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# TNF-ALPHA PRODUCTION BY ALVEOLAR MACROPHAGES IN MINERAL-DUST-INDUCED FIBROSIS

A thesis submitted to the School of Graduate Studies

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## ABSTRACT

Macrophages are the predominant cell type in the chronic inflammatory reaction associated with the development of mineral-dust-induced fibrosis. Macrophages are potent producers of cytokines, which have recently emerged as potentially important mediators in regulating inflammatory states. I investigated cytokine production by alveolar macrophages (AM), with a special emphasis on tumor necrosis factor-$\alpha$, in experimental lung fibrosis induced by mineral dusts. Exposure to fibrigenic dusts had a bimodal effect on TNF-$\alpha$ production by AM. First, supperssed TNF-$\alpha$ production after 1 and 3 weeks of exposure. Decond, after 6 weeks of exposure TNF-$\alpha$ production was high. By contrast, interleukin-1 (IL-1) and interleukin-6 (IL-6) production was increased in animals with inflammation with and without fibrosis. Potentiations in IL-1 and IL-6 production were associated with the early stage of the inflammatory reaction and were inversely correlated with TNF-$\alpha$ changes. Interestingly, alterations in TNF-$\alpha$ production were associated with definite shifts in the distribution size of AM suggesting that the overall production of TNF-$\alpha$ may be regulated by the specific class of AM present at sites of inflammation. To our knowledge, our study brings the first evidence for a negative modulation of TNF-$\alpha$ during inflammatory reactions leading to lung fibrosis. Furthermore, experiments with neutralizing antibody to TGF-$\beta$ suggest a role for TGF-$\beta$ in down-regulating TNF-$\alpha$ production in our system.

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## DEDICATION

My thesis is dedicated to my family, my husband and especially to my brother who made me realise that every instant of life is worth it.

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| | |
|---|---|
| AM | alveolar macrophage |
| BAL | bronchoalveolar lavage |
| cAMP | adenosine 3', 5'-cyclic monophosphate |
| DMEM | Dulbelcco's modified Eagles medium |
| FBS | fetal bovine serum |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| ICAM | intercellular adhesion molecule |
| IFN-$\gamma$ | interferon-gamma |
| IL-1 | interleukin-1 |
| IL-6 | interleukin-6 |
| KD | kilo dalton (molecular weight |
| LAK | lymphocyte activated killer cells |
| LPS | lipopolysaccharide |
| MDP | muramyl dipeptide |
| mRNA | messenger ribonucleic acid |
| NK | natural killer cells |
| O.D. | optical density |
| PBS | phosphate buffered saline solution |
| PMN | polymorphonuclear leukocytes |
| TGF-$\beta$ | transforming growth factor-beta |
| TNF-$\alpha$ | tumor necrosis factor-alpha |

# 1. INTRODUCTION

## 1.1 LUNG INFLAMMATION AND FIBROSIS

The lungs and particularly the airways, because of their close contact with the environment, are sites of deposition, absorption and clearance of inhaled infectious and toxic agents. Lung inflammation and fibrogenesis are protective events that take place to destroy, wall-off and eradicate invading organisms, injurious agents and damaged tissues (Roitt et al., 1989; Elias, 1990).

### 1.1.1 Acute Inflammation

The acute form of inflammatory reaction is clinically characterized by the classical signs of pain, heat, redness, swelling and loss of function (Dinarello, 1988). Inflammation is generally a beneficial, non-specific response to injury that resolves. The changes that are implicated in inflammatory response can be divided into vascular and cellular events (Roitt et al., 1989). Vascular events are mainly characterized by vasodilation and increased blood flow to the infected or injured area. Capillary permeability increases, possibly due to contraction of endothelial cells, and exudation of fluids, including plasma proteins. The coagulation system and complement system may also be activated during the vascular events ( Kuhn , 1988). The cellular events are characterized by the involvement of resident macrophages as well as cells that are attracted from blood due to increased permeability of blood vessels. Cells that enter the lung include polymorphonuclear leukocytes (PMN), lymphocytes

and monocytes/macrophages. The accumulation and subsequent activation of these leukocytes are the central point in the pathogenesis of various forms of inflammation (Kuhn, 1988).

### 1.1.2 Chronic Inflammation

Chronic inflammatory reactions may take place when the elimination of foreign material is ineffective (Sibille and Reynolds, 1990). This occurs in certain types of infectious diseases such as tuberculosis, or following exposure to some chemicals, drugs or mineral dusts. Granulomatous inflammation is a special form of chronic inflammation characterized by the presence of organized collections of macrophages (Adams, 1989). Granuloma is a focalized cellular reaction against materials that are retained in tissues for a long time. Macrophages are the main component of the granuloma but other cell types can be present such as fibroblasts, neutrophils, eosinophils, lymphocytes and mast cells. The role of the granuloma is to sequester and, if possible, destroy the foreign invader.

### 1.1.3. Pulmonary Fibrosis

When chronic inflammatory reactions take place, it may result in abnormal tissue remodeling and fibrosis. Such irreversible fibrotic reaction, which is characterized by uncontrolled fibroblast proliferation and abnormal collagen deposition, results in lung distortion and damage (McDonald, 1991). Lung fibrosis can occur in chronic obstructive disease, systemic and localized sclerosis, many chronic infections, exposure to mineral dusts (Rom et al.,

1991), and various chemicals, including bleomycin (Piguet et al., 1989), paraquat and ozone. Unfortunately, when initiated, fibrosis is an irreversible process and to date the factors that control and modulate lung inflammation and pulmonary fibrosis are poorly understood.

## 1.2 ASBESTOS AND SILICA-INDUCED PULMONARY FIBROSIS

### 1.2.1 Asbestos

Asbestos is a collective name given to a group of naturally occurring fibrous minerals. All the asbestos minerals are silicates consisting of sheets of silicate tetrahedra in which oxygen is covalently bound to either one or two silicon atoms. Chrysotile ($Mg_6(OH)_8Si_4O_{10}$), the principal asbestiform of commerce which accounts for almost 95% of the world production of asbestos has interleaving layers of magnesium oxide-hydroxide between the silica layers (reviewed by Miller, 1978). Asbestosis is a disabilitating disease characterized by a chronic inflammatory reaction and interstitial fibrosis which results in obliteration of alveoli, alveolar ducts and respiratory bronchioles. Asbestosis develops many years after the first exposure and is characterized by a slow progressive fibrosis often associated with the excessive deposition of collagen. The degree of pathogenicity of asbestos appears to be related to the size (long fibers being more pathogenic than short ones (Lemaire 1985; Davis and Jons, 1988)), chemical composition (Morgan et al., 1977) and surface properties of fibers (Bonneau et al., 1986).

### 1.2.2 Silica

Silica has a well known chemical composition ($SiO_2$). Silica occurs mainly in three crystal forms or isomers: quartz, tridymite and cristobalite with quartz being the most common form. When silica is immersed in fluids, silicic acid is liberated. Silicic acid reacts readily with membrane phospholipids and it was postulated that such effect may be primarily responsible for its toxicity. Silica particles which are almost symmetric and less than 5 $\mu$m in diameter can easily penetrate to the terminal respiratory tract. The tetrahedral structure of silica appears to be essential for fibrogenic activity of the mineral (Allison, 1974). Compared with the lesions of asbestosis, silicotic fibrosis is well defined and characterized by fibrotic nodules that vary in size from a few miliimetres to larger conglomerate areas of massive fibrosis. Silicotic nodules have a concentric arrangement consisting of hyalinized collagen fibers in a central region and reticulin fibers on the periphery, with some fibroblastic activity at the edges of early lesions (Ziskind, 1976). As mentioned for asbestosis, silicosis has a slow onset and develops many years after the first exposure. Despite their differences in terms of shape and physico-chemical characteristics, both asbestos and silica cause pulmonary fibrosis when inhaled in sufficient quantity. As yet there is no definitive evidence that they do so by any common mechanisms.

### 1.2.3 Animal Models

Various experimental models of asbestos and silica-induced fibrosis have been developed in rodent and non-rodent species with the rat species being by

far the most studied (reviewed by Lemaire, 1993). The rat represents a good model (Davis, 1979; Lemaire, 1993). Definite technical advantages of this model include: a) the rat relative resistance to pulmonary disease (Goodman 1979), b) the stability of rat lung structure over a 2-year adult lifespan (Pinkerton et al., 1982) c) the sampling of sufficient numbers of lung cells following bronchoalveolar lavage (7-10 x $10^6$/rat) (Lemaire 1985) or enzymatic digestion (162 x $10^6$) (Day et al., 1987), d) the relatively pure population of alveolar macrophages (AM) (99%) that can be obtained by bronchoalveolar lavage and which is ideal for studies aimed at assessing macrophage cell biology in relation to fibrosis and e) the large data base that has been developed from both inhalation and acute studies with that species. This can be used as a reference for comparison of data and relevance of some specific cellular parameters to disease. Although few markers are available for rat cells compared to the mouse system, many murine reagents cross-react with rat determinants.

The rat has been used successfully to reproduce the fibrotic lesions induced by silica (Reiser, 1983; Heppleston, 1986; Absher et al., 1989). Fibrosis was obtained in rats after inhalation or intratracheal injection of silica. For its part, asbestos inhalation in rats caused deposits of granulation tissue composed mainly of macrophages and fibroblasts with few giant cells, around the terminal and respiratory bronchioles. With increasing time the lesions evolved toward peribronchiolar fibrosis and consisted mainly of a cellular fibrous

tissue. It was later followed by widespread alveolar fibrosis and resulted in a progressive thickening of alveolar septa (Davis and Jons, 1988). With time, experimental lesions corresponding to advanced human asbestosis could be observed in rats (Davis et al., 1986). Early lesions consisting of granulation tissue with fibroblastic proliferation at bifurcations of distal airways as well as peribronchiolar fibrosis were also observed following a single intratracheal instillation of asbestos (Lemaire and Lemay, 1985).

Inhalation as a method of exposure to mineral dusts, is considered more "physiologic" and results in more homogeneous distribution of particulates than intratracheal instillation. In addition, it usually involves much lower dose rates and generally lower total lung burdens than do studies using intratracheal instillation. In the latter, when initial dust lung burden is high, particle clearance processes may be overwhelmed resulting in marked increase in dust retention. Interpretation of data may be an area of some controversy for those concerned with dust exposure, distribution and retention, as compared to human exposure. These limitations have been discussed in detail by Pritchard et al. (1985) in a review. However, intratracheal instillation offers a convenient technique for descriptive studies comparing many samples of dusts and has been shown, in various animal species, to reproduce asbestotic and silicotic fibrosis. It is clear that intratracheal instillation is a valuable tool for mechanistic studies aimed at determining the cellular responses which trigger the induction of the fibrotic process.

## 1.3 ROLE OF ALVEOLAR MACROPHAGES

Alveolar macrophages originate from the bone marrow, circulate in the blood as monocytes and migrate inside the lung where they may or may not divide (Sibille and Reynolds, 1990). The population of AM is morphologically and functionally heterogeneous (Shellito and Kaltreider, 1984; Lemaire and Lemay, 1985; Ferro et al., 1987). In fact, AM separated on a Percoll density gradient, are functionally different for IL-1 production, expression of Ia antigen, phagocytosis, and prostaglandins. It is important to note that these differences appear to be correlated with the maturation/differentiation state of AM. Their diameter varies from 12 $\mu$m to 40 $\mu$m (Cohen and Cline, 1971). Normal AM from rodents display smooth or ruffled surface membranes with ruffled cells indicating a high capacity for phagocytic and chemotactic activity ( Warheit et al., 1984a). Alveolar macrophage morphology is also characterized by a lobulated nucleus and vacuolated cytoplasm containing several mitochondria and electron dense secondary lysosomes (Sibille and Reynolds, 1990).

In mineral dust-induced fibrosis the causative agent can be followed after it has gained access to the respiratory system. The first lung responses to mineral dusts exposure is a persistent inflammatory reaction in which the macrophage is an essential participant. Asbestos fibers and silica particles have been shown to deposit on alveolar duct bifurcations. This is followed by an influx of alveolar macrophages which accumulate at sites of dusts deposition (Chang et al., 1988). Attraction of AM to the site of deposition may result

from activation of complement, particularly C5a (Warheit et al., 1985) and/or production of leukotriene $B_4$ (Garcia et al., 1989). Overall, when considering data on inflammatory cell involvement during the development of silica- and asbestos-induced fibrosis, one of the most consistent features is the presence of macrophage infiltration. The weight of available evidence favours the opinion that macrophages act as primary effector cells in these reactions, while the other cell populations including lymphocytes, neutrophils and mast cells as secondary effectors. Because particles or fibers are phagocytosed by macrophages, such process is likely to alter macrophage functions in many ways. Therefore, research has focused mainly on the role of the macrophage and its relationships to the subsequent development of fibrotic process. Notably, macrophages have the ability to produce a variety of growth factors and cytokines which can modulate the activities of fibroblasts, the effector cells of fibrogenesis.

## 1.4 INVOLVEMENT OF CYTOKINES

Cytokines play a prominent role as mediators of inflammatory and immune responses (Balkwill and Burke, 1989). The importance of cytokines in fibrosis has been reviewed recently (Kovacs, 1991). It is likely that certain classes of cytokines play a role in dictating and modulating the progression and outcome of inflammatory reactions.

### 1.4.1 Tumor Necrosis Factor-$\alpha$

**a. Protein**

Tumor necrosis factor alpha, also known as cachectin, has been described as an important mediator of inflammatory responses (Sherry and Cerami, 1988; Beutler and Cerami, 1990). It was first described as a necrotic agent for its ability to induce haemorrhagic necrosis of certain solid tumors (Carswell et al., 1975). TNF-$\alpha$ was cloned and characterized in 1985 (Frausen et al., 1985; Pennica et al., 1985; Shirai et al., 1985). It is a pleiotropic molecule that can be produced by a number of cells including monocytes, macrophages, lymphocytes, natural killer cells, glomerular mesanglial cells, astrocytes, microglial cells of the brain and Kupffer's cells of the liver (Camussi et al., 1990). The TNF-$\alpha$ molecule is initially synthesized as a precursor of 233 amino acids (a.a.) for human (Shirai et al., 1985) and 235 a.a. for mouse (Frausen et al., 1985; Pennica et al., 1985). The mature protein, mainly produced by macrophages, ( Fish and Gifford, 1983), has a molecular weight of 17 Kd and contains 157 a.a. for the human protein (Shirai et al., 1985) and 156 a.a. for the mouse (Frausen et al., 1985; Pennica et al., 1985). The TNF-$\alpha$ molecule is highly conserved within the mammalian species, with approximately 80% homology between the amino acid sequences of human, mouse and rabbit (Beutler and Cerami, 1990 ). The gene that encodes for TNF-$\alpha$ protein is located within the major histocompatibility complex gene (Muller et al., 1987). The TNF-$\alpha$ gene is also closely linked to the lymphotoxin gene also

known as TNF-$\beta$ (Nedwin et al., 1985a) and is located on chromosome 6 in human (Shirai et al., 1985) and on chromosome 17 in mouse (Muller et al., 1987).

**b. Modulation of Production**

TNF-$\alpha$ molecule is not stored inside the macrophage but rather synthesized following cell activation. It was demonstrated that the macrophage contains a pool of TNF-$\alpha$ mRNA that is not expressed as a protein. After stimulation with lipopolysaccharide (LPS) (Camussi et al.,1991), the macrophage will increase the quantity of TNF-$\alpha$ mRNA that will be mobilized for translation which will lead to an active production of TNF-$\alpha$ protein (Camussi et al.; Beutler et al., 1986). Besides LPS, there are other factors that have been reported to induce TNF-$\alpha$ production, including muramyl dipeptide (MDP) (Bahr and Chedid, 1986), interferon gamma (IFN-$\gamma$) (Nedwin et al., 1985b), granulocyte-macrophage colony stimulating factor (GM-CSF) (Kleinerman et al., 1988) and interleukin-1 (IL-1) (Camussi et al., 1990). TNF-$\alpha$ itself may, in an autocrine manner, trigger its own production (Camussi et al., 1990). TNF-$\alpha$ protein is produced by the macrophages as a soluble factor and acts as a mediator for activation of other cells. There is also a cell-associated form of TNF-$\alpha$ protein that has been described (Krigler et al., 1988). The major role of this cell-associated TNF-$\alpha$ appears to be related to the destruction of sensitive targets. The cell-associated protein exists in two forms: TNF-$\alpha$

attached to its receptor (Luettig et al., 1989) and a 26 Kd transmembrane protein that is the precursor of the 17Kd TNF-$\alpha$ mature protein (Bakouche et al., 1990). There are also certain inhibitors of TNF-$\alpha$ production that could act as physiological modulators of TNF-$\alpha$ action. These include interleukin-6 (IL-6) (Fletcher et al., 1990), transforming growth factor-$\beta$ (TGF-$\beta$) (Chantry et al., 1989) and prostaglandins (Warren, 1990). In addition, a soluble fragment of the TNF-$\alpha$ receptor molecule has been found to inhibit TNF interaction with target cells (Kohno et al., 1990; Gray et al., 1990).

**c. TNF-$\alpha$ Receptor**

TNF-$\alpha$ interacts with the target cell by forming a stable trimer that binds to a TNF-$\alpha$ receptor (Aggarwal et al., 1985). TNF-$\alpha$ receptors have been identified on all nucleated cells. Two types of receptors have been found for human and for mouse TNF. Both receptors have an intracellular domain and an extracellular domain. The type 1 receptor (55 Kd) is present mainly on cells of epithelioid origin, while type 2 receptor is expressed by cells of myeloid origin (Shepherd, 1991) although some cells, notably HL60 and U937, express both types of receptors (Brockhaus et al., 1990; Loetscher et al., 1990). It was reported that TNF-$\alpha$ receptor expression increased during monocyte differentiation (Michishita et al., 1990). Interferon gamma as well as interleukin-1 have been found to increase the expression of TNF-$\alpha$ receptor on several cells (Shepherd, 1991; Drapier and Wietzerbin, 1991). However, IFN-

gamma apparently decreases surface TNF-$\alpha$ receptor expression on primary macrophages (Shepherd, 1991), suggesting that its modulatory effect on TNF-$\alpha$ receptor expression may vary depending on the cell type.

**d. TNF-$\alpha$ and Inflammation**

TNF-$\alpha$ exerts many pro-inflammatory activities. TNF-$\alpha$ has been shown to be associated with fever induction (Wassen, 1990) and PMN infiltration (Warrenet al., 1991; Matsushima et al., 1988). TNF acts on endothelial cells by inducing the production of intercellular adhesion molecules (ICAM) ( Wassen, 1990) and increasing their adherence to neutrophils, monocytes, and lymphocytes. Such events are critical steps in the inflammatory cell recruitment at the site of infection or injury (Gamble et al., 1985; Pohlman, 1986). In addition, TNF-$\alpha$ has been found to induce lung granulomas (Kasahara et al., 1989). Furthermore, injection of anti-TNF-$\alpha$-antibody prevented development of granulomas during infection of mice with *Mycobacterium bovis* (Kindler et al., 1989). Moreover, TNF-$\alpha$ can induce the production of a variety of inflammatory mediators, including IL-1 (Dinarello et al., 1986; Buchan et al., 1988), IL-6 (VonAsmuth et al., 1991; McIntosh et al., 1989), and prostaglandins (Akama et al., 1990) by macrophages, fibroblasts and endothelial cells. Therefore, TNF-$\alpha$ is an important mediator involved in inflammation and in cell-to-cell communication.

