INVESTIGATION OF ENDOTOXIN-INDUCED CYTOKINE EXPRESSION IN THE AIRWAYS IN VITRO AND IN VIVO

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ABSTRACT

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria, is a potent inflammatory stimulus responsible for a number of clinically critical airway conditions including gram-negative pneumonia, gram-negative septic lung injury and septic adult respiratory distress syndrome (ARDS). These conditions are all characterized by activation of alveolar monocytes/macrophages and massive infiltration of polymorphonuclear leukocytes (PMNs). While it is generally agreed that alveolar macrophages have a central role in the initiation of neutrophil accumulation and that the contents released from PMNs play a major part in causing tissue injury, the molecular mechanisms underlying these processes remain incompletely understood. Recently, some cytokines (polypeptide hormones) such as interleukin-1 (IL-1), tumor necrosis factor α (TNFα), interleukin-8 (IL-8) and interleukin-6 (IL-6), have been implicated in these processes, yet the expression of these cytokines by airway cells in response to LPS still remains to be fully elucidated. Thus, both in vitro and in vivo studies were undertaken to investigate the effect of LPS on cytokine gene expression and protein production by airway cells and tissues.

To investigate the regulation of cytokine expression in alveolar macrophages by LPS and extracellular matrix (ECM) components, LPS-

stimulated rat alveolar macrophages were maintained on different substrates: plastic, collagen and airways fibroblast-derived ECM (fECM). It was found that adherence to plastic induced IL-1ß expression and that LPS further enhanced this expression in a time-dependent manner. In contrast, significant expression of IL-6 mRNA was observed only in LPS-stimulated alveolar macrophages. Adherence to collagen or fECM evoked a stronger IL-1ß mRNA expression as compared to adherence to plastic. Only cells cultured on fECM, however, retained maximal responsiveness to LPS stimulation over a period of five days in culture.

To determine whether LPS can directly act on airway-derived fibroblasts, nasal, bronchial and lung fibroblasts were exposed to LPS and expression of several cytokines including granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-8 and IL-6 was assessed. While fibroblasts of all three anatomic sites produced significantly increased amounts of cytokines in response to IL-1, only upper airway-derived nasal fibroblasts could synthesize significant quantities of cytokines upon LPS stimulation.

Furthermore, the interaction of airways structural epithelial cells and fibroblasts with monocytes/macrophages was examined. Human peripheral blood monocytes were cultured with either nasal epithelial cell- or fibroblast-derived conditioned medium, and survival, proliferation and differentiation of monocytes then analyzed. Both macrophage colony-stimulating factor (M-CSF) and particularly, GM-CSF released by airway structural cells were found to dramatically promote survival and differentiation, but not proliferation, of monocytes.

To investigate the temporal sequence of cytokine induction upon LPS exposure in vivo, a rat model of acute lung inflammation was established. Kinetics and cellular origin of cytokines were examined. LPS markedly evoked an early expression of TNFα and macrophage inflammatory protein-2 (MIP-2; the rodent functional equivalent of human IL-8), followed by IL-1ß and IL-6 but not RANTES (a T cell/monocyte chemotactic cytokine) or transforming growth factor β_1 (TGFβ_1) expression. Alveolar macrophages represented the most significant source of cytokines shortly after LPS challenge. At later times, the infiltrating PMNs were the most significant source of cytokines in the lung.

In conclusion, these data suggest that (a) the alveolar macrophage can respond directly to LPS stimulation by elaborating and releasing cytokines; (b) this cytokine response can be modulated by the extracellular environment in which alveolar macrophages reside; (c) only the fibroblast population in the upper airway, in close proximity to the external environment, is capable of responding to LPS exposure by cytokine release. This particular fibroblast population may be thus directly involved in the initiation of acute airways inflammation; (d) monocytes/macrophages and airway structural cells communicate with each other. This interaction is accomplished in part through cytokines released from the structural cells; and (e) LPS evokes a sequential and specific cytokine response in the airways in vivo. TNFα and MIP-2 appear to play a major early role in eliciting the neutrophilic response. Consistent with in vitro findings, alveolar macrophages serve as a significant source of cytokines in vivo prior to recruitment of PMNs. Thereafter, PMNs serve as the other significant source of cytokines in vivo, suggesting that these cells likely contribute to the amplification of the response to LPS in an autocrine and paracrine fashion.

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PREFACE

This dissertation consists of seven chapters. Chapter 1, the introduction, describes the basic concepts and advances in the field that the thesis deals with and the objectives of the study. Chapters 2-6, the core part of the thesis, are composed of five individual published scientific papers. Each of them addresses a specific facet on the theme of cytokine induction by LPS in the airways. Following these chapters is the summary chapter where the major messages and the overall implications are drawn from the findings presented in the preceding papers, in conjunction with additional data presented in the appendices. The references cited in the introduction and summary chapters are listed in the references section at the end of the thesis.

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List of Abbreviations

ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
C-C	cysteine-cysteine
CD	cluster designation
cDNA	complementary deoxynucleic acid
CSFs	colony-stimulating factors
ECM	extracellular matrix
EGF	epidermal growth factor
ELAM-1	endothelial-leukocyte adhesion molecule-1
EpCM	epithelial cell conditioned medium
FCM	fibroblast conditioned medium
fECM	fibroblast-derived ECM
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
g	gram
gp	glycoprotein

h	hour
ICAM-1	intercellular adhesion molecule-1
IgA	immunoglobulin A
IGFs	insulin-like growth factors
IH	immunohistochemistry
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
ISH	in situ hybridization
kDa	kilodalton
LBP	lipopolysaccharide binding protein
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
MCAF	macrophage chemotactic activating factor
MCP-1	macrophage chemoattractive protein-1
M-CSF	macrophage colony-stimulating factor

MHC	major histocompatibility complex
min	minute
MIP-1	macrophage inflammatory protein-1
MIP-2	macrophage inflammatory protein-2
mRNA	messenger ribonucleic acid
ng	nanogram
OSM	oncostatin M
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PDGF	platelet-derived growth factor
pg	picogram
PMN	polymorphonuclear leukocyte
RANTES	regulated on activation, normal T expressed and secreted
SSC	saline sodium citrate
TGFß	transforming growth factor ß
TNFα	tumor necrosis factor α
VCAM-1	vascular cell adhesion molecule-1

Chapter one

INTRODUCTION

1.1 LINES OF AIRWAYS DEFENSE

1.1.1 Components of the defense system

The airways are divided into upper and lower airways. The former is composed of the nasal cavity and paranasal sinuses, the nasopharyngeal passage and the trachea, whereas the latter comprises the conducting airway structures including bronchi, bronchioles and terminal bronchioles, and the terminal airway portion including respiratory bronchioles, alveolar ducts and alveoli.

The airways are directly exposed to the exterior environment. There exist a vast array of microorganisms in the air that is constantly inhaled into the airways. Although normally a large number of these microbes exist in the naso-oro-pharyngeal secretions, only a very small number of bacteria can be detected in the lower airway. In addition to microbes, there are also dust particles, noxious gases, and antigens which, if small enough (<3 μm), can make their way down to the lower airways, becoming potentially detrimental to the host (Reynolds, 1992). An efficient defense system is therefore needed to defend the upper airway, and to prevent the penetration of injurious agents from the upper airways into the lower airways.

The airways defense system consists of three mechanisms: mechanical, phagocytic and immune. These mechanisms commonly act in an orchestrated fashion. The mechanical mechanism is mainly fulfilled by the anatomic barriers, associated with powerful nerve-mediated reflexes, mucocilliary clearance and secretory IgA in the upper airways where air gets heated, humidified and filtered. These processes most often result in clearance or confinement of large particulates (>10 μm) and most microorganisms (Reynolds, 1992). In the conducting airways below the trachea but above the terminal respiratory lower airways, in addition to mucus, cilia, and secretory Ig-A, there exist bronchus-associated lymphoid tissues, lymphocytes and bronchial alveolar macrophages on the surface of the bronchus as well as submucosal plasma cells and mast cells. Further down, particularly in the terminal airways, the alveolar surface lining substance surfactant-rich lipoprotein layer, immunoglobulins, and certain complement factors as well as alveolar macrophages, all reinforce the defense system against any foreign intruders.

1.1.2 Cellular basis of defense

Cellular components constitute an important part of the efficient airway defense system. They include nonparenchymal and parenchymal (structural) elements. The former includes alveolar macrophages and lymphocytes and other submucosal inflammatory cell types whereas the latter mainly comprises epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts.

The alveolar macrophage is the most abundant nonparenchymal cell type in the airways, which outnumbers the lymphocyte in a ratio of 5-10 to 1 (Crystal, 1991). Alveolar macrophages are mobile cells and play a pivotal role in airways nonspecific defenses through phagocytosis, microbial killing, and clearance of foreign substances, as well as in specific immune responses through antigen presentation to T lymphocytes in the context of MHC I and II surface molecules.

In addition to the above, a large number of mediators such as cytokines, released by these cells profoundly regulate functions of themselves and of other cell types, thus amplifying or modulating the protective mechanism (Crystal, 1991). Lymphocytes play an important role in antigen-specific responses. These are mediated chiefly via the activation of T lymphocytes, development of cellular cytotoxicity, communication of T cells with B cells, release of cytokines and antibody production.

The airways structural cells also play a direct role in airways defense. This is particularly true of nasal and bronchial epithelial cells. In addition to being mechanical barriers and providing mucus secretion and antibody presentation functions, these cells are capable of elaborating anti-bacterial proteins such as lactoferrin and lysozyme, as well as arachidonic acid metabolites and cytokines (Bernstain, 1992; Reynolds, 1992). In addition, the airway vascular endothelial cells are capable of releasing regulatory molecules such as angiotensin II and prostaglandins as well (Gail and Lenfant, 1982). Lastly, the fibroblast population of the airways represents the most abundant type of structural or parenchymal cells, constituting about 50% of total pulmonary parenchymal cells (Raghu and Kavanagh, 1991). It has long been recognized that these fibroblasts play a key role in maintaining the structural and functional integrity of the airways and in reparative processes (Raghu and Kavanagh, 1991). More recently, it has become increasingly evident that these cells may operate as inflammatory effector cells via matrix elaboration, production of oxygen species, complement factors, prostaglandins and cytokines, and perhaps antigen presentation (Elias et al., 1992).

When a new strain or species emerges, or large quantities of microbes invade or are accidentally introduced into the airways, or when the defense mechanisms become compromised, additional defense mechanisms come into play. Neutrophils represent one of these important additions to the resident airway defenses and they move more slowly in the airways vascular compartment than those at other vascular sites and are in stationary contact with the capillary endothelium due to a low capillary pressure (Parsons et al., 1992). Therefore, when needed, these neutrophils can gain easy access from the blood stream into airways tissue under the influence of soluble factors including cytokines released from alveolar macrophages and perhaps structural cells, thus mounting an acute inflammatory response against the injurious agent (Reynolds, 1992).

1.2 CYTOKINES IN THE AIRWAYS

1.2.1 Cytokines: an Overview

Cytokines are glycoproteins or small peptides with molecular weight ranging from 6000-60,000 kDa, that mediate intercellular communication almost exclusively in a paracrine or autocrine fashion. Most cytokines are not expressed at a significant level under normal circumstances. Instead, they are produced and quickly released upon stimulation by exogenous stimuli. These features differentiate cytokine hormones from neuro-endocrine hormones (Oppenheim et al., 1993; Kelley 1993; Wang et al., 1993). A few cytokines including M-CSF, TGFß, insulin-like growth factors (IGFs), and stem cell factor, however, are constitutively expressed, playing a role in normal physiologic processes (Kelly 1993; Oppenheim et al., 1993). Cytokines play a central role in the regulation of developmental, hematopoietic, immune, inflammatory and reparative processes, and act at very low concentrations of 10^{-10} - 10^{-13} mol/L (1 pg/ml) (Oppenheim et al., 1993). Approximately 60 cytokines to date have been found and characterized and an ever-growing number of novel cytokines are being added to this list.

Most nucleated cells in the airways are able to express multiple cytokines, and receptors for more than one cytokine. Cytokines act via binding to specific high affinity receptors ($K_d = 10^{-9}$ - 10^{-12} M). Occupancy of as few as 50 receptors can be sufficient to activate cells (Oppenheim et al., 1993).

A given cytokine may display pleiotropic effects since numerous cell types may possess receptors for the same cytokine. In addition, some cytokines act in a redundant or overlapping manner, eliciting similar biological effects. This may be attributed to: different cytokines function through induction of a similar set of other functional cytokines (Oppenheim, et al., 1993); the individual receptor for these cytokines comprises two peptide subunits, a distinct ligand-binding unit and a signal transducing unit common for a group of cytokines. For example, the receptors for IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM) and interleukin-11, all utilize gp130 as a signal transducer while receptors for interleukin-3 (IL-3), interleukin-5 (IL-5) and GM-CSF use KH97 protein to transduce a common signal (Kishimoto et al., 1992; Yin et al., 1993); some structurally nonhomologous cytokines such as IL-1α and IL-1ß may bind to the same receptor, presumably mediated by homologous receptor binding domains (Aksamit and Hunninghake, 1993), or there might be promiscuous receptors that recognize different cytokines which possess a small degree of structural homology, for example, macrophage chemotactic activating factor (MCAF) and macrophage inflammatory protein-1 (MIP-1) in the "C-C" chemokine family, can compete for RANTES receptors on monocytes (Wang et al., 1993).

A cytokine may induce other cytokines, forming cytokine cascades and networks which often lead to swift amplified cytokine responses and effects. For instance, cytokines such as IL-1 and TNFα can potently stimulate expression of a wide spectrum of other cytokines including IL-6, IL-8, and CSFs, which are generally referred to as "second wave" cytokines. These processes operate through reciprocal, additive and synergistic mechanisms.

A cytokine response can not only be swiftly turned on, but also turned off quickly so as to conform to appearance and disappearance of the insult. Switching off is usually associated with a transient expression of mRNAs for cytokines due to a short period of transcription, perhaps secondary to turning on of transcriptional repressor factors, and/or by a short half-life of mRNA. Indeed, the mRNAs of many cytokines such as IL-1, IL-6 and GM-CSF have been found to possess AU-rich sequences in the 3′ untranslated regions, presumably involved in mRNA turn-over (Balkwill, 1991). Not only cytokine mRNA expression is a self-limited event, but cytokine-mediated effects are under tight control of negative regulatory mechanisms (Abbas, et al., 1991; Balkwill, 1991). These mechanisms include: concurrent induction of negative regulatory cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10), TGFß and perhaps IL-6 and of other soluble inhibitory molecules such as prostaglandin E_2 (PGE_2) which counteract the effects of stimulatory cytokines such as TNFα and IL-1; decreased receptor expression and increased receptor-ligand complex internalization, and induction of receptor antagonists such as interleukin-1 receptor antagonist (IL-1ra); induction of cytokine binding proteins such as α_2-macroglobulin; induction of soluble cytokine receptors such as those for IL-4, IL-1 and TNFα; enzymatic degradation of cytokines; and generation of auto-antibodies against cytokines.

Cytokines that can be produced by the airway cells, fall into several major categories according to their functions although these categories are general and not mutually exclusive, and some of them will be discussed separately:

A. primary inflammatory cytokines: IL-1, TNFα, IL-6

B. chemoattractive cytokines: IL-8, MIP-2, MCP-1, MIP-1, RANTES

C. hematopoietic cytokines: GM-CSF, G-CSF, M-CSF, IL-3

D. growth factors: TGFß, PDGF, EGF, IGFs

1.2.2 IL-1

There are two forms of IL-1, IL-1α and IL-1ß. Both are synthesized as 31 kDa precursors and the mature forms are 17 kDa in size. IL-1α and IL-1ß are nonhomologous but bind to the same receptor, hence triggering similar biologic responses. The majority of secretory forms of IL-1 released by monocytes is IL-1ß whereas IL-1α appears to remain cell-associated (Kelley, 1990; Aksamit and Hunninghake, 1993). The formation of the mature form of IL-1ß is mediated by enzymatic cleavage by the IL-1ß converting enzyme, a member of the cysteine protease family, specific for the IL-1ß, but not IL-1α, precursor (Howard et al., 1991; Dinarello, 1993).

IL-1 has broad effects on a vast array of cells modulating immune and inflammatory processes. As a proinflammatory cytokine, IL-1 is a direct inducer of components of the acute phase response, adhesion molecule expression on endothelial cells and T and B lymphocyte activation and/or proliferation and differentiation. IL-1 is also a potent trigger for expression of other cytokines and many biologic effects of IL-1 involve these IL-1-mediated cytokine cascades.

Monocytes/macrophages are a major source of IL-1. Alveolar macrophages, bronchial epithelial cells and neutrophils produce IL-1, primarily in response to exogenous stimuli such as LPS, under in vitro and in vivo conditions (Aksamit and Hunninghake, 1993). However, alveolar macrophages seem to produce much less IL-1ß in response to LPS than peripheral blood monocytes. Interestingly, respiratory fibroblasts express increased IL-1ß mRNA but not soluble protein, likely due to the deficiency of IL-1ß converting enzyme (Elias et al., 1989; Elias, et al., 1992). In some animal models of pulmonary fibrosis, expression of IL-1 mRNA in the lung was hardly detectable in contrast to TNFα expression, suggesting a more prominent role for TNFα than IL-1 in this type of lesion (Piguet et al., 1990; Piguet et al., 1989). Thus, the expression and the relative contribution of IL-1 in vivo remain to be clarified.

1.2.3 TNFα

The mature form of TNFα protein is 17 kDa in size. As its name indicates, TNFα is cytotoxic for a variety of tumors. Like IL-1, TNFα is also a potent multifunctional and proinflammatory cytokine capable of inducing acute phase responses, endothelial adhesion molecule expression, neutrophil and T/B cell activation, and cytokine expression by diverse types of cells. TNFα usually operates in concert with IL-1ß. TNFα is also a principal mediator of endotoxic shock (Ulich, 1993a).

The alveolar macrophage appears to be a major source of TNFα in the airways, particularly upon exposure to LPS (Ulich, 1993a). In contrast to the pattern seen for IL-1 secretion, alveolar macrophages produce much more TNFα than peripheral blood monocytes in response to LPS. There have been in vitro data suggesting that both vascular endothelial and smooth muscle cells may also be potential sources of TNFα in the airways (Ulich, 1993a).

TNFα seems to play a key role in a number of respiratory conditions including sepsis-associated lung injury (Tracey et al., 1988), adult respiratory distress syndrome (Tracey et al., 1988), gram negative pneumonia (Ulich, 1993a), immune complex alveolitis (Warren et al., 1989), and silica/bleomycin-induced pulmonary fibrosis (Piguet, 1990; Phan and Kunkel, 1992; Piguet, 1989). Antibody against TNFα has been subsequently shown to prevent or attenuate lung injury associated with these conditions. Recently, soluble TNFα

receptors have also been shown to antagonize LPS-induced septic shock (Mohler et al., 1993) and LPS-induced neutrophilic pneumonia (Ulich, 1993b).

1.2.4 IL-6

The mature IL-6 molecule is approximately 21 kDa in size. IL-6 is a major inducer of acute phase proteins and fever as compared to IL-1 and TNFα (Gauldie, 1991) and is a potent B cell growth and antibody secretion factor. It also has stimulatory effects on T cell functions and maturation of hematopoietic stem and progenitor cells. While it stimulates growth of plasmacytoma and myeloma cells in vitro, IL-6 has been used as an anti-tumor agent to inhibit some forms of leukemia and abrogate melanoma metastases through induction of cellular immune responses (Katz et al., 1993).

