres have intrinsically more biological activity.

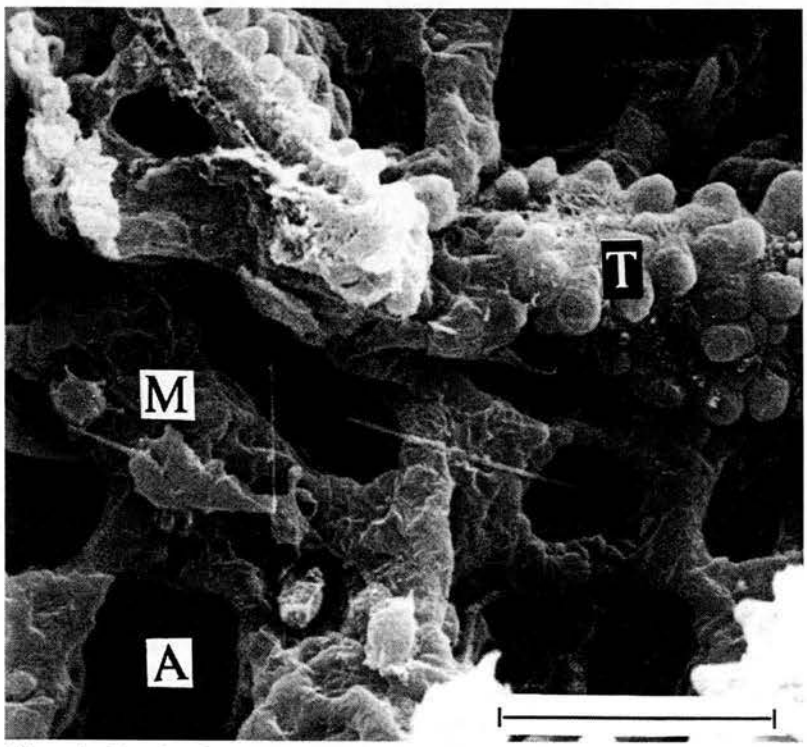


Figure 1 Scanning electron microscope image of the distal airspace of a rat that had been inhaling a cloud of 1000 fibres/ml of amosite asbestos for seven hours. An alveolar duct is shown with a portion of terminal bronchiolar epithelium present (T) and openings into alveoli (A). Asbestos fibres can be seen on the alveolar duct surface, both free and partially phagocytosed inside alveolar macrophages (M). (Scale bar = 40 μm.)

*Table 3 Biological activity of long and short fibre samples of amosite asbestos in animal studies**

	Amosite fibres	
Response measure	*Short*	*Long*
Inhalation % with lung tumours (rats)	0	32·5
Mean fibrosis score (rats)	0	15·6
Intraperitoneal instillation		
% with mesotheliomas (rats)	0	88
Inflammation score (mice)	−/+	++++

*Data summarised from experiments described in Davis *et al*[9] and Donaldson *et al*.[10]

ROLE OF CHEMICAL CHARACTERISTICS OF THE FIBRE SURFACE

Thus the role of fibre size has been well investigated and shown to be important, but less attention has been given to the chemistry of fibres. Of particular interest in this respect is erionite. This naturally occurring fibrous mineral of the zeolite family has been implicated in a high prevalence of mesothelioma in the small Turkish village of Karain, where it is present in the air owing to the weathering of rock.[16] In animal studies erionite produced an incidence of mesothelioma far higher than any other fibre, even though the dimensions of the fibres were not greatly different from those of fibres with less pathological potential.[17] Possibly therefore erionite has a composition that contributes unusually to its pathogenicity. Further evidence that the fibre surface can contribute to the biological activity comes from a study by Brown *et al*,[18] who substituted hydrocarbon chains of different length on the surface of amosite fibres without altering the number or length of the fibres. This treatment appears to alter the ability of the fibres to cause mesothelioma after instillation into the pleura.