### e. TNF and Fibrosis

A role TNF-$\alpha$ has been suggested in silica- and bleomycin-induced pulmonary fibrosis (Piguet et al., 1990). Increased levels of TNF were found in silicotic nodules of mice but the cellular origin of TNF was not determined in that study. With regard to TNF production by AM in experimental silicosis, conflicting data have been reported. While no change in spontaneous and LPS-induced TNF production was observed in mice that have received a high dose of silica (250 mg/kg, intratracheal instillation) (Bissonnette and Rola-Pleszczynski, 1989), increases in both spontaneous and LPS-induced TNF release by AM were found in rats treated with silica at doses greater than 50 mg/kg (Driscoll et al., 1990). On the other hand, no spontaneous release of TNF-$\alpha$ by AM was observed following inhalation of silica in rats (Mohr et al., 1991) while TNF-$\alpha$ production induced by low concentrations of LPS was greatly enhanced.

## 1.4.2 Other Cytokines

### a) Interleukin-1

Interleukin-1 (IL-1) is another major inflammatory mediator. IL-1 can be produced by several cells but mainly by macrophages and keratinocytes. Two forms of IL-1 have been cloned and expressed: IL-1-$\alpha$ and IL-1-$\beta$ (Auron et al., 1984; Lomedico et al., 1984). The gene location for both forms of IL-1 are located on chromosome 2 for human protein (Durum and Oppenheim, 1989;

Keley, 1990). Our laboratory found that rat alveolar macrophages produce mainly IL-1-$\alpha$ (unpublished). The two forms of IL-1 are initially synthesized as 31 kD precursor polypeptides (termed pro-IL-1) and share only small stretches of amino acid homology. Pro-IL-1 has weak biological activity and no signal peptide sequence. It is unclear how it is processed to a 22- or 17-kD active molecule. Processing may take place in the lysosomal fraction or elsewhere. A significant amount of translated pro-IL-1 is found in the cell membrane as a 22-kD molecule. The primary extracellular form of IL-1 is 17 kD and is vulnerable to extracellular proteases which yield biologically active IL-1 peptides with molecular weights of 11-, 4- and 2-kD. It is interesting to note that both forms share similar activities and bind to a single common receptor (Keley, 1990; Sims et al., 1988). The secreted protein has a molecular weight of 17 kd. IL-1 has been shown to exert potent pro-inflammatory activities including induction of fever (Dinarello, 1988), neutrophil activation (Warren, 1990), stimulation of AM chemotaxis (Laplante et al., 1986) and induction of production of other inflammatory mediators (Durum and Oppenheim, 1989). Although TNF-$\alpha$ shares only 3% amino acid homology with IL-1 $\beta$ and acts on an independent receptor (Dinarello, 1988) many of the biological properties of IL-1 and TNF-$\alpha$ overlap. Moreover, IL-1 and TNF-$\alpha$ often act synergistically and many of the changes seen during inflammatory reactions may be the net result of the biological activities of both IL-1 and TNF-$\alpha$.

With regard to experimentally induced fibrosis, relatively little work has been performed on the role of cytokines in mineral dust-induced lung fibrosis. Increases in IL-1 release by AM have been reported in various models of silicosis (Oghiso and Kubota, 1986; Struhar et al., 1989b; Driscoll et al., 1990; Kusaka, 1990). Enhanced production of IL-1 was correlated with an increase in Ia-positive AM (Oghiso and Kubota, 1986; Struhar et al., 1989b) consistent with a local amplification of cell-mediated immunity in fibrosis. Increased release of IL-1 from AM has also been reported following asbestos exposure by inhalation (Hartman, 1984) or intratracheal instillation (Lemaire, 1991). These observations are compatible with a role of IL-1 in some stages of the inflammatory process, possibly associated with further AM recruitment and activation. However, IL-1 was not detected in silicotic nodules of a murine model of silicosis (Piguet et al., 1990).

**b) Interleukin-6**

IL-6 is a cytokine produced by various types of cell including macrophages, fibroblasts, lymphocytes and endothelial cells (VanSnick, 1990; Terebuh et al., 1992; Aarden et al., 1987). IL-6 is synthesised as a 212- a. a. precursor that is processed into a 184-a.a. secreted product (Warren, 1990). It was cloned in 1986 (Hirano et al., 1986). The IL-6 gene is located on chromosome 7 in human ( Durum and Oppenheim, 1989). IL-6 has been associated with B-cell differentiation, induction of acute phase proteins,

promotion of myeloma/plasmacytoma/hybridoma cells, and proliferation and differentiation of T-cells (Hirano et al., 1990).

Recently, IL-6 has been identified as a potential mediator of inflammation. However, its role in local acute inflammation has not yet been defined. IL-6 was reported to decrease endotoxin-induced serum levels of TNF-$\alpha$ in mice (Aderka et al., 1989) as well as IL-1 mRNA levels in spleen and liver of endotoxemia rats ( Ulich et al., 1991a). Recent experimental evidence has showed that IL-6 significantly inhibits the acute neutrophilic exodus caused by an intratracheal injection of LPS ( Ulich et al., 1991b). Taken together the data points to an inhibitory function for IL-6 in acute inflammation.

There is very little information available on the role of IL-6 in lung fibrotic reaction. IL-6 has been detected in cells from silicotic nodules but not in serum (Piguet et al., 1990). In addition, IL-6 levels have been reported to be increased following *in vitro* exposure to coal dust (Gosset et al., 1991).

**c) Transforming growth factor-$\beta$**

TGF-$\beta$ is a protein of 25 kD consisting of two identical 12.5 kD subunit joined by disulfide bonds (Keley, 1990). TGF-$\beta$ is synthesized as a precursor protein that is cleaved into a 112 a.a. mature protein. The gene for TGF-$\beta$ is located on chromosome 19 in the humans and on chromosome 7 in the mouse ( Durum and Oppenheim, 1989). TGF-$\beta$ is produced constitutively by many cell types including AM (Kelley et al., 1991). TGF-$\beta$ has been located in many

tissues such as placenta, bovine kidney, bones, and platelets ( Durum and Oppenheim, 1989). Many different activities of TGF-$\beta$ on target cells have been reported. TGF-$\beta$ is a chemotactic and mitogenic factor for fibroblasts and enhances collagen, fibronectin and collagenase synthesis ( Durum and Oppenheim, 1989). TGF-$\beta$ was found to inhibit proliferation of endothelial cells, T and B lymphocytes, early haematopoietic stem cells, fetal hepatocytes and keratinocytes and has also been related to the suppression of NK and LAK cell development ( Durum and Oppenheim, 1989; Wahl et al. 1989). Finally, TGF-$\beta$ has been identified as modulator of tissue repair by initiating fibroblasts proliferation and chemotactic activity ( Wahl et al., 1989). In fact, TGF-$\beta$ has been identified in wound sites and reported to be produced in such sites by macrophages (reviewed by Wahl et al., 1989).TGF-$\beta$ can also modulate the production of other cytokines at transcriptional and post translational levels. TGF-$\beta$ has been shown to down-regulate IL-1 and TNF-$\alpha$ production by a prostaglandin and cAMP independent pathway (Chantry et al., 1989). It seems that the effect of TGF-$\beta$ on the production of other cytokine is variable because under certain conditions the levels of IL-1 mRNA increased (Wahl et al, 1989). Similarly, both induction and inhibition of IL-6 synthesis as well as inhibition have been observed after TGF-$\beta$ stimulation (Turner et al., 1990).

A role for TGF-$\beta$ in pulmonary fibrosis has been suggested by studies of bleomycin-induced fibrosis (Khalil et al., 1989). TGF-$\beta$ mRNA levels were

significantly elevated in mice exposed to bleomycin. This elevation in TGF-$\beta$ mRNA was shown to precede changes in the gene expression for collagen and fibronectin (Hoyt and Lazo, 1988) (Kelley et al., 1991). To date, the putative role of TGF-$\beta$ in asbestos- and silica-induced lung fibrosis has not been investigated except for one recent study that showed no significant variation of TGF-$\beta$ during experimental silicosis (Piguet et al., 1990).

## 2. OBJECTIVES

The mechanisms that are involved in dust-induced lung inflammation and fibrosis remain undefined. The early molecular events that initiate and sustain granuloma formation as well as those that dictate the progression of these reactions toward irreversible fibrosis are poorly understood. It is now appreciated that cytokines play a prominent role as mediators of inflammatory and immune reactions (Roitt et al., 1989). Accumulating evidence based mostly on *in vitro* experiments suggests that cytokines play a predominant role in fibrosis. However, relatively little work has been performed with regard to the role of cytokines in experimental fibrosis, notably mineral dust-induced fibrosis. There is little information concerning some cytokines such as IL-6 and TNF in silica and asbestos-induced pulmonary fibrosis. In one study of experimental silicosis, the source of cytokines has not been clearly identified and relatively few studies have examined, in a sequential fashion, the production of these cytokines by inflammatory AM exposed *in vivo* to fibrogenic agents. Moreover, there is strong evidence in support of a network of interacting cytokines maintaining a proper balance which has been only partially discovered. To date, the relative contribution of various cytokines in inflammatory reactions remains unclear. Previous observations from our laboratory indicate that intratracheal instillation of various silicate fibers induced different types of lung inflammatory reactions which range from resolving granuloma to irreversible fibrosis ( Lemaire, 1985; Lemaire et al., 1985; Lemaire

et al., 1989; Lemaire, 1991) We took advantage of this animal model to investigate the sequential production of IL-1, IL-6 and TNF-$\alpha$ in relation to AM morphology and lung histopathological changes.

## 2.1 Specific Objectives

Using an in vivo model of asbestos- and silica-induced lung inflammation and fibrosis:

1. to determine the production of TNF$\alpha$, IL-1 and IL-6 by alveolar macrophages during the development of chronic inflammation and fibrosis.
2. to investigate the modulation of TNF-$\alpha$ production from AM by other cytokines.

# 3. MATERIALS AND METHODS

## 3.1 ANIMALS:

Male Wistar rats weighing between 250 and 275 g were purchased from Charles River Canada, Inc. (St-Constant, Québec). These rats were derived from a pathogen-free colony. The animals were shipped behind filter barriers and were housed in a horizontal laminar flow isolator (Johns Scientific Inc.,Toronto). They were fed with Purina Rat Chow (Ottawa, Canada) and water *ad libitum* and they were used one week later.

## 3.2 ASBESTOS FIBERS AND SILICA PARTICLES:

UICC standard sample of chrysotile B (21% were longer than 10 $\mu$) was obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa (Timbrell, 1970). Very short 4T30 chrysotile fibers (100% were less than 5 $\mu$m) were isolated from Johns-Manville grade 4T30 chrysotile as described by Jolicoeur et al. (1981). Except for differences in mean length and in specific surface area (short 4T30, 38$m^2$/g; UICC, 26.8$m^2$/g), these 2 chrysotile asbestos samples had similar chemical and structural properties (Lemaire et al., 1985). These fiber preparations were provided by the Laboratoire de Caractérisation de l'amiante (SNA), Université de Sherbrooke, Sherbrooke. Silica particles (5 $\mu$) (Min-U-Sil) were obtained from Pennsylvania Glass-Sand Corp., Pittsburg, PA. Each sample was autoclaved for 45 minutes and resuspended in sterile phosphate buffered saline (PBS) (pH 7.4)

with a Dounce glass homogenizer (Fisher Scientific, Ottawa) before intratracheal instillation into the animals.

### 3.3 INDUCTION OF LUNG GRANULOMA AND FIBROSIS:

Rats were lightly anaesthetized with a mixture of Ketamine-xylazine (85%-15%, 100mg/Kg). The trachea was exposed surgically, and fiber and particle samples were injected intratracheally in a final volume of 0.5 ml as described previously (Lemaire et al., 1985). Groups of 5 animals each received saline or 5 mg of UICC chrysotile B, short chrysotile 4T30, or silica, and animals were killed periodically (1, 3 and 6 weeks) after instillation.

### 3.4 OBTENTION AND CULTURE OF BRONCHOALVEOLAR CELLS:

Bronchoalveolar cells were obtained by bronchoalveolar lavage as described previously (Lemaire, 1985). Briefly, animals received sodium pentobarbital, 30 mg/rat, i.p. (M.T.C. Pharmaceuticals Canada Packers Inc., Cambridge, Ontario.). The abdominal aorta was severed to prevent the presence of blood in the lungs and the trachea was cannulated. A total volume of 48 ml PBS (pH 7.4) was infused in 8 ml aliquots into the lungs. A volume of 44 ml was recovered in control rats. The bronchoalveolar lavage fluid (BAL) was centrifuged at 200g for 10 minutes at 4°C. The cell pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Burlington, Ontario). Cells were counted using a haemacytometer chamber and

the viability was determined by trypan blue exclusion. For comparison, values of total cell counts in lavage fluid at each time studied were corrected for the corresponding fluid recovery at that particular interval, and all values were calculated as cell counts per 44 ml of lavage fluid recovered. To generate supernatants for TNF-$\alpha$ quantification, BAL cells ($0.2 \times 10^6$) from control and treated rats were incubated in 0.2 ml of DMEM without serum for 18 h at 37°C in the presence on absence of LPS (1 $\mu$g/ml) (Sigma Chemical Co. St-Louis, MO). For determination of IL-1 and IL-6, BAL cells were resuspended in RPMI-1640 supplemented with 0.5 % FBS (Grand Island Biological Co. (Gibco)) and incubated under the same conditions. Culture supernatants were collected by centrifugation and frozen at -80°C until assayed.

### 3.5 ANALYSIS OF CELL SIZE AND POPULATIONS

Differential counts of lavage cells were made from cytocentrifuge smears prepared with $2.5 \times 10^4$ cells and stained with Wright-Giemsa stain. Two hundred cells were counted. In addition, the diameter of 200 AM collected from control and treated rats was measured with a calibrated reticle (Leitz, Wetzlar, Germany) in the eyepiece of a light microscope using a 40X objective. The proportion of AM with a diameter of 10 to 45 $\mu$m was determined for each rat.

## 3.6 CELL LINES

### 2.6.1 L929 Cells

L929 tumorigenic murine fibroblast cell line was obtained from the American Type Culture Collection (ATCC) (CCL 1). They were grown in DMEM supplemented with 10% horse serum (Gibco/BRL) and penicillin. These cells were adherent to plastic and were subcultured three times a week by detaching the cell monolayer with a 0.5% trypsin solution (Gibco/BRL). L929 cells were maintained in an atmosphere of 5% $CO_2$ - 95% air at 37°C.

### 3.6.2 B9 Cells

B-9 murine hybridoma cell line was obtained from Peter M. Lansdorp, Terry Fox Laboratory, Cancer Research Center, Vancouver, B.C., Canada. These cells are dependent on IL-6 for proliferation (Helle et al., 1988). B9 cells were grown in suspension in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco/BRL) supplemented with 5% FBS, $5x10^{-5}$ M 2- mercaptoethanol, 0.8% Hepes and Gentamicin. A source of IL-6 should be added to the medium to allow the B-9 cells to grow and rmIL-6 (50 U/ml, Genzyme Co.,Cambridge, USA) or conditioned medium from P388D cells (5%) was used. $P388D_1$ cells produce several cytokines including IL-6 when stimulated with muramyl dipeptide (MDP). Therefore, IL-6-containing supernatants were prepared by incubating $P388D_1$ cells with MDP (20 $\mu$g/ml) for 6 days in an atmosphere of 5%$CO_2$-95% air at 37°C. Conditioned medium was dialysed overnight,

supplemented with 1% FBS and filtered through a 0.45 $\mu$m filter (Millex, Millipore). Frozen aliquots were stored at -80°C. The B9 cell population doubles every 13 hours and cells were seeded twice a week at a 1/10 dilution. Cells were grown in a humidified atmosphere of 5% $CO_2$ - 95% air at 37°C.

### 3.6.3 D10(N4)M Cells

D10(N4)M cells (a gift from Dr. S. Hopkins, University of Manchester, U.K.) are a subclone of a murine T cell line (D10.G4.1) (Kaye et al., 1983). D10(N4)M cell proliferation is dependent on the presence of IL-1 and IL-2 in the medium. The cells were grown in IMDM containing 10% FBS, 0.8% hepes, $5x10^{-5}$ M 2-mercaptoethanol, Gentamicin, P388 supernatant (5%, as a source of IL-1 as described above) and EL4 cell supernatant (10%, as a source of IL-2). EL4 supernatant was prepared by incubating $10^6$ cells/ml with Concanavalin A (Con A; 10 $\mu$g/ml) (Sigma) and phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) (Sigma) for 24 hours. Culture supernatants were then centrifuged, filtered and kept frozen at -80°C. D10(N4)M cells were subcultured three times a week at a density of $0.5x10^6$/10ml.

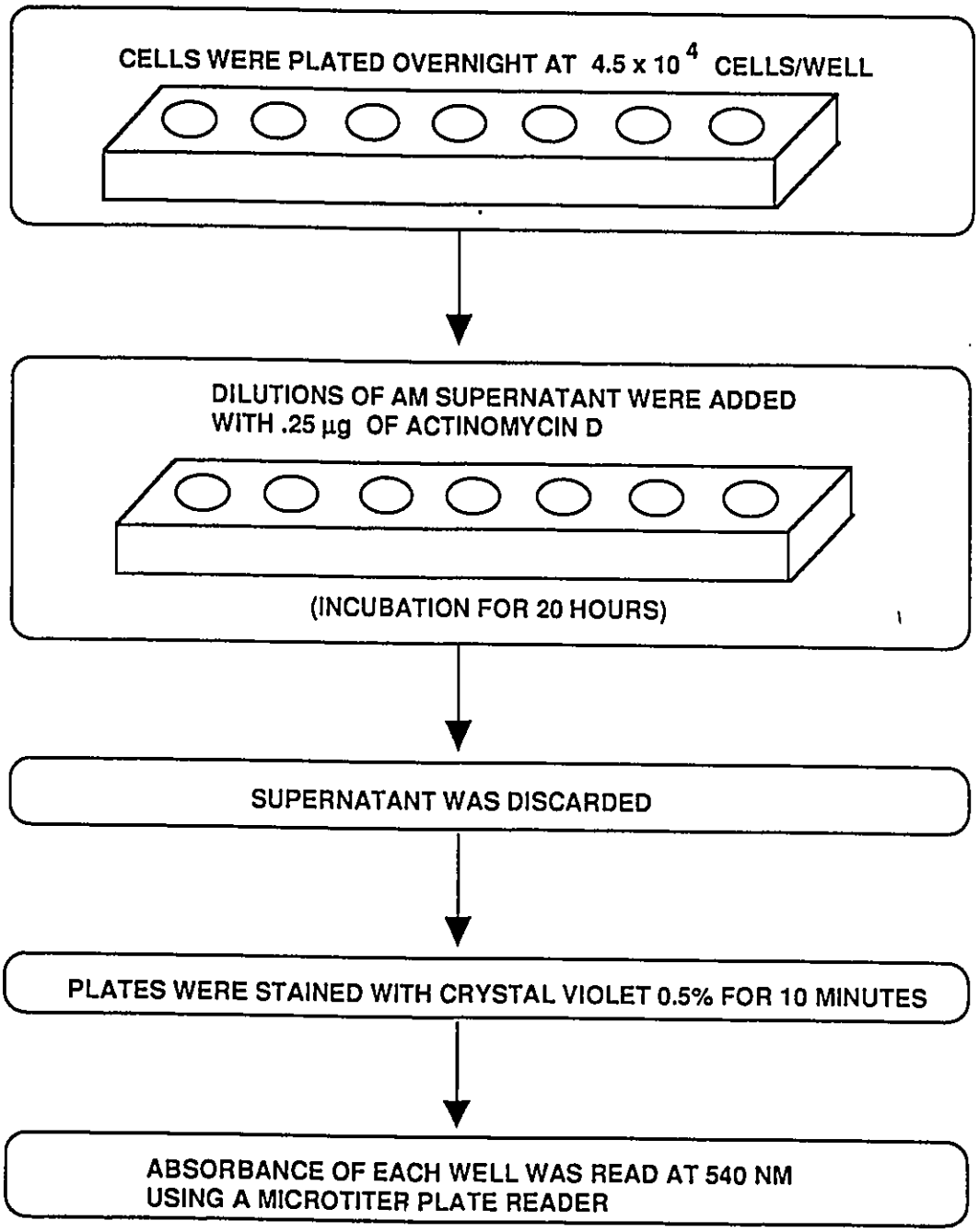
## 3.7 TUMOR NECROSIS FACTOR-$\alpha$ ASSAY

TNF-$\alpha$ activity present in the alveolar macrophage supernatants was measured according to a modified L929 fibroblast lysis assay (Flick and Gifford, 1984) as illustrated in Figure 1. L929 cells were seeded into a flat bottom

**Figure 1:** This is a schematic illustration of the technique used to measure TNF-$\alpha$ production by AM. This cytotoxic assay using L929 as target cells is described in the Materials and Methods.

figure 1

## TNF ASSAY



96-well microtiter plate (Falcon) at a density of $4.5 \times 10^4$/well in 200$\mu$l of DMEM containing 10% horse serum , penicillin and 0.8% Hepes. The plates were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% $CO_2$-95% air. During this first step L929 cells form an adherent cell monolayer on the bottom of the well. Supernatants were then removed and replaced by 200$\mu$l DMEM without serum (Kramer and Carver, 1986) containing actinomycin D (1.25 $\mu$g/ml) (Boehringer Mannheim). Actinomycin D is used to stop L929 proliferation. Serial dilutions of test supernatant or murine recombinant TNF-$\alpha$ (generously provided by Genetech, San Francisco) were also added to each well in triplicate. After incubation for 20 hours, the supernatants were discarded and plates were washed with warm PBS. The remaining adherent viable cells were stained with crystal violet (0.5% in 2% methanol) for 10 min. The microtiter dishes were rinsed with water and the absorbance of each well was read at 540 nm using an automated Bio-Tek microplate reader (Mandel Scientific Co. Lachine, Qué, Canada). TNF bioassay was validated by the use of a rabbit antimurine TNF-$\alpha$ antibody (Genzyme Corporation, Boston, MA) which completely neutralized the cytotoxicity of AM-conditioned media. Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{O.D. L929} - \text{O.D. sample}}{\text{O.D. L929}} \times 100$$

Each assay was standardized with murine recombinant TNF-$\alpha$ ($2.6 \times 10^7$ U/mg)

and TNF-$\alpha$ units were calculated by probit analysis.