A number of cell types including mononuclear cells, endothelial cells and fibroblasts produce IL-6 in response to exogenous stimuli or endogenous mediators such as IL-1 or TNFα (Gauldie, 1991). The alveolar macrophage is a rich source of IL-6 upon stimulation with LPS whereas lung fibroblasts produce far more IL-6 than alveolar macrophages in response to IL-1 stimulation.

Traditionally, IL-6 has been regarded as a proinflammatory cytokine. In fact, IL-6 may also be regarded as an antiinflammatory cytokine capable of inducing acute phase antiproteases both systemically and locally (Richards et al., 1991; Gauldie, 1991; Richards et al., 1993). Noticeably, unlike IL-1/TNFα, IL-6 is unable to significantly induce other cytokines. In contrast, it seems to inhibit production of proinflammatory cytokines such as TNFα (Aderka and Vilcek, 1989) and to attenuate LPS-elicited neutrophilia in the lung (Ulich, 1991a). Nevertheless, the local effect of IL-6 at tissue sites still awaits to be fully elucidated.

1.2.5 IL-8/MIP-2 and RANTES

IL-8, MIP-2 and RANTES all belong to a family of chemotactic cytokines, collectively designated as chemokines, characterized by their small molecular weight ranging from 8 to 11 kDa (Oppenheim, 1993). Based on the relative position of the first two of their four cysteine residues, IL-8 and MIP-2 belong to the "C-X-C" subfamily whereas RANTES belongs to the "C-C" subfamily. IL-8 and MIP-2 are nonhomologous molecules but both are potent neutrophil chemoattractants, with the former being produced mainly by human cells and the latter by rodent cells.

IL-8 and MIP-2 are produced by a variety of cells in the airways including alveolar macrophages, neutrophils, alveolar epithelial cells, endothelial cells and fibroblasts (Oppenheim et al., 1993; Standiford et al., 1992; Driscoll et al., 1993). Like IL-6, these cytokines appear to be "second wave" cytokines which are produced in response to stimuli such as LPS and IL-1, dependent on the target cell type, and have more specialized activities and little inductive effects on other cytokines (Oppenheim et al., 1993). These neutrophil chemoattractive cytokines may potentially play an important role in acute inflammatory responses, ARDS and fibrotic disorders in the airways.

RANTES, chemotactic to T cells, monocytes and eosinophils, has been shown to date to be produced by monocytes, T cells and synovial fibroblasts (Kameyoshi et al., 1992; Schall et al., 1992; Rathanaswami, et al., 1993). Its expression and activities in the airways remain to be discerned.

1.2.6 GM-CSF and M-CSF

Several cytokines, GM-CSF, granulocyte colony-stimulating factor (G-CSF), M-CSF and IL-3 also known as multilineage CSF, which stimulate growth and differentiation of hematopoietic precursor cells, are collectively called colony-stimulating factors (CSFs) (Abbas et al., 1991). Among them, GM-CSF is a 22 kDa protein acting on all leukocyte lineage precursors and G-CSF is an approximately 19 kDa protein acting primarily on marrow progenitors already committed to develop into granulocytes, and M-CSF is 40 kDa dimer molecule acting mainly on the precursor cells committed to mature to monocytes.

Outside the bone marrow, GM-CSF and M-CSF similar to IL-6 and the chemokines, can be produced by alveolar macrophages in response to LPS stimulation, and by endothelial cells, epithelial cells and fibroblasts in response to IL-1 or TNFα (Gauldie et al., 1993a). Airway epithelial cells appear to produce several-fold less M-CSF than airway fibroblasts (Jordana et al., 1991).

The production of CSFs including G-CSF by respiratory structural cells and their effects on progenitor cell differentiation in vitro suggest that these cytokines may be involved in local differentiation and proliferation of leukocyte progenitors present in the airway milieu. In addition, recombinant or airway structural cell-derived GM-/G-CSFs promote survival and activation of granulocytes (Jordana et al., 1991). These cytokines enhance survival by inhibiting cells from undergoing apoptosis (Cox et al., 1992). These lines of evidence suggest that airway structural cell-derived CSFs represent "second wave" cytokines largely produced upon stimulation by proinflammatory cytokines and may contribute to the accumulation of inflammatory cells infiltrating the airways under the influence of chemoattractive cytokines (Gauldie et al., 1993b).

1.2.7 $TGF\beta_1$

$TGF\beta_1$ is a member of the TGFß family including $TGF\beta_{1-5}$, with a molecular weight of 25 kDa. $TGF\beta_1$ is a unique cytokine in that it remains biologically inactive after secretion until it undergoes proteolytic cleavage (Kelley, 1990; Kelley, 1993). In addition, its amino acid sequence is highly conserved across species. Together with $TGF\alpha$, TGFß was first discovered as a molecule capable of inducing a transformed phenotype in non-neoplastic cells, but it has, however, a wide spectrum of biologic activities including growth stimulation or inhibition of many cell types, promotion of extracellular matrix production and inhibition of activation of T cells as well as counteraction of the effects of proinflammatory cytokines.

Alveolar macrophages, neutrophils, and endothelial, epithelial and fibroblast cells can release $TGFß_1$. In addition, $TGFß_1$ has been found to be constitutively present in the airways in vivo. However, elevated TGFß mRNA expression and protein localization are found associated with fibrotic responses in airway tissues and elsewhere (Kelley, 1993).

1.3 Cellular Communication in the Airways

1.3.1 Cytokines and cellular communication

During the airways response to an insult, different cellular components function in an orchestrated manner. Cytokines are the paramount soluble factors mediating communication between cells (Kelley, 1990).

The notion of cytokine cascades (Kelley, 1990; Strieter et al., 1993a; Abbas et al., 1991) suggests that different cell types are sequentially or simultaneously triggered to release certain primary cytokines, usually proinflammatory cytokines. These cytokines act on the cells that released them (autocrine), or on adjacent cells of other types (paracrine) or, in some cases, travel and act on cells at a distant site (endocrine). This results in direct functional alterations, production of "second wave" cytokines, and/or further release of the same cytokines from these cells. Cytokines exert their stimulatory or inhibitory effects on target cells in a synergistic or additive fashion to achieve an amplified and fine-tuned response. The consequences of a response are likely determined by the nature of the causative agent, the degree to which the target cells are involved and/or impaired, and subsequently, the types and perhaps the quantities of the cytokines that are "turned on". The causative agent may be eliminated in an inflammatory response leaving no residual tissue damage. Alternatively, a fibrotic response develops to confine the agent and/or to repair the damaged tissue (Raghow, 1991).

Cellular communication in the airways, under the influence of cytokine cascades, may be exemplified by the response to a nonimmune stimulus: (a) the injurious agent enters airway tissues causing hemorrahage, activation of complement cascade, release of arachidonic acid metabolites and platelet degranulation, establishing the earliest chemoattractive forces for inflammatory cells (Raghow, 1991); (b) alveolar macrophages become activated, releasing proinflammatory cytokines including IL-1, TNFα and chemokines such as IL-8. Alveolar macrophages have been recently proven to be central in eliciting the ensuing cytokine and cellular events. Depletion of alveolar macrophages by a chemical approach strikingly reduces TNF induction and neutrophil influx in LPS-challenged lungs (Berg et al., 1993); (c) the initial wave of neutrophils and/or mononuclear cells migrate towards the injury along a gradient of chemoattractive substances such as IL-8 or macrophage chemoattractive protein-1 (MCP-1), thus establishing an early inflammatory response (Strieter et al., 1993a); (d) concurrently, alveolar macrophage-derived IL-1/TNFα act on structural cells including epithelial cells and fibroblasts to release the "second wave" cytokines including chemokines and CSFs. These chemokines further recruit and activate neutrophils and/or mononuclear cells as well as alveolar macrophages whereas CSFs may promote differentiation of local inflammatory progenitor cells and survival and growth of mature infiltrating cells (Jordana et al., 1991). In addition, in vitro data suggest that recruited neutrophils may release the above cytokines thereby generating autocrine/paracrine loops (Lloyd and Oppenheim, 1992). The final outcome of these cellular interactions is the amplification and/or perpetuation of the inflammatory response; (e) at this point, the response may subside and eventually resolve. The fine-tuning and resolution of inflammation likely involve negative regulatory cytokines including IL-6, IL-4, IL-10 and TGFβ_1 (Baumann and Gauldie, 1994; Lotz and Guerne, 1991; Aderka et al., 1989; Kelley, 1993; De Wall Malefyt et al., 1992). If the response continues and severe tissue damage occurs, there might be a "follow-up" expression of growth factors such as TGFß, platelet-derived growth factor (PDGF) and IGFs by both inflammatory and structural cells, leading to enhanced proliferation of structural cells and deposition of extracellular matrix proteins from these cells. As a result, fibrosis may ensue.

1.3.2 Adhesion molecules

In addition to cytokines, cellular communication also involves direct cell-cell contact, mediated through mutual cell adhesion molecules or receptors on both stromal cells including endothelial cells and fibroblasts, and leukocytes. These play an important role in adherence of leukocytes to the vascular endothelium, transmigration through the endothelium, and directed movement in the tissue.

Endothelial cells possess several surface adhesion molecules involved in endothelial cell-leukocyte interaction: intercellular adhesion molecule-1 (ICAM-1; a member of the immunoglobulin supergene family), E-selectin or endothelial leukocyte adhesion molecule-1 (ELAM-1), P-selectin and vascular cell adhesion molecule-1 (VCAM-1). Leukocytes such as neutrophils mainly express CD11/CD18 complex molecules including CD11a/CD18, CD11b/CD18 and CD11c/CD18 (members of β_2 integrin family), L-selectin and ICAM-1 (Albelda and Buck, 1990; Spragg and Smith, 1992).

Compelling evidence indicates that ICAM-1 on endothelial cells is a counter-receptor for both CD11a/CD18 and CD11b/CD18 on neutrophils. Although it is constitutively expressed on endothelial cells, ICAM-1 is upregulated by exposure to IL-1, TNFα and/or LPS (Strieter et al., 1993b). P-selectin is constitutively synthesized and intracellularly stored in endothelial cells as well as platelets, and can be rapidly mobilized to the cell surface. New synthesis of both P-selectin and E-selectin in endothelial cells is also induced by IL-1 and TNFα (Bevilacqua and Nelson, 1993; Spragg and Smith, 1992). On the other hand, CD11/CD18 molecules are constitutively expressed on leukocytes. Moreover, neutrophils have additional stores of CD11b/CD18 in their granules and activation of these cells brings about a rapid translocation of this molecule to the cell surface (Strieter et al., 1993b). Hence, the interaction between adhesion molecules on endothelial cells and those on leukocytes contributes to adhesion of leukocytes to endothelial cells and extravasation into the tissue during inflammation.

Using a P-selectin "knock-out" mouse model, it has been shown that the initial margination or "rolling" of neutrophils on endothelial cells depends mainly on P-selectin expressed on endothelial cells (Mayadas et al., 1993). In animal models, pretreatment with antibodies against CD11b, CD18 or ICAM-1 has been shown to significantly inhibit neutrophil-mediated lung injury, suggesting an essential role for adhesion molecules on neutrophils and endothelial cells in acute lung inflammation (Barton et al., 1989; Winn et al., 1993; Strieter et al., 1993b). In addition, it is speculated that inducible expression of ICAM-1 on airway interstitial fibroblasts may play a role, in combination with chemokines, in localizing and immobilizing leukocytes within the tissue.

1.3.3 Extracellular matrix proteins

Cellular communication is mediated not only by soluble cytokines and direct cell-cell contact, but also through the extracellular matrix (ECM).

The airway tissue is rich in ECM accounting for more than one third of the total dry mass (Raghow, 1992). The ECM of the lung parenchyma comprises collagen (60-70%), elastin (25-35%), glycosaminoglycans (<1%) and other glycoproteins such as fibronectin and laminin (Raghow, 1992). These ECM components can be produced by a variety of cell types but largely by epithelial and endothelial cells and particularly by parenchymal fibroblasts.

Leukocytes also have adhesion receptors for ECM proteins. These receptors belong to the β_1 integrin subfamily, including the classical receptors for fibronectin and laminin, $\alpha_5\beta_1$ and $\alpha_6\beta_1$, respectively (Albelda and Buck, 1990). Activated neutrophils express increased numbers of these receptors (Abramson et al., 1991). Thus ECM proteins within the basement membrane and parenchyma likely play a role, in concert with adhesion receptors, in directing leukocyte movement and localization.

It is well known that ECM can profoundly affect the function of ECM-producing structural cells, including proliferation, movement, and ECM production. In addition, ECM may regulate the activity of inflammatory cells that reside within the matrix by modulating cytokine gene expression and/or cytokine presentation.

There is increasing evidence that adherence to ECM induces expression of certain cytokines by mononuclear cells including GM-CSF, M-CSF, IL-1 and TNFα (Nathan and Sporn, 1991). However, the effect of ECM on cytokine expression by alveolar macrophages remains to be elucidated. ECM can also change the response of cells to cytokines. For instance, different amounts of fibronectin or collagen used to coat culture dishes can dictate whether endothelial cells will proliferate or differentiate in response to fibroblast growth factor (FGF) (Ingber and Folkman, 1989). ECM may also serve as an anchor for cytokines so that cytokines can be efficiently stored or presented to cells. Cytokines known to interact with ECM are FGF, GM-CSF, IL-3 and TGFβ (Nathan and Sporn, 1991). Binding to ECM may also protect cytokines from proteolysis, as has been shown for FGF (Schubert, 1992). Taken together, it appears that cytokines can alter ECM production by structural cells, and that ECM can in turn influence cytokine expression and presentation and cellular responses. Thus, structural and inflammatory cells, soluble cytokines, adhesion receptors and insoluble ECM proteins, act in a cohesive context to optimize the tissue response to insult.

1.4 Lipopolysaccharide and Airways Inflammation

1.4.1 Structure and metabolism of LPS

LPS (endotoxin) is a component of the outer membrane of gram-negative bacteria. A great deal of attention has been paid to this fascinating molecule because of its potent multiple effects on immune and inflammatory effector cells.

LPS is made up of lipid A and a complex polysaccharide (Figure 1). The polysaccharide moiety contains the inner core, outer core and O-antigen repeating oligosaccharide. KDO (3-deoxy-D-manno-octulosonic acid) and heptose constitute the inner core (Raetz et al, 1991). The O-antigen determines antigenic specificity of LPS and is highly variable among species. Most of biological activities of LPS reside in the lipid A and core region (Freudenberg and Galanos, 1990).

LPS is released mainly as a consequence of bacterial death and lysis. Certain amounts of LPS can be also shed from live bacteria (Freudenberg and Galanos, 1990).

It appears that circulating LPS undergoes complex formation with lipoproteins, transferrin and LPS-binding protein (Freudenberg and Galanos, 1990; Tobias et al., 1992). Binding to high density lipoprotein (HDL) delays LPS clearance (Freudenberg and Galanos, 1990). The data concerning the

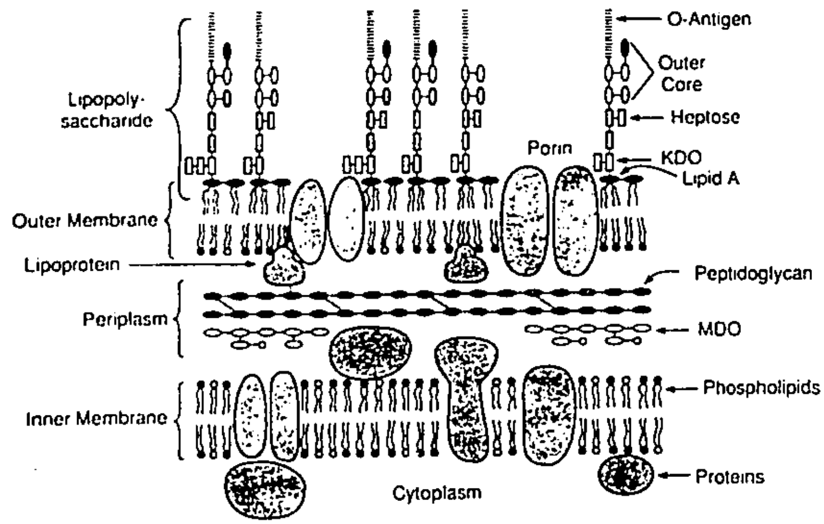

Figure 1. Schematic representation of LPS structure. LPS is a component in the outer membrane of Gram-negative bacteria. It consists of lipid A and a polysaccharide moiety. The latter comprises KDO (3-deoxy-D-manno-octulosonic acid), heptose, outer core sugar residues and O-antigen repeating oligosaccharide (From Raetz et al., 1991).

detailed kinetics of these reactions are still scarce.

Circulating LPS can be taken up by various organs, but mainly by the liver, spleen and adrenal glands. Cells including sinusoidal cells and hepatocytes in the liver, and macrophages and granulocytes in other sites can localize LPS (Freudenberg and Galanos, 1990).

LPS remains chemically intact in the circulation until partitioned into tissues. Macrophages and granulocytes have been found to possess several enzymes that cleave lipid A in LPS (Freudenberg and Galanos, 1990). Degraded LPS is excreted in the bile and feces, and in small amounts through urine. It may be also excreted in macrophages through the lungs.

1.4.2 LPS binding protein and receptors

The LPS-binding protein (LBP) is a 55 kDa serum glycoprotein expressed and secreted from the liver. Human LBP is composed of 456 amino acids. The amino acid sequences of LBP appear to be considerably conserved between species. For instance, the LPB molecules for human and rabbit are 69% homologous. Normal serum contains about 5-10 μg/ml of LBP whereas acute phase serum contains more than 200 μg/ml, suggesting LBP is one of the hepatic acute phase proteins (Tobias et al., 1992). Bronchoalveolar lavage (BAL) samples from ARDS patients were found to contain much higher levels of LBP than those of normal subjects.

LPS-binding protein forms a high affinity complex with LPS which binds to the LPS-LBP complex receptor, CD14, causing activation of cells (Figure 2). The requirement of LBP for the optimal response of cells is exemplified by the observation that TNFα production by peritoneal or alveolar macrophages in response to LPS, particularly at low doses, was many orders of magnitude higher in the presence of LBP (Tobias et al., 1992).