There is also clear evidence in vitro that simply altering the protein that is attached to the fibre surface will change the biological activity. Treating asbestos fibres with rat immunoglobulin G in vitro dramatically increased its ability to stimulate macrophages to release the important inflammatory mediators superoxide anion[19] and tumour necrosis factor.[15] Presumably this is a direct effect mediated via the macrophage membrane receptors for the Fc portion of immunoglobulin G. Further research on the chemical nature of the surface of fibres and of the receptors responsible for the interactions of fibres with the cell surface are clearly warranted. In this regard Brown *et al*[20] recently implicated the fibronectin tripeptide receptor in the binding of amosite asbestos to cells in vitro.

The postulated role of free radicals in the toxicity of fibres[21 22] has focused interest in the role of iron at the fibre surface. Because of the ability of iron to enhance and catalyse the reactions of free radicals it has been suggested that fibres may act as a source of iron in tissue, leading to injury by free radicals. The evidence that iron is important has come from studies where iron chelators have ameliorated the toxicity of fibres to cells in vitro.[21 23 24] Recently iron from crocidolite asbestos has been shown to be important in the causation of breaks in DNA strands[25] and in the formation of 8-hydroxydeoxyguanosine (RC Brown, unpublished findings). Although iron may indeed be important in the toxic effects of types of fibre with a substantial content of iron, other fibres, such as chrysotile asbestos and some fibrous glasses, have little or no iron. In other cases it is the iron, mobilised from the fibre, that causes a short term effect; the relevance of this to the long term effect of fibres in vivo is not clear as such fibres become coated with biological material, which itself may contain iron. Indeed, many fibres form ferruginous (asbestos) bodies, at least in man, so that iron is deposited on the fibre rather than leached from it. Iron derived from fibres is therefore unlikely to be an important unifying factor in fibre toxicity, but the relative importance of the surface and the dimensions of fibres has yet to be determined fully.

One area where fibre chemistry is likely to be an important factor in determining biological activity relates to the ability of fibres to persist in the lung once deposited there. Fibres that dissolve or break down in the lung are less likely to be dangerous because they may become shorter; short fibres are less active than longer fibres and are more efficiently cleared. In addition, epidemiological studies in workers exposed to the relatively soluble chrysotile asbestos suggest that this particular fibre is much less harmful in man than are other forms of asbestos.

Experiments with chrysotile fibres that have been treated to mimic the dissolution effects of residence in the lungs have shown that they do indeed have decreased biological activity[26] and are removed faster by the clearance systems of the lung.[11] As most glassy fibres are likely to be more soluble in the lung than even chrysotile asbestos possibly these will represent less hazard.

A substantial body of research at present is concerned with the persistence of various fibre types within the lung. In animal experiments even "soluble" fibres may persist in the lungs of rodents for a substantial portion of the animal's lifespan and thus are able to cause disease, whereas the same fibres persisting for the same time may not have the same effect in humans with their longer lifespan. This raises the possibility that "false positive" results might occur in animals because fibres are cleared by purely physicochemical means unrelated to the animals' lifespan. Negative results in such animal experiments should therefore be more reassuring than similar results with purely chemical toxins.

Mechanisms of disease caused by fibres

A substantial body of research has been directed at the interactions between fibres and cells in the hope of understanding the mechanism whereby deposited fibres cause the principal diseases associated with exposure.

EPITHELIAL CELLS

Brody *et al*[27] showed that inhaled chrysotile asbestos fibres could be found inside epithelial cells, and this was interpreted as a mechanism whereby fibres were translocated to the interstitium. Hobson *et al*[28] have also shown the uptake of fibres by airway epithelial cells in vitro. The effect of the interaction of fibres with epithelial cells is both frank toxicity[14 24] and, in some studies, a low level of sublethal genetic damage.[29 30] As well as acting as an initiator in causing direct DNA damage, asbestos is able to increase the frequency of

transformation effects caused by other carcinogens at doses at which asbestos alone has no effect.[31 32] These observations lead to the contention that asbestos acts as a tumour promoter, and indeed several different studies have found that asbestos has activities similar to those of classical promoters.[33]

Increased proliferation of epithelial cells is an early event following inhalation exposure to chrysotile asbestos in rats.[34] Increased cell proliferation is a common effect of promoters[33] and is likely to represent the restorative response to epithelial damage, combined with the response to local accumulation of inflammatory cell derived growth factors. A role for epithelial hyperplasia in the development of neoplasia associated with fibres therefore appears likely.