### 3.8 INTERLEUKIN-1 ASSAY

IL-1 activity produced by alveolar macrophages was determined according to Hopkins and Humphreys (1989) as described in Figure 2. D10 (N4)M cells (36) ($10^4$) were cultured in 96-well microculture plates in a final volume of 200 $\mu$l of complete medium (RPMI 1640 containing 10% FBS, 5 x $10^{-5}$M 2-mercaptoethanol, Hepes (0.8%), Concanavalin A (5 $\mu$g/ml) and recombinant IL-2 (30 U/ml) with serial dilutions of AM supernatants. Appropriate controls contained medium, IL-2, Con A and LPS alone or in combination. Cultures in triplicate were incubated for 66 h at 37°C, pulsed with 1 $\mu$Ci/well [$^3$H]thymidine (New England Nuclear, Dupont, Mississauga, Ontario) and harvested at 72 h. Bioassay data were calculated as net cpm by the following formula: net cpm = (cpm of D10 cells + Con A + IL-2 + sample)-(cpm of D10 cells + Con A + IL-2). Incorporation of [$^3$H]thymidine in the presence of sample dilutions was compared with that in the presence of dilutions of a standard recombinant IL-1 preparation (Genzyme, $10^8$ U/mg) and IL-1 units were calculated by probit analysis. IL-1 bioassay was validated by the use of a goat antimurine IL-1 $\alpha$ (generously provided by Dr. R. Chizzonite, Hoffman-La Roche Inc. Nutley, NS) which completely neutralized the activity found in AM supernatants.

### 3.9 INTERLEUKIN-6 ASSAY

IL-6 activity was determined according to Aarden and colleagues (Helle

**Figure 2:** The IL-1 assay is represented on this diagram which describes the different steps used in the determination of the IL-1 production. This proliferative assay is using D10 Cells as targets and is described more precisely in the materials and methods section.

figure2

## IL-1 ASSAY

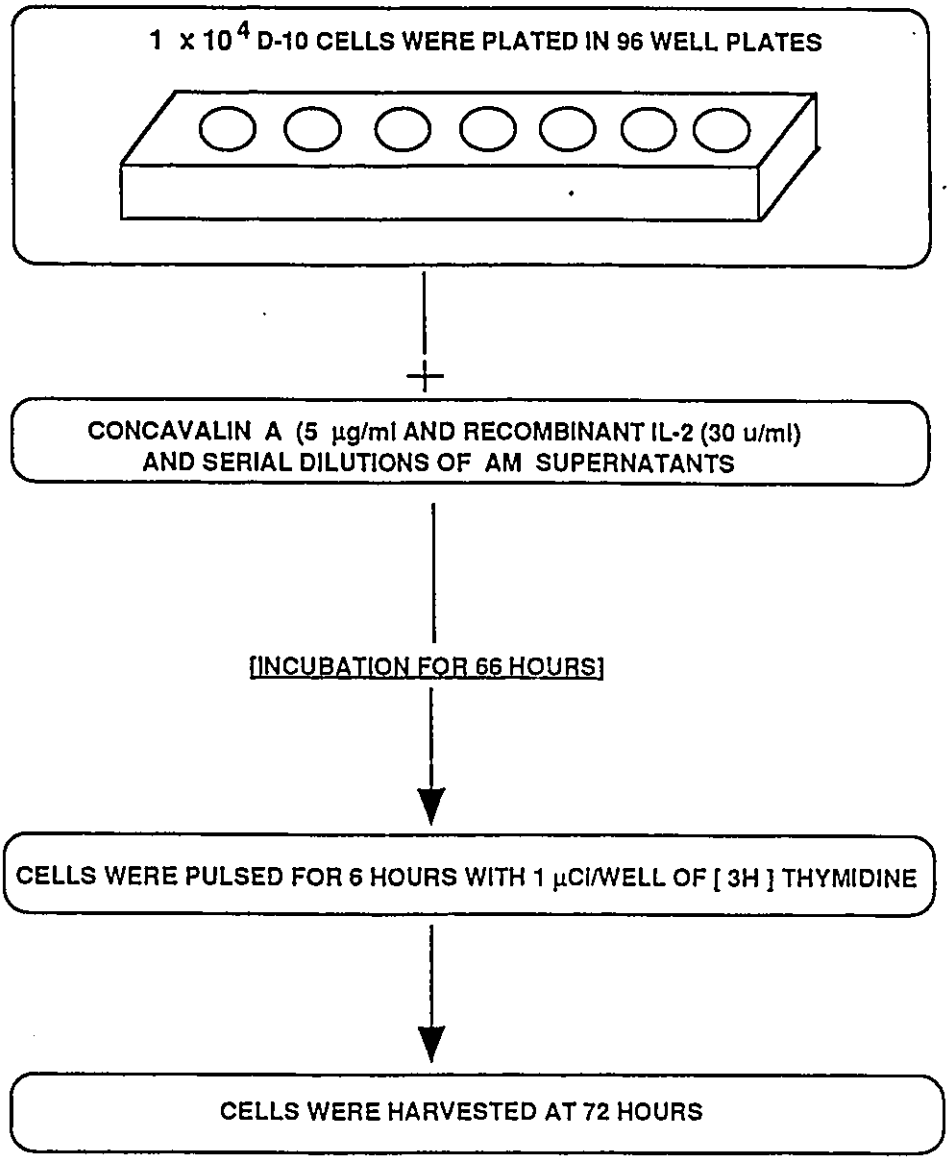
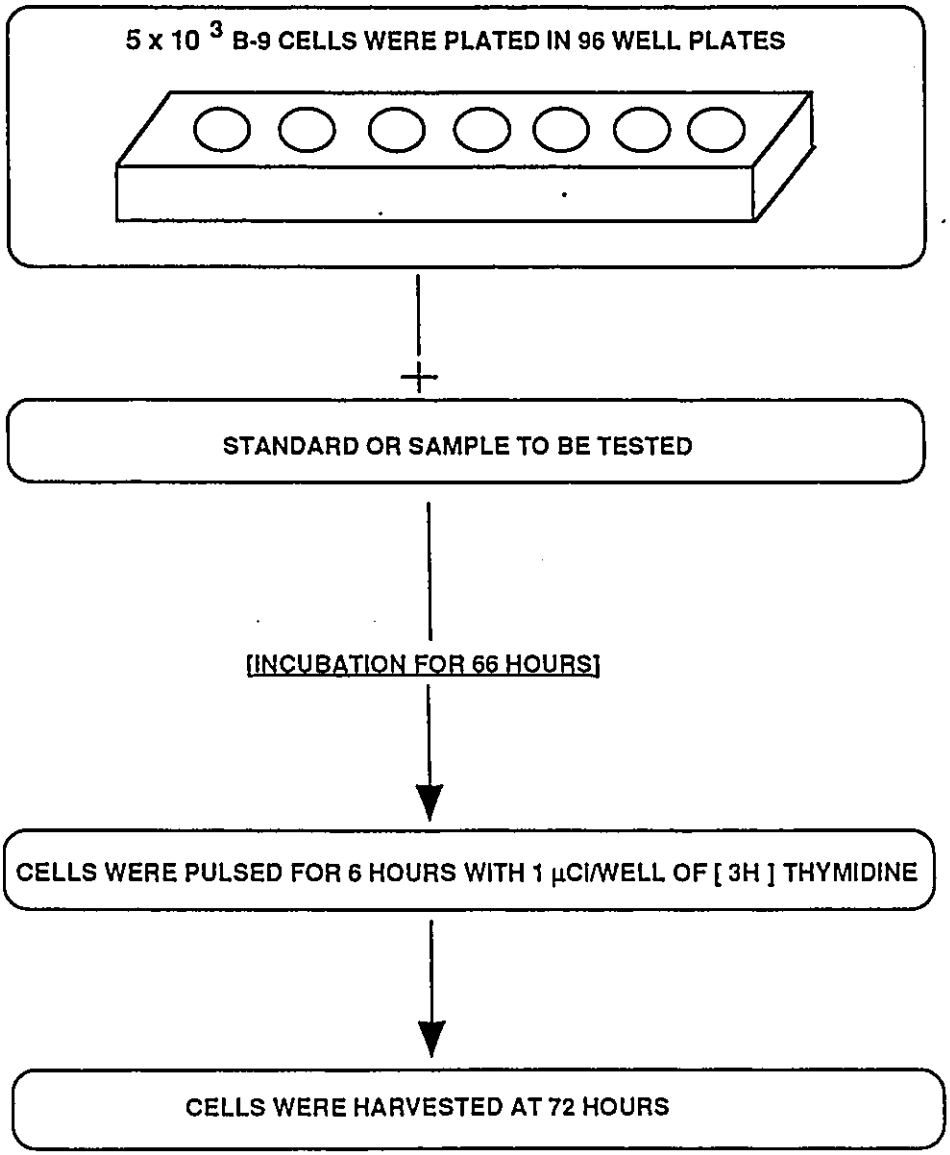


**Figure 3**: Representation of the IL-6 assay that has been used to detect IL-6 production from AM. This is a proliferative assay using B9 Cells as target cells.

figure3

## IL-6 ASSAY



et al., 1988; Aarden et al., 1987) using the B-9 proliferation assay as described in Figure 3. Briefly, B-9 cells were washed and centrifuged in IL-6 free medium. Cells were then plated in triplicate at $5x10^3$/200 $\mu$l in flat bottom wells in the presence of test samples at various dilutions. Tritiated thymidine (1 $\mu$ Ci/well) was added after 66 h of incubation and cultures were harvested at 72 h with a Skatron filtration apparatus. Bioassay data were calculated as net cpm by the following formula: net cpm = (cpm of B9 cells + sample) - (cpm of B9 cells). IL-6 units were calculated by probit analysis. Sample dilution curve was related to a standard curve generated with recombinant murine IL-6 (Genzyme, $10^8$ U/mg) and IL-6 units were calculated by probit analysis.

### 3.10 NORTHERN HYBRIDIZATION ANALYSIS

Measurement of messenger RNA for TNF-$\alpha$ was performed by Huixin Yang from our laboratory. Total cellular RNA was obtained using the acid guanidinium thiocyanate-phenolchloroform extraction procedure of Chomczynski and Sacchi, (1987). RNA samples ( 10 $\mu$g) were electrophoresed through 1% agarose-formaldehyde/MOPS gels and transferred by capillary action to Pall Biodyne A nylon membranes (Pall Canada Ltd, Mississauga, Ontario, Canada) as described by Fourney et al., 1988. The cDNA probe for human TNF-$\alpha$ (585 bp) was prepared by Hind 111 and Ava 1 digestions from plasmid pc DV (ATCC, No 39894) and the human $\beta$-actin probe (850 bp) was

obtained from Dr. Izaguirre ( Federal Center for AIDS, Ottawa, Ont.). Probe DNA fragments (25 ng) were radiolabeled with $\alpha$-$^{32}$P dCTP (3000 Ci/mmol; Dupont/NEN Canada Ltd, Mississauga, Ontario, Canada) to specific activity $5x10^8$ dpm/$\mu$g using commercial kits (Promega, Madison WI) for random hexadeoxyribonucleotide synthesis (Feinberg and Vogelstein, 1983). Unincorporated dNTPs were removed by chromatography through sephadex G50 syringe columns (Stratagene Cloning Systems, LaJolla CA). Hybridizations were carried out at 65°C in a mechanical incubator (Robins Scientific Corp.,Sunnyvale CA) in the presence of 1.5 x SSPE (0.15 M NaCl, 0.01 M $NaH_2PO_4$, 0.001 M EDTA)/ 7% SDS (sodium dodecyl sulfate)/10% PEG (polyethylene glycol)(m.w. 8000) supplemented with 200 $\mu$g/ml sonicated and denatured herring sperm DNA, 500 $\mu$g/ml heparin and 2 ng/ml $^{32}$P-labelled probe in a procedure adapted from Budowle and Baechtel (1990). Blots were washed at high stringency in 0.1 x SSC (0.15 M NaCl, 0.015 M $Na_3$ Citrate)/0.1% SDS (65 C) and subjected to autoradiography for 3 to 5 days at -76°C under Kodak XAR film sandwiched between Dupont Cronex Lightning Plus intensifying screens. Following hybridization with TNF-$\alpha$ cDNA probe, membranes were washed 3 x with 1 mM EDTA pH 8.0/0.5% SDS for 20 min at 95 C, then rinsed with 0.1 x SSC/0.1% SDS at room temperature and re-hybridyzed with $^{32}$P-labelled $\beta$-actin cDNA probe. Quantitative assessment of mRNA expression levels was accomplished by scanning autoradiograms on a Molecular Dynamics Model 300A Laser Densitometer, and by performing image analysis and volume

integrations using the image Quant V3.0 software (Molecular Dynamics, Sunnyvale CA).

### 3.11 LUNG HISTOPATHOLOGY

A median longitudinal section of the upper left lobe (1 mm thick) was fixed by immersion in a phosphate-buffered 4% formaldehyde-1% glutaraldehyde solution for histologic examination. After fixation, lung tissue was embedded in paraffin and representative sections (5 $\mu$m) were cut and stained with hematoxylin-eosin or Masson's trichrome. Preparation of the sections and analysis of data were performed in collaboration with Dr. G. Wenckebach.

### 3.12 STATISTICAL ANALYSIS

Results are expressed as mean values ± standard error of the mean (SEM). The statistical significance between treated and control groups was determined using a one-way ANOVA and Bonferroni test (Instat) ($p<0.05$).

Probit analysis was used in the biological assays in order to calculate the quantity of cytokines that were produced. This method was used because several dilutions were tested and probit analysis eliminate extreme dilution that will not fit on the curve.

## 4. RESULTS

The first objective of my project was to evaluate TNF-$\alpha$ production by alveolar macrophages during the development of chronic inflammation and fibrosis. To achieve this I took advantage of an animal model previously developed in our laboratory in which either resolving granuloma or fibrosis could be induced. The experimental protocol used is summarized in Figure 4. Briefly, rats received a single intratracheal instillation of saline (control) or 5mg (in saline) of the following materials: UICC chrysotile B, 4T30 short chrysotile or silica. At various times after treatment (1, 3, and 6 weeks), bronchoalveolar lavages were performed. Cells were recovered by centrifugation, counted and the viability was determined by trypan blue exclusion. Differential analysis of BAL cell populations was determined on cytocentrifuge preparations stained with Wright-Giemsa solution. The alveolar macrophage supernatants were prepared as described in the materials and methods and were used to determine TNF-$\alpha$, IL-1 and IL-6 production in relation to histopathological changes.