CD14 is a surface protein anchored to membrane glycosylphosphatidyl-inositol found mainly in monocytes and macrophages including alveolar macrophages (Tobias et al., 1992). While IL-1 and TNFα induce an increase in CD14 on mature monocytes, INFτ and IL-4 have been shown to downregulate its expression (Zieglar-Heitbrock and Ulevitch, 1993). Increased immunostaining for CD14 has been observed on alveolar macrophages recovered by BAL from patients with sarcoidosis (Zieglar-Heitbrock and Ulevitch, 1993). Neutrophils normally possess about 10 fold less membrane-bound CD14 than monocytes but can be induced to express considerably more upon exposure to stimuli such as TNFα and GM-CSF (Wright et al., 1991). The interaction of LBP/LPS complex with CD14 on neutrophils evokes TNFα release in vitro (Haziot et al., 1993a). Recent evidence also shows that this interaction is one of the mechanisms whereby LPS translocates into the intracellular compartment becoming enzymatically deacylated in neutrophils (Luchi and Munford, 1993). The CD14-mediated intracellular signal transduction pathways are still largely

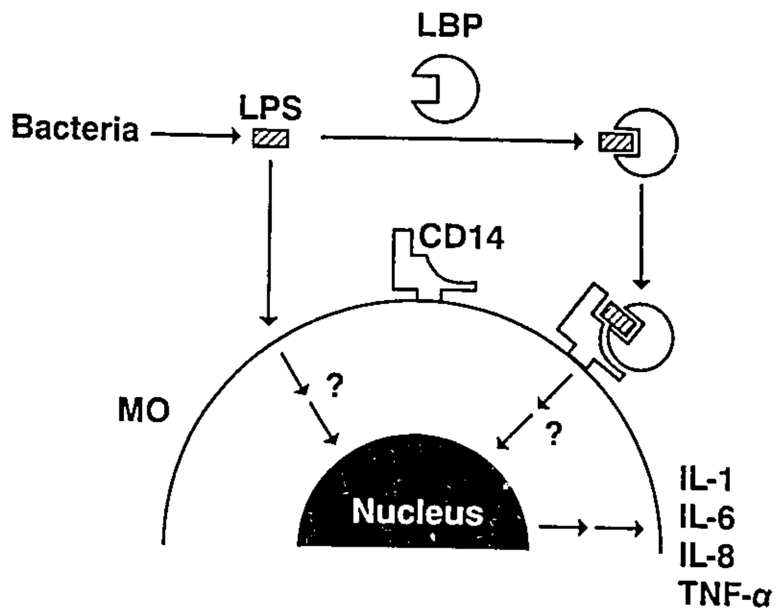

Figure 2. Schematic representation of the interaction of LPS and LPS-binding protein (LBP) with LPS receptor CD14 on macrophages (MO) (From Tobias et al., 1992).

unknown.

The discovery on the involvement of the membrane-anchorage CD14 in LPS-LBP complex-triggered cellular response facilitated the unravelling of the link between the soluble form of CD14 and LPS responsiveness of endothelial cells which do not express CD14. Free soluble forms of CD14 can be detected in plasma or culture supernatants of monocytes and neutrophils as a result of shedding of the membrane-anchorage form (Haziot et al., 1993a). It has been found that without LBP or serum, endothelial cells respond to high concentrations of LPS in the presence of recombinant soluble CD14. However, the response, particularly to low doses of LPS, is drastically enhanced by serum or LBP in a dose-dependent manner (Frey et al., 1992; Haziot et al., 1993b). Nevertheless, the binding site for this LPS- or LPS/LBP-soluble CD14 complex on endothelial cells remains to be identified.

While CD14 may serve as an important membrane-bound receptor for the LPS-LBP complex, several other membrane molecules on monocytes/macrophages also have the ability to bind to LPS, including CD11a-b/CD18 complex, an 80 kDa protein and a lectin-like protein (Cavaillon and Haeffner-Cavaillon, 1990). These structures may in part explain the responsiveness of monocytes to high doses of LPS in the absence of LBP or serum. Interestingly, interaction of the LPS/LBP complex with the membrane-bound CD14 enhances CD11b/CD18 activity on neutrophils, implying an amplifying feedback loop for LPS reaction (Wright et al., 1991). Recently, it has been shown that there is an increased expression of CD14 and CD11/CD18 during differentiation of peripheral blood monocytes to macrophages in vitro, associated with increased production of TNFα upon stimulation by LPS (Gessani et al., 1993).

1.4.3 LPS in the airways

The ubiquity of LPS is attributable to the wide existence of airborne gram-negative bacteria and their break-down products. LPS has been found to be associated with environmental and occupational dusts (Haglind et al., 1981). Thus, inhalation is one of the ways by which LPS potentially impacts upon the respiratory system.

In addition, the airway system, particularly the upper airway, is one of the organs most heavily colonized with bacteria (Johanson, 1988). Although most of these bacteria are gram-positive under normal circumstances, colonization with gram-negative bacteria may occur in most individuals with underlying diseases, compromised systemic or airway defense conditions or imbalance of normal colonization brought about by antibiotic treatment for other airway infections. Not only the quality of bacterial flora but also the distribution of these bacteria can change. For instance, normally there should not be a significant number of bacteria in the distal airways, but in the case of chronic bronchitis, persistent bacterial floras are found at these sites (Johanson, 1988). These factors can all contribute to the development of gram-negative bacterial infections and lung injury subsequent to LPS release from the bacteria.

The respiratory system is also susceptible to systemic gram-negative bacterial sepsis. Lung injury associated with this condition and other types of gram-negative pneumonia may cause adult respiratory distress syndrome (ARDS), a life-threatening condition with 50% or even higher mortality (Brigham and Meyrick, 1986; Hanson-Flaschen and Fishman, 1989).

1.4.4 LPS-mediated cytokine induction in the airways

A histologic hallmark of gram-negative pneumonia is the conspicuous accumulation of neutrophils, very similar to that found in the early acute stage of ARDS, particularly ARDS associated with gram-negative sepsis (Pierce, 1988; Bachofen and Weibel, 1988). The influx of neutrophils is usually accompanied by diffuse perivascular and interstitial edema and fibrin deposition and is considered to play a central role in the tissue injury chiefly via the release of oxidants and proteases. In animal models of ARDS, a single intravenous infusion of LPS brings about morphologic changes in the airway tissue resembling those seen in ARDS, including neutrophil sequestration in the airway microcirculation, interstitial infiltration and activation of neutrophils, and damage to endothelial and type I alveolar epithelial cells (Brigham and Meyrick, 1986). In contrast, the intratracheal administration of LPS appears to cause a much more severe intraalveolar accumulation of neutrophils (Nelson et al., 1989; Remick et al., 1990). However, in both cases, these pathologic alterations appear to be self-limited, reminiscent of the clinical course of gram-negative pneumonia and some cases of ARDS, unless repeated doses of LPS are administered, which usually leads to emphysematous changes in certain animal species (Brigham and Meyrick, 1986; Stolk et al., 1992).

The mechanisms underlying LPS-induced pathologic changes, particularly neutrophil recruitment in the airways, still remain to be fully understood. However, increasing evidence suggests that cytokines including TNFα, IL-1 and IL-8 play an important role.

Ample in vitro evidence indicates that LPS may directly induce cytokine release on a number of cell types in the airways. Alveolar macrophages release great amounts of TNFα, IL-1 and IL-8 upon exposure to LPS (Kelley, 1990; Strieter et al., 1990), but neutrophils have also recently been shown capable of releasing cytokines in response to LPS (Palma et al., 1992; Strieter et al., 1992). Pulmonary endothelial cells also respond to LPS by releasing cytokines (Albelda et al., 1989). It is less clear whether LPS can directly induce airway epithelial cells and fibroblasts to release cytokines (Standiford et al., 1990; Koyama et al., 1991). Fibroblast heterogeneity attributable to species difference and distinct anatomic sites and fibroblast subpopulations (Elias et al., 1992), and different experimental conditions may explain the discrepancies in the data. In some studies, serum-free culture conditions were used. The crucial requirement of serum for the responsiveness to LPS of cells that do not normally express CD14 was not recognized until very recently (Frey et al., 1992; Haziot et al., 1993b). In the context of respiratory systems, however, it has been clearly shown that LPS does not stimulate the pulmonary parenchyma-derived fibroblasts (Rolfe et al., 1991).

Regardless of the above data in vitro, the cellular sources and kinetics of cytokines in LPS-induced acute airway inflammation in vivo are far less clear. Elevated levels of TNFα and IL-8 have been found in BAL fluids of patients with septic ARDS (Tracey et al., 1988; Miller et al., 1992). Studies in animal models of LPS-induced acute lung inflammation demonstrate increased mRNA levels for TNFα, IL-1 and IL-6 in total lung tissues (Ulich et al., 1991b) or MIP-2 in total trachea tissues (Huang et al., 1992). Recent evidence also shows that both alveolar macrophages and infiltrating neutrophils in BAL fluids of LPS-challenged rat lungs are significant sources of IL-1 mRNA expression (Ulich et al., 1992).

1.5 PURPOSE OF THE STUDY

Cytokines have been implicated in the underlying mechanisms of a number of clinically critical respiratory conditions where LPS is involved. Thus, the objectives of this study were to investigate the ability of LPS to induce a certain set of cytokines in airways cells and the regulation of these events. Both in vitro and in vivo approaches were utilized.

Inasmuch as the alveolar macrophage is one of the first cells to encounter LPS and the most potent resident defense cell in the airways, I embarked on examining IL-1ß and IL-6 expression by alveolar macrophages in response to LPS in vitro. To specifically address the issue of ECM regulation on effector activities of alveolar macrophages, the expression of IL-1ß and IL-6 mRNAs was evaluated in cells cultured on several matrix substances, with or without LPS. Due to difficulties in securing sustained access to human alveolar macrophages, rat alveolar macrophages were utilized for this study. The findings in this study resulted in the publication in chapter two.

Having examined the effect of LPS on cytokine expression by alveolar macrophages, I proceeded to investigate the direct effect of LPS on cytokine induction by airways structural cells. This endeavour was at variance with the traditional concept that only alveolar macrophages are involved in the initiation of LPS-induced acute inflammation. The upper airways, in contrast to the lower airways, are normally heavily colonized with bacteria, hence constantly exposed to LPS contaminants. Therefore, it is hypothesized that fibroblast populations at different anatomic sites in the airways may differ in their capability to respond to LPS. To test this hypothesis, I examined the expression of the "second order" cytokines GM-CSF, IL-6 and IL-8 in nasal, bronchial and lung primary fibroblast lines challenged in vitro with either an "exogenous" stimulant, LPS or an "endogenous" cytokine, IL-1. The findings from this investigation are presented in the publication in chapter three.

The tissue response to LPS likely involves communication between cells of different types, especially interactions between inflammatory and structural cells. While different types of signals may be involved in these processes, I chose to examine the interactions between inflammatory cells and epithelial cells and fibroblasts in vitro that are mediated by cytokines. Since the human peripheral blood monocyte represents an easily accessible supply of monocyte/macrophage cell types, attention was focused on the effect of airway epithelial cell- or fibroblast-derived cytokines, particularly the CSFs, on survival, proliferation, and differentiation of peripheral blood monocytes. The results from this study are detailed in the publication in chapter four.

The examination of the effect of LPS on cytokine induction in both inflammatory and structural cells in vitro prompted the investigation on effects of LPS in vivo in order to provide further understanding of the biological activities of LPS in the respiratory system. A rat model of LPS-induced acute lung inflammation was established, and inflammatory and cytokine responses characterized. This investigation of cytokine induction by LPS in the lung in vivo was expected to provide information on (a) the nature and kinetics of inflammatory infiltrates; (b) the profile and kinetics of cytokines expression in the whole lung; (c) the specificity of cytokine response; (d) the primary cellular sources of cytokines and, finally (e) the temporal correlation of cellular influx with the expression of a given cytokine. The results of this study are presented in two separate publications in chapters five and six.

Chapter two

LPS INDUCTION OF CYTOKINES IN AIRWAYS INFLAMMATORY CELLS IN VITRO

The following article entitled "IL-1ß and IL-6 gene expression in alveolar macrophages: modulation by extracellular matrices" is published in The American Journal of Physiology. 6:L600-605, 1992. It deals with the effect of LPS on cytokine expression in the primary airway defense cells, alveolar macrophages, and its regulation by extracellular matrix in vitro.

The work presented in this study was performed by the author of the thesis with the help from lab technicians with cytokine bioassays. This paper was written by the author of the thesis. The supervision from Drs. Gauldie and Jordana resulted in the multiple authorship in this paper.

IL-1β and IL-6 gene expression in alveolar macrophages: modulation by extracellular matrices

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Xing, Zhou, Manel Jordana, and Jack Gauldie. IL-1β and IL-6 gene expression in alveolar macrophages: modulation by extracellular matrices. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L600–L605, 1992.—Interleukin-1 (IL-1) and interleukin-6 (IL-6) are two cytokines involved in a variety of host inflammatory reactions. The alveolar macrophage (AM), a predominant cell source for IL-1 and IL-6, exists in a microenvironment in which there are abundant extracellular matrix (ECM) components, and it is likely that ECM may participate in the inflammatory response in the lung by modulating the effector activities of AMs. To investigate this hypothesis, we cultured rat AMs on different substrates including plastic, collagen, and airways fibroblast-derived ECM (fECM) and assessed IL-1β and IL-6 gene expression in these cells. Our study demonstrates that cytokine gene expression in AMs is affected by the conditions of culture. IL-1 gene expression is stimulated by adherence to plastic and exposure to endotoxin, whereas IL-6 mRNA is detectable only in the cells stimulated by endotoxin. Coating the plastic with collagen or fECM modifies cytokine gene expression. At early time points, collagen enhances gene expression. At later times (5 days), actin and cytokine gene expression are predominantly maintained in the endotoxin-stimulated cells cultured on fECM. These findings suggest an extracellular environment-directed mechanism of regulation of cytokine expression in alveolar macrophages.

fibroblast-derived extracellular matrix; collagen; lipopolysaccharide

THE ALVEOLAR MACROPHAGE (AM), a major defense cell in the lung, participates in immune and inflammatory reactions through the release of a number of regulatory peptide hormones or cytokines. Among important cytokines released by these cells are interleukin-1 (IL-1) and interleukin-6 (IL-6). IL-1 is involved in a wide range of physiological and pathological processes, including inflammation, fibrosis, and coagulation (21), whereas IL-6 plays a crucial role in the regulation of inflammation through the induction of acute-phase protein synthesis by the liver and the augmentation of immunoglobulin synthesis (11, 12). IL-1α and IL-1β are two well-characterized nonhomologous cytokines, sharing the same bioactivity and receptors (21, 24). The two species are often coregulated in macrophages (10). However, between the two, IL-1β accounts for >90% of the mRNA and released active protein (21). A number of factors are able to trigger the expression of IL-1 and IL-6. We have previously shown that in vivo administration of bleomycin or an infective agent can increase the spontaneous production of IL-1 and IL-6 by AM in vitro (20, 22). Endotoxin is a factor capable of enhancing IL-1 and IL-6 expression by monocytes or macrophages (18, 27). In addition, adherence itself is able to trigger freshly isolated macrophages to express IL-1 and IL-6 (18, 27).

AMs are normally in contact with extracellular matrix (ECM) components comprised of collagen, elastin, proteoglycans, fibronectin, and laminin (6). Disruption, remodeling, and/or accumulation of ECM occur during lung inflammatory reactions (4, 31). The role of ECM in inflammation remains largely unknown, although it has long been recognized that ECM is important in regulating cell differentiation and survival as well as response to growth factors (13, 16, 23, 29). More recently, it has been demonstrated that individual components of the ECM are able to regulate the gene expression of some cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF) (30), macrophage colony-stimulating factor (M-CSF), and tumor necrosis factor (TNF) (8) and the secretion of IL-1 (7, 19), suggesting that a conditioned environment can affect the release of inflammatory mediators. However, there is no documentation on effects of the complete matrix produced by fibroblasts on cytokine gene expression, particularly IL-1 and IL-6 expression by AM, and it is unclear whether the effects of ECM on cells will remain unchanged through a period of time. In this report, we examine the expression of IL-1β and IL-6 mRNA in AM cultured on a common substrate, plastic, and the modulation of expression of these cytokines by fibroblast-derived ECM and pure collagen.

METHODS

Isolation and culture of alveolar macrophage. Lung lavage was performed on male or female Sprague-Dawley rats weighing 150–250 g (Biobreeding, Ottawa, Canada), and AMs were isolated from resultant suspensions as described previously (20). The cells were cultured in RPMI 1640 medium (Roswell Park Memorial Institute) with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 1% penicillin/streptomycin (GIBCO), 1% bicarbonate, 1% *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 1% L-glutamine. Cells (4–6 × 10^6/dish) were incubated in 100-mm Falcon plates at 37°C in an incubator containing 5% CO_2 in air. Cell viability was >97%, and over 95% of attached cells were macrophages as determined by trypan blue exclusion and Giemsa staining. The cells were cultured on plastic, collagen, or fibroblast-derived ECM (fECM) for the periods of time specified, in the absence or presence of lipopolysaccharide (LPS) (from *Escherichia coli* 055:B5, Sigma Chemical, St. Louis, MO).

Fibroblast extracellular matrix and collagen membrane. Human fibroblast primary cell lines were established from nasal tissue obtained by biopsy as previously described (32). Cells (5×10^5/dish) were seeded into 100-mm Falcon plates and cultured with RPMI 1640 containing 10% FBS until reaching confluency. ECM was prepared as described by Fridman et al. (9). Briefly, the cell monolayer was permeabilitized by exposure to 0.5% Triton X-100 for 5 min and solubilized with 0.025 N ammonium hydroxide for 2 min. Firmly attached ECM was extensively washed five times with phosphate-buffered saline (PBS). Collagen membrane was prepared with Vitrogen 100 collagen (type I collagen; Collagen, Palo Alto, CA) as previously described (28). Briefly, collagen-covered plastic surface was treated with 3.5% NH_4OH for 25 min and 2.5% glutaraldehyde for 10 min, respectively, and washed with distilled water three times.

IL-1 and IL-6 bioassay. IL-1 activity was measured by a mitogen costimulator assay (20). Briefly, thymocytes were isolated from the thymus of 4-to 6-wk-old C3H/HeJ female mice and cultured in 96-well plates with diluted AM supernatants in the presence of phytohemagglutinin (Difco Laboratories, St. Louis, MO). Cells were incubated for 72 h. In the last 24 h, 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was pulsed to each well. IL-1 activity was calibrated by thymocyte growth detected by thymidine incorporation. In our hands, using 10% FBS, recombinant rat IL-6 cannot be detected in this assay. At lower concentrations of FBS, IL-6 can be stimulatory. IL-6 activity was detected by B9 hybridoma growth factor assay (1). Briefly, 2,500 B9 cells/well were cultured in 96-well plates with diluted AM supernatants and Icove's modified Dulbecco's medium for 72 h. [^{3}H]thymidine (0.2 μCi) was added to each well at 48 h. The assay was calibrated with purified recombinant rat IL-6 (11), and its sensitivity was 10 pg of IL-6.

RNA extraction and Northern blot analysis. Total cellular RNA was extracted from attached cells by a single-step method with some modifications (5). Cells were dislodged, transferred to Eppendorf tubes, and lysed in 0.5 ml of the guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). RNAs were extracted with 2 M sodium acetate, pH 4, water-saturated phenol and chloroform, and precipitated in isopropanol alcohol. For Northern blot analysis, RNA (10 μg/lane) was denatured and size electrophoresed through a 1% agarose formaldehyde gel overnight. The RNA was transferred onto the nitrocellulose membrane and baked in vacuo at 80°C for 2 h. Specific mRNAs were detected by hybridizing blots with cDNA probes [rat IL-1β, linearized *Hind* III fragment of Ped 1218 (kindly provided by Dr. Tsutomu Nishida, Cellular Technology Institute, Kawauchi-cho, Tokushima, Japan); rat IL-6, *Pst* I fragment of PTZ 19R (11); and chicken β-actin (Oncor, Gaithersburg, MD)]. Blots were prehybridized at 42°C for 4 h in a hybridization buffer (40% formamide, 2× saline sodium citrate (SSC), 1 mM EDTA, 5× Denhardts, and 100 μg/ml of salmon sperm DNA) and hybridized at 42°C for 12–16 h in the same buffer containing cDNA probes labeled with [α-^{32}P]dCTP using a random priming method (Pharmacia LKB, Uppsala, Sweden). Blots were washed with 2×SSC at 42–55°C and autoradiographed for 1–2 days. After autoradiography, the blot was stripped of the signal in 50% formamide and 0.5% SSC at 70°C for 30 min and reautoradiographed to ensure complete stripping before reprobing. The size of RNA transcripts was determined according to the position of the signal relative to RNA standards. In most cases, amounts of total RNA in each lane of the gel were assessed by monitoring 28S and 18S RNA by ethidium bromide staining and/or β-actin mRNA hybridization using a chicken cDNA probe.