MACROPHAGES AND INFLAMMATION

Most deposited fibres are likely to be phagocytosed by alveolar macrophages if they remain on the lung surface for long enough (fig 2). Early research showed the ability of fibres to injure macrophages in vitro, and shortening of the fibres by milling reduced the toxic effects.[35] At that time fibre mediated death of macrophages was seen as an important pathogenic step. But, although fibres (mostly asbestos) were able to kill macrophages after phagocytosis a dose-response relationship could be seen, and low concentrations of fibre were not frankly toxic. As the roles of the macrophage became better understood and the subtleties of its interactions with other cells via various mediators became clear, research converged on the sublethal effects of low concentrations of fibres. The idea that fibres at low dose could stimulate macrophages to change their secretory repertoire has therefore come to predominate.

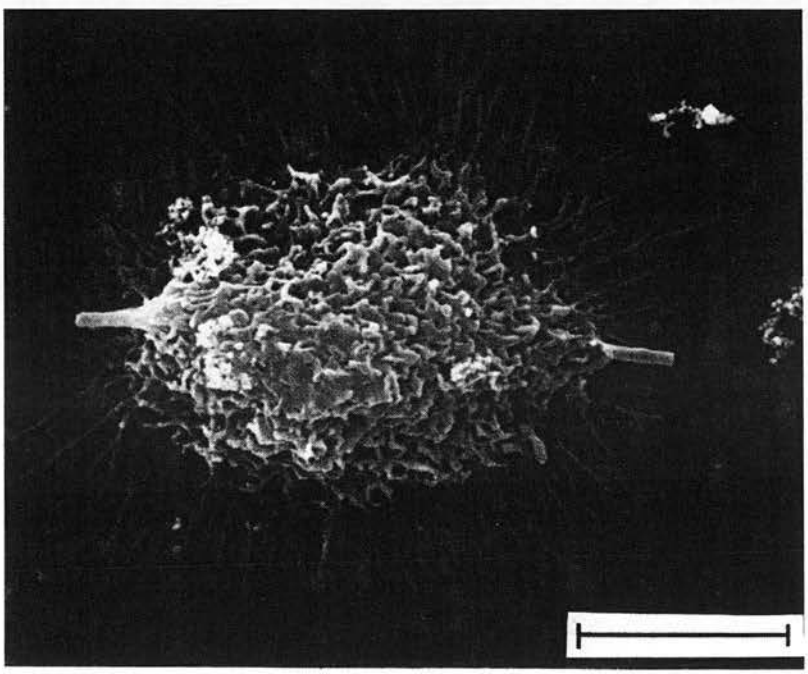


Figure 2 Scanning electron microscope image of an alveolar macrophage from the lungs of a rat exposed to amosite asbestos (see legend to fig 1). A single long fibre, or two shorter fibres, can be seen protruding from the alveolar macrophage, which appears to be activated. (Scale bar = 7·5 μm.)

Particular attention has been given to proteases, cytokines, and oxidants because these have an obvious potential for mediating inflammation and pathological change. Table 4 shows the various secretory products of macrophages that have been found to be increased by exposure to fibres in vitro or in vivo.