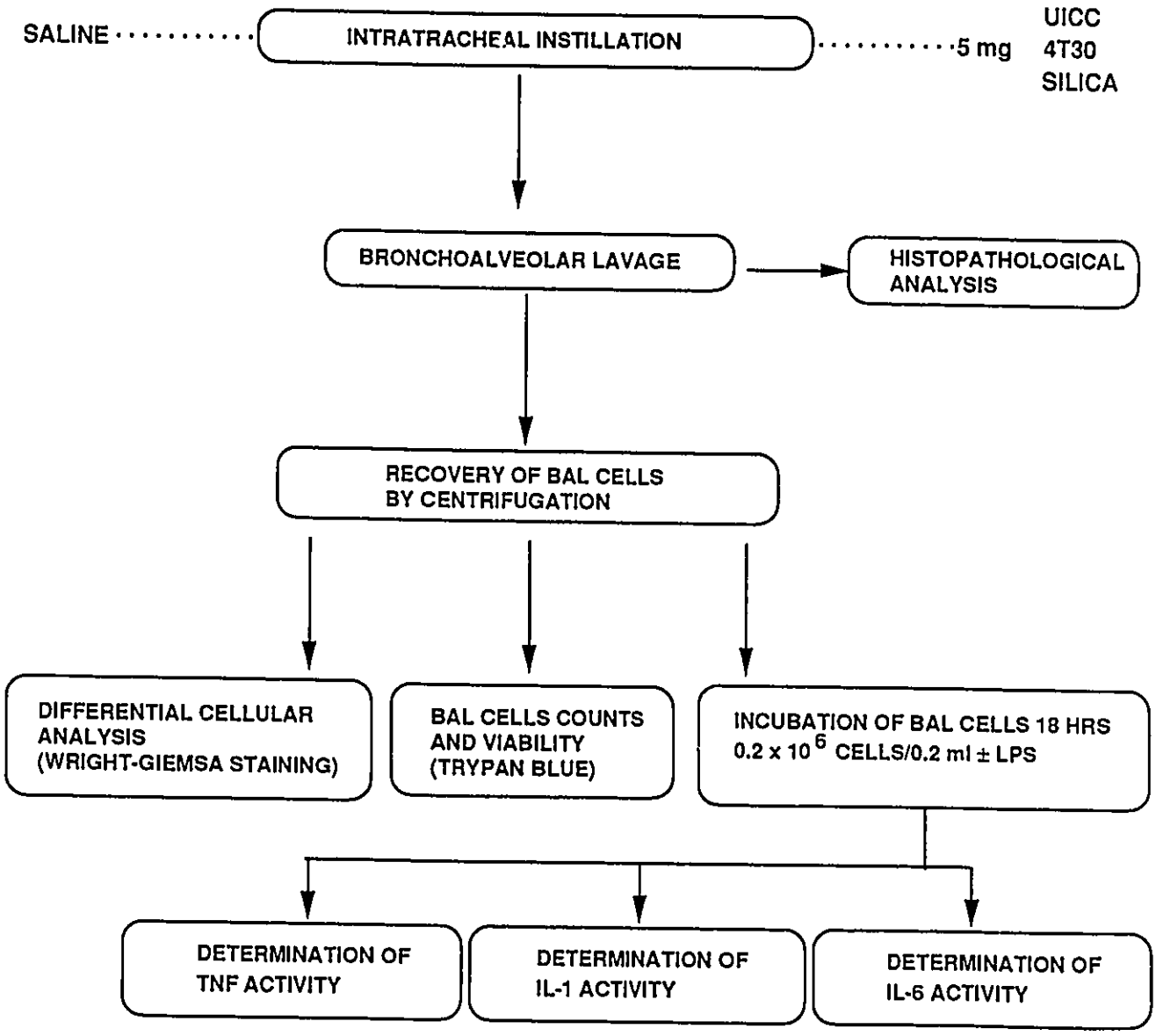
### 4.1 LUNG HISTOPATHOLOGICAL CHANGES

The two types of chrysotile asbestos fibers used as well as the silica particles induced different inflammatory responses (Table 1). Treatment with short 4T30 chrysotile fibers was associated with granulomatous reactions characterized by accumulation of macrophages, epithelioid and giant cells which caused multifocal thickening and alveolar distortion (Figure 5b). These

**Figure 4:** Illustration of the different steps used during the *in vivo* experimental protocol. The different techniques mentioned in this figure are described in the Materials and Methods section.

figure 4

## EXPERIMENTAL PROTOCOL



changes were seen at 1, 3, and 6 weeks. There was no evidence of fibrosis at any time-point studied. Previous work from our laboratory indicated that these granulomatous reactions resolved or decreased after a longer period of time (8 months) (Lemaire, 1991). On the other hand, UICC chrysotile fibers which are long asbestos fibers, caused severe fibrotic lesions that were already apparent 1 week after treatment. These lesions were characterized by focal fibroblastic proliferation and collagen deposition as evidenced by Masson's trichrome (Figure 5C). The lesions were predominantly located in and around terminal bronchioles and tended to increase with time. Silica, which is also known as a fibrotic agent both in animals and in humans (Dauber et al., 1980; DeShazo, 1982), resulted in a delayed-type reaction compared to UICC chrysotile B. The reaction was characterized at an early time point (1 week) by interstitial pneumonitis with infiltration of neutrophils and small macrophages. A significant granulomatous reaction was seen at 3 and 6 weeks. At this stage the inflammatory lesion was composed mostly of epithelioid macrophages, lymphocytes a and few giant cells (Figure 5D).

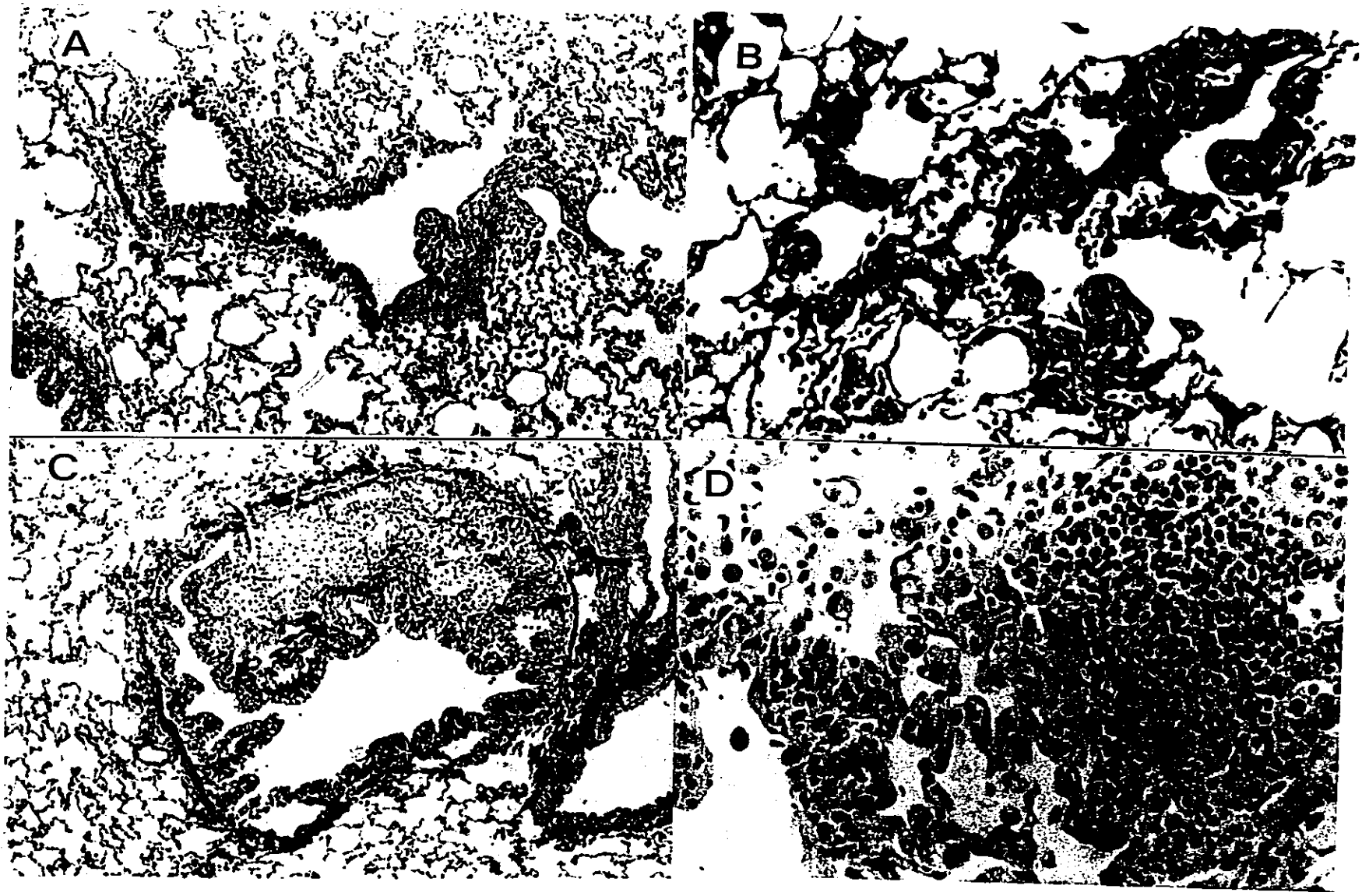
**Table 1.** ***Lung histopathological changes induced by Asbestos and Silica***

| Treatment | Lung histology | | |
|---|---|---|---|
| | 1 week | 3 weeks | 6 weeks |
| Saline (5)* | normal | normal | normal |
| 4T30 (5) | granuloma | granuloma | granuloma |
| UICC (5) | granuloma + fibrosis (+) | granuloma + fibrosis (+) | granuloma + fibrosis (++) |
| Silica (5) | pneumonitis | granuloma (+) | granuloma (+) |

*Number of animals examined.(n)
(+) Presence of lesion
(++) More extensive lesion

**Figure 5:** Lung histopathological changes in exposed rats. A) Histopathology of normal lung tissue. B) Rats exposed to 5mg of 4T30 chrysotile asbestos. C) Bronchiole from rat following exposure to UICC chrysotile asbestos and D) Histopathological changes following silica ($SiO_2$) exposure.

Figure 5

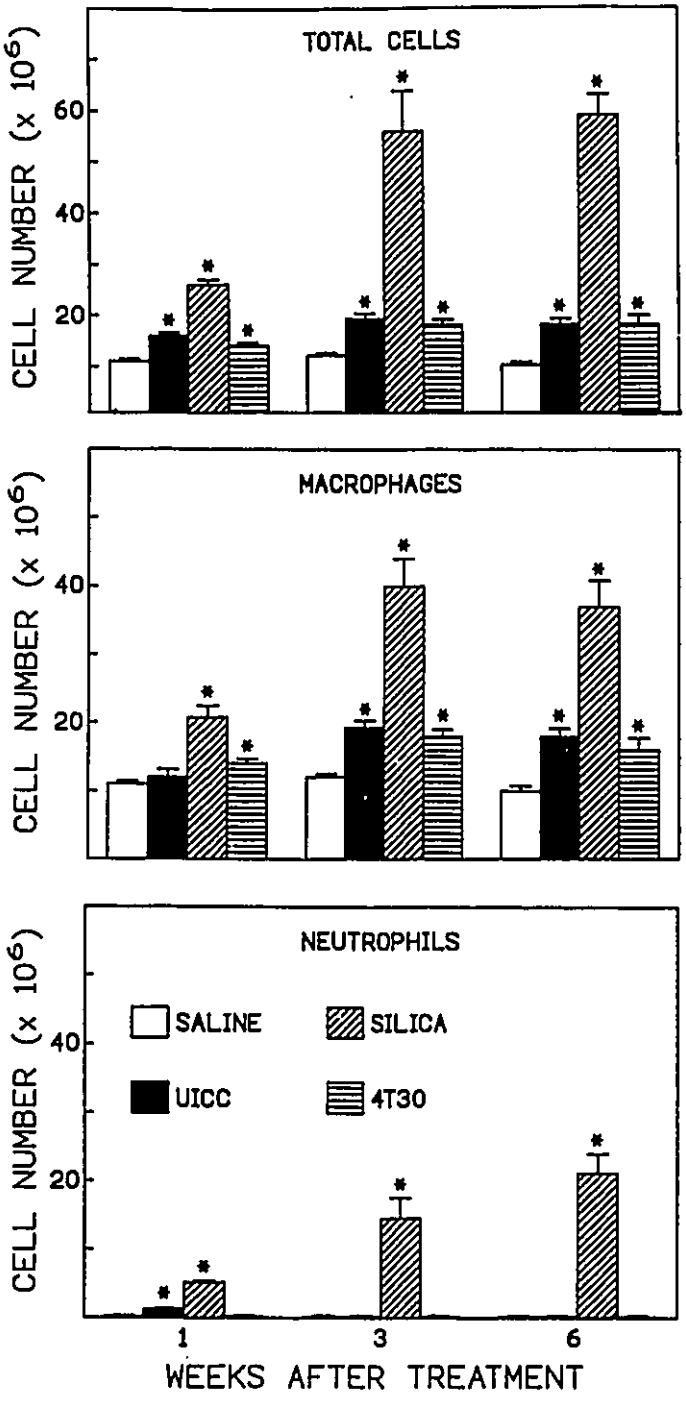


## 4.2 BRONCHOALVEOLAR CELL RECOVERY AND DIFFERENTIAL ANALYSIS OF CELL POPULATIONS

Alterations in cell populations and numbers are good indicators of an on-going inflammatory response. A significant increase in the total number of cells recovered in lavage fluid was observed in all animals that were exposed to, UICC, silica or 4T30 (Figure 6). Differential analysis indicated that lavage fluid from saline-injected rats consisted of 99% macrophages with no eosinophils and very few neutrophils (PMN) and lymphocytes. No changes in the cell populations of these control animals occurred throughout the study. Injection of UICC chrysotile B caused a small and transient increase in PMN which was present at 1 week only. At later time points (3 and 6 weeks), the inflammatory cell population was composed mainly of macrophages (Figure 6). By contrast, a significant increase in PMN was observed in animals treated with silica. Accumulation of PMN was observed at all time points investigated and the absolute number of PMN and macrophages was maximal at 3 weeks. Treatment with very short 4T30 chrysotile caused a small increase in macrophages but no influx of PMN was observed. Cellular changes in response to both chrysotile asbestos exposure were characterized accumulation of macrophages. Mixed PMN and AM accumulations were observed during the chronic inflammatory reaction to silica.

**Figure 6:** Analysis of cells recovered from BAL fluid. Total cell counts of lavage fluid and differential analysis of BAL cell populations at various intervals following intratracheal instillation of saline, UICC, silica, or 4T30. Values are mean ± SEM of at least 4 animals per group. * Significantly different from control at $p < 0.05$.

figure 6


TOTAL CELLS
MACROPHAGES
NEUTROPHILS
CELL NUMBER (x 10⁶)
SALINE
SILICA
UICC
4T30
1
3
6
WEEKS AFTER TREATMENT

## 4.3 TNF-$\alpha$ PRODUCTION BY INFLAMMATORY ALVEOLAR MACROPHAGES

### 4.3.1 In Vitro Production

In initial experiments, the L929 cytotoxic assay described by Flick and Gifford (1984) was set up to measure the production of TNF-$\alpha$ by normal AM. The assay was standardized with various concentrations of recombinant murine TNF-$\alpha$ (0.05 pg/well to 8.0 pg/well). Various concentrations of L929 target cells and different incubation times were tested. A linear dose-response curve was obtained with recombinant TNF-$\alpha$ using 5 x $10^4$ target cells/well and incubation for 20 h (Figure 7). Using these conditions, production of TNF-$\alpha$ by normal rat AM was evaluated. Bronchoalveolar lavage was performed and bronchoalveolar cells (99% AM) were incubated either alone or in the presence of different concentrations of lipopolysaccharide (LPS), a known activator of macrophages (Beutler et al., 1986). Unstimulated AM did not produce detectable levels of TNF activity. On the other hand, when AM were incubated with concentrations of LPS ranging from 0,1 $\mu$g/ml to 20 $\mu$g/ml, TNF-$\alpha$ was produced in a dose-dependent manner (Figure 8A). TNF-$\alpha$ production was linear up to 2 $\mu$g/ml LPS and tended to level off at higher concentration (5 to 20 $\mu$g/ml) (Figure 8A). One microgram per ml was found to be optimal and was chosen for further investigation.

To determine the time course of TNF-$\alpha$ production, AM were incubated with LPS (1 $\mu$g/ml) for 2,4,6,12 and 24 h. Culture media from LPS-stimulated

**Figure 7:** Standard curve obtained using recombinant TNF in TNF-$\alpha$ assay. The curve was plotted in logarithmic units and corresponds to the % cytotoxicity of 0.05pg/well to 8pg/well of recombinant TNF using 5 x $10^4$ L929 cells and an incubation time of 20 h. Values represent mean ± SEM of 15 different standard curves.

figure 7

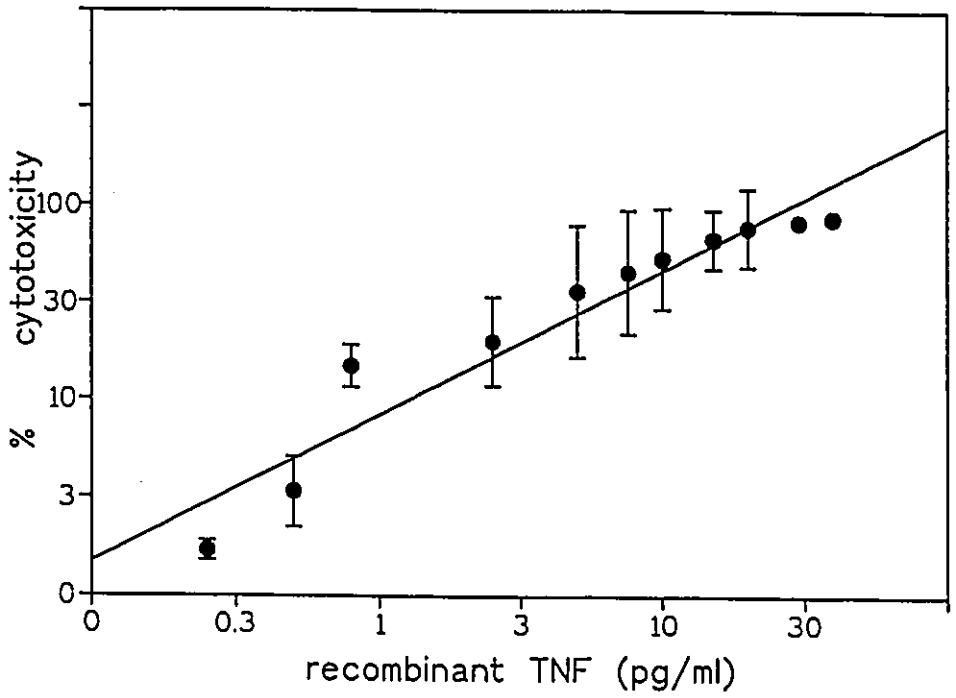
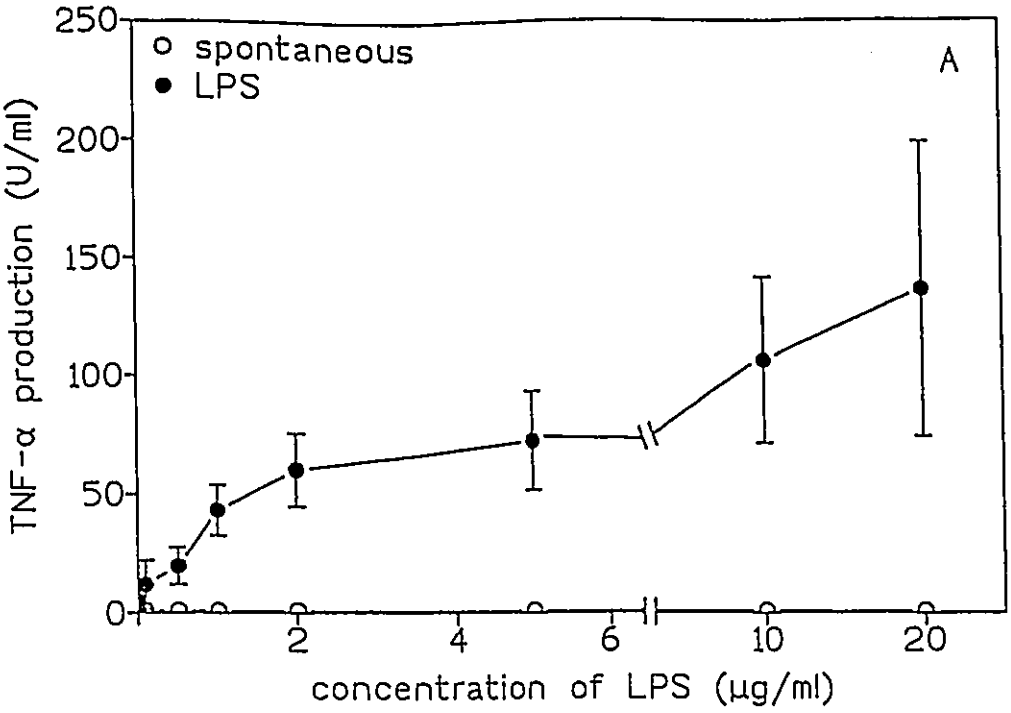
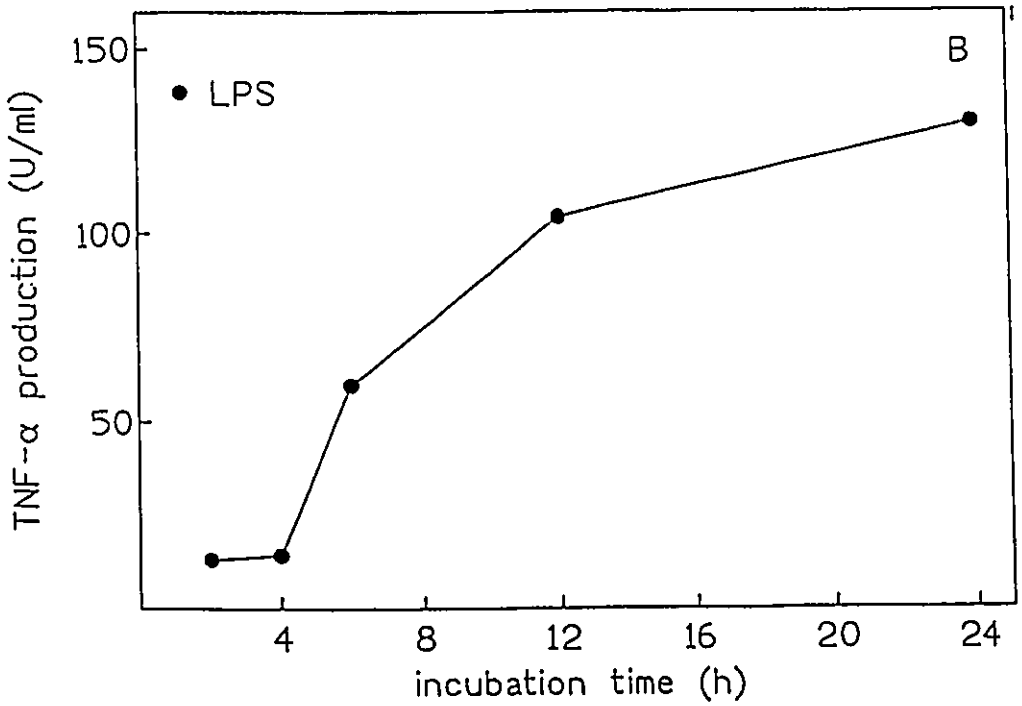


**Figure 8**: A) Augmentation of TNF-$\alpha$ production by AM incubated for 18h with different concentrations of LPS. The results are the mean ± SEM of at least 5 different experiments. B) Kinetics of LPS (1 $\mu$g/ml)-induced TNF production by AM. Values represent the results of one representative experiment.