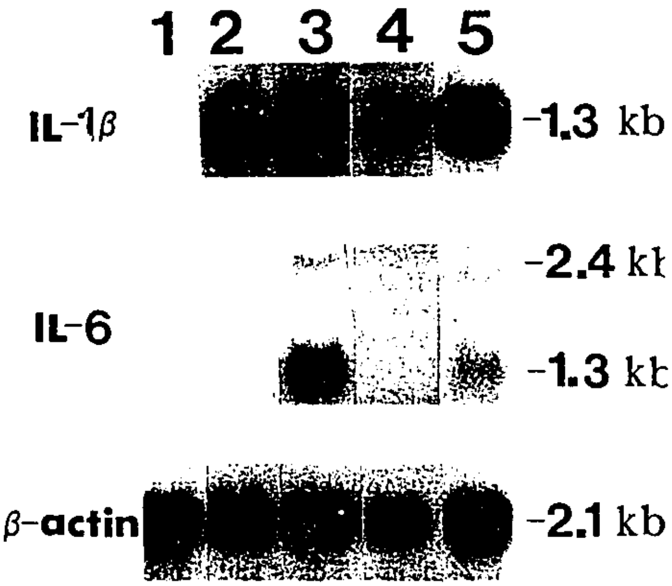

Fig. 1. Expression of interleukin-1β (IL-1β) and interleukin 6 (IL-6) mRNA in alveolar macrophage under nonadherent and adherent conditions. *Lanes*: *1*, starting population; *2*, 10-h adherent cells; *3*, 10-h adherent cells stimulated by 5 μg/ml lipopolysaccharide (LPS); *4*, 10-h nonadherent cells; *5*, 10-h nonadherent cells stimulated by 5 μg/ml LPS. β-actin probe was used for standardization.

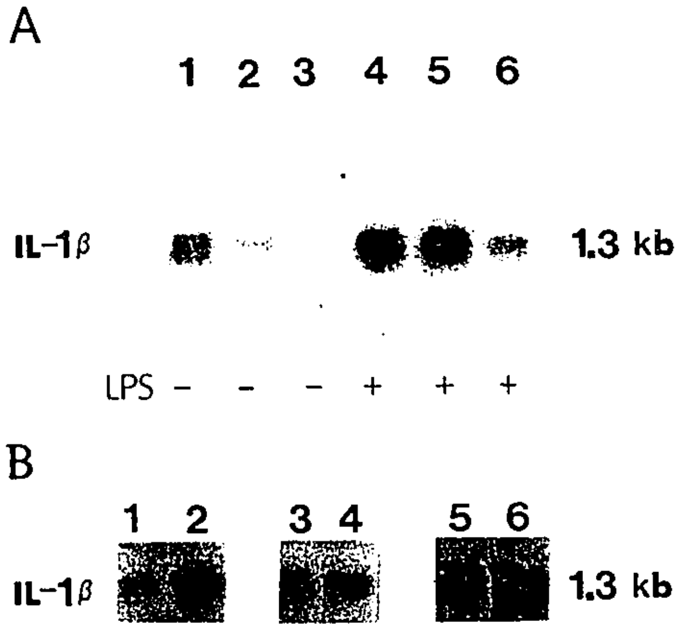

Fig. 2. *A*: dynamic changes of interleukin 1β (IL-1β) expression in alveolar macrophage (AM) on plastic. Cells were stimulated with 5 μg/ml lipopolysaccharide (LPS) for 2 h in *lane 4* and for last 3 h in *lanes 5* and *6*. *Lanes*: *1* and *4*, 2 h; *2* and *5*, 24 h; *3* and *6*, 5 days. *B*: expression of IL-1β mRNA in AM on different substrates at 2 h. *Lanes*: *1* and *2*, plastic; *3* and *4*, fibroblast-derived extracellular matrix; *5* and *6*, collagen. *Lanes 1, 3,* and *5*: without LPS; *lanes 2, 4, and 6*: with 5 μg/ml LPS. Equal amounts of loaded RNA were verified by ethidium bromide-stained 28S and 18S RNA in gel.

Oligo(dT) hybridization was performed to standardize the hybridizable RNA on some blots with oligo(dT) probe labeled with [τ-^{32}P]ATP using an end-labeling method (17). Autoradiograph quantitation was performed by laser densitometry.

RESULTS

Expression of IL-1β and IL-6 gene by AM cultured on plastic. There was no detectable IL-1β or IL-6 mRNA in the freshly isolated AM starting preparations (*lane 1*, Fig. 1). Adherence of AM to plastic for a period of 10 h induced only IL-1β expression, but both IL-1β and IL-6 could be induced by LPS. IL-1β, however, was not totally adherence dependent because cells, prevented from attachment by culturing in polyallomer tubes on a rocker, expressed detectable IL-1β at 10 h after culture. In addition, in the presence of LPS, these cells displayed an increased IL-1β and a weak but detectable IL-6 message, which was composed of 2.4- and 1.3-kb species in a ratio of approximately 1:5 (27), suggesting that striking IL-6 expression by AM may be dependent on specific stimuli like endotoxin. It was noted that actin expression did not seem to be altered under all these conditions. To demonstrate the changes in cytokine expression by AM in vitro, AMs were cultured on plastic in the presence and absence of LPS for 2 h, 24 h, and 5 days and probed for IL-1β (Fig. 2*A*). In the absence of LPS, IL-1β expression diminished with time so that no detectable signal was found in *day 5* preparations. Under these adherent conditions, the response of AM to LPS was also decreased with the passage of time. A similar in vitro IL-1 expression has been shown in human AM (3) and monocytes (2).

Modulation of cytokine and actin gene expression by ECM. Modulation of cytokine gene expression was examined in AM seeded onto fECM and collagen I-coated plastic as well as plastic alone. A weak IL-1β signal was found in AMs cultured on plastic at 2 h as above, and these cells demonstrated an augmented signal upon LPS challenge at this time (Fig. 2*B*). Conversely, fECM by itself was able to slightly increase IL-1β transcripts in the absence of LPS, and further LPS stimulation did not further markedly enhance IL-1β expression. Collagen elicited the strongest IL-1β signal even in the absence of LPS. The presence of LPS led to a further but small increase of IL-1β in these cells. In a separate series of experiments, quantitative measurements of IL-1β and IL-6 gene expression were made on the preparations of AM cultured for 2 h in the presence of LPS (Fig. 3, *A* and *B*). The IL-1β and IL-6 transcripts were present at greater than twofold levels for AM on collagen compared with plastic. However, the cells cultured on fECM, in the presence of LPS, did not express higher amounts of mRNA for IL-1β and IL-6 than those cultured on plastic. Actin mRNA expression remained unchanged under these different culture conditions at 2 h. At *day 5*, however, estimates of the amounts of electrophoresed mRNAs were detected by ethidium bromide staining and were assessed by oligo(dT) probing (17), since actin expression was found to be altered (Fig. 4, *A* and *B*). By this time, while the intrinsic expression of IL-1β was markedly decreased, so that the signal could be hardly detected in the cells cultured on either substrate in the absence of LPS, the responsiveness of cells to LPS with regard to cytokine gene expression was markedly altered. In contrast to 2-h cultures, 5-day LPS-stimulated AMs cultured on fECM (*lane 4*, Fig. 4*A*) expressed the highest levels of IL-1β and IL-6 in comparison to those on plastic or collagen. Noticeably, after LPS stimulation, the cells on collagen no longer demonstrated strikingly higher levels of IL-1β expression over the cells on plastic and even expressed much lower level of IL-6 message than those on plastic, in sharp contrast to the gene expression profile of

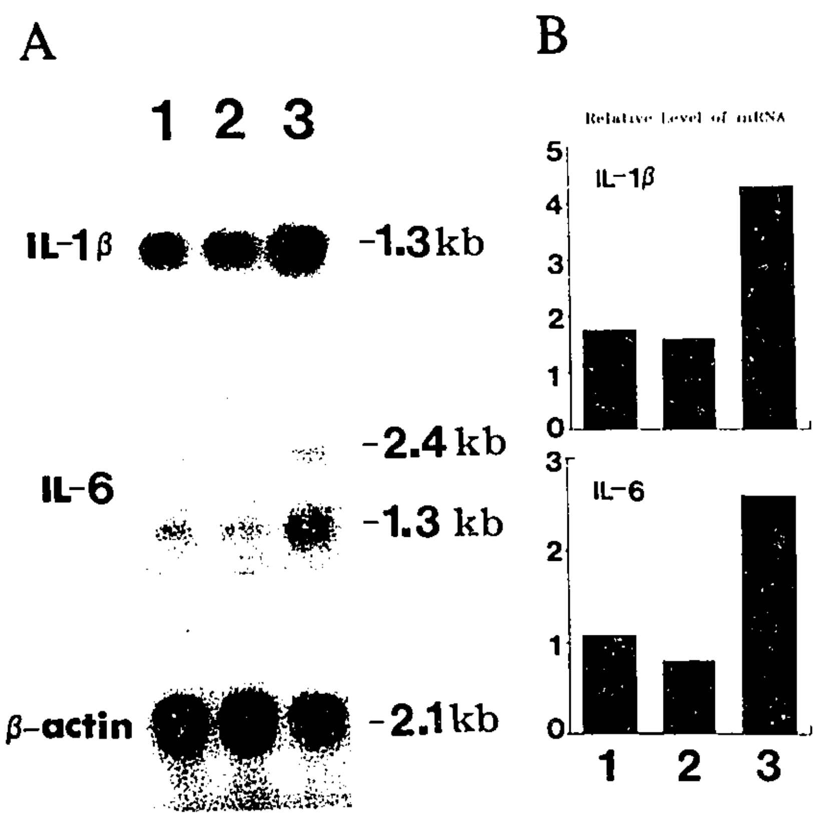

Fig. 3. *A*: expression of interleukin-1β (IL-1β) and interleukin-6 (IL-6) mRNA in alveolar macrophage on different substrates in presence of 5 μg/ml lipopolysaccharide at 2 h. *Lanes*: *1*, plastic; *2*, fibroblast-derived extracellular matrix; *3*, collagen. *B*: densitometric measurements of specific mRNAs. Quantitation for IL-6 was carried out on 1.3-kb message. Input of mRNAs was normalized by actin density.

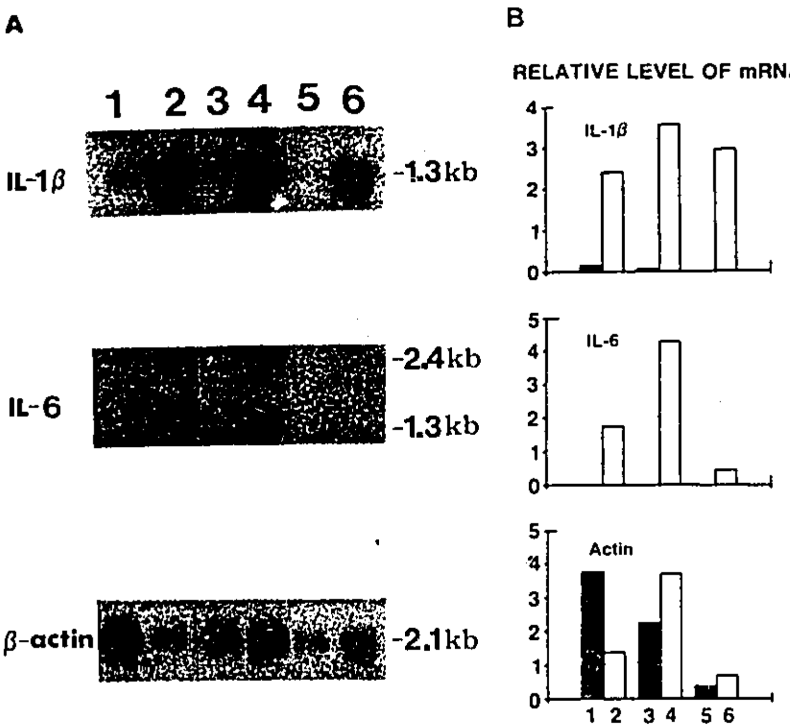

Fig. 4. *A*: expression of interleukin-1β (IL-1β), interleukin-6 (IL-6), and β-actin mRNA in alveoloar macrophage on different substrates at *day 5*. Lipopolysaccharide (LPS) was added 3 h before termination of experiments. *Lanes*: *1*, plastic; *2*, plastic + LPS (5 μg/ml); *3*, fibroblast-derived extracellular matrix (fECM); *4*, fECM + LPS; *5*, collagen; *6*, collagen + LPS. *B*: densitometric measurements of specific mRNAs. Quantitation for IL-6 was made on 1.3-kb message. Input of mRNAs was normalized by oligo(dT) density.

these cells at the 2-h time point. Compared with AM on fECM, the LPS stimulated-cells cultured on collagen showed induction of ~10-fold less IL-6 mRNA (Fig.4*B*). However, IL-1β expression was not similarly affected by the different culture conditions (Fig.4*B*). In addition to the relatively high level of cytokine expression in AM on fECM, the expression of an intracellular matrix protein, actin, was also well maintained in these cells (Fig. 4*A*), and on the contrary, a marked decrease in actin mRNA transcripts was noticed in AM cultured on collagen in the absence of LPS (*lane 5*) or on plastic and collagen in the presence of LPS (*lanes 2* and *6*).

Modulation of IL-1 and IL-6 production by ECM. AMs were incubated in the absence and presence of LPS for 24 h, and supernatants were collected and assayed for IL-1 and IL-6 activity. On the average, AM on collagen produced about twice as much the IL-1 activity in the absence or presence of LPS as that by those on plastic or fECM (Table 1), and IL-6 production by the cells on collagen was about twice and three times the levels by the cells on plastic and fECM, respectively. This was consistent with the early induction of these cytokine genes by collagen.

Table 1. *IL-1 and IL-6 activity from rat AM cultures*

Substrates	IL-1, U/ml		IL-6, ng/ml	
	−LPS	+LPS	−LPS	+LPS
Plastic	54-100	90-172	9.0-17.1	17.6-24.0
fECM	48-110	67-142	2.7- 8.3	10.0-23.3
Collagen	103-159	212-240	20.0-33.3	36.0-54.0

Results represent ranges from two separate experiments. Bioactivity was assayed in the supernatants of 24-h cultures. IL-1, interleukin-1; IL-6, interleukin-6; fECM, fibroblast-derived extracellular matrix; LPS, lipopolysaccharide.

DISCUSSION

Few reports have examined both IL-1 and IL-6 gene expression by macrophages. Our study shows that although both IL-1β and IL-6 mRNA transcripts were undetectable in freshly isolated AM populations, the expression of IL-1β could be easily induced in vitro by a number of conditions or stimuli, including nonadherent cell culture, adhesion, and/or LPS stimulation. Similar induction of IL-1 gene expression was found in nonadherent human monocytes (18) and AM in vitro (3). In contrast, significant induction of IL-6 gene expression was achieved only when endotoxin was applied to adherent AMs, whereas in nonadherent AMs, endotoxin only elicited a weak IL-6 signal (Fig. 1). This phenomenon may imply a difference between in vivo IL-1β and IL-6 gene expression as to the requirement or ease of stimulation.

It appears that the expression of cytokine genes can be modulated not only by the action of adherence to plastic and the presence of soluble factors such as endotoxin, but also by insoluble ECM through direct stimulatory effects and/or modulation of responsiveness of the cell to endotoxin. We found that within a short period of time in culture, in general, AMs on plastic responded better to LPS than those on fECM, showing enhanced cytokine expression, whereas AMs on collagen expressed the highest levels of the cytokines in the absence or presence of LPS, most likely due to the stimulatory ability of collagen itself (Fig. 2*B*). The reason why the cells on fECM responded, to a lesser degree, to LPS at 2 h, remains unclear (Fig. 2*B*), but it may reflect interference by fECM with LPS receptors on AM during the early adherence stage in this approach. Nevertheless, it appears that the overall intrinsic responsiveness of the cell on different substrates to the exogenous challenge by endotoxin was not significantly altered within 2 h of culture time. The early induction of IL-1β and IL-6 gene expression in AM by collagen is in agreement with the finding of the early enhancement of TNFα gene expression in monocytes by collagen (18). More recently, collagen matrix was also found to be able to enhance phagocytosis of bacteria by monocytes (26). However, little is known about whether the early inducibility of cytokine expression in cells by collagen can remain consistent days after culture. Here, we document that, after 5 days of culture, in the absence of LPS, the cells cultured on fECM or collagen no longer expressed significant IL-1β mRNA in contrast to those at 2 h of culture. In addition, the pattern of the responsiveness to LPS of AMs, in terms of IL-1β and IL-6 gene expression, was considerably changed, and this responsiveness appeared to be exogenously determined by the extracellular substrate. This was demonstrated by the sustained high expression of both IL-1β and IL-6 as well as actin mRNAs in AMs cultured on fECM and the low profile cytokine expression in AMs cultured on plastic and collagen, in response to LPS (Fig. 4). From our results, it is tempting to assume that the makeup and surface morphology of substrates may become important in maintaining the responsiveness to stimuli such as LPS of the cells that are cultured in vitro over a period time with increased expression of cytokines. Since the matrix from the fibroblast is more constitutionally natural it may have a greater capability to maintain AM in a more responsive state to LPS, resulting in a relatively higher induction of IL-1β and IL-6, compared with plastic, which apparently is foreign to AM, and collagen, which merely represents a single matrix component. We have found some evidence to support speculation that the collagen membrane we used in the experiment had different surface morphology from fECM. Alveolar macrophages cultured on this matrix spread minimally, as revealed by scanning electron micrographs (data not shown). On the contrary, AMs on fECM spread considerably and had highly developed microvilli, perhaps in part contributing to the maintenance of the greater potential of cells to express cytokines in response to LPS. The same rationale may also explain the maintenance of actin mRNA in the cells cultured on fECM and the decrease of actin mRNA in AM cultured on collagen. The change of actin mRNA expression may also reflect a difference in demands of reorganization of cytoskeleton and cell mobility. Similar actin alteration has been found in other cells under different circumstances (14, 15) and may be potentially used as a marker of decreased cellular function. Decreased expression of β-actin has been found accompanied with diminished production of collagen I and III by fibroblasts cultured in collagen gel (25). These findings suggest that insoluble extracellular substances may play a role in altering the effector activities of AMs and modulate their responsiveness to exogenous stimuli during the course of inflammation.

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Chapter three

LPS INDUCTION OF CYTOKINES IN AIRWAYS STRUCTURAL CELLS IN VITRO

The following article entitled "Lipopolysaccharide induces expression of granulocyte/macrophage colony-stimulating factor, interleukin-8, and interleukin-6 in human nasal, but not lung, fibroblasts: evidence for heterogeneity within the respiratory tract", has been published in The American Journal of Respiratory Cell and Molecular Biology. 9:255-263, 1993. This paper represents a follow-up study following that presented in the preceding chapter, investigating whether LPS could directly act on airway structural fibroblasts to induce cytokine production in vitro.

This paper was written and all work in this paper was carried out by the author of the thesis. The help, advice, or contribution to the generation of the IL-6 promoter-luciferase DNA construct from Braciak T. and Ohtoshi T., and the supervision from Drs. Gauldie and Jordana, resulted in the multiple authorship of this paper.