Stimulation of macrophages by fibres to secrete their products, along with the epithelial events outlined above, culminates in inflammation, characterised by recruitment of more macrophages and neutrophils.[41] A hypothesis can be proposed about the events that occur as a consequence of this localised accumulation of inflammatory cells at the terminal bronchiolar and proximal alveolar region. Oxidants cause local injury and so contribute to loss of epithelial integrity, allowing passage of cells and proteins from the interstitium to the alveolar space; fibres and macrophages and their products may also gain access to the interstitium. Macrophage molecules that stimulate the mesenchymal cells (platelet derived growth factor, tumour necrosis factor, etc) can then signal an increase in production of fibroblasts and their matrix products, leading to fibrosis of the small airway walls and the alveolar walls. Oxidants and growth factors may contribute to "promoting" effects, such as proliferation, and so contribute to neoplastic change.

THE PLEURA AND MESOTHELIAL CELLS

Because of the association between asbestos exposure and mesothelioma, the effects of fibres on pleural mesothelial cells have been the subject of many investigations. Mesothelial cells in culture have been reported to be particularly susceptible to the toxic effects of fibres, far more so than epithelial cells or fibroblasts.[42] Mesothelial cells phagocytose fibres, leading to chromosomal abnormalities[43] and an increase in unscheduled DNA synthesis as evidence of DNA repair following damage.[44]

With regard to pleural fibrosis, the macrophage events described above in relation to alveolar fibrosis could occur in the most peripheral alveolar units. This would be in close proximity to, and may even be continuous with, the subpleural connective tissue and could lead to localised pleural fibrosis.

Although no clear role for the interactions between mesothelial cells and the leucocyte population of the pleural cavity has emerged, changes in the activity of the pleural

Table 4 Some secretory products produced by macrophages exposed to asbestos

Asbestos fibre	*Secretory product*	*Reference*
Chrysotile	Lysosomal hydrolase	36
Crocidolite	Chemoattractant	37
Chrysotile	Platelet derived growth factor	38
Chrysotile	Superoxide anion	39
Chrysotile	Arachidonic acid metabolites	40
Amosite	Tumour necrosis factor	15

leucocytes after deposition of asbestos fibres in the lung have been described.[45] Such changes could contribute to the development of pleural disease.

FIBRES IN COMBINATION WITH OTHER AGENTS

The clear multiplicative effects of smoking and asbestos exposure in causing lung cancer,[46] and the activity of asbestos as a promoter as seen experimentally, raise the question of combined exposures to fibres and other agents. This has not yet been studied in depth but Pinkerton *et al*[47] have shown that low dose exposure to ozone results in a dramatic slowing down of the clearance of asbestos from the lung. Davis *et al*[48] have shown that inhaling asbestos fibres in combination with other dust, such as titanium dioxide or quartz, may produce substantially more lung and pleural tumours than inhaling fibres alone. Thus we need to consider fibre exposure in relation to concomitant environmental exposures to oxidants and dusts.

Future research on respirable industrial fibres

Almost all that is known about the mechanisms of the diseases caused by inhaled fibres is based on experience with asbestos. The dissemination of a set of standard samples of asbestos (Unione Internationale Contre Cancer (UICC) samples produced by the British and South African Medical Research Councils) was a vital step in allowing interlaboratory comparisons to be made of the biological activity of at least these asbestos samples. Whereas elucidation of the cell biology of asbestos bioeffects has moved ahead rapidly no standard samples of non-asbestos fibres have been generally available to the scientific community. Such a standard set should be well characterised with regard to dimensions and surface chemistry. The lack of standard non-asbestos fibres has meant that progress has stalled with regard to very basic questions on the similarities and differences between asbestos and other fibres. The UICC samples were made in 1969 before the role of fibre length was fully appreciated; they were made by milling commercial asbestos to ensure that each sample contained mostly respirable fibres. These fibres, however, were mostly short and the resulting activity of the UICC series is therefore far from optimal for biological research. Nearly all the experiments carried out so far have used samples containing less than 1% of the long active fraction.