FIGURE 8


250
200
150
100
50
0
spontaneous
LPS
A
TNF-α production (U/ml)
2
4
6
10
20
concentration of LPS (μg/ml)


150
100
50
LPS
B
TNF-α production (U/ml)
4
8
12
16
20
24
incubation time (h)

AM contained no significant TNF-$\alpha$ activity at 2 and 4 h. By 6 h, however, TNF production was detectable and maximal activity was obtained between 12 and 24 h (Figure 8B). Therefore, an 18 h interval was chosen for further experiments.

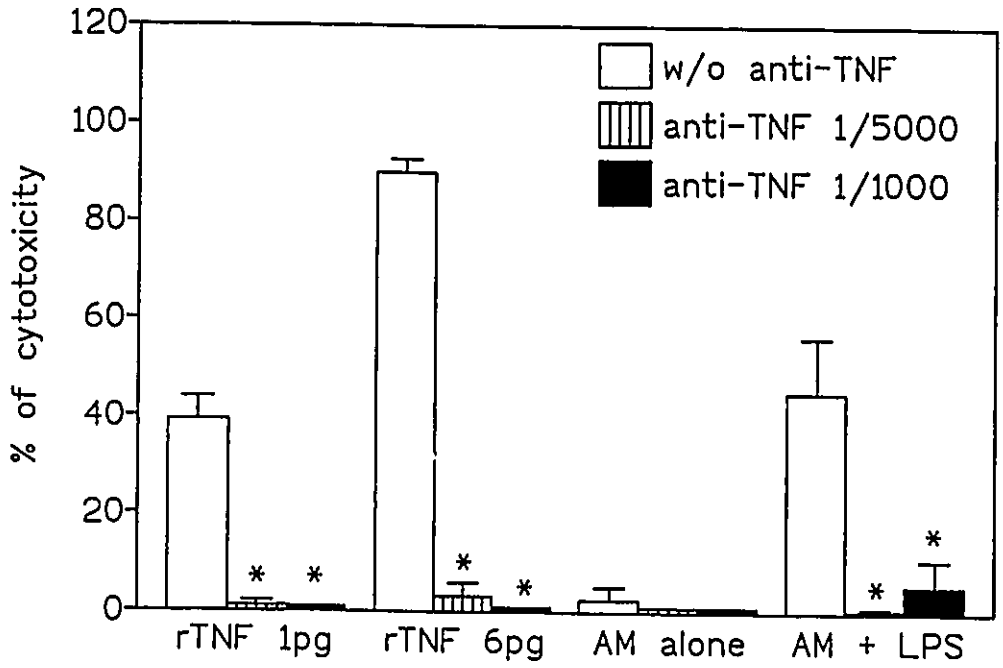
In order to validate our assay for TNF measurement in culture supernatants, samples were incubated with two concentrations of neutralizing antibody against murine TNF-$\alpha$ (Genzyme) and TNF activity was determined. As shown in Figure 9, anti-TNF at dilutions 1/1000 and 1/5000 completely neutralized the lytic activity observed indicating that the cytotoxic effect of AM supernatants on L929 cells was due to TNF.

### 4.3.2 Ex Vivo Production

Bronchoalveolar cells obtained from rats exposed *in vivo* to various dusts were incubated *in vitro* for 18 h in the presence and absence of LPS, and TNF production (referred to as *ex vivo*) was measured. Basal as well as LPS-induced TNF-$\alpha$ production by total cells were determined in all groups at various intervals after dust exposure. There was no significant spontaneous production of TNF-$\alpha$ by unstimulated AM from control and treated rats except for a small increase at 6 weeks in the UICC chrysotile B group (63 U/ml) (Figure 10). LPS-induced TNF-$\alpha$ production by AM obtained from saline treated rats did not vary significantly throughout the study. Similarly, no significant change in LPS-induced TNF-$\alpha$ release was found with AM from the 4T30 group. By contrast,

**Figure 9:** Effect of a neutralizing anti-TNF antibody. Normal AM ($1 \times 10^6$/ml) were incubated for 18 h in the presence of two different dilutions of anti-TNF-$\alpha$ with or without LPS (1-$\mu$g/ml). In order to verify whether anti-TNF-$\alpha$ was blocking TNF in our assay, I also incubated recombinant TNF with the antibody dilutions. Values represent mean ± SEM of 3 experiments. * Significantly different from control at $p < 0.05$.

figure 9


120
100
80
60
40
20
0
% of cytotoxicity
w/o anti-TNF
anti-TNF 1/5000
anti-TNF 1/1000
*
*
*
*
*
*
rTNF 1pg
rTNF 6pg
AM alone
AM + LPS

AM from rats exposed to fibrogenic UICC chrysotile B displayed a biphasic TNF-$\alpha$ response (Figure 10). TNF-$\alpha$ release was decreased at 1 and 3 weeks (43 and 71 U/ml respectively, compared to 106 and 145 U/ml for controls) but was potentiated 6 weeks after treatment (264 U/ml compared to 152 U/ml for controls) (Figure 10). AM obtained from silica treated rats also produced lower levels of TNF-$\alpha$ than controls. However, in contrast to UICC, inhibition of TNF-$\alpha$ was still present at 6 weeks (Figure 10).

## 4.4 VIABILITY AND FUNCTIONAL STATUS OF INFLAMMATORY BAL CELLS:

We investigated whether decreased levels of TNF-$\alpha$ in AM supernatants from UICC- and silica-exposed animals were related to a cytotoxic effect of these dusts. As shown in Figure 11, this possibility was ruled out because the viability of BAL cells as measured by trypan blue exclusion was over 92% for each group at all times. Furthermore, AM from asbestos- and silica-treated rats were found to be quite functional for the production of another inflammatory cytokine, IL-1 (data not shown).

## 4.5 EFFECT OF NEUTROPHILS (PMN) ON TNF-$\alpha$ PRODUCTION

During the inflammatory reaction induced by silica, the presence of neutrophils might contribute to lower levels of TNF-$\alpha$ either through absorption

**Figure 10**: Sequential analysis of spontaneous and LPS-induced TNF-$\alpha$ release by BAL cells from rats treated with UICC chrysotile, 4T30 chrysotile or silica. TNF-$\alpha$ was assessed on triplicate samples of BAL supernatants as described in Materials and Methods. Values represent mean ± SEM of at least 4 animals per group. * Significantly different from control at $p < 0.05$.

figure 10

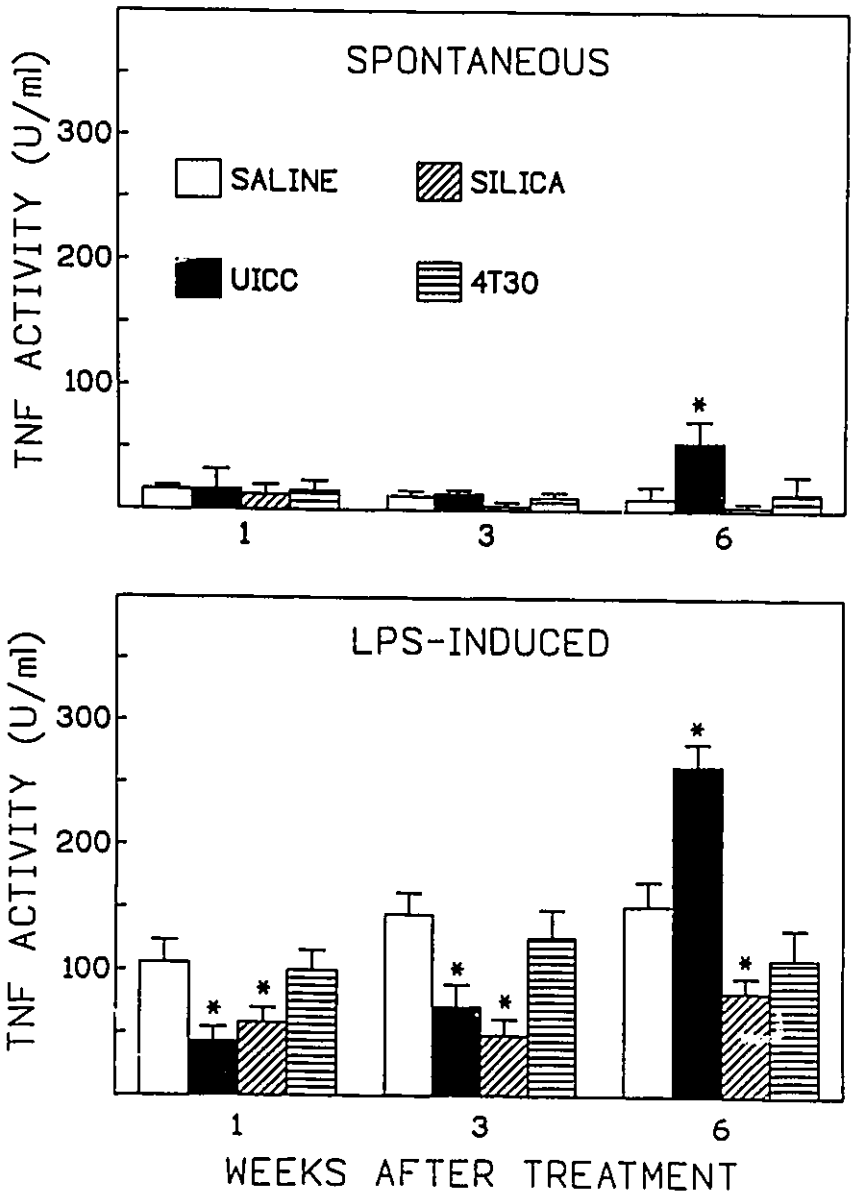
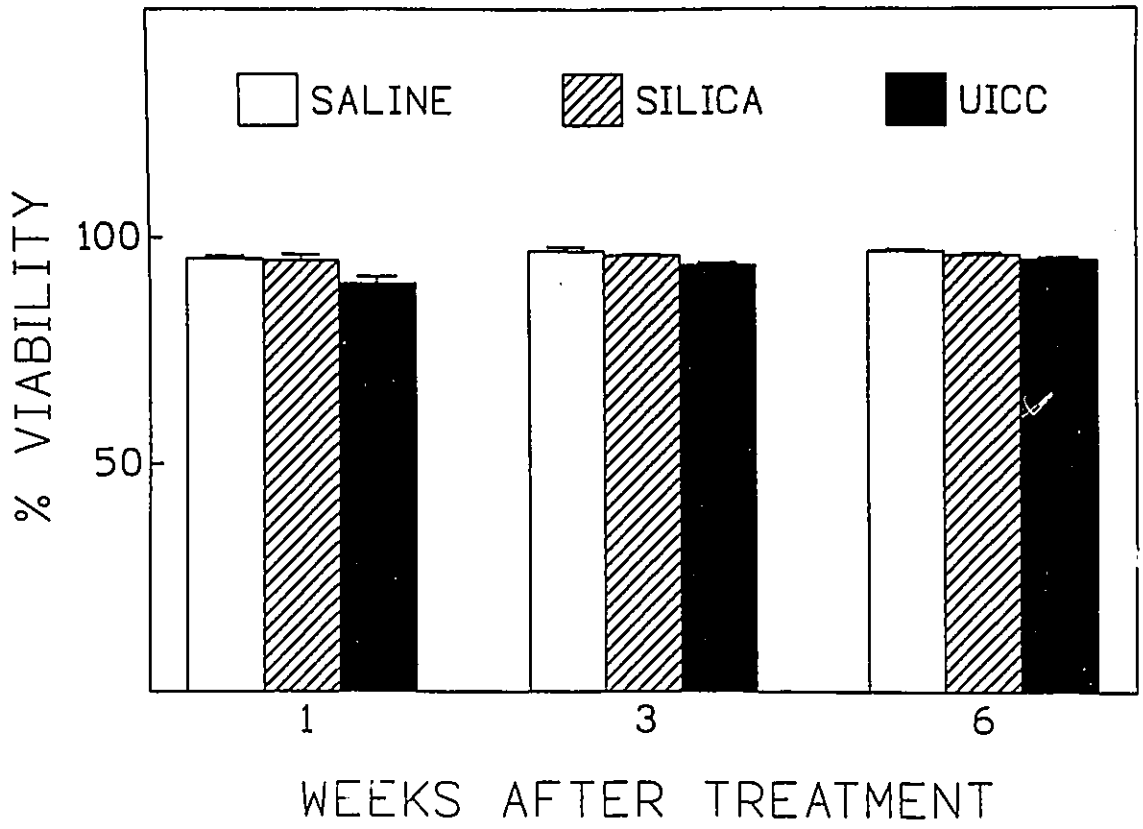

SPONTANEOUS
SALINE
SILICA
UICC
4T30
TNF ACTIVITY (U/ml)
300
200
100
1
3
6
*
LPS-INDUCED
TNF ACTIVITY (U/ml)
300
200
100
1
3
6
WEEKS AFTER TREATMENT

**Figure 11**: Viability of BAL cells obtained from animals treated with saline, UICC chrysotile asbestos, or silica. Viability was determined by trypan blue exclusion. Values represent mean ± SEM of at least 9 rats.

figure 11

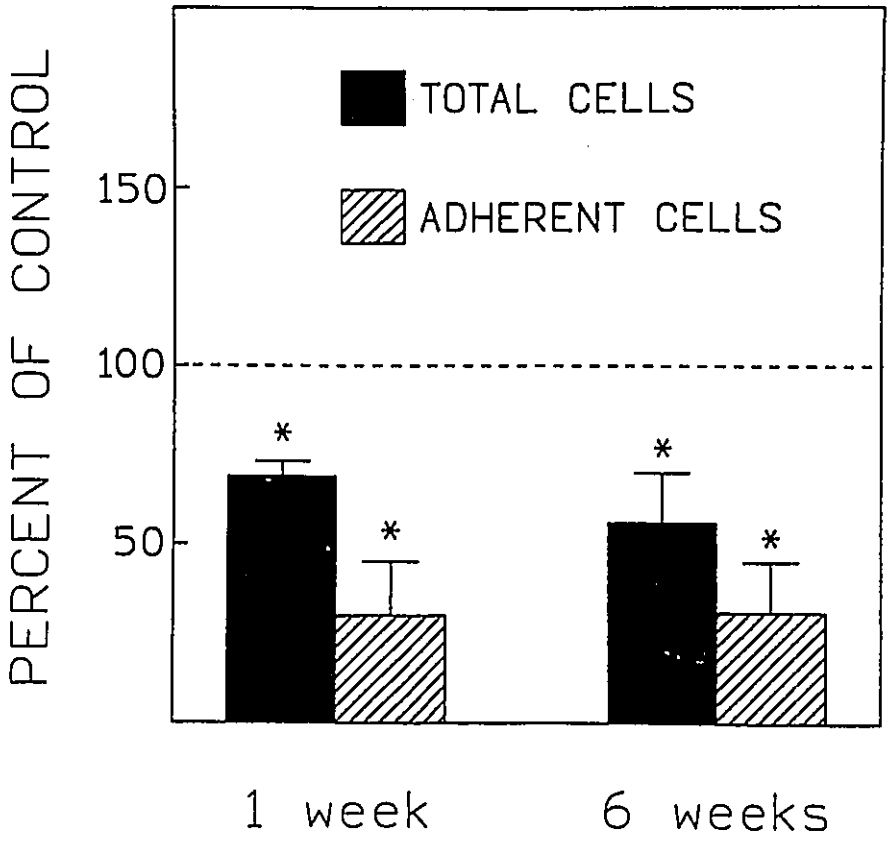

SALINE
SILICA
UICC
% VIABILITY
100
50
1
3
6
WEEKS AFTER TREATMENT

of TNF which is known to act on PMN (Krigler et al., 1988) or by direct inhibition of TNF-$\alpha$ production. A second *in vivo* protocol was undertaken to investigate these possibilities. The total BAL cell population was depleted of PMN by adherence to plastic for 20 min. These conditions were found to be optimal for preferential adherence of AM to the culture dish. Non-adherent cells contained an enriched population of PMN (65%) (the other cells being AM) whereas adherent cells consisted mostly of AM (85%). TNF-$\alpha$ production was measured in total and depleted cell fractions. As observed for total BAL cells from silica-treated rats, adherent AM depleted of PMN also produced significantly less TNF-$\alpha$ than controls in response to LPS (Figure 12). Adherent AM even produced less TNF-$\alpha$ than total cells 1 and 6 weeks after treatment suggesting a positive influence, if any, of PMN or other non-adherent AM populations on TNF-$\alpha$ production. These differences, however, did not reach statistical significance. Therefore rapid absorption by PMN of newly released TNF-$\alpha$ could not account for the lower levels of TNF-$\alpha$ measured.

To further determine whether PMN might directly inhibit TNF-$\alpha$ production, we incubated an enriched population of neutrophils (65% PMN and 35% AM) from silica treated rats with normal macrophages. Non-adherent cells were incubated at 1:1 ratio with freshly isolated AM for 18 h at 37°C in the presence or absence of LPS (1 $\mu$g/ml). Appropriate controls consisted of similar numbers of AM and PMN incubated alone with or without LPS. Conditioned media from total BAL cells,adherent AM, non-adherent cells and PMN-AM co-

**Figure 12:** TNF-$\alpha$ release by total cells and adherent cells. BAL cells ($10^6$/ml) were incubated in the presence of LPS for 18 h and TNF-$\alpha$ activity was measured in culture supernatants as described in Materials and Methods. Values represent mean ± SEM of 4 animals per group. * Significantly different from control at $p < 0.05$.

figure 12


PERCENT OF CONTROL
150
100
50
TOTAL CELLS
ADHERENT CELLS
*
*
*
*
1 week
6 weeks

cultures were then collected and TNF-$\alpha$ activity was determined. Co-culture of PMN, obtained 1 and 6 weeks following silica treatment, with normal AM had no significant effect on LPS-induced TNF-$\alpha$ production (Figure 13) thus ruling out the participation of PMN as negative regulators of TNF-$\alpha$ production.