Lipopolysaccharide Induces Expression of Granulocyte/Macrophage Colony-stimulating Factor, Interleukin-8, and Interleukin-6 in Human Nasal, but Not Lung, Fibroblasts: Evidence for Heterogeneity within the Respiratory Tract

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Fibroblasts play an indirect augmenting effector role in the inflammatory response by releasing growth and differentiation factors and other inflammatory mediators after activation by inflammatory cytokines such as interleukin (IL)-1, but whether direct activation occurs by exogenous agents such as endotoxin (lipopolysaccharide, LPS) remains controversial. Using a number of primary human airways tissue-derived fibroblast lines, we demonstrate that in contrast to IL-1α, LPS significantly induced gene expression and production of granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-8, and IL-6 only in nasal but not bronchial or lung tissue-derived fibroblasts. Enhanced expression was dose- and time-dependent, and the minimal stimulatory dose was 10 ng LPS/ml. Polymyxin B entirely abrogated increased cytokine expression by LPS. Actinomycin D treatment largely inhibited expression, and LPS markedly increased an IL-6 gene promoter-driven luciferase reporter response in transfected nasal fibroblasts, suggesting enhanced expression may involve transcriptional regulation. Secondary protein or IL-1 synthesis requirement seemed unlikely since cycloheximide superinduced LPS-stimulated cytokine expression and anti-IL-1α/β antibodies failed to abrogate the response. Thus our data show that GM-CSF, IL-8, and IL-6 are directly inducible in nasal fibroblasts by LPS, and establish heterogeneous responsiveness to LPS by different fibroblast populations in the airways.

Granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, and IL-6 are cytokines with a broad spectrum of activities involved in acute and chronic inflammatory responses (1–10). Activated mononuclear cells, particularly monocytes, have generally been considered the primary source of these cytokines. However, it has recently been shown that fibroblasts and other tissue structural cells can also produce these cytokines, especially in response to challenge by IL-1 and tumor necrosis factor-α (TNF-α) (11–13), suggesting a role for these cells as a secondary source of mediators in the amplification of the inflammatory response (14–16). In addition, there is evidence that fibroblasts may be the direct target of environmental agents. For example, certain viruses, such as measles or rubella, and some bacteria have been shown to stimulate IL-6 expression in infected skin fibroblasts (17, 18). Whether bacterial products, such as lipopolysaccharide (LPS), can directly elicit cytokine responses in fibroblasts remains controversial (13, 19–25). Whereas there is evidence of heterogeneity in metabolic and proliferative features among fibroblasts from different tissue sites (26), or from the same anatomic site of tissues undergoing chronic inflammatory responses (27), relatively little is known about fibroblast heterogeneity with regard to cytokine expression. Since the respiratory tract is a site of direct interaction with infective organisms or their products, we wished to compare cytokine expression by primary fibroblast lines derived from upper (nasal) and lower (lung) airway sites after exposure to either IL-1 or LPS.

Our results demonstrate that synthesis of GM-CSF, IL-8, and IL-6 was upregulated by exposure to LPS in fibroblasts derived from nasal tissue but not in fibroblasts derived from lung and bronchial tissues. This is in contrast to IL-1, which upregulated cytokine expression in all cells. This observation indicates that fibroblasts from tissues in closer proximity to the external environment might be involved not only in the amplification but also in the initiation of the inflammatory response.

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Abbreviations: enzyme-linked immunosorbent assay, ELISA; fetal calf serum, FCS; granulocyte/macrophage colony-stimulating factor, GM-CSF; interleukin, IL; lipopolysaccharide, LPS; phosphate-buffered saline, PBS; sodium dodecyl sulfate, SDS; tumor necrosis factor-α, TNF-α.

Am. J. Respir. Cell Mol. Biol. Vol. 9. pp. 255–263, 1993

Materials and Methods

Reagents and Probes

LPS, actinomycin D, polymyxin B, and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). LPS preparations were dissolved and subjected to sonication in phosphate-buffered saline (PBS) (4 mg/ml). Budesonide (Astra Draco AB, Lund, Sweden) was made into a stock solution in 30% ethanol in PBS. Human recombinant IL-1α expressed in *Escherichia coli* was a kind gift from Dr. J. Saklatvala (Strangeways Research Labs, Cambridge, UK). The human IL-8 oligonucleotide probe has the sequence 5'-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3' and is specific for human IL-8 mRNA (24). The human GM-CSF cDNA probe, provided by Genetics Institute (Cambridge, MA), was an EcoRI to AhaIII fragment (800 bp) derived from pSP 64 (28). The human IL-6 cDNA probe was a 1.031 kb EcoRI fragment excised from Bluescript. The human IL-1β cDNA probe was a Bam HI fragment (600 bp) from pCD 1218 obtained from DNAX Research Institute (Palo Alto, CA). The human β-actin oligonucleotide probe was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Neutralizing murine anti-human IL-1α and IL-1β monoclonal antibodies were obtained from G. D. Searle (Skokie, IL). Neutralizing goat anti-human IL-6 serum was provided by Dr. S. Poole (The National Institute for Biological Standards and Control, South Mimms, Hertfordshire, UK).

Cell Source and Culture

Primary fibroblast lines were established from human biopsy tissue and characterized as previously described (2, 3, 27) and were used only between the third and eleventh passage. Nasal polyp tissues were removed at polypectomy, and normal nasal tissues were obtained from nonallergic patients at the time of surgery for mechanical obstruction or cosmetic correction. Normal lung fibroblast lines were derived from normal areas of surgical lung specimens from patients undergoing resective surgery for cancer or hiatus hernia repair. Fibrotic lung fibroblast lines were established from histologically proven fibrotic lung tissue of patients with active interstitial lung disease. Bronchial fibroblast lines were derived from histologically normal areas of surgical bronchus specimens from patients undergoing resection for lung cancer. These studies conformed to guidelines for human experimentation and were approved by the Chedoke McMaster Hospital's ethics committee.

Before use, fibroblasts (1.5 to 2 × 10^6) were plated in 100-mm culture dishes (Corning, Corning, NY) and allowed to grow to confluence in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (GIBCO, Grand Island, NY). Since it has been shown that the optimal response elicited by LPS in leukocytes (29, 30) or endothelial cells (31) requires the presence of serum, fibroblasts were subsequently stimulated with LPS or IL-1α in media containing 10% FCS. At specified time points, supernatants were collected and centrifuged for cytokine protein assays, and cell monolayers were trypsinized and pelleted for RNA extraction.

RNA Preparation and Hybridization Analysis

Total cellular RNA was extracted from fibroblasts using a slightly modified single-step method (32). Briefly, after centrifugation, cell pellets were lysed in 0.5 ml of guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). RNA was extracted with 2 M sodium acetate, pH 4, water-saturated phenol and chloroform, and precipitated in isopropanol alcohol overnight. For Northern blotting, total RNA (25 μg/lane) was separated by a 1% agarose formaldehyde gel, transferred onto a BIOTRANS nylon membrane (Pall Biosupport Corp., East Hills, NY), and fixed by exposure to ultraviolet light for 2 min. Blots were prehybridized for 15 min at 55°C in 50 mM Pipes, pH 6.8, 100 mM NaCl, 50 mM NaH_2PO_4, 1 mM EDTA, and 5% sodium dodecyl sulfate (SDS). Hybridization was performed at 55°C overnight in the same solution with γ-[^{32}P]ATP-5'-end labeled human oligonucleotide IL-8 or actin probe, or human GM-CSF or human IL-6 cDNA probe labeled with α-[^{32}P]CTP by a random primer method (Pharmacia LKB, Uppsala, Sweden). The blots were stringently washed and exposed to Kodak XAR film at −70°C for 1 to 2 days. The same blots were then stripped of the signal for reprobing by washing in 0.1% SDS at 70°C for 2 to 3 h and reautoradiographed to assure complete stripping. RNA loading in each lane of the gel was assessed by monitoring the density of actin message and/or ethidium bromide–stained 28S and 18S rRNA in the gel.

Fibroblast Transfection and Luciferase Assay

Preparation of DNA constructs containing rat IL-6 gene 5' flanking promoter regions linked to a luciferase reporter gene has been previously described (33). One of these constructs, designated P7, was used to transfect nasal or lung fibroblasts. The *cis*-acting elements present in this fragment (see Figure 6B) are highly homologous to those found in the human IL-6 gene (33, 34). Fibroblast transfection and luciferase assays were performed by methods previously described (33). Briefly, 2 × 10^6 fibroblasts were transfected with a DNA calcium phosphate precipitate (20 μg of the P7 DNA construct). The cells were incubated for 7 h, washed with 10 mM Hepes buffer (pH 7.3), and cultured in media containing 10% FCS for 36 to 46 h. The cells were then stimulated with 10 μg LPS/ml or 5 ng IL-1α/ml for 24 h in media containing 2% FCS. Cell contents were then extracted and assayed for luciferase activity using a luminometer (Lumat LB 9501; Berthold, Wildbad, Germany). Amounts of the samples added for the measurement of luciferase activity were normalized to protein concentrations in the cellular extracts determined with a Bio-Rad protein assay kit.

Cytokine Assays

Human IL-8 and GM-CSF contents in fibroblast supernatants were measured by enzyme-linked immunosorbent assays (ELISA) (Genzyme Corp., Boston, MA). The sensitivity of these assays are 4 and 4.7 pg/ml for GM-CSF and IL-8, respectively. Human IL-6 activity was assessed by a modified B9 hybridoma growth factor assay (35, 36). Briefly, 2,500 B9 cells/well were cultured in 96-well plates (Inter-Med NUNC, Roskilde, Denmark) in MEM F11 medium containing 5% FCS, 1% penicillin/streptomycin, and 5 × 10^{-5} M mercaptoethanol with diluted fibroblast supernatants for 72 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma) dissolved in PBS at 5 mg/ml was then added to each well (10 μl/well), and plates were incubated at 37°C for 4 h. Triton (10% in 0.5 M HCl) was added to lyse the cells. The plates were read on an ELISA reader. The assay was calibrated with purified recombinant rat IL-6 (9) and human IL-6 standards, and the sensitivity of this assay was 0.5 pg/ml of IL-6.

Metabolic Labeling and Immunoprecipitation of IL-6 Protein

Nasal and lung fibroblasts (0.2 × 10^6/well) were washed 3 times with PBS and cultured in methionine-free medium supplemented with 1% dialyzed FCS and [^{35}S]methionine (100 μCi/ml) (New England Nuclear, Boston, MA) in the absence or presence of LPS or IL-1α for 24 h. The supernatants were collected and incubated with protein A-Sepharose (Pharmacia LKB) and goat antibody against human IL-6 at 4°C overnight on a rotor. The beads were then washed, boiled for 5 min, and separated on a 12% polyacrylamide gel. After staining, the gel was treated with Enlightening (NEN Research Product, Boston, MA) for 30 min, dried, and exposed to Kodak XAR film for 24 h.

Statistical Analysis

Data, whenever applicable, are expressed as means ± SD. The significance of differences in results was analyzed by an unpaired *t* test. The differences were considered statistically significant when *P* values were < 0.05.

Results

Effect of LPS on Cytokine Gene Expression by Nasal and Lung Fibroblasts

Unstimulated lung and nasal fibroblasts expressed weak or undetectable specific message for GM-CSF, IL-8, and IL-6. We and others have previously shown that IL-1 can stimulate cytokine expression in fibroblast lines. The nasal and lung fibroblast lines used here all responded to IL-1α challenge with expression of GM-CSF, IL-8, and IL-6 mRNAs peaking at approximately 12 h after stimulation, and this induction occurred in a dose-dependent manner with a minimal stimulatory dose of 0.5 ng IL-1α/ml (data not shown). We then examined the effect of LPS on lung and nasal fibroblasts. LPS was used at a concentration of 10 μg/ml for most experiments in order to ensure maximal responses elicited by LPS in all cell lines tested, since a dose of 1 μg LPS/ml generally elicited a maximal response in nasal cells. Neither normal (NL) nor fibrotic (FL) lung fibroblast lines responded, with regard to cytokine gene expression, to LPS exposure in a significant manner (Figure 1). In contrast, the same dose of LPS markedly upregulated the expression of GM-CSF, IL-8, and IL-6 in four nasal fibroblast lines, two derived from normal nasal mucosa (NN) and two from nasal polyps (NP). Different levels of basal expression of cytokine mRNAs seen in unstimulated fibroblast lines reflect the effect of FCS and heterogeneity among fibroblast lines from different patients

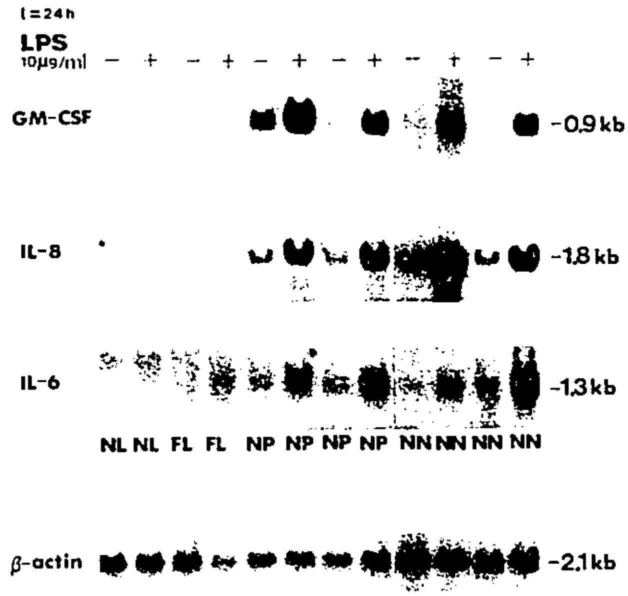
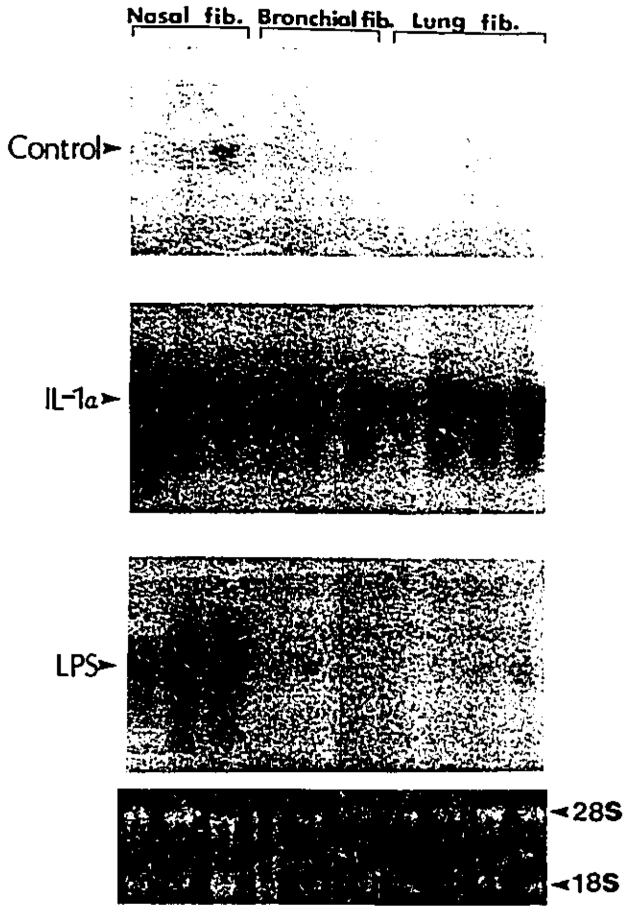


Figure 1. Expression of GM-CSF, IL-8, and IL-6 mRNA in a normal lung fibroblast line (NL), a fibrotic lung fibroblast line (FL), two different nasal polyp fibroblast lines (NP), and two different normal nasal fibroblast lines (NN), cultured with or without 10 μg/ml LPS (055:B5 *E. coli*) for 24 h. Equivalent amounts of total RNA were loaded and this was verified by ethidium bromide-stained 28S and 18S rRNA in the gel and by β-actin mRNA message (*bottom*).

(27). To further demonstrate the site-specific responsiveness of airways fibroblasts to LPS challenge, additional different primary fibroblast lines (four from nasal and nine from lung) were tested, and similar findings were obtained. Figure 2 shows IL-8 mRNA expression in further fibroblast lines, different from those shown in Figure 1, three from nasal polyp, three from normal bronchial, and four from fibrotic lung tissues (27). Whereas IL-1α strikingly enhanced IL-8 expression in all respiratory fibroblast lines tested regardless of the site of origin, LPS significantly induced IL-8 message only in nasal-derived fibroblasts.

Figure 3A shows the kinetics of the LPS effect on cytokine gene expression in nasal polyp fibroblasts. Enhanced signals for IL-8 and IL-6 were first detected at 30 min upon LPS stimulation and peaked at 24 h. Enhanced expression of GM-CSF was first detected at 3 h and reached a plateau between 24 and 48 h. A similar kinetic study was performed with a lung fibroblast line, and no significantly increased cytokine mRNA expression was found at any time point examined (data not shown). The effect of different doses of LPS is shown in Figure 3B. Enhanced GM-CSF, IL-8, and IL-6 transcripts were seen in cells stimulated by concentrations from 10 ng/ml to 10 μg/ml of LPS, and a dose of 1 μg/ml generally induced a maximal response.

The effect of LPS on nasal fibroblasts was further studied using LPS samples from different strains or species of bacteria including 055:B5 *E. coli*, 011:B5 *E. coli*, *Salmonella typhimurium*, *Salmonella minnesota*, and *Klebsiella pneu-*



moniae. Whereas all LPS samples tested led to induction of GM-CSF, IL-8, and IL-6 mRNAs, the potency of individual LPS samples differed. LPS from *S. typhimurium* was the most potent inducer of cytokine gene expression (Figure 4).

Regulation of LPS-induced Cytokine Expression in Nasal Fibroblasts

To demonstrate the specificity of LPS action on nasal fibroblasts, LPS was preincubated with a specific LPS inhibitor, polymyxin B, and added to fibroblast cultures. As shown in Figure 5, stimulation of GM-CSF, IL-8, and IL-6 expression by LPS was completely blocked by polymyxin B.

In addition, we investigated the effect of cycloheximide, a protein synthesis inhibitor, in this same system. We found that cycloheximide together with LPS resulted in superinduction of GM-CSF, IL-8, and IL-6 in comparison with levels induced by LPS alone (Figure 5).

To determine negative or inhibitory regulatory factors in LPS-induced cytokine expression, nasal fibroblast cultures were pretreated with two synthetic corticosteroids, budesonide or dexamethasone, for 10 h and then incubated with LPS in the continuous presence of steroid for an additional

Figure 2. IL-8 mRNA expression in three normal bronchial fibroblast lines (Bronchial fib.), and three nasal polyp (Nasal fib.) and four fibrotic lung (Lung fib.) fibroblast lines different from those used in the experiment shown in Figure 1. Fibroblasts were cultured without (Control) or with either LPS (055:B5 *E. coli*, 10 μg/ml) or IL-1α (5 ng/ml) for 24 h. Amounts of loaded total RNA in the gel for LPS-stimulated samples were confirmed by ethidium bromide–stained 28S and 18S rRNA (*bottom*).