Both this dimensional problem and the absence of standard samples of the manmade fibres have been partially remedied by the United States Thermal Insulation Manufacturer's Association (TIMA). This organisation and the equivalent European bodies have sponsored a series of animal experiments on their products. To do this they isolated fibres with target mean dimensions of 20 μm length and 1 μm diameter from commercial mineral wools. Such fibres could be expected to be extremely biologically active if dimension is the main determinant of activity. As well as carrying out their own programme of research with these materials the Thermal Insulation Manufacturers Association has established a repository, which is making a range of respirable vitreous and ceramic fibres and one long fibre sample of crocidolite available to bona fide researchers. This generous step should greatly improve fibre research.

The asbestos experience has been the driving force for most research into the newer fibres and this has engendered a cautious approach to any respirable fibre. Currently, the idea that any long, thin, durable fibre has the potential to be pathogenic dominates the study of fibre pathology (fig 3) but there is no general consensus on secure definitions of long, thin, or durable; the question of the role of chemical composition, particularly as it affects durability, continues to be vexing. The next 20 years of research into the physicochemistry of fibres and their interactions with cells should reveal whether "guilt by association" with asbestos is justice for respirable industrial fibres. A ban on fibres would be impractical with the present state of knowledge, as non-fibrous substitutes do not exist for all known applications. In addition, the energy efficiency of domestic and other buildings and industrial processes would be substantially reduced, with consequent increases in air pollution. A thorough understanding of the characteristics of fibres that control their toxicity may allow industry to design safer fibres, to the benefit of themselves, the workforce, and society.

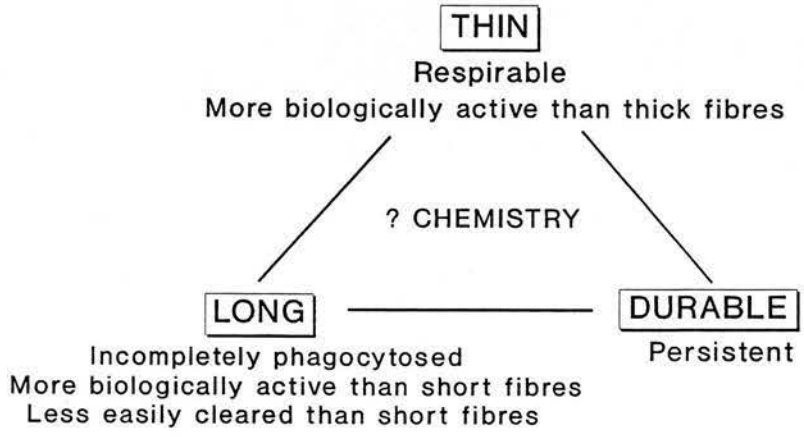


Figure 3 The pathogenicity of fibres: diagram showing the three properties that are currently considered to underlie pathogenicity, indicating how each property is likely to have its biological effects.

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Section IV
Chapter 4

MODULATION OF QUARTZ TOXICITY BY ALUMINUM

Geraldine M. Brown and Kenneth Donaldson

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I. MECHANISMS OF QUARTZ TOXICITY

The biological activity of silica, the most common form of which is α-quartz, is related to interactions between the surface of quartz particles and cells,[1] leading to cell damage[2] or cell activation.[3] Although the precise mechanisms are not yet fully elucidated, these quartz/cell interactions are believed to contribute, in the long term, to the tissue derangements which result from chronic inhalation of quartz in its pure form or in mixed dusts, both in man and in experimental animal models. Substances such as aluminum which can bind to the quartz particles and so reduce the reactivity of the quartz surface are therefore of potential importance in limiting quartz toxicity. This has implications for both hazard assessment of mixed dusts containing quartz and aluminum and for therapeutic intervention following silica inhalation.