## 4.6 BIPHASIC CHANGES IN TNF-$\alpha$ GENE EXPRESSION BY AM FROM ASBESTOS RATS

The suppression of TNF-$\alpha$ production observed 3 weeks following treatment with UICC chrysotile B and silica could be related to an inhibition of TNF-$\alpha$ production per se or the presence of TNF-$\alpha$ inhibitors interfering with TNF effect on L929 cells. In this respect, a soluble fragment of the TNF-$\alpha$ receptor molecule has been found to inhibit TNF-$\alpha$ action on cells (Kohno et al., 1990; Coray 1990). In order to investigate this further, the pattern of mRNA expression for TNF-$\alpha$ in response to LPS was examined in AM from saline and UICC exposed rats 3 and 6 weeks following treatment. These time-points were chosen because BAL cell populations from these animals contained almost exclusively AM. The mRNA levels were found to reflect the profile of TNF-$\alpha$ release. Indeed mRNA expression following LPS stimulation was lower in AM exposed to asbestos compared to controls at 3 weeks (Figure 14). By 6 **the inflammatory response.**

**Figure 13:** Effect of enriched populations of neutrophils (non-adherent cells) from silica-treated rats on LPS-induced TNF-release by normal AM. Freshly isolated BAL cells (99% AM) from normal rats (0.5 x $10^6$/ml), non-adherent cells from silica-treated rats (0.5 x $10^6$/ml), and mixed populations of cells (non-weeks, however, TNF-$\alpha$ mRNA levels rose significantly over control indicating that AM had become activated for TNF-$\alpha$ production at this stage of adherent cells from silica treated rats (0.5 x $10^6$/ml) + AM (0.5 x $10^6$/ml)) were incubated in the presence of LPS (1 $\mu$g/ml) for 18 h and TNF-$\alpha$ activity determined in culture supernatants as described in Materials and Methods. Values represent mean ± SEM of 3 different experiments.

figure 13

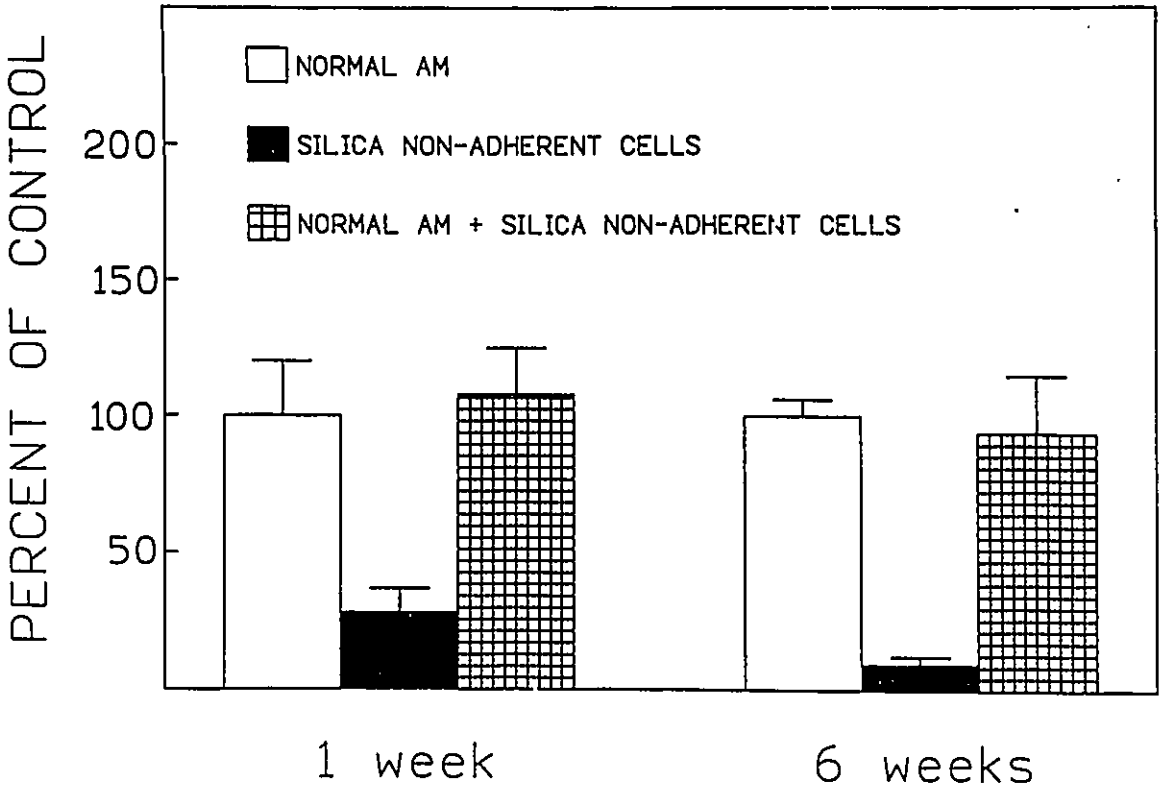
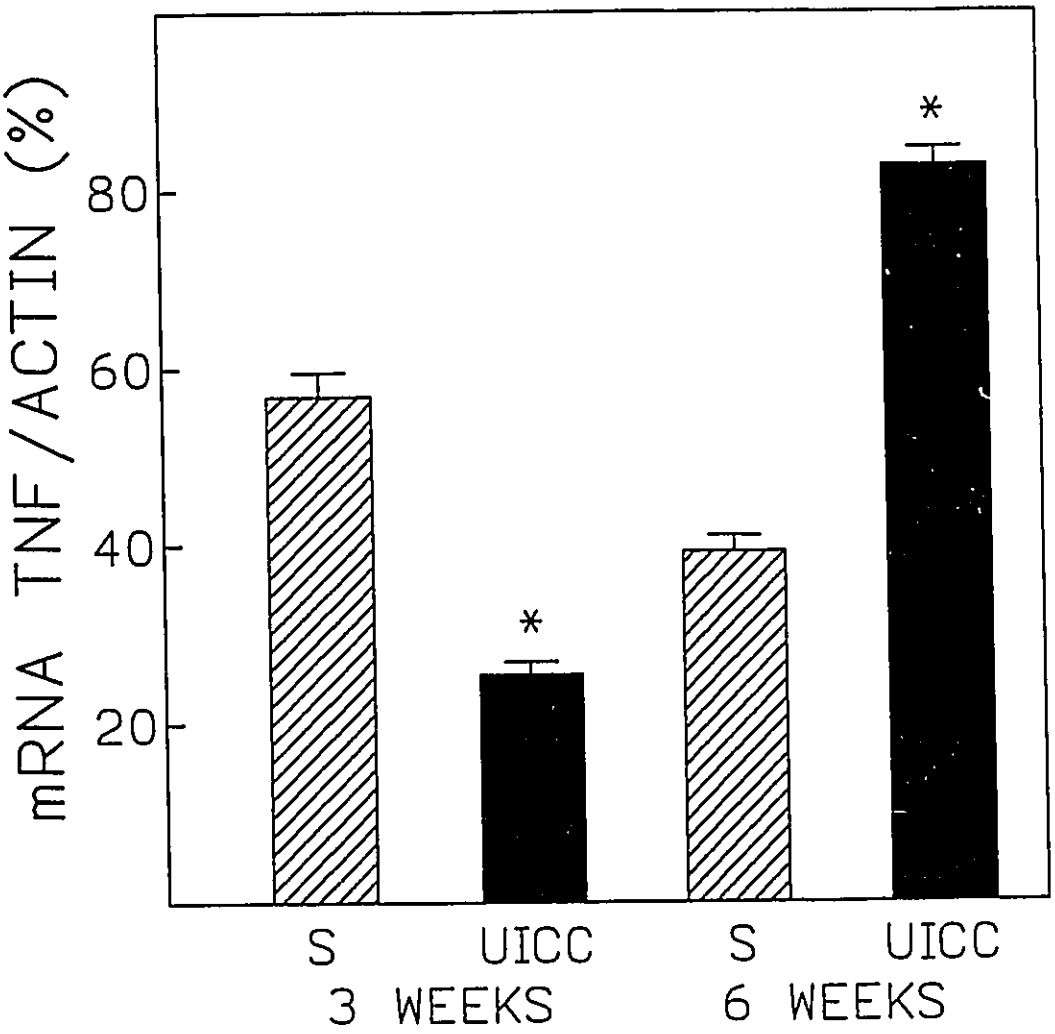

NORMAL AM
SILICA NON-ADHERENT CELLS
NORMAL AM + SILICA NON-ADHERENT CELLS
PERCENT OF CONTROL
200
150
100
50
1 week
6 weeks

**Figure 14:** Northern hybridization analysis of TNF-$\alpha$ mRNA in cultured BAL cells. BAL cells obtained from rats 3 and 6 weeks following intratracheal instillation of saline (S) or UICC chrysotile asbestos (UICC) were cultured for 18 h in the presence of LPS. The graph shows the relative expression of TNF-$\alpha$ as determined by laser densitometric scanning after standardizing the mRNA amounts according to the expression of a constant gene, $\beta$-actin. Values represent mean ± SEM of 3 separate experiments. * Significantly different from control at $p < 0.05$.

Figure 14

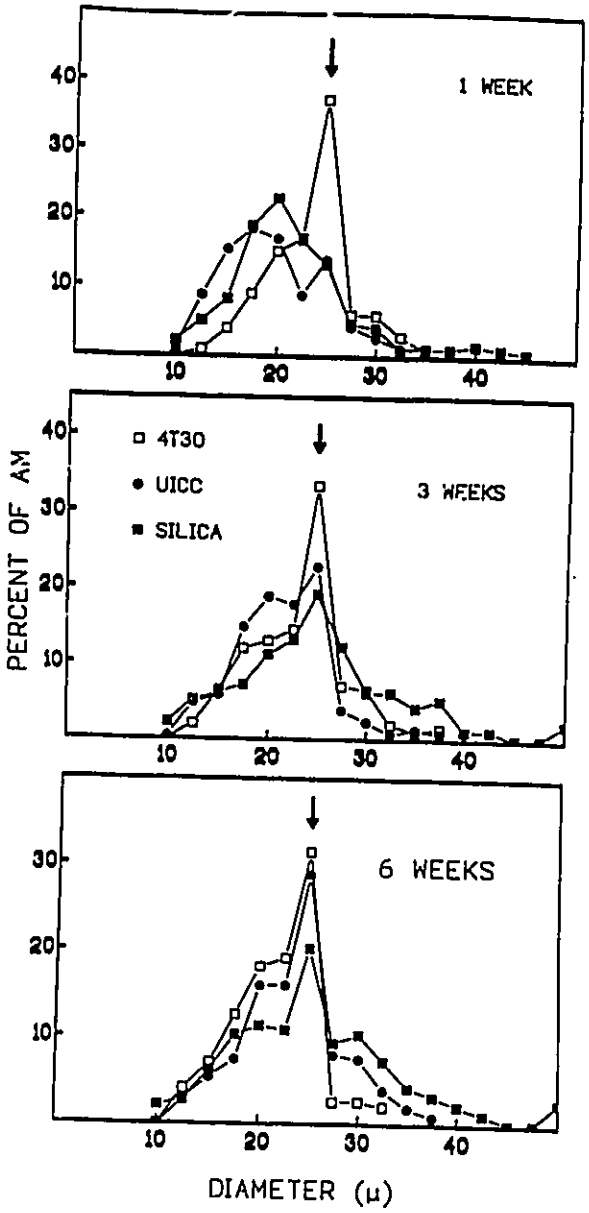


## 4.7 TNF-$\alpha$ PRODUCTION AND AM POPULATION

To further analyze the modulation of TNF-$\alpha$ production during inflammation we investigated the macrophages themselves. Cell preparations showed a heterogeneous population with respect to cell size and shape. Since increased cell size has been related to the stage of activation or maturation of AM (Shellito and Kaltreider, 1984), we analyzed the mean diameter of 200 AM from each rat. The distribution profile of the mean cell diameter of AM from saline and 4T30-treated rats was not significantly different at 1, 3, or 6 weeks (Figure 15). In contrast, AM from UICC- and silica-exposed animals displayed a significant shift in their distribution profile characterized by an increase in the proportion of AM with a small diameter (15 - 20 $\mu$) and a concomitant decrease in the proportion of AM with an average diameter of 25 $\mu$ (Figure 15). Interestingly, the decrease in the proportion of 25 $\mu$ AM seen at 1 and 3 weeks correlated with the suppression of TNF-$\alpha$ production. By 6 weeks, this proportion was still lower in the silica group (in which TNF-$\alpha$ was suppressed) but reached the control values in the UICC group (which displayed at that time enhanced TNF-$\alpha$ production). Therefore, sequential changes in TNF-$\alpha$ production appear to be related to the type of AM present at sites of inflammation.

**Figure 15:** Distribution profile of AM size in control and treated rats. Cell diameter was measured on 200 AM as described in Materials and Methods. Results are expressed as the percentage of AM with a given diameter and values were calculated as means ± SEM of at least 4 animals per group. The standard errors up to 3.4% overall are not expressed in the figure for clarity. For the same reason, distribution profile of saline-treated rats which was identical to that of 4T30-treated animals, is not illustrated.

figure 15


1 WEEK
3 WEEKS
6 WEEKS
4T30
UICC
SILICA
PERCENT OF AM
DIAMETER (μ)
10
20
30
40

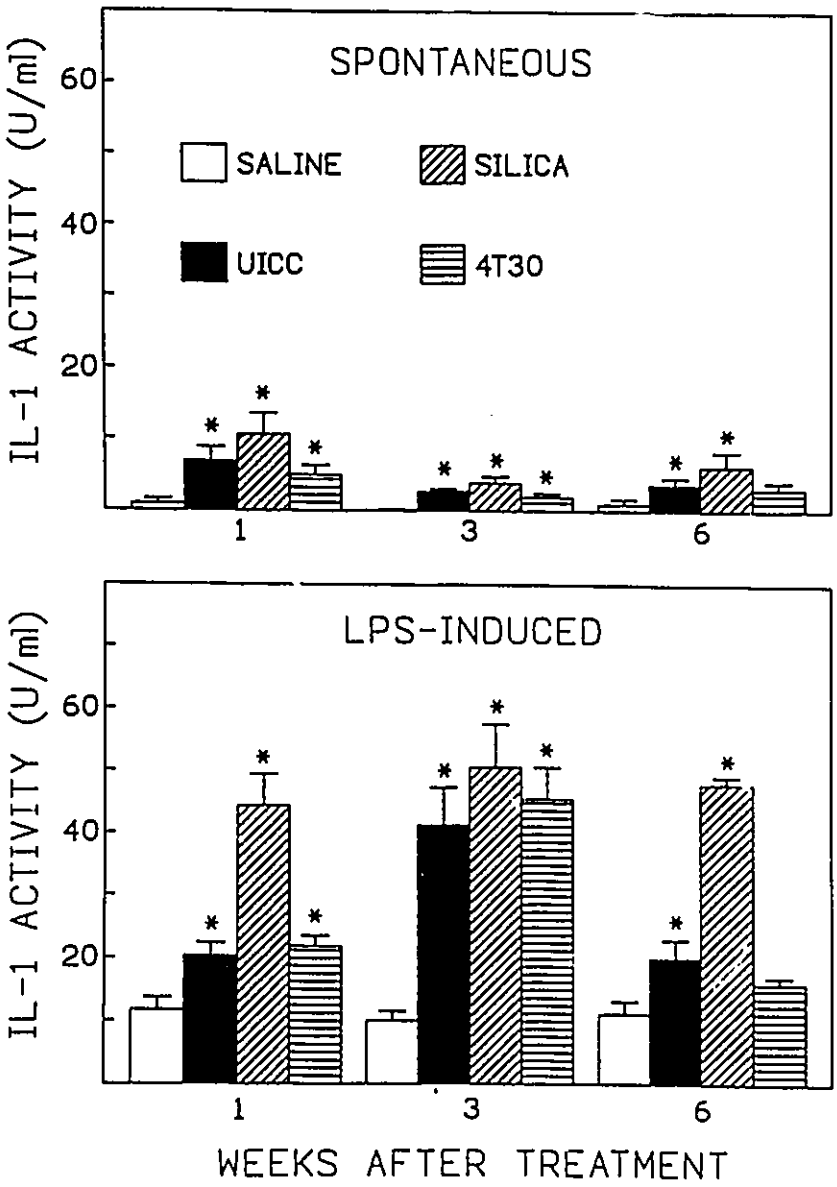
## 4.8 EFFECTS OF ASBESTOS AND SILICA EXPOSURE ON IL-1 AND IL-6 SECRETION BY BAL CELLS

Since other cytokines are produced by AM and might have an effect on TNF-$\alpha$ production by inflammatory alveolar macrophages, I measured the release of two other cytokines in our *in vivo* model. Using proliferation assays, the production of IL-1 and IL-6 by unstimulated and LPS-induced AM was determined. The secretion level of IL-1 by unstimulated AM from control was very low and remained unchanged throughout the study. However, significantly higher levels of IL-1 activity were detected in AM culture supernatants from animals treated with UICC chrysotile B, short 4T30 chrysotile and silica (Figure 16). The order of magnitude for spontaneous release was silica > UICC chrysotile B > 4T30 chrysotile. In all cases the maximal release was observed 1 week after treatment. In contrast to UICC and silica, the stimulatory effect of 4T30 chrysotile was less prolonged and was no longer apparent at 6 weeks.

The release of IL-1 by AM in response to LPS was also determined in all groups. LPS-induced IL-1 release was seen in AM from control animals. In addition, BAL cells obtained from rats treated with the silicates released much higher levels of IL-1. Overall, the kinetics of IL-1 release in response to LPS paralleled that seen with unstimulated BAL cells but the magnitude of response was significantly greater (Figure 16).

**Figure 16:** Spontaneous and LPS-induced IL-1 release by BAL cells from rats exposed to asbestos or silica. IL-1 was measured as described in Materials and Methods. IL-1 activity was determined in triplicate and values represent mean ± SEM of at least 4 animals per group. * significantly different from control at $p < 0.05$.

figure 16


SPONTANEOUS
SALINE
SILICA
UICC
4T30
IL-1 ACTIVITY (U/ml)
60
40
20
1
3
6
LPS-INDUCED
IL-1 ACTIVITY (U/ml)
60
40
20
1
3
6
WEEKS AFTER TREATMENT

The kinetics of IL-6 production by AM from control and treated rats was also investigated. Culture supernatants from unstimulated AM of control animals contained little or no detectable IL-6 activity. Spontaneous release of IL-6 increased rapidly following exposure to mineral dusts with the maximal increase seen at 1 week (Figure 17). The magnitude of response was similar to that found for IL-1 with silica being more potent than UICC and 4T30 chrysotile in inducing IL-6 release. As well, the kinetics of response were comparable to those of IL-1.

AM produced significant levels of IL-6 (56 U/ml) when stimulated with LPS and these levels were further significantly increased following *in vivo* treatment with the mineral dusts (Figure 17). The pattern of LPS-induced IL-6 secretion paralleled that of unstimulated AM for the various groups. Maximal increases were seen at 1 week in response to asbestos but IL-6 release tended to return towards control levels by 6 weeks. In contrast, maximal release of IL-6 was still observed 6 weeks after treatment with silica.

Overall, IL-1 and IL-6 production by inflammatory alveolar macrophages was inversely correlated with TNF-$\alpha$. These results suggest a role for these cytokines in modulating TNF-$\alpha$ production.