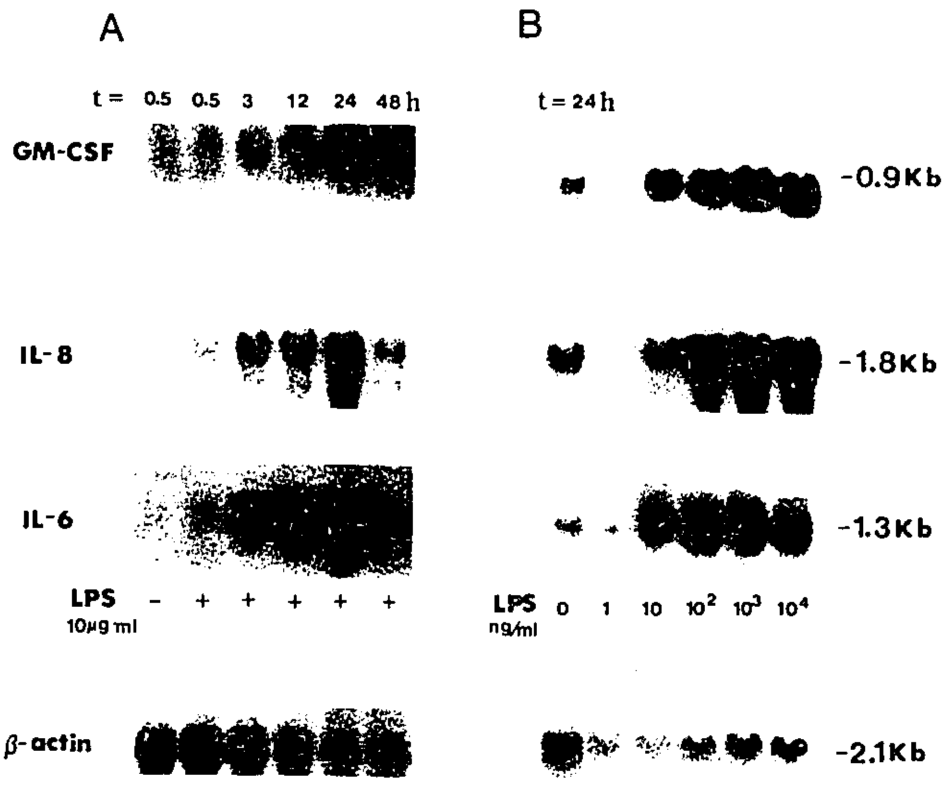


Figure 3. (A) Kinetics of GM-CSF, IL-8, and IL-6 mRNA expression in nasal polyp fibroblasts stimulated with 10 μg/ml LPS (055:B5 *E. coli*) for 0.5, 3, 12, 24, and 48 h. (B) Gene expression of GM-CSF, IL-8, and IL-6 in nasal polyp fibroblasts stimulated with different concentrations of LPS (055:B5 *E. coli*) for 24 h. Equal amounts of total RNA were loaded and this was shown by ethidium bromide-stained 28S and 18S rRNA in the gel and by β-actin mRNA message (*bottom*).

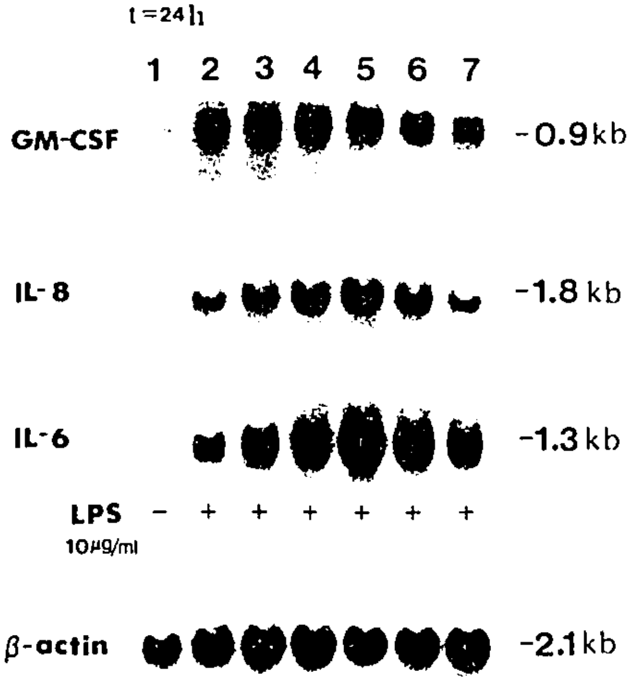


Figure 4. GM-CSF, IL-8, and IL-6 mRNA expression in nasal polyp fibroblasts stimulated by different LPS samples for 24 h. Lane 1, unstimulated; 2, LPS from 055:B5 *E. coli*, trichloroacetic acid extract; 3, LPS from 055:B5 *E. coli*, phenol extract; 4, LPS from 011:B5 *E. coli*, phenol extract; 5, LPS from *S. typhimurium*, phenol extract; 6, LPS from *S. minnesota*, phenol extract; 7, LPS from *K. pneumoniae*, phenol extract. Equal amounts of total RNA were loaded and this was confirmed by ethidium bromide staining and β-actin mRNA expression (*bottom*).

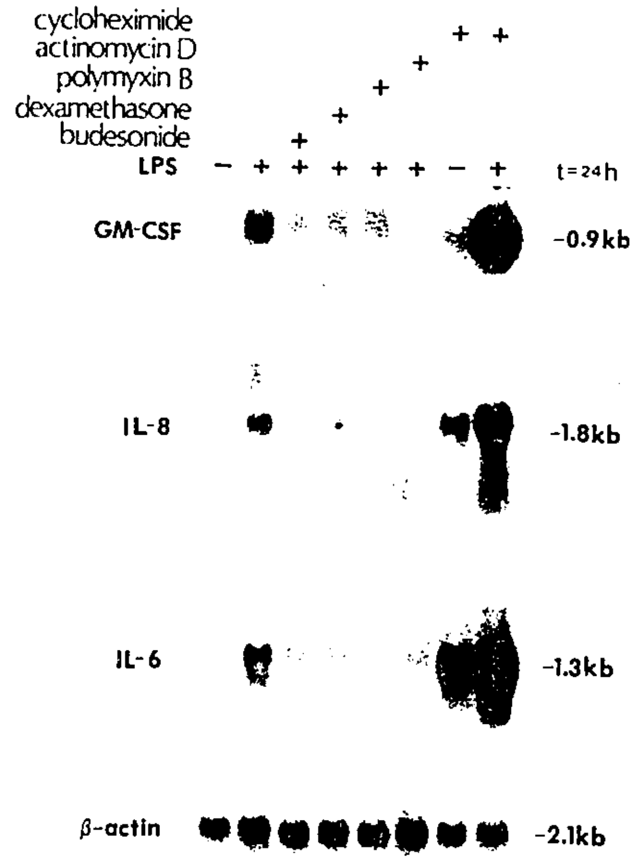


Figure 5. Regulation of LPS-induced GM-CSF, IL-8, and IL-6 gene expression in nasal polyp fibroblasts. Cells were treated with different agents for 24 h (10^{-6} M budesonide, 10^{-6} M dexamethasone, 10 μg/ml polymyxin B, 5 μg/ml actinomycin D, or 10 μg/ml cycloheximide) in the absence or presence of 100 ng/ml of LPS (*S. typhimurium*). Cells were pretreated with budesonide or dexamethasone for 10 h before the addition of LPS. For polymyxin B treatment, LPS was preincubated with polymyxin B at 37°C for 30 min and added into the cultured cells. Equal amounts of total RNA were loaded and this was confirmed by ethidium bromide-stained 28S and 18S rRNA and by β-actin mRNA message (*bottom*).

24 h. As shown in Figure 5, preincubation of nasal fibroblasts with either steroid markedly inhibited LPS-stimulated GM-CSF, IL-8, and IL-6 expression.

To investigate the possible role of transcriptional regulation in nasal fibroblast cytokine induction by LPS, cells were simultaneously incubated with LPS and actinomycin D. Fibroblasts expressed minimal amounts of specific message for GM-CSF, IL-8, and IL-6 in response to LPS when treated with actinomycin D (Figure 5). As a second approach to determine whether transcriptional regulation was involved, nasal and lung fibroblasts were transfected with a DNA construct containing 276 bp of the rat IL-6 promoter sequence fused to a reporter luciferase gene. The transfected cells were stimulated with LPS and assayed for luciferase activity. Figure 6A shows that stimulation by LPS induced about 4 times as much luciferase activity as that produced by unstimulated nasal fibroblasts, presumably mediated through the *cis*-acting regulatory elements present in this construct (Figure 6B). In contrast, LPS had no effect on luciferase activity in transfected lung fibroblasts.

IL-1 Is Not Involved in LPS-induced Cytokine Expression in Nasal Fibroblasts

The observation that LPS-induced cytokine mRNA peaked at 24 h whereas the peak cytokine mRNA induction by IL-1α occurred at 12 h raises the possibility that a newly synthesized protein(s) or a secondary cytokine such as IL-1 is involved in LPS-mediated cytokine induction. However, this is unlikely since cycloheximide treatment led to increased expression of all three cytokines in the presence of LPS (Figure 5) and the presence of a mixture of neutralizing monoclonal anti-IL-1α and anti-IL-1β antibodies failed to decrease bioactive IL-6 production by LPS-stimulated nasal fibroblasts (Table 1). Furthermore, we chose to examine IL-1β mRNA expression in these fibroblasts since IL-1β accounts for > 90% of expressed IL-1 mRNA and active protein in LPS-stimulated monocytes (37). We found no evidence of IL-1β mRNA expression seen in LPS-stimulated nasal fibroblasts under conditions where such expression in nasal fibroblasts was seen with IL-1α stimulation (Figure 7).

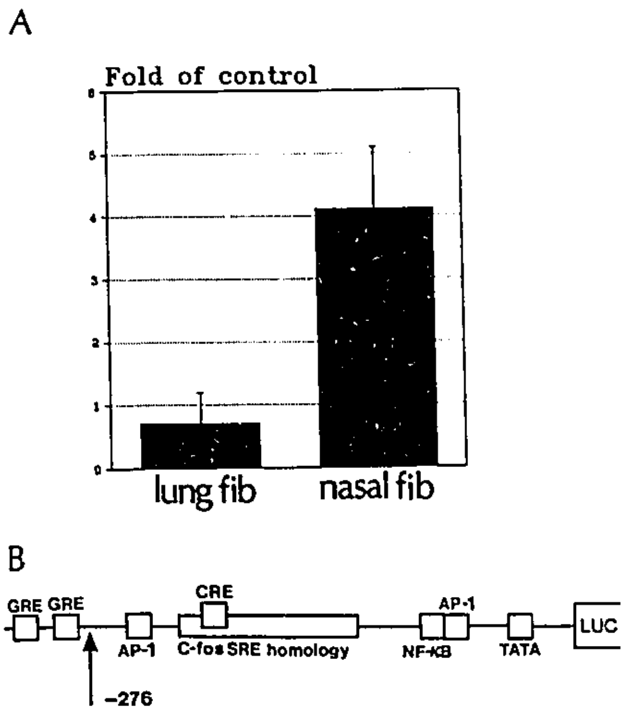


Figure 6. (A) Luciferase activity in IL-6 promoter-luciferase DNA construct-transfected lung (lung fib) and nasal (nasal fib) fibroblasts stimulated by LPS (*S. typhimurium*, 10 μg/ml) for 24 h. IL-1α induced about 7 times as much luciferase activity as that by unstimulated cells. The construct included 276 bp upstream of the IL-6 transcriptional start. Results are expressed as fold of unstimulated control and represent means ± SD from three separate experiments ($n = 3$) using one representative normal lung or nasal polyp fibroblast line. Similar results were obtained with three other lung or nasal fibroblast lines. (B) Illustration of relative locations of the transcriptional control elements in the 5′ IL-6 promoter region and luciferase reporter gene (LUC) in the construct used. The 5′ deletion point is labeled (*arrow*). GRE = glucocorticoid response element; CRE = cAMP response element; SRE = serum response element; NF-κB = NF-κB binding element; AP-1 = activator protein-1 binding element.

TABLE 1

*Effect of anti-IL-1 antibodies on LPS-induced IL-6 activity by nasal fibroblasts**

Culture Condition	IL-6 (ng/ml)
Medium control	4.0 ± 0.6
Medium + anti-IL-1α/β	3.9 ± 0.6
Medium + IL-1α/β (1 ng/ml)	153.0 ± 25.2
Medium + IL-1α/β + anti-IL-1α/β	5.5 ± 2.5
Medium + LPS	64.3 ± 12.4
Medium + LPS + anti-IL-1α/β	83.7 ± 4.0†

* Nasal fibroblasts were cultured with or without LPS (1 μg/ml) and/or neutralizing anti-IL-1α and -IL-1β antibodies (1:50) for 24 h. Supernatants were assayed for IL-6 activity by B9 assay. Results are expressed as means ± SD from three experiments in triplicate samples.

† No statistically significant difference from medium + LPS ($P = 0.12$).

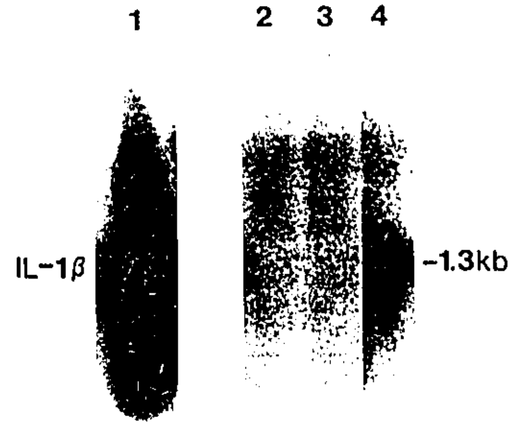


Figure 7. IL-1β gene expression by nasal fibroblasts. Lane 1, positive control (human peripheral blood monocytes stimulated with fibroblast-conditioned medium for 16 h). Lanes 2 and 3, nasal polyp fibroblasts cultured without LPS and with 10 μg/ml LPS (055:B5 *E. coli*) for 24 h. Lane 4, same nasal polyp fibroblasts stimulated with 20 ng/ml of IL-1α for 12 h. Equivalent amounts of total RNA were loaded and this was verified by ethidium bromide-stained 28S and 18S rRNA in the gel.

Cytokine Protein Production by LPS-stimulated Nasal Fibroblasts

To determine cytokine synthesis and secretion, supernatants from a number of cultured nasal and lung fibroblast lines were assayed for cytokine content. Table 2 shows a representative result with one nasal polyp fibroblast line and one fibrotic lung fibroblast line. In agreement with the enhanced mRNA expression, LPS stimulated nasal fibroblasts to produce statistically significantly greater amounts of GM-CSF, IL-8, and IL-6 over control. In contrast, LPS failed to significantly increase cytokine production in lung fibroblasts.

To complement B9 bioassay for IL-6 and to visualize the differential production of cytokine protein by LPS-stimulated nasal and lung fibroblasts, the newly synthesized IL-6 protein was metabolically labeled, immunoprecipitated, and separated by SDS polyacrylamide gel electrophoresis. Figure 8 shows that LPS dramatically increased the *de novo* synthesis of IL-6 protein in nasal fibroblasts. This was comparable with that induced by IL-1α. On the contrary, LPS caused only marginally increased IL-6 production from lung fibroblasts in contrast to IL-1 stimulation. The different species of IL-6 protein seen, ranging mostly from 23 to 30 kD, likely represent differently glycosylated forms of IL-6 (38).

Discussion

Inflammation is the response developed in the tissue upon encountering potentially injurious chemical, physical, or biologic agents. This response includes the release of a substantial number of mediators, among which cytokines are considered to have a major regulatory role. It is now clear that fibroblasts and other tissue structural cells may actively participate in this process via the release of cytokines such as GM-CSF, IL-6, and IL-8 (14–16). It is also known that

TABLE 2
*Cytokine production by nasal and lung fibroblasts**

Cytokine	GM-CSF (*pg/ml*)		IL-8 (*ng/ml*)		IL-6 (*ng/ml*)	
LPS	−	+	−	+	−	+
NP	117 ± 17	913 ± 104†	3 ± 1	62 ± 22†	128 ± 45	1,233 ± 186†
FL	147 ± 32	242 ± 85	7 ± 3	12 ± 6	250 ± 53	307 ± 101

* Fibroblasts were cultured in the absence or presence of 10 μg/ml LPS (055:B5 *E. coli*) for 24 h. Supernatants were assayed for GM-CSF, IL-8, and IL-6. Results are expressed as means ± SD of three separate experiments using one nasal polyp fibroblast line (NP) and one fibrotic lung fibroblast line (FL) representative of a number of fibroblast lines examined.
† $P < 0.05$ versus control.

IL-1 and TNF-α, derived mostly from monocytes/macrophages, are powerful upregulators of these fibroblast effector activities (13, 39, 40).

LPS is an abundant component of the outer membrane of most gram-negative bacteria (41). Systemic administration of LPS elicits a marked inflammatory response, and this agent has been shown to be a powerful stimulant for monocytes/macrophages to release cytokines *in vitro* (42, 43). Although there is evidence demonstrating that LPS stimulates cytokine expression in human endothelial cells (44, 45), smooth muscle cells (46), and keratinocytes (47, 48) as well as bovine bronchial epithelial cells (49), data examining the effect of LPS on fibroblasts are conflicting. LPS has been shown to enhance IL-6 expression in a human skin fibroblast strain (FS-4) (19) and to induce IL-1 and IL-6 activities in human gingival fibroblast lines (21) as well as IL-8 expression in human synoviocytes (22). Moreover, LPS induced factor B of the complement pathway in human skin fibroblasts (20) and induced competence genes JE and KC in 3T3 fibroblasts (23). In contrast, LPS failed to induce IL-6 activity in a human embryonic skin fibroblast strain (E_6SM) (18) or IL-8 expression in human skin (24) and lung fibroblasts (13), nor did LPS increase complement C3 or C5 production

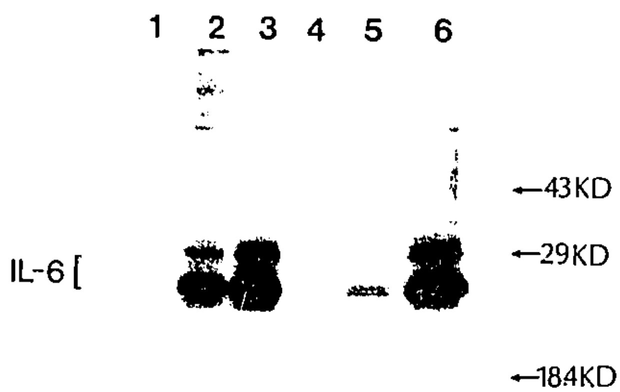


Figure 8. Metabolically labeled IL-6 protein from nasal or lung fibroblasts cultured for 24 h, analyzed by polyacrylamide gel electrophoresis. Lane 1, unstimulated nasal fibroblasts; 2, nasal fibroblasts stimulated with 10 μg/ml LPS (*S. typhimurium*); 3, nasal fibroblasts stimulated with 5 ng/ml IL-1α; 4, unstimulated lung fibroblasts; 5, lung fibroblasts stimulated with 10 μg/ml LPS (*S. typhimurium*); 6, lung fibroblasts stimulated with IL-1α (5 ng/ml). Equal amounts of supernatants and anti-IL-6 antibody were added and this was confirmed by staining the gel.

in a human lung fibroblast line (25). A possible reason accounting for these variable observations may be heterogeneity in the response to LPS of fibroblasts from different sites and/or from tissues in different states, healthy versus diseased.