II. MODIFICATION OF THE QUARTZ SURFACE BY ALUMINUM

Modification of the quartz surface by reaction with aluminum was first indicated in the study of Denny et al.,[4] who observed reduction in the solubility of quartz in the presence of metallic aluminum powder. Reduced solubility of quartz was also shown by Dale and Kings[5] using aluminum chloride solution and, indirectly, by Ulmer,[6] who demonstrated markedly enhanced particle agglomeration following the addition of aluminum to a suspension of quartz. Other indicators of quartz surface activity which have been modified by treatment with aluminum are electron diffraction[7] and zeta potential.[8]

The foregoing studies provided circumstantial evidence that quartz particles could be coated with aluminum. The first actual demonstration of aluminum on the quartz surface was that of Bremner,[9] who

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stained the aluminum with aurintricarboxylate. The use of X-ray photoelectron spectrophotoscopy also demonstrated that aluminum strongly bonded to the quartz surface by means of an oxygen bridge[10] and, more recently, Wallace et al.[11] demonstrated an aluminosilicate coating on the surface of quartz particles using energy dispersive X-ray analysis.

III. REDUCTION OF QUARTZ BIOACTIVITY BY ALUMINUM

A. FIBROTIC RESPONSE

The first evidence that aluminum could reduce the toxicity of quartz particles was provided in work by Denny et al.[12] who demonstrated that concomitant inhalation of quartz and aluminum powder resulted in reduced lung fibrosis in rabbits. These results were questioned when experiments by Belt and King[13] failed to show a protective effect of aluminum on quartz toxicity in experiments where the dust was administered by intratracheal injection. However, the same authors did subsequently demonstrate an ameliorative effect of aluminum powder on quartz toxicity[14,15] using a more physiological exposure regimen where rats were exposed to aerosols of the two minerals. Between 1950 and the late 1960s further experimental studies were carried out in America and Europe investigating interactions between quartz and diverse forms of aluminum such as metallic aluminum, aluminum hydroxide, aluminum chloride, and aluminum chlorhydroxyallantoinate (reviewed in Reference 16). These studies demonstrated conclusively that the development of quartz-induced lung damage, measured as the number and severity of fibrotic lesions, altered lung weight or increased amounts of collagen per lung, in rats, guinea pigs, and rabbits, could be reduced and in some cases eliminated entirely by simultaneous treatment with aluminum.

B. BRONCHOALVEOLAR INFLAMMATORY RESPONSE

Evaluation of the mechanisms involved in the modulation of quartz toxicity by aluminum have been facilitated in recent years by the introduction of bronchoalveolar lavage as a research tool. This has enabled quantification of the inflammatory response in the airspaces of lungs exposed to quartz and so has permitted more detailed analysis of how aluminum acts to reduce quartz toxicity. The first work investigating the bronchoalveolar response to aluminum-coated quartz was reported by Begin et al.[17] using a soluble form of aluminum, aluminum lactate. Native quartz and aluminum-coated quartz were administered to sheep by intratracheal injection, and the lung response was assessed over a period of 60 d. The coated quartz elicited a markedly reduced influx of inflammatory leukocytes into the bronchoalveolar space; biochemical markers of inflammation in the lavage fluid, which increased in response to untreated quartz, were also less in the sheep treated with aluminum-coated quartz. The protective effect of aluminum in the sheep model persisted for up to 10 months and was associated with increased clearance of quartz from the lung.[18] Further studies in sheep[19,20] and in rats[21] demonstrated that the inflammatory response to quartz in the lung could be ameliorated even when the aluminum was administered substantially later than the quartz and the inflammatory response was well established. It was notable that there was no suppressive effect of aluminum on the lung response to a bacterial challenge.[21] Thus the suppressive effect of late administration of aluminum on the quartz alveolitis was deemed to be due to interaction of aluminum with quartz particles either following uptake of aluminum into the phagolysosome of live macrophages containing quartz particles or extracellularly when quartz was released by dead macrophages.