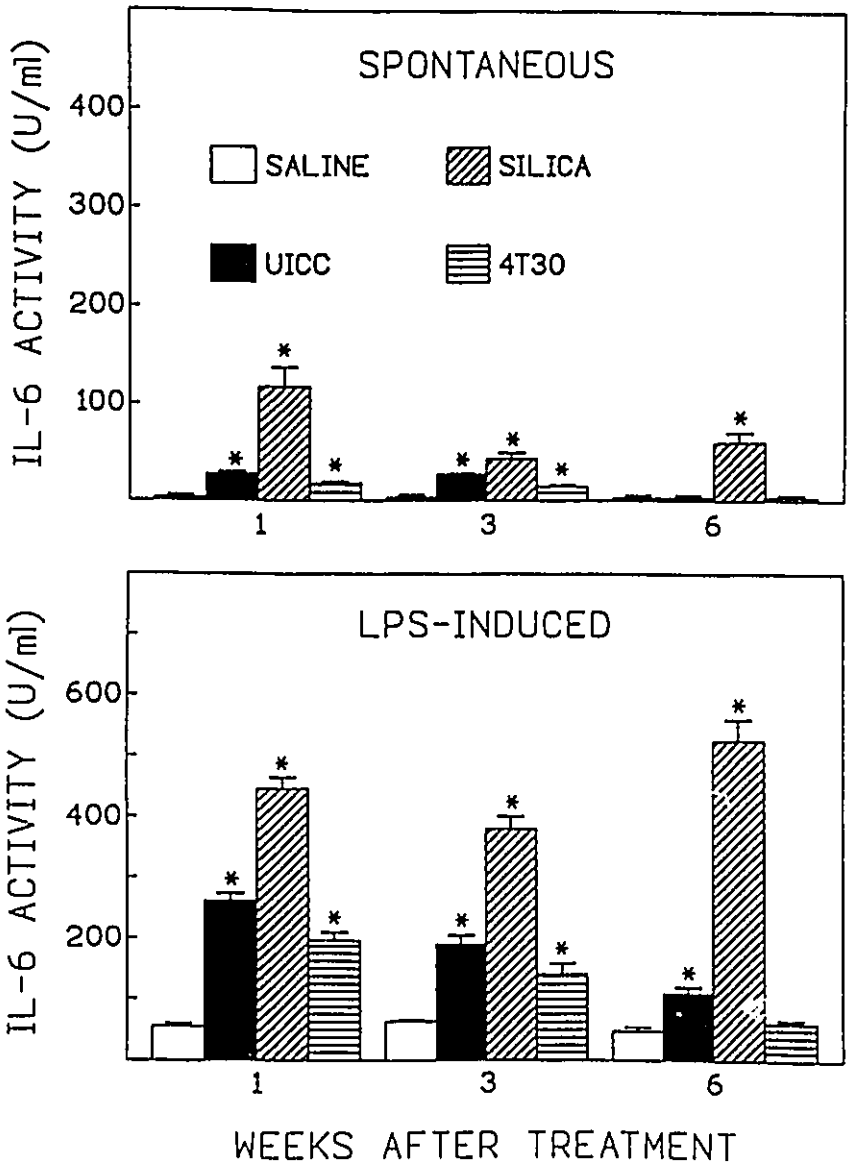
## 4.9 MODULATION OF TNF-$\alpha$ BY OTHER CYTOKINES IN VITRO

### 4.9.1 Effect of IL-1

In order to examine a possible modulatory role of IL-1 on TNF-$\alpha$

**Figure 17:** Kinetics of spontaneous and LPS-induced IL-6 release by BAL cells from rats treated with asbestos and silica. IL-6 activity was measured as described in materials and methods. IL-6 determinations were done in triplicate and values represent mean ± SEM of at least 4 animals per group. * significantly different from control at $p < 0.05$.

figure 17


SPONTANEOUS
SALINE
SILICA
UICC
4T30
IL-6 ACTIVITY (U/ml)
400
300
200
100
1
3
6
LPS-INDUCED
IL-6 ACTIVITY (U/ml)
600
400
200
1
3
6
WEEKS AFTER TREATMENT

production in our model, *in vitro* experiments have been done. Normal AM were incubated with different concentrations of recombinant IL-1 with or without LPS. IL-1 did not induce measurable TNF activity from AM over a wide range of concentrations (5 to 50 pg/ml) (Figure 18). Similarly, when AM were stimulated with LPS and different concentrations of IL-1, TNF-$\alpha$ production was not altered significantly.

### 4.9.2 Effect of IL-6

Interleukin-6 has been described as a negative regulator of TNF-$\alpha$ production *in vivo* (Keley, 1990) as well as *in vitro* on human leukocytes (Scuderi et al., 1990), monocytes and histiocytic cell line U937. Since IL-6 is present in our inflammation model, i decided to investigate the direct effect of IL-6 on TNF-$\alpha$ production. Normal AM were incubated *in vitro* with various concentrations of murine recombinant IL-6 with LPS. AM incubated with different concentrations of IL-6 did not produce any detectable TNF activity. However, LPS-induced TNF-$\alpha$ production was inhibited significantly (up to 57%) by IL-6 (Figure 19). Inhibition was observed at a relatively low concentration of IL-6 (10 pg/ml).

### 4.9.3 Effect of TGF-$\beta$

Another cytokine which is believed to play a key role in the development of bleomycin-induced pulmonary fibrosis is TGF-$\beta$ ( Khalil et al, 1989). Since

**Figure 18:** Effect of recombinant IL-1 on TNF-$\alpha$ release. Different concentrations of rIL-1 were incubated *in vitro* with normal AM ($1 \times 10^6$/ml) with or without LPS (1 $\mu$g/ml) for 18 h. TNF-$\alpha$ activity was determined as described in the Materials and Methods. Values represent mean ± SEM of 3 different experiments.

figure 18

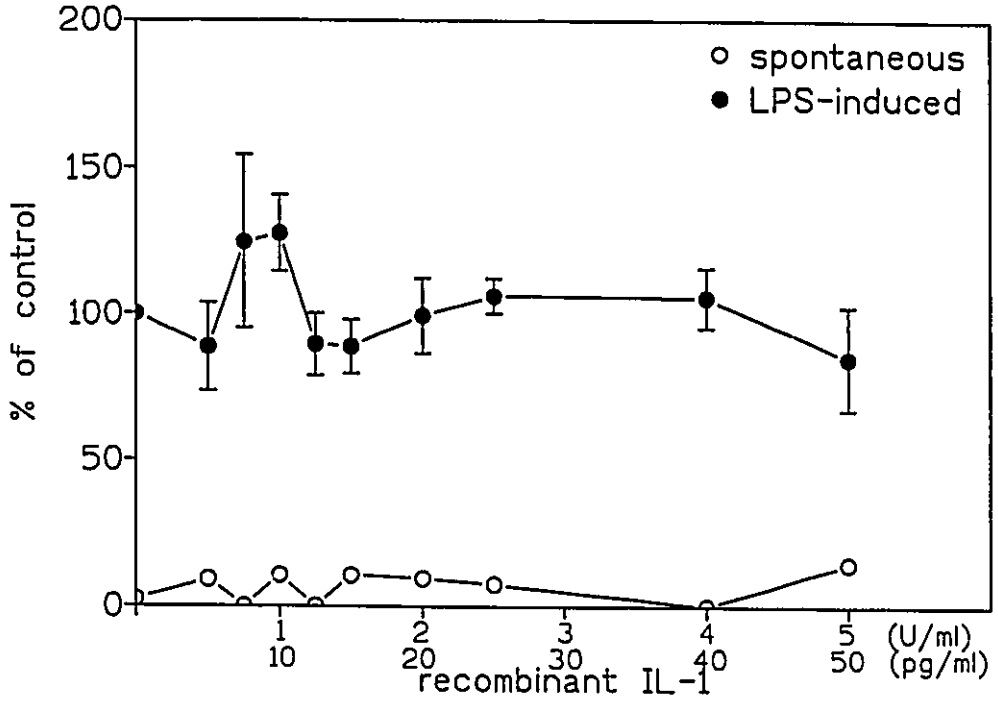
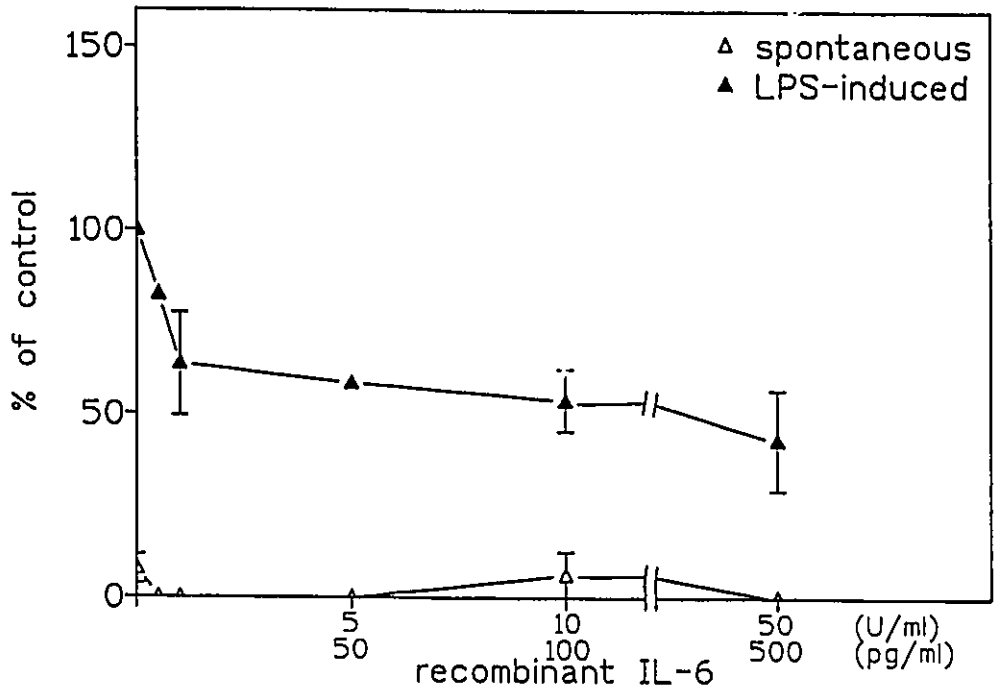

200
150
100
50
0
% of control
spontaneous
LPS-induced
1
10
2
20
3
30
4
40
5
50
(U/ml)
(pg/ml)
recombinant IL-1

**Figure 19**: Effect of IL-6 on TNF-$\alpha$ production. Different concentrations of rIL-6 were used in the presence of normal AM (1 x $10^6$/ml) with or without LPS (1 $\mu$g/ml) and incubated for 18 h. TNF-$\alpha$ activity was determined using the L929 lysis assay described in the Materials and Methods. Values represent mean ± SEM of 3 separate experiments.

figure 19


150
100
50
0
% of control
spontaneous
LPS-induced
5
50
10
100
50
500
(U/ml)
(pg/ml)
recombinant IL-6

higher production of TGF-$\beta$ by macrophages has been reported in some models of fibrosis (Khalil et al., 1989), we investigated whether TGF-$\beta$ could inhibit TNF-$\alpha$ production by normal AM. As shown in Figure 20, TGF-$\beta$ is a potent inhibitor of LPS-induced TNF release by AM. Inhibition was obtained with concentrations as low as 1 ng/ml and maximal inhibition (65%) was almost achieved with 2.5 ng/ml. Such inhibition was blocked in the presence of anti-TGF-$\beta$ (not shown).

### 4.9.4 Effect of Prostaglandin $E_2$ on TNF-$\alpha$ production

Prostaglandin $E_2$ ($PGE_2$) is a third candidate that could down regulate TNF-$\alpha$ production (Scuderi et al., 1990). To investigate this, normal AM were incubated with LPS in the presence of various concentrations of $PGE_2$ (0-350 $\mu$g/ml). Such concentrations were chosen because other experiments from our laboratory have shown that AM incubated under similar conditions release in the culture medium up to 300 $\mu$g/ml $PGE_2$. As shown in Figure 21, $PGE_2$ at a concentration of 50 $\mu$g/ml inhibited TNF-$\alpha$ release from AM by approximately 45%.

## 4.10 *IN VIVO* IMPLICATION OF THESE CYTOKINES IN TNF-$\alpha$ PRODUCTION

In order to further delineate the role of these various mediators on the *in vivo* suppression of TNF-$\alpha$, we performed a last set of experiments in which the effects of anti-TGF-$\beta$, and indomethacin, an inhibitor of prostaglandin synthesis,

**Figure 20:** Effect of TGF-$\beta$ on TNF-$\alpha$ production. Normal AM ($1 \times 10^6$/ml) were incubated *in vitro* with different concentrations of rTGF-$\beta$ in the presence or absence of LPS (1 $\mu$g/ml). TNF-$\alpha$ activity was determined as described in Methods. Values represent mean $\pm$ SEM of at least 3 different experiments.

FIGURE 20

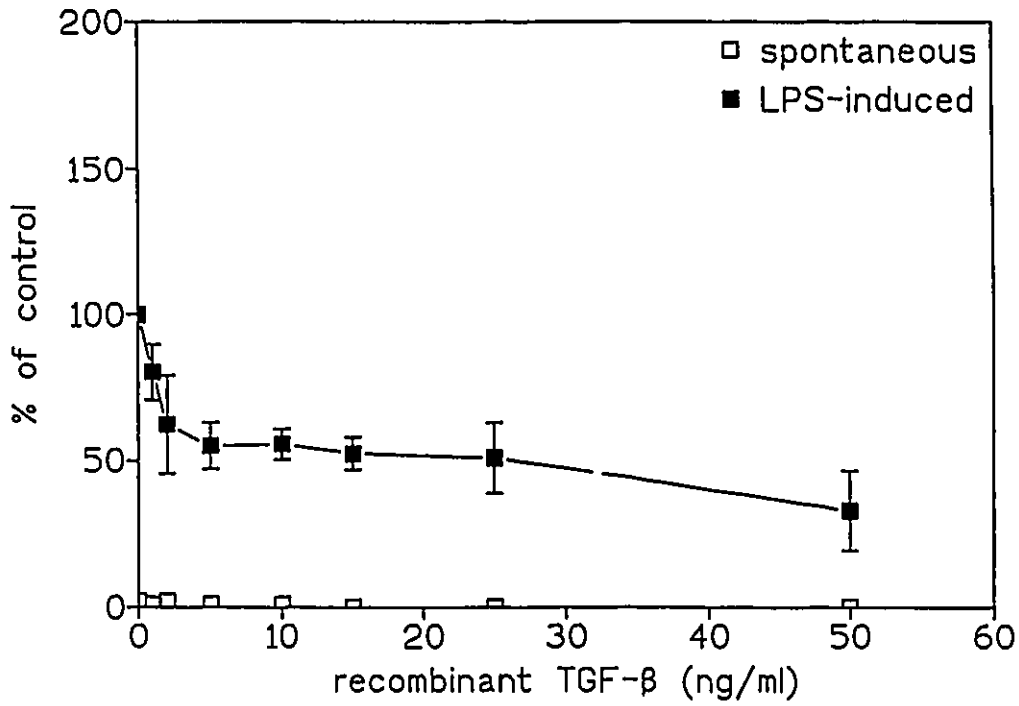
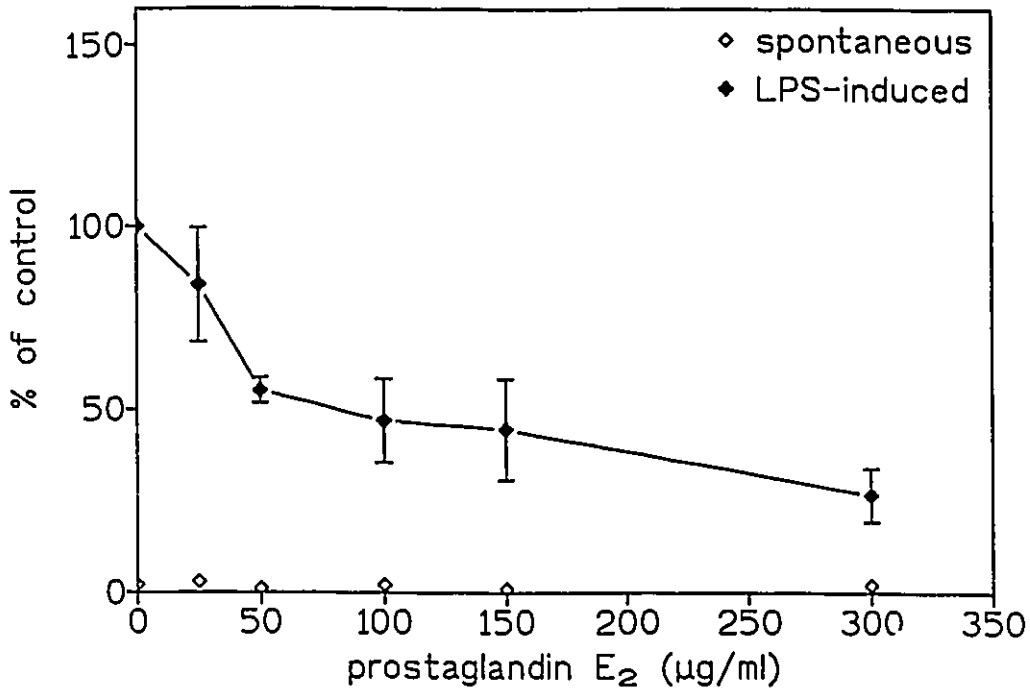

200
150
100
50
0
% of control
spontaneous
LPS-induced
0
10
20
30
40
50
60
recombinant TGF-β (ng/ml)

**Figure 21**: Effect of $PGE_2$ on TNF-$\alpha$ release. Different concentrations of $PGE_2$ were incubated *in vitro* for 18 h in the presence of normal AM ($1 \times 10^6$/ml) with or without LPS (1 $\mu$g/ml). TNF-$\alpha$ activity was measured as described in materials and methods. Values represent mean ± SEM of 3 different experiments.