We report here that whereas IL-1 stimulates the synthesis of GM-CSF, IL-8, and IL-6 by respiratory fibroblasts regardless of the site of origin or the state of the tissue, LPS stimulates the synthesis of these cytokines only in nasal fibroblasts but not in bronchial or lung fibroblasts. The responsiveness of nasal fibroblasts and unresponsiveness of lung fibroblasts to LPS were confirmed with a total of eight nasal fibroblast lines (three normal and five polyp) and 11 lung fibroblast lines (five normal and six fibrotic) as well as three normal bronchial fibroblast lines. Thus, these results also indicate that LPS responsiveness was site, but not disease, specific. Furthermore, we examined the effect of LPS derived from different strains or species of bacteria on nasal fibroblasts and found these samples elicited qualitatively similar effects, with those from *K. pneumoniae* and *S. typhimurium* being the least and the most potent, respectively. This further confirmed the specificity of the response and established a universal responsiveness of nasal fibroblasts to LPS derived from different species of gram-negative bacteria. Different potencies of LPS samples in inducing IL-6 gene expression in skin fibroblasts were also documented by Helfgott and colleagues (19). Our finding that fibroblasts from either normal or fibrotic lung tissues were not capable of significant cytokine responses to LPS is in agreement with the observation by Rolfe and associates that LPS could not upregulate IL-8 mRNA in primary lines of normal lung fibroblasts (13).

Recently, four LPS receptor-like structures such as CD14 have been described on macrophages (33, 34), and some of these LPS binding domains appear to be present on other inflammatory cells (30). Also, it has been shown that the lipid A region of LPS is important in the binding of LPS to its receptors on macrophages (41, 42), and polymyxin B specifically inhibits the effect of LPS by forming a stable complex with the lipid A portion of LPS (51). Although this has not been studied in fibroblasts, our observation that polymyxin B entirely neutralized the stimulatory effect of LPS on GM-CSF, IL-8, and IL-6 gene expression by nasal fibroblasts suggests that a similar mechanism might be operating in these cells and also indicates that the response of nasal fibroblasts is specific to LPS. The optimal cytokine response of macrophages to LPS was found to be serum dependent, likely due to the presence of LPS binding protein(s) in serum (29, 30, 52). We found a similar dependence on serum for the maximal LPS response of nasal fibroblasts (data not shown). This is also in support of a recent observation that cells lacking surface CD14 require the presence of soluble CD14 in serum to respond to LPS (53).

In addition, the effect of LPS on nasal fibroblasts was unlikely mediated by secondary cytokine synthesis such as IL-1 because cycloheximide treatment caused superinduction of cytokine messages, and anti-IL-1 neutralizing antibodies did not interfere with the enhanced IL-6 production nor was IL-1β mRNA detected. Induction or superinduction of cytokine mRNAs by cycloheximide in unstimulated or LPS-stimulated nasal fibroblasts was likely due to increased mRNA stability for these cytokines. Cycloheximide has been reported to stabilize mRNAs for GM-CSF, IL-6, or IL-3 in human lymphocytes or fibroblasts, possibly through inhibiting the synthesis of RNA degrading proteins (54–56).

Increased transcription was likely involved in the induction of cytokines by LPS in nasal fibroblasts since actinomycin D largely inhibited the cytokine gene expression in LPS-stimulated nasal fibroblasts, and by transfecting nasal fibroblasts with an IL-6 gene promoter construct linked to a luciferase reporter gene, we showed that LPS led to a several-fold increase in expression of luciferase activity in these cells, indicating the activation of the transcriptional regulatory element(s) present in the construct, after LPS stimulation (33). However, confirmation of transcriptional activation will require a further approach such as nuclear run-on analysis. We are currently investigating the relative contributions of various regulatory elements of the IL-6 promoter in the LPS-induced IL-6 response by nasal fibroblasts. This approach may provide an opportunity to look into the mechanism by which lung fibroblasts fail to respond to LPS with regard to cytokine expression. In addition, as others have shown with LPS- or TNF-α-stimulated fibroblasts (19, 42, 50), we show that the stimulation of cytokines in nasal fibroblasts by LPS could be abrogated by exposure to steroids.

The observation that nasal fibroblasts released enhanced GM-CSF, IL-8, and IL-6 in response to LPS suggests a distinct role for these cells in the initiation of the inflammatory response. Since airborne gram-negative bacteria and their breakdown products including LPS are ubiquitous, particularly in association with environmental and occupational dusts (57, 58), the inhalational exposure is likely another way by which LPS impacts upon the respiratory tract in addition to intravascular exposure. The nasal mucosa is the first respiratory tissue that environmental agents encounter and, thus, it is likely exposed to a great deal of potentially injurious agents including microorganisms and LPS. We suggest that the ability of nasal fibroblasts, and perhaps other tissue structural cells, to release proinflammatory cytokines in response to LPS, in addition to IL-1, may represent an additional defense mechanism. The high prevalence of nasal inflammatory conditions such as sinusitis, rhinitis, and nasal polyposis may reflect the outstanding ability of the nasal tissue to directly mount an inflammatory response. Interestingly, we also found that in addition to nasal fibroblasts, two normal human primary skin fibroblast lines cultured under the same conditions as nasal fibroblasts could respond to LPS by expressing increased GM-CSF and IL-8 mRNAs, although the expression was less enhanced as compared with nasal fibroblasts (data not shown). This is reminiscent of the protective function of the skin as a first barrier of the body. On the other hand, the inability of fibroblasts in lower respiratory sites, bronchus and lung, to release cytokines in response to LPS, and the ability of these cells to respond to IL-1α, suggests that the effector contribution of these cells to the inflammatory response is largely dependent upon stimulation by the signals such as IL-1 and TNF-α released from such cells as activated alveolar macrophages, the predominant and powerful resident defense cells in the lower respiratory site. Indeed, using a co-culture model, Rolfe and co-workers have elegantly provided the evidence that the expression of IL-8 in lung fibroblasts was primarily dependent upon stimulation by IL-1 and TNF released from the alveolar macrophages cultured in the presence of LPS (13). However, based upon our results, we cannot rule out the possibility that fibroblasts in the lower respiratory sites may respond to LPS by regulating genes other than the cytokines shown here.

In summary, our findings demonstrate for the first time that human nasal fibroblasts, as opposed to bronchial and lung fibroblasts, markedly release increased amounts of GM-CSF, IL-8, and IL-6 in response to LPS. These results establish heterogeneity in the responsiveness to LPS of different fibroblast populations within the respiratory system, perhaps reflecting distinct contributions of these cells to respiratory inflammation.

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Chapter four

INTERACTION OF AIRWAYS INFLAMMATORY AND STRUCTURAL CELLS IN VITRO

The following article entitled "Human upper airway structural cell-derived cytokines support human peripheral blood monocyte survival: a potential mechanism for monocyte/macrophage accumulation in the tissue" is published in The American Journal of Respiratory Cell and Molecular Biology. 6:121-218, 1992. It attempts to provide evidence that the effector macrophages and structural cells in the airways can act upon each other through the direct communication, resulting in functional changes. In this paper, attention was focused on the effect of the upper airway structural cell-derived cytokines on the survival, proliferation and differentiation of monocytes.

Most of the work included in this publication was done, and the paper was written, by the author of the thesis. Dr. T. Ohtoshi contributed to establishing epithelial cell cultures, Dr. P. Ralph carried out the ELISA's for GM-CSF and M-CSF in the supernatants, and Drs. J. Gauldie and M. Jordana provided supervision to this study, thus resulting in the multiple authorship in this paper.

Human Upper Airway Structural Cell-derived Cytokines Support Human Peripheral Blood Monocyte Survival: A Potential Mechanism for Monocyte/Macrophage Accumulation in the Tissue

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A central feature of chronic airway inflammation is accumulation of monocyte/macrophages in the tissue. It is well known that circulating monocytes are short-lived cells whereas tissue macrophages are longer-lived cells. One mechanism that may account for accumulation of inflammatory cells includes enhanced survival and/or differentiation of these cells. Recent studies imply that signals released by tissue structural cells may be crucial in these events. To investigate this notion, human blood monocytes were cultured with either culture medium alone as a control, human nasal epithelial cell-conditioned medium (EpCM), or fibroblast-conditioned medium (FCM) for more than 1 wk. Survival of monocytes in medium alone was 17% at day 7, whereas survival of those cultured with 50% of EpCM or FCM was 62% and 64%, respectively. The effect of EpCM and FCM was dose dependent. Preincubation of either conditioned medium (CM) with an antibody against granulocyte/macrophage colony-stimulating factor (GM-CSF) or an antibody against macrophage colony-stimulating factor (M-CSF) resulted in a partial abrogation of the survival-enhancing effect, to an average of 50% and 30%, respectively. Complete inhibition was obtained by preincubation of the CM with a combination of both antibodies. The effect of CM represented true survival because CM only induced a low profile of [^{3}H]thymidine incorporation and, furthermore, less than 0.3% of the cells cultured with CM underwent DNA synthesis as assessed by autoradiography. In addition, ultrastructural observations demonstrated that most monocytes cultured with either CM but not with control culture medium assumed ultrastructural features of macrophages by day 8 of culture. Thus, our findings indicate that one mechanism by which monocyte/macrophages accumulate in the airways may involve enhanced survival mediated by GM-CSF and M-CSF released by airway fibroblasts and epithelial cells.

The sustained accumulation of monocyte/macrophages in the tissue is a central feature of chronic inflammation (1). Recent evidence indicates that in addition to monocyte migration from the bloodstream, proliferation and progenitor differentiation at the local site may contribute to the accumulation of these cells in the lung (2–5). Human peripheral blood monocytes, like other leukocytes, are short-lived cells while in the circulation with a half-life ranging from several hours to 2 days (6, 7). Shortly after entering the tissue, however, monocytes differentiate to macrophages, which have a longer life-span (2, 6, 7). The elements mediating this process of prolonged monocyte survival and differentiation remain largely unknown.

We and others have recently shown that tissue structural cells (TSC) including fibroblasts and epithelial and endothelial cells may contribute to the regulation of the inflammatory response (8–10). This contribution is accomplished by the production of differentiating and growth-promoting cytokines as well as of matrix molecules active on inflammatory cell function. Here, we sought to investigate whether TSC, specifically human upper airways fibroblasts and epithelial cells, regulate the survival of human peripheral blood monocytes *in vitro*. Our studies demonstrate that conditioned medium (CM) from human nasal epithelial cells or fibroblasts markedly prolongs the survival of monocytes *in vitro*. On the basis of experiments with specific neutralizing antibodies, we show that this survival-enhancing effect is mediated mainly by granulocyte/macrophage colony-stimulating factor (GM-CSF) and to a lesser degree by macrophage colony-stimulating factor (M-CSF) in the CM. We also show that this increased survival is not due to cell replication as these CM have little effect on monocyte proliferation. Fur-

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Abbreviations: conditioned medium, CM; epithelial cell-conditioned medium, EpCM; fibroblast-conditioned medium, FCM; granulocyte/macrophage colony-stimulating factor, GM-CSF; interleukin, IL; macrophage colony-stimulating factor, M-CSF; phosphate-buffered saline, PBS; recombinant human, rh; tissue structural cells, TSC.

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thermore, a morphologic examination of the surviving cells demonstrates that the majority of these cells have the ultrastructural characteristics typical of macrophages. Together, these results provide evidence for a potential mechanism contributing to the *in vivo* accumulation of mononuclear phagocytes at inflammatory sites of the airway.

Materials and Methods

Cytokines and Antibodies

Recombinant human (rh) GM-CSF, with specific activity of 9.3 × 10^6 U/mg, was obtained from Genetics Institute (Cambridge, MA). Recombinant human granulocyte colony-stimulating factor (G-CSF) was purchased from Amersham (Buckinghamshire, UK). Recombinant human M-CSF with specific activity of 5 × 10^5 U/ml, and rabbit anti-human M-CSF serum (neutralizing titer of 3 × 10^6 NU/ml) were obtained from Cetus Corp. (Emeryville, CA). Rat anti-human GM-CSF monoclonal antibody was kindly provided by Dr. John S. Abrams (DNAX Research Institute, Palo Alto, CA); 1 μg/ml of this antibody neutralizes 2 ng/ml of human GM-CSF. Rabbit anti-human interleukin (IL)-8 was obtained from Genetics Institute. Rabbit anti–rh IL-6 serum was made in our laboratory.

Monocyte Isolation and Culture

Heparinized blood was obtained from normal volunteers. Cold phosphate-buffered saline (PBS)-diluted blood was layered on Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 1,800 rpm for 25 min at room temperature. The resultant mononuclear cell layer was washed with cold PBS and resuspended in RPMI 1640 medium with 10% fetal bovine serum (GIBCO, Grand Island, NY), 1% penicillin/streptomycin (GIBCO), and 1% bicarbonate (hereafter referred to as supplemented RPMI). Cell viability was > 95%. Routinely, 3 × 10^6 cells were seeded in 35-mm Falcon culture dishes. After 1 h of incubation, nonadherent cells were washed off with four changes of PBS. The number of attached cells was determined at this point. Usually, 7 to 15% of the originally plated cells were attached. More than 90% of the remaining adherent cells were shown to be monocytes on the basis of Wright-Giemsa staining and CD14 immunocytochemical staining (5). Duplicate cultures of monocytes were incubated in supplemented RPMI medium, alone or with various concentrations of CM or cytokines, in a 5% CO_2 incubator at 37° C. Fresh culture medium was added at day 4. For the [^{3}H]thymidine incorporation assay, the mononuclear cell suspension was further layered over 49.2% Percoll (Pharmacia) (11) and centrifuged at 1,500 rpm for 15 min at room temperature. Monocytes were washed and resuspended in supplemented RPMI medium. More than 90% of monocytes were viable after this purification.

Epithelial Cell CM (EpCM) and Fibroblast CM (FCM)

We have established a number of human primary fibroblast lines from biopsies of nasal tissues as previously described (12). Normal nasal FCM was generated from 48-h cultures in supplemented RPMI medium, pooled, centrifuged, and stored at −20° C in aliquots until use. Human primary epithelial cell cultures were established from biopsies of nasal polyp and inferior turbinate tissues as described before (5), and nasal polyp EpCM was generated as described above. Most experiments were carried out with normal nasal FCM and nasal polyp EpCM because of much greater access to nasal polyp tissues as well as greater ability of polyp epithelial cells to reach monolayers. The CM from nasal polyp fibroblasts caused qualitatively similar effects on monocyte survival compared with normal nasal FCM (data not shown).

Survival Assessment

At days 3, 6, and 7 or 8, both detached and attached cells were collected. The attached monocytes were dislodged by vigorous pipetting with PBS and gentle scraping. The total number of cells in each culture was counted. Cell viability was determined by trypan blue exclusion, and survival was calculated by using the formula: 100 × [(number of cells recovered) × (percentage of cells excluding trypan blue)]/[number of initially attached cells] (13).

[^{3}H]Thymidine Incorporation and *In Situ* Autoradiography

Monocytes (0.5 to 1 × 10^5) in 250 μl of supplemented RPMI with or without CM or recombinant cytokines were plated into each well of 96-well plates (InterMed NUNC, Denmark). Cultures were pulsed with 1 μCi/well [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) at day 4, subjected to two freeze-thawing cycles 20 h later, and harvested on a cell harvester (MA Bioproducts, Walkersville, MD). [^{3}H]Thymidine incorporation was detected by beta-counting. The resultant cpm was expressed as the mean of four replicates for each condition.

For autoradiography, cells cultured in 35-mm Falcon dishes were pulsed with 2.5 μCi/ml [^{3}H]thymidine at day 5 for 20 h, washed with PBS twice, and fixed with 10% buffered formalin for 10 min. The attached monolayer of cells was covered with Kodak NTB2 emulsion and exposed for 4 days at 4° C. The dishes were developed in Kodak developer for 4 min and fixed in Kodak fixer for 5 min. The labeling index was expressed as the percentage of cells with > 15 grains/nucleus. At least 600 cells were randomly counted on each dish, and the results represent the mean of duplicate dishes per condition.

Immunoassay of GM-CSF and M-CSF in CM

Human GM-CSF in the CM was detected by an enzyme-linked immunosorbent assay (Genzyme Corp., Boston, MA). The sensitivity of this assay is 4 pg GM-CSF/ml (2 pg = 1 U). Human M-CSF content of CM was measured by a radioimmunoassay, and the sensitivity of this assay is 0.2 ng/ml (20 pg = 1 U).

Electron Microscopy Studies

Monocyte cultures at days 1, 4, and 8 were processed as described previously for scanning and transmission electron microscopy (13).

Statistical Analysis

The significance of differences in results was analyzed by means of an unpaired *t* test.

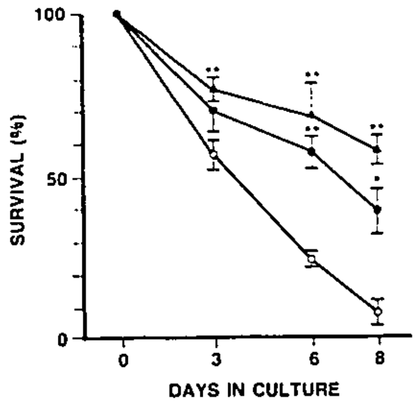


Figure 1. Time course of monocyte survival. Monocytes were cultured with RPMI alone (*open circles*), 50% epithelial cell-conditioned medium (EpCM) (*closed circles*), or 50% fibroblast-conditioned medium (FCM) (*triangles*). Values represent mean ± SD of survival (n = 3). * $P < 0.05$ and ** $P \leq 0.01$ versus RPMI alone.

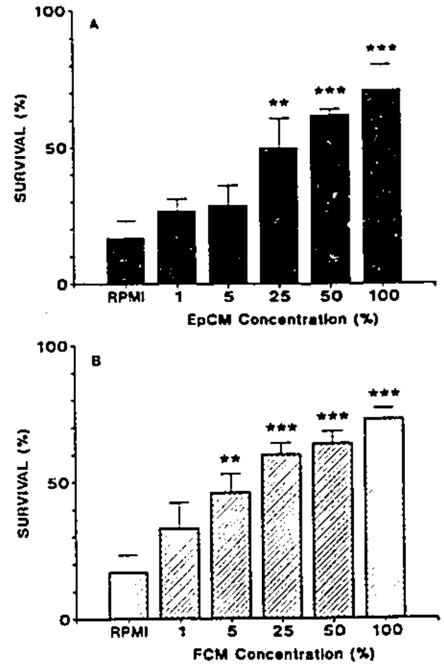


Figure 2. Effect of EpCM (A) and FCM (B) on monocyte survival. Monocytes were cultured with various concentrations of conditioned medium (CM) and assessed for survival at day 7. Values represent mean ± SD (n = 3). ** $P \leq 0.01$ and *** $P < 0.005$ versus RPMI alone.

Results

Survival of Monocytes Cultured with EpCM and FCM

Figure 1 shows the survival of monocytes cultured under different experimental conditions over time. Survival of monocytes cultured with supplemented RPMI alone was 56%, 24%, and 8% at days 3, 6, and 8, respectively. In contrast, survival of monocytes cultured with FCM was 77%, 68%, and 58% at the same time points. Culture of monocytes with EpCM produced similar results, albeit somewhat lower compared with FCM: 70%, 57%, and 39%. As shown in Figure 2, the survival-enhancing effect of both FCM and EpCM was concentration dependent. A maximal effect was seen with monocytes cultured in 100% CM. In fact, under these conditions, most of the monocytes remained alive for at least 15 days (data not shown).

Cytokine Content in the CM and Effect of Recombinant Human Cytokines on Monocyte Survival

Because there is evidence that rhGM-CSF or rhM-CSF can each enhance human monocyte survival (14, 15), we measured the content of these two cytokines in the CM that we used in these experiments. Pooled EpCM contained 100 pg/ml of GM-CSF and 605 pg/ml of M-CSF; pooled FCM contained 218 pg/ml of GM-CSF and 1,770 pg/ml of M-CSF.