C. *IN VITRO* BIOACTIVITY

The mechanisms whereby the pathogenicity of quartz is ameliorated by aluminum have been addressed in a number of studies which investigated specific aspects of quartz bioactivity. In one study, inhibition of the cytochrome C-oxidase system of lung homogenates by silicic acid was suppressed by aluminum chloride.[22] Nolan et al.[8] demonstrated a link between particle surface charge and bioactivity by the observation that change in zeta potential was correlated with a reduced interaction with red blood cells (RBC). There was a concomitant reduction in hemolysis of the RBC, possibly due to the binding of aluminum to ionized silanol groups on the quartz surface which are thought to be involved in the interaction between quartz particles and components of cell membranes.[8]

Specific effects of aluminum on quartz:cell membrane interactions were recently demonstrated by Cao et al.[23] who showed that membrane dehydration in rabbit erythrocytes exposed to quartz was reduced by coating the quartz with aluminum; this was associated with a reduction in the quartz-mediated membrane

fluidity and permeability of guinea pig macrophages by aluminum-coated quartz. Further studies by Cao et al.[24] demonstrated conformational changes, measured by Raman spectroscopy, in membrane phospholipids of liposomes exposed to quartz, which were blocked by coating the quartz with aluminum. Thus, the protective effect of aluminum is likely to be related to its ability to reduce the interaction between quartz particles and lipid components of the cell membrane. This may account for the reduced phagocytosis by rat macrophages of aluminum-coated, compared with native quartz particles.[6] It does not, however, explain the increased pulmonary clearance of aluminum-coated quartz reported by Begin et al.[18] and others.[16]

Two key aspects of quartz bioactivity relevant to the development of silicotic lung disease, cytotoxicity, and cell activation have also been investigated *in vitro* and shown to be modified by aluminum. The cytotoxic effect of quartz on guinea pig macrophages was reduced.[23,25] This was related, in rat macrophages, to the concentration of aluminum on the quartz surface as measured by electron spectroscopy for chemical analysis (ESCA) and by Auger spectroscopy.[26] The suppressive effects of aluminum on quartz-mediated lung inflammation and fibrosis may be related to changes in the type of inflammatory mediator secreted by macrophages following phagocytosis of native and aluminum-coated quartz. Englen et al.[27] demonstrated a shift in the type of arachidonic acid product secreted by bovine macrophages exposed *in vitro* to DQ12 quartz. Native quartz caused release of lipoxygenase products, while aluminum-coated quartz caused release of cyclooxygenase products. Effects of aluminum-coating of quartz on the secretion of other inflammatory mediators by macrophages have not yet been assessed.

IV. ASSESSMENT OF QUARTZ TOXICITY IN MIXED DUSTS CONTAINING QUARTZ

The interest in effects of aluminum on the toxicity of quartz in mixed dusts was initiated, in part, by the demonstration that aluminum could be leached from rock dusts and could subsequently coat quartz particles, thus reducing their solubility.[9] On the basis of these findings he postulated that the toxicity of quartz in mixed dusts, such as coal mine dusts containing aluminum-bearing minerals, might be less than predicted for quartz alone due to coating of the quartz by aluminum.

Epidemiological studies of coal miners in Britain substantiate the notion that the harmfulness of a mixed dust containing quartz is not simply related to the quartz content of the dust. While important colliery-related differences between exposure to respirable dust and prevalence of pneumoconiosis have been demonstrated,[28] these are not always related to differences in the quartz content of the coal mine dust. Walton et al.[28] speculated that the presence of other mineral components of the respirable dust cloud (possibly aluminum-bearing clays) could perhaps lessen the impact of the toxic effects of the quartz. In some instances, however, the quartz content of a coal mine dust has had a strong bearing on the harmfulness of that dust.[29,30] These studies suggest that when the quartz content of a mixed dust is sufficiently high (above around 10%), then its toxicity is no longer masked by other minerals in the dust.

A number of experimental studies have demonstrated that coal mine dust and its components could indeed reduce the pathogenic effects of quartz.[31,32] The work of Martin et al.,[33] however, suggested that the protective effect of aluminum may be transient and that, unless there is continual replacement of the aluminum, the fibrotic effect of the quartz will eventually be expressed. Martin's work is in contradiction with that of Begin et al.[18] and Policard et al.,[16] who reported increased quartz clearance from the lung and hence, presumably, reduced quartz toxicity in the presence of aluminum.