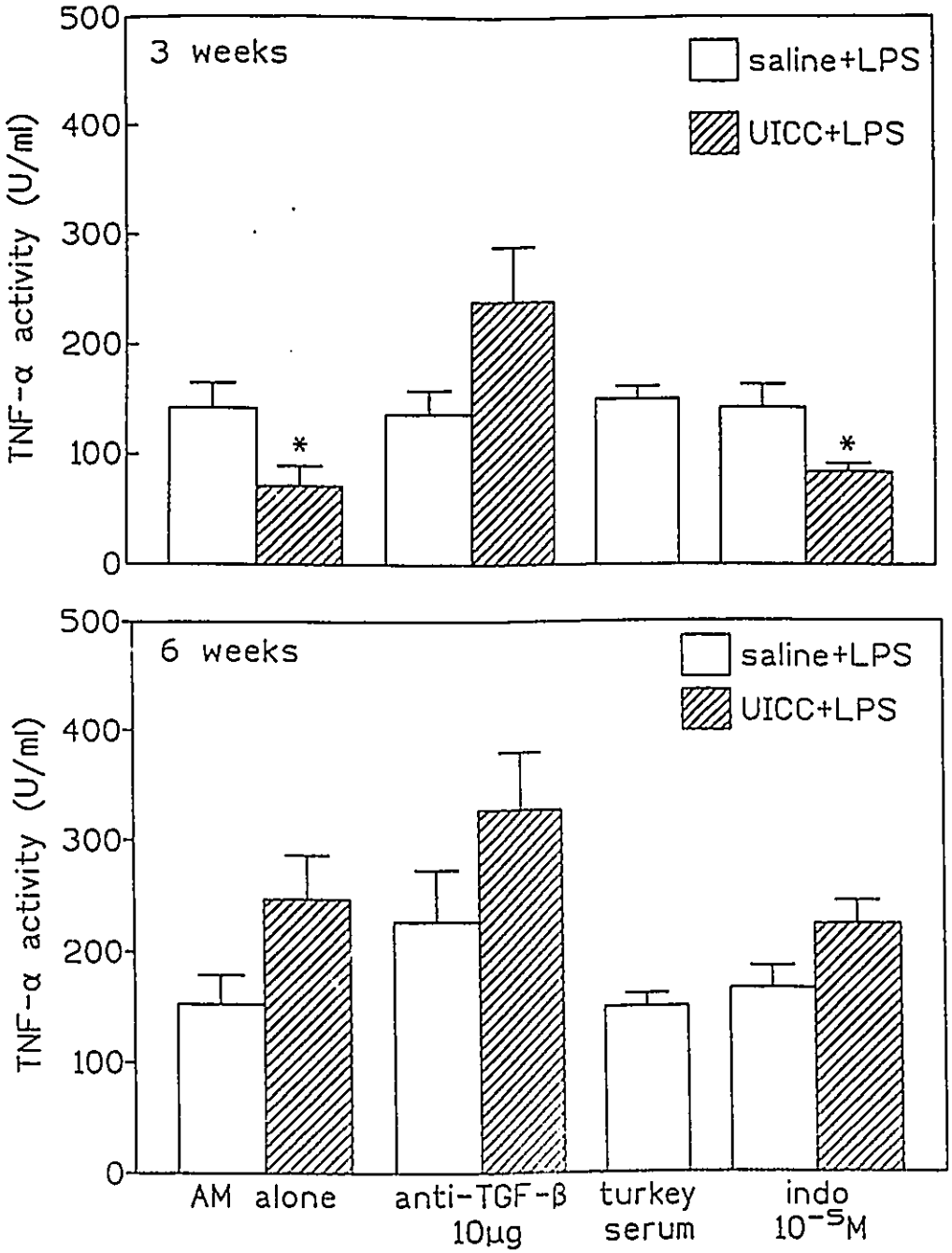
FIGURE 21


spontaneous
LPS-induced
% of control
150
100
50
0
0
50
100
150
200
250
300
350
prostaglandin $E_2$ (µg/ml)

were determined on the *ex vivo* production of TNF-$\alpha$. At that stage, investigation of the role of IL-6 was precluded due to the lack of an appropriate antibody against rat IL-6. Macrophages from animals exposed to UICC chrysotile asbestos for 3 and 6 weeks were incubated in the presence and absence of anti-TGF-$\beta$ or indomethacin. Treatment of AM with anti-TGF-$\beta$ but not with turkey serum (used as a control for the anti-TGF-$\beta$) abrogated the UICC-induced TNF-$\alpha$ suppression seen at 3 weeks (Figure 22). By contrast, $10^{-5}$M indomethacin, which decreases $PGE_2$ levels in AM, did not significantly reverse TNF-$\alpha$ suppression (Figure 22). Treatment with either anti-TGF-$\beta$ or indomethacin did not have any significant effect at 6 weeks when TNF-$\alpha$ production was higher.

**Figure 22:** Implication of cytokines in TNF-$\alpha$ production by inflammatory AM from rats that were exposed to UICC asbestos chrysotile. AM ($1 \times 10^6$/ml) were incubated *ex vivo* for 18 h with LPS (1 $\mu$g/ml) + 10 $\mu$g of anti-TGF-$\beta$, with LPS (1 $\mu$g/ml) + Turkey serum (as a control for anti-TGF-$\beta$), or with LPS (1 $\mu$g/ml) + indomethacin $10^{-5}$M. TNF-$\alpha$ activity in AM supernatants was determined as described in Materials and Methods. Values represent mean ± SEM of at least 4 animals per group.

figure 22


3 weeks
saline+LPS
UICC+LPS
TNF-α activity (U/ml)
500
400
300
200
100
0
*
*
6 weeks
saline+LPS
UICC+LPS
TNF-α activity (U/ml)
500
400
300
200
100
0
AM alone
anti-TGF-β 10μg
turkey serum
indo $10^{-5}$M

## 5.DISCUSSION

In the present work we have attempted to delineate the relative contribution of three cytokines, namely TNF-$\alpha$, IL-1 and IL-6 in the progression of inflammatory reactions toward either resolution or tissue damage such as irreversible lung fibrosis. Our results demonstrate that bidirectional modulation of TNF-$\alpha$ production occurred during the development of inflammatory response associated with fibrosis. In contrast, spontaneous as well as LPS-induced release of IL-1 and IL-6 were increased in animals with inflammation with and without fibrosis. Changes in IL-1 and IL-6 were associated with the early stages of the inflammatory reaction and might be part of a protective mechanism aimed at setting appropriate inflammatory cell responses in order to overcome the insult. In this regard, ample opportunity exists for these cytokines to exert such function. IL-1 has been shown to induce neutrophil accumulation (Ulich et al., 1991) and to stimulate various macrophage functions (Laplante et al., 1986; Dinarello et al., 1987; Dinarello et al., 1983). While the actions ascribed to IL-1 are predominantly pro-inflammatory, recent evidence indicates that IL-6 inhibits neutrophilic accumulation (Aderka et al., 1989) as well as endotoxin-induced TNF-$\alpha$ and IL-1 (Aderka et al., 1989; Ulich et al., 1991b). Thus, IL-1 may be primarily responsible for activation of the inflammatory cell machinery whereas IL-6 production may down-regulate this response in an attempt to terminate the reaction. Therefore within our experimental conditions, IL-1 and IL-6 may be classified as cytokines that

modulate primarily the inflammation component of the overall inflammatory reaction.

I investigated in more detail the alterations in TNF-$\alpha$ production that were associated with the fibrotic reaction but were absent in animals displaying inflammation that eventually resolves. The initial changes were characterized by a significant reduction in TNF-$\alpha$ levels following activation of inflammatory AM with LPS. Decreased levels of TNF-$\alpha$ were not related to decreased viability of BAL cells exposed to asbestos or silica. In silica-exposed animals, low levels of TNF-$\alpha$ could not be explained either by negative regulatory effects of neutrophils which represented a significant feature of the inflammatory response. This, coupled with our observations that inflammatory cells from asbestos exposed rats, which were composed exclusively of macrophages, also released lower levels of TNF-$\alpha$, ruled out the participation of other cell populations in mediating that effect. In this respect, AM culture supernatants might contain TNF-$\alpha$ inhibitor(s) that could inhibit TNF-$\alpha$ effects on L929 cells used for TNF-$\alpha$ bioassay. Thus a soluble fragment of the TNF-$\alpha$ receptor molecule has been found to inhibit TNF-$\alpha$ action on cells (Gray et al., 1990; Kohno et al., 1990). However, such possibility was ruled out by our observation that lower TNF-$\alpha$ activity found in AM culture media from rats exposed to asbestos for 3 weeks was correlated with a concomitant decrease in the levels of TNF-$\alpha$ mRNA in AM. Therefore, our results are consistent with a down-regulation of TNF-$\alpha$ gene expression at this stage of the inflammatory

response.

This study also brings some evidence on the mechanisms involved in TNF-$\alpha$ suppression. Accumulating evidence points to a network of interacting cytokines maintaining a proper balance and it is likely that specific cytokine interplay takes place during inflammatory reactions. Based on the observations that IL-1 and IL-6 levels were inversely correlated with TNF-$\alpha$ changes in our *in vivo* system, we reasoned that other cytokines might be involved in TNF-$\alpha$ inhibition. Our data further demonstrate that in our experimental system, at least *in vitro*, IL-6, TGF-$\beta$ and prostaglandins have the capacity to inhibit TNF-$\alpha$ production from normal AM. Further experimentation with neutralizing antibody against TGF-$\beta$ supports a role for that cytokine in down-regulating TNF-$\alpha$ production at some stage of the inflammatory reaction. Our data however, cannot rule out the participation of IL-6 in mediating such effect and further experiments utilizing a neutralizing antibody against rat IL-6 will be required to address this issue.

My observation that LPS-stimulated AM from animals exposed to asbestos produced significantly more TNF-$\alpha$ than controls at a later stage (6 weeks) of the inflammatory reaction indicates that AM became activated locally. However, the mechanisms by which inflammatory AM were activated for enhanced TNF-$\alpha$ production remain open. Cytokines such as IL-2 and INF-$\gamma$, which are strong signals for TNF-$\alpha$ production (Dunham et al.,1990; Strieter et al., 1989) could potentially represent the initiative agents for TNF-$\alpha$ production.

However, these cytokines are mainly produced by activated T-lymphocytes which were absent from the bronchoalveolar milieu of rats exposed to asbestos and silica. Nevertheless the participation of these cytokines cannot be completely ruled out since interstitial lung lymphocytes were not assessed in the experimental model. Other activators of TNF-$\alpha$ production including GM-CSF (Lindermann et al.,1988) and complement component C5a (Caivaillon et al., 1990) may also represent likely candidates. In this regard, GM-CSF was found to induce TNF-$\alpha$ production in the histiocytic cell line U-937 (Lindermann et al., 1988), although it had no effect on human AM (Thomassen et al., 1989). In agreement with this latter study, we observed that GM-CSF was unable to induce *in vitro* TNF-$\alpha$ release by rat AM (unpublished), thus casting doubts on the role of GM-CSF as a triggering agent.

To my knowledge, my study presents the first evidence for a negative modulation of TNF-$\alpha$ during inflammatory reactions leading to lung fibrosis. Variable data have been reported with regard to TNF-$\alpha$ production in experimentally induced pulmonary fibrosis. Enhanced TNF-$\alpha$ mRNA was found in whole lung tissue of bleomycin- and silica-treated mice (Piguet et al.,1989; Piguet et al., 1990). However, the cellular source of TNF-$\alpha$ was not determined in those studies and it is possible that pulmonary TNF-$\alpha$ originated from cells other than AM. With respect to AM, lack of spontaneous TNF-$\alpha$ production was reported in mice exposed to a high dose of silica (250 mg/kg) or asbestos (25 mg/kg) (Bissonnette and Rola-Pleszczynski, 1989). Although, an increase

in LPS-induced TNF-$\alpha$ was seen at the 2-week interval only after treatment with asbestos in such murine model, no change in LPS-induced TNF-$\alpha$ was observed following exposure to silica. In contrast, increases in both spontaneous and LPS-induced TNF-$\alpha$ release by AM were found in Fisher 344 rats exposed to silica at doses greater than 50 mg/kg (Driscoll et al., 1990). The discrepancies between these studies as well as ours may be related to differences in the experimental conditions used, such as animal species, doses of dusts, or AM populations analyzed. With respect to the latter, previous observations indicated that the overall AM population is composed of several subpopulations that differ in their ability to produce various cytokines, including TNF-$\alpha$ (Shellito and Kaltreider, 1984; Everson and Chandler, 1992), and that TNF-$\alpha$ production may be a feature of macrophages at a higher activation/maturational state (Martinet et al., 1988; Beutler et al., 1986; Chensue et al., 1989). In concordance with this, TNF-$\alpha$ response in our study was correlated with definite shifts in AM size that may reflect the stage of AM activation or differentiation. Thus, increase in the proportion of AM with a small diameter (immature AM) was associated with suppression of TNF-$\alpha$ and an enhanced capacity to release IL-1 and IL-6, whereas increased production of TNF-$\alpha$ and low release of IL-1 and IL-6, were related to a greater proportion of larger AM (more mature AM). Therefore, the overall TNF-$\alpha$ production may be regulated by the specific class of AM present during the inflammatory response. In agreement with this, increased production of TNF-$\alpha$ during bleomycin-induced

lung fibrosis was observed in a discrete subpopulation of AM (Everson and Chandler, 1992). When the total unfractionated AM population was assessed, no change in TNF-$\alpha$ was noticed in that study.

The physiological relevance of bidirectional regulation of TNF-$\alpha$ during inflammatory reactions is unknown. Interestingly, decreased production of TNF-$\alpha$ by mononuclear cells has been observed during the lung inflammatory response associated with cystic fibrosis (Elborn et al., 1992) and in primary biliary cirrhosis, a disease characterized by immunoregulatory disturbances and fibrosis (Broomé et al., 1992). In the first study, reduced TNF-$\alpha$ production did reverse during the course of the disease (Elborn et al., 1992). In the animal model I used, inhibition of LPS-induced TNF-$\alpha$ production was also reversible and was followed by enhanced TNF-$\alpha$ production at a later time (6 weeks). These findings are consistent with a bidirectional regulation of TNF-$\alpha$ secretion which might represent a physiologic regulatory mechanism of the overall inflammatory response. The biological relevance of TNF-$\alpha$ in immune homeostasis is poorly understood, and whether TNF-$\alpha$ should be considered as a mediator of pathogenesis or rather could represent a protective mechanism remains an unresolved issue (Jacob, 1992). In fact, bidirectional modulation of TNF-$\alpha$ production might subserve these two functions. Thus TNF-$\alpha$ has been found to be associated with PMN infiltration (Warren et al., 1991; Matsushima et al., 1988) and to induce lung granulomas (Kasahara et al., 1989). Furthermore, injection of anti-TNF-$\alpha$-antibody markedly prevented development

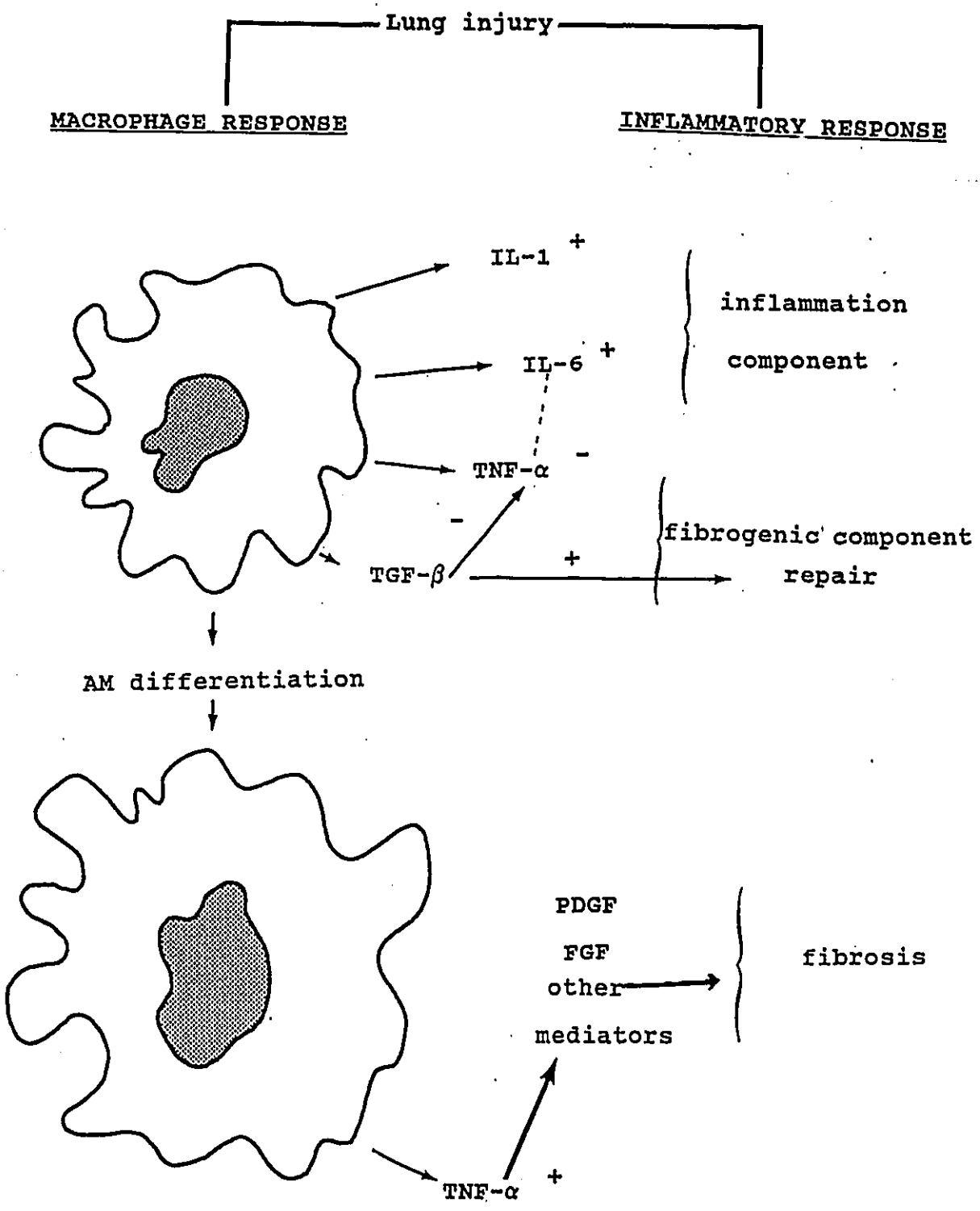
of granulomas during infection of mice with *Mycobacterium bovis* (Kindler et al., 1989). Therefore down-regulation of TNF-$\alpha$ at a given stage of the inflammatory response may represent an anti-inflammatory action. On the other hand, TNF-$\alpha$ has been found to enhance silica-induced collagen deposition and lung fibrosis, and such effect was blocked by anti-TNF-$\alpha$ antibody (Piguet et al., 1989). However, TNF-$\alpha$ treatment by itself was not sufficient to increase total collagen content in the lung indicating that TNF-$\alpha$ action requires the participation of co-factor(s) or other mediators. Since TNF-$\alpha$ has been shown to inhibit wound healing(Rapala et al., 1991), decreased TNF-$\alpha$ production may represent an attempt to promote repair of damaged lung tissue. Thus inhibition of TNF-$\alpha$ may result in higher production of fibrogenic cytokines or may increase their fibrogenic effects (Steenfos et al., 1989), while up-regulation of TNF-$\alpha$ at further stages of the reaction may contribute to maintain the inflammatory response. Therefore, repeated episodes of up- and down-regulation of TNF-$\alpha$ may be requisite for the setting of appropriate cytokine and cell responses which would result in lung damage and fibrosis due to an overexpression of repair processes normally beneficial to the host.

Overall, these results suggest the existence of a cytokine network that is modulating inflammatory reaction and fibrogenesis. Figure 23 illustrates this network. In this schema, IL-1 is produced by AM as a primary inflammatory mediator. As mentionned above, IL-1 can attract AM and other cells to the site of injury and induce the production of other cytokines. IL-6 is also considered

as a primary mediator in this model and may exert anti-inflammatory effects. At early stages of the inflammatory response, I noticed that TNF-$\alpha$ production was inhibited, and I presented evidence that this effect could be mediated by TGF-$\beta$. Such inhibition of TNF-$\alpha$ may be necessary to promote repair of the injured tissues since TNF-$\alpha$ was found to inhibit wound healing as well as the effects of growth factors (Steenfos et al., 1989). TGF-$\beta$ production may also be required at this stage of the reaction since it has been shown to be chemotactic for fibroblasts and to an induce collagen and fibronectin production (Wahl et al., 1989). Differentiation of AM was observed with time in this model and was correlated with an enhanced production of TNF-$\alpha$. At this stage, mature AM appear to become competent for TNF-$\alpha$ production which could in turn act synergistically with other growth factors to promote fibroblast activity. Although, it has been shown that TNF-$\alpha$ alone could not induce fibrosis (Piguet et al., 1989), TNF-$\alpha$ was found to enhance silica-induced collagen deposition and its effect was blocked by an anti-TNF-$\alpha$ antibody (Piguet et al.,1989). Thus, TNF-$\alpha$ may be an essential co-factor for the onset of fibrosis by other growth factors.

**Figure 23:** Schematic representation of the implication of cytokines during lung inflammation and fibrosis.

Figure 23


Lung injury
MACROPHAGE RESPONSE
INFLAMMATORY RESPONSE
IL-1
+
IL-6
+
TNF-α
-
inflammation
component
-
TGF-β
+
fibrogenic component
repair
AM differentiation
PDGF
FGF
other
mediators
fibrosis
TNF-α
+

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