V. ALUMINOTHERAPY IN HUMAN SUBJECTS

The use of aluminum inhalation in humans for the treatment of established silicosis was implemented in Canada very soon after the initial reports of success in animal studies. Improved lung function was subsequently reported in patients treated with metallic aluminum[34–36] and hydrated alumina.[37] However, these studies, for the most part, did not control for a possible placebo effect by using mock dust inhalation, and later work showed that improvements in both subjective and objective lung function were similar in silicotics and pneumoconiotics subdivided randomly into treatment and placebo groups.[38] Between the years 1945 and 1979, aluminum powder inhalation was used as prophylaxis against silicosis in the gold mining industry of Ontario.[39] The effect of this treatment resulted in a decrease in the incidence of new

cases exhibiting the first radiological signs of silicosis following implementation of aluminum therapy in 1944/45. The summary indicated that there were no ill effects due to aluminum inhalation and that not a single case of silicosis was found where exposure to silica and aluminum treatment was concurrent. Nevertheless, the report concluded that aluminum therapy was not a substitute for sound dust control and that reducing dust levels in mines was a preferable method of disease reduction. Despite this, aluminum therapy continued to be used and later studies again indicated that aluminum treatment could be beneficial in prophylaxis or in the treatment of early lesions of pneumoconiosis,[40] although it did not affect established lesions.[41]

VI. HARMFUL EFFECTS OF ALUMINUM

The benefits of using aluminum therapy in the prophylaxis of silicosis or mixed dust pneumoconiosis must be balanced with the health risks associated with aluminum exposure. Experimental studies have demonstrated toxic effects of aluminum powder in the lungs of rats[15,42] and hamsters.[43] The major limitation of these studies was that the dusts were administered, at high doses, by intratracheal instillation. However, pulmonary aluminosis was also described in humans exposed to aluminum pyrotechnic flake,[44] finely powdered aluminum,[45,46] and, possibly, aluminum fibers.[47]

In addition to lung toxicity, aluminum powder affects cognitive test performance,[48] and there are now fears that aluminum exposure may contribute to the development of Alzheimer's disease.[49]

VII. SUMMARY

From *in vitro* work with quartz and isolated cells, it appears that the well-documented protective effect of aluminum may be mediated by its ability to bind to the surface of quartz particles, probably by interaction with ionized silanol groups on the quartz surface. This in turn attenuates the interaction between quartz particles and components of the cell membrane, thus ameliorating the cytotoxic and/or stimulatory effects of the quartz and therefore preventing the inflammatory and, ultimately, the fibrotic response in the lung. *In vivo* experiments have confirmed the decreased pathogenicity of aluminum-treated quartz. In addition, it has been demonstrated that late treatment with aluminum lactate can reduce the evidence of toxicity to a previous quartz exposure.

This implies that there might be a potential for therapeutic intervention in situations where quartz exposure has been substantial and no alternative therapy exists. However, the risk of neurotoxic effects of aluminum should still preclude the use of prophylactic aluminotherapy except in cases such as acute progressive silicosis with its otherwise bleak prognosis. The controversy over the duration of the protective effect of aluminum suggests that, in such cases, prolonged treatment with aluminum postexposure might be necessary to prevent recurrence of quartz toxicity. Thus the use of aluminum treatment, with its attendant risks, should never replace the imperative of reducing quartz exposure in the workplace.

The presence of aluminum-bearing clays in mixed dusts containing quartz may have a bearing on the predicted toxicity of that dust and should therefore be considered in setting limits for occupational exposure levels. However, the long-term protective effect of aluminum on silicosis is not known, thus complicating hazard assessment in the workplace. In addition, in certain situations the quartz content of a mixed dust, if sufficiently high, can contribute substantially to the harmfulness of that dust, despite the presence of aluminum-bearing minerals.

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