We then examined the effect of recombinant human cytokines on monocyte survival. As shown in Table 1, rhM-CSF (400 U/ml) or rhGM-CSF (200 U/ml) each increased monocyte survival from 10% to 15% in the supplemented RPMI control cultures to about 40%, and a combination of these cytokines increased survival to about 62%. The addition of rhG-CSF (50 U/ml) to monocyte cultures had no effect at all on survival.

Effect of Specific Antibodies on the Survival-enhancing Activity of CM

Figure 3 shows the effect of FCM preincubated with specific neutralizing monoclonal anti-GM-CSF and anti-M-CSF antibodies on monocyte survival. Anti-M-CSF antibody decreased the survival induced by FCM by an average of 30%. Preincubation of FCM with anti-GM-CSF antibody alone resulted in greater inhibition of the survival-enhancing effect (50%). It is apparent that only preincubation of the FCM with both anti-GM-CSF and anti-M-CSF resulted in total abrogation of the effect. These antibodies also had a similar

TABLE 1

*Effects of recombinant human cytokines on monocyte survival**

Culture Conditions	Survival (%)
RPMI	10–15
FCM (25%)	56–62
M-CSF 100 U/ml	22–35
M-CSF 400 U/ml	42–45
GM-CSF 50 U/ml	28–32
GM-CSF 200 U/ml	37–44
M-CSF 50 U/ml + GM-CSF 10 U/ml	25–31
M-CSF 100 U/ml + GM-CSF 50 U/ml	60–63
M-CSF 400 U/ml + GM-CSF 100 U/ml	58–67
G-CSF 50 U/ml	8–12
EpCM (25%)	39–45
EpCM + anti-IL-6 (1:100)	41–45
EpCM + anti-IL-8 (1:50)	40–48
EpCM + anti-GM-CSF (1:50)	17–23

Definition of abbreviations: FCM = fibroblast-conditioned medium; M-CSF = macrophage colony-stimulating factor; GM-CSF = granulocyte/macrophage colony-stimulating factor; EpCM = epithelial cell-conditioned medium.

* Survival was assayed at day 7. The results represent ranges of several experiments.

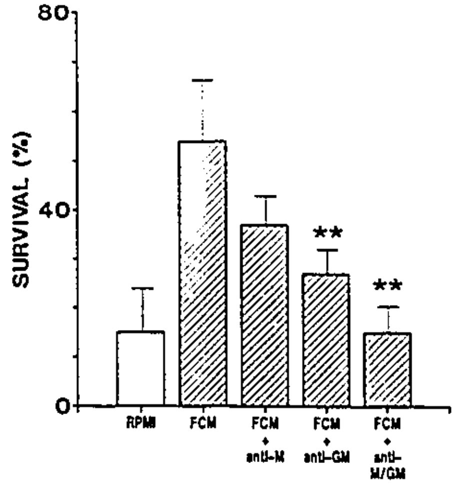


Figure 3. Effect of FCM preincubation with specific neutralizing antibodies on monocyte survival. FCM (25%) was preincubated with anti-macrophage colony-stimulating factor (anti-M-CSF) (1:2,500), anti-granulocyte/macrophage colony-stimulating factor (anti-GM-CSF) (1:50), or both at 37° C for 60 min, and then added to monocyte cultures. Survival was examined at day 7. Results are mean ± SD (n = 3). ** $P \leq 0.01$ versus FCM alone.

TABLE 2

Monocyte [^{3}H]thymidine incorporation and DNA labeling index

Culture Conditions	Thymidine Incorporation*	DNA Labeling Index†
RPMI	544 ± 56	0
FCM		
5%	718 ± 81	0
25%	768 ± 188	0
50%	739 ± 17	0.03
100%	1,190 ± 556	0.17
EpCM		
5%	751 ± 241	0
25%	1,086 ± 352	0
50%	1,713 ± 609	0.08
100%	1,475 ± 162	0.28

Definition of abbreviations: FCM = fibroblast-conditioned medium; EpCM = epithelial cell-conditioned medium.

* Thymidine incorporation was examined at day 5. The results are expressed as mean ± SD from three separate experiments.

† Autoradiography was performed at day 6. A total of 600 to 1,500 cells were counted for thymidine uptake. The results represent the average percentage of cells that have taken [^{3}H]thymidine from four separate experiments.

inhibitory effect on the survival induced by EpCM, and full abrogation of the effect required the presence of both anti-GM-CSF and anti-M-CSF except that anti-GM-CSF inhibited a larger part of the effect induced by EpCM than by FCM (data not shown). The same antibodies were able to fully abrogate the survival-enhancing effects of rhGM-CSF or rhM-CSF (data not shown). In contrast, preincubation of CM with neutralizing antibodies against human IL-6 or IL-8 had no inhibitory effect (Table 1).

Monocyte [^{3}H]Thymidine Incorporation and *In Situ* Autoradiography

To investigate whether proliferation could contribute to the prolonged survival of monocytes induced by CM, thymidine incorporation was examined. As shown in Table 2, only concentrations of 50% and 100% of EpCM and 100% of FCM induced a small increase in thymidine incorporation ranging from 1,190 to 1,713 cpm, which is considerably low compared with incorporation detected in a proliferating fibroblast culture starting with an equal number of cells (> 10^4 cpm). rhGM-CSF and rhM-CSF had a similarly small effect on thymidine incorporation (data not shown). In addition, autoradiographic studies at day 6 showed that there were no labeled cells detected in cultures with supplemented RPMI medium alone and only an average of 0.17% and 0.28% labeled cells were detected in cultures with 100% of FCM and EpCM, respectively, whereas a similar analysis showed approximately 15% of proliferating fibroblasts well labeled.

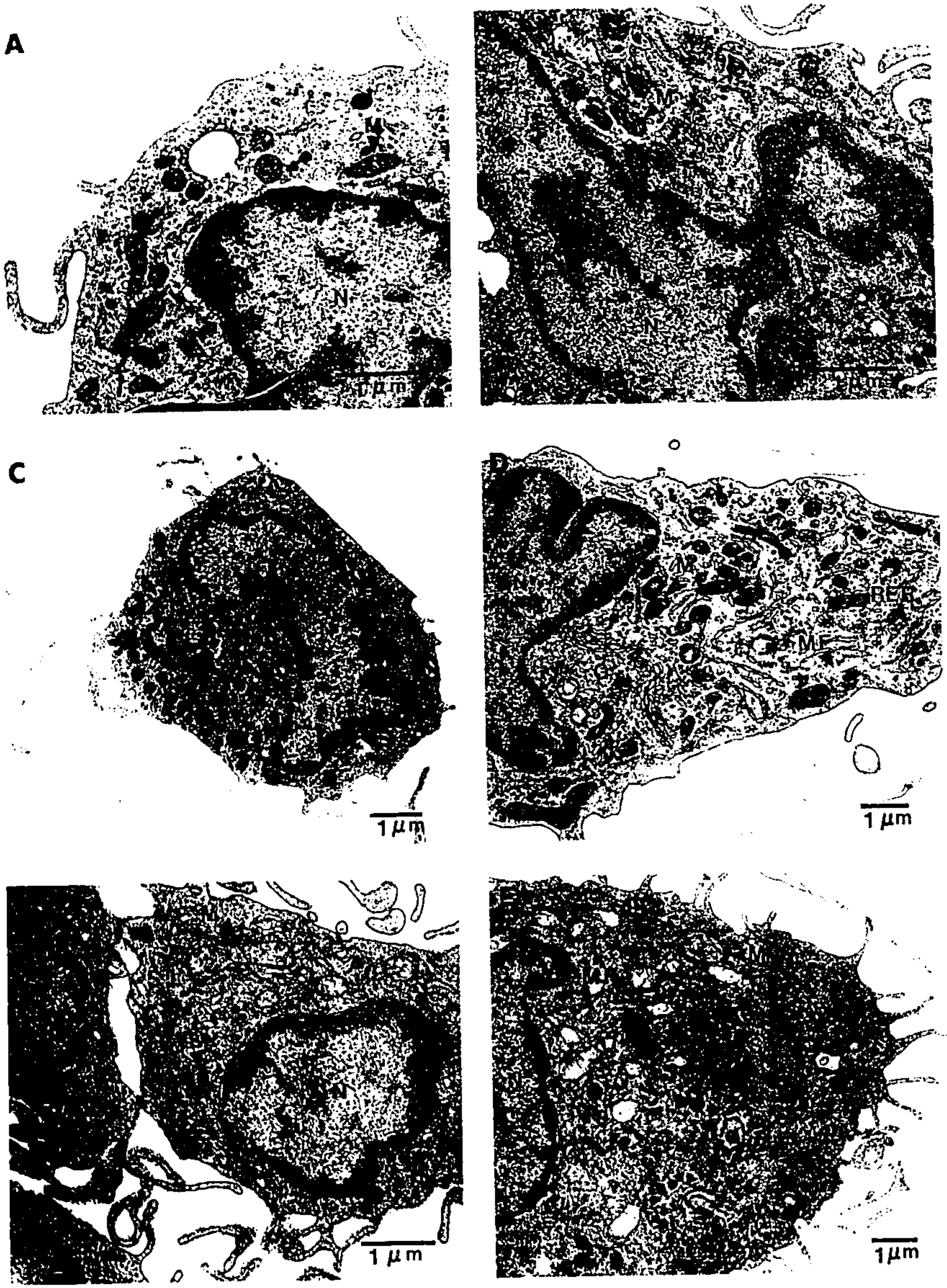
Morphologic Examination

Scanning electron microscopy examination revealed that cells cultured with CM, particularly FCM, spread better and possessed larger sizes and more developed microvilli compared with those in supplemented RPMI alone. Figures 4A and 4B represent a transmission electron micrograph of cells at day 1 and demonstrate morphologic features typical of monocytes. These include a horseshoe-like nucleus located slightly eccentrically with a thicker rim of heterochromatin, relatively scanty cytoplasm, and few organelles. At day 4, some transitional changes are seen (Figures 4C and 4D), particularly in cells cultured in FCM, such as an enlarged cytoplasm and better developed organelles. At day 8, a considerable number of the cells in supplemented RPMI still retained monocyte features (Figure 4E). In contrast, the majority of cells cultured in FCM displayed macrophage characteristics. These include a highly eccentric nucleus with a thinner peripheral rim of heterochromatin, a greater cytoplasm/nuclear ratio, and increased numbers of secondary lysosomes, Golgi areas, rough endoplasmic reticula, and free ribosomes (Figure 4F). In addition, an increase in microfilaments could be seen. Cells cultured in EpCM showed features similar to FCM-stimulated cells.

Discussion

Monocyte/macrophage accumulation in the tissue is a central feature of chronic inflammation. It is believed that monocytes, which are short-lived cells while in the circulation, enter the tissue at an accelerated rate during inflammation (16) where they become differentiated long-lived cells (7). Thus, the number of tissue macrophages at a given point likely involves a balance between ingress of monocytes and time of residence in and removal from the tissue. The mechanisms underlying the transition of monocytes to macrophages and the accumulation in the tissue of these cells are not clear but are likely to involve signals, such as cytokines and extracellular matrix components produced by the TSC. In this regard, various cytokines produced by TSC, including IL-6 (9, 17), IL-8 (18), and the colony-stimulating factors (12), have been implicated in the regulation of inflammatory events in the tissue.

Nasal polyps are grape-like structures that arise from the posterior and sphenoid sinus mucosae and eventually occlude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the nares (19). Nasal polyposis occurs as frequently in patients with atopy as in the general population. Pathologically, the condition is characterized by the presence of a sustained inflammatory infiltrate and of structural abnormalities including varying degrees of basement membrane thickening and fibrosis in the polyp stroma (20, 21). Thus, nasal polyps represent a model of human chronic inflammation that offers abundant and easily accessible tissue specimens.

In order to investigate whether human airways TSC-derived cytokines are directly involved in the tissue accumulation of human monocyte/macrophages, we examined the *in vitro* effects of CM produced by primary lines of fibroblasts and epithelial cells established from nasal polyp tissues on the survival and differentiation of peripheral blood monocytes. Our results (Figures 1 and 2) show that CM from fibroblasts and epithelial cells markedly prolong the survival of monocytes in a dose-dependent fashion. An examination of the data in Table 2 shows that both FCM or EpCM induced only a small increase in monocyte thymidine incorporation and only a marginal increase in labeled cells by autoradiography, indicating that cell proliferation could not account for this enhanced survival.

Previous studies have examined the effect of specific recombinant human cytokines on human monocyte survival and proliferation *in vitro.* Becker and associates (15) showed that rhM-CSF promoted the survival and differentiation of monocytes in serum-free culture conditions. Erickson-Miller and colleagues (22) documented similar findings and also indicated that only 3% of the monocytes cultured in the presence of rhM-CSF underwent DNA synthesis as assessed by bromodeoxyuridine incorporation. Furthermore, Elliot and co-workers (14) showed that a low dose of rhGM-CSF (150 U/ml) increased monocyte survival but not proliferation, and that a larger dose of rhGM-CSF (450 U/ml) induced only a small increase in thymidine incorporation and DNA labeling index.

Because we had previously shown that human upper airways fibroblasts and epithelial cells release GM-CSF and that these cells, particularly fibroblasts, release M-CSF (5, 12), we proceeded to examine the relative contribution of these cytokines to the effect induced by the various CM. Both FCM and EpCM contained similar amounts of GM-CSF, and FCM contained greater amounts of M-CSF as compared with EpCM. We examined the effect on monocyte survival of rhGM-CSF and rhM-CSF, alone and in combination, at doses similar to or smaller than those found in the CM. As shown in Table 1, each cytokine enhanced monocyte survival, and a combination of both cytokines further enhanced this effect to levels similar to those elicited by FCM. The greater content of M-CSF in FCM may explain the observation that this CM induced a greater effect on monocyte survival as compared with EpCM. We further evaluated the ability of specific neutralizing antibodies to abrogate the effect induced by both FCM and EpCM. We found that preincubation of either CM with an anti-GM-CSF antibody resulted in a substantial decrease in the survival-enhancing effect of these CM. Preincubation with an anti-M-CSF antibody resulted in a smaller but consistent inhibition of the effect induced by the CM. Only preincubation of CM with a combination of antibodies resulted in a complete abrogation of enhanced monocyte survival. These results suggest that GM-CSF and M-CSF are both required for the optimal maintenance of monocyte survival at tissue sites. In addition, this monocyte survival-enhancing effect is not exclusive to cells of the upper respiratory tract as either normal or fibrotic lung fibroblast-derived CM containing GM-CSF and M-CSF also enhance the survival of monocytes (data not shown).

We have previously reported that human lung fibroblasts and bronchial epithelial cells prolong the survival of human eosinophils *in vitro* and that this effect is fully mediated by GM-CSF (13, 23). In addition, we have recently found that bronchial epithelial CM prolongs the survival of human neutrophils and that this effect is mediated by a combination of G-CSF and GM-CSF (G. Cox *et al.*, manuscript submitted). These observations, together with the results reported here, suggest that the specific profile of cytokines synthesized by airways structural cells may affect the nature of the inflammatory infiltrate at a particular tissue site.

Because there is evidence that monocytes usually develop macrophage morphologic features after a period of time in culture (24), we examined the effect of FCM and EpCM on this process of differentiation. Our data indicate that the vast majority of the cells cultured with either CM showed, over time, ultrastructural features characteristic of macrophages (25). The temporal coincidence of these two processes suggests that they might be in fact inseparable. It is likely, but not certain, that the same cytokines that promote monocyte survival cause monocyte differentiation. However, whether monocytes survive because they differentiate into macrophages, or vice versa, remains to be clarified.

In summary, these studies demonstrate that monocytes survive much longer when cultured *in vitro* with the products released by the cells with which they are closely associated *in vivo*, and this action is mediated by GM-CSF and M-CSF. The perpetuation of the inflammatory response likely involves multiple interactions between several cell types. In this regard, it is well known that monocyte/macrophage-derived cytokines, such as IL-1 and tumor necrosis factor, are able to enhance cytokine release by fibroblasts (26). Some of these cytokines could, as we have shown here, contribute to further monocyte/macrophage tissue accumulation by virtue of their ability to prolong the survival and induce the differentiation of monocytes.

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Figure 4. Transmission electron micrographs of monocytes cultured with RPMI (*left column*) or 50% FCM (*right column*), at days 1 (A and B), 4 (C and D), and 8 (E and F). N = nucleus; M = mitochondria; RER = rough endoplasmic reticulum; G = Golgi apparatus; MF = microfilament; L = lysosome; SL = secondary lysosome. (Magnification: A, B, and E, ×12,000; C, D, and F, ×8,000.)

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Chapter five

LPS INDUCTION OF CYTOKINES IN THE AIRWAYS IN VIVO (1)

The following paper entitled "Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-α in endotoxin-challenged lung tissue" has been published in the American Journal of Pathology. 143:1009-1015, 1993. This is part of the follow-up study to further the in vitro observations on LPS cytokine induction in airway cells, examining the in vivo effect of LPS on cytokine induction in the airways. This paper focuses on the effect of LPS on expression TNFα mRNA and protein in the airways as assessed by Northern analysis, in situ hybridization and immunochemistry.

This study was performed and the paper was written by the author of the thesis. Among other co-authors in this paper, Dr. H. Kirpalani participated in performing in situ hybridization; Mrs. D. Torry provided the protocol for immunochemistry; Drs. Jordana and Gauldie provided supervision over the project.

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Short Communication

Polymorphonuclear Leukocytes as a Significant Source of Tumor Necrosis Factor-α in Endotoxin-Challenged Lung Tissue

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The kinetic expression and potential cellular source of tumor necrosis factor-α (TNF-α) in lipopolysaccharide- (LPS) induced acute lung inflammation was investigated using a rat model by Northern blot analysis,* in situ *hybridization and immunohistochemistry. LPS induced a polymorphonuclear leukocyte infiltrate in the lung that peaked between 6 and 24 hours. TNF-α messenger (m)RNA was strongly induced by LPS in whole lung tissues shown by Northern analysis. Both alveolar macrophages and polymorphonuclear leukocytes (PMNs), purified from bronchoalveolar lavage fluids of LPS-treated rats, were shown to express TNF-α mRNA by Northern analysis. However, PMNs displayed several times more TNF-α mRNA, relative to actin mRNA, than alveolar macrophages at 6 and 12 hours. By* in situ *hybridization, most of the cells positive for TNF-α mRNA at 6 and 12 hours seemed to be PMNs located within the tissue near bronchioles or vessels. By immunohistochemistry, TNF-α protein was localized mainly to alveolar macrophages at early times (1 to 3 hours) after LPS challenge, and thereafter, PMNs seemed to be the predominant source of TNF-α protein as more than 90% of total intraalveolar positive cells at 6 and 12 hours were PMN. Thus, our data provide the first* in vivo *evidence that PMNs can serve as a significant source of TNF-α at sites of acute inflammation. (Am J Pathol 1993, 143:1009–1015)

Endotoxin (lipopolysaccharide, LPS) is an abundant component of the outer membrane of gram-negative bacteria capable of inducing severe lung injury in gram-negative sepsis and gram-negative bacterial pneumonia, which are among the most common predisposing causes of adult respiratory distress syndrome.[1] The influx of polymorphonuclear leukocytes (PMNs) into airway tissues is a pathological hallmark of LPS-induced lung injury, and the release of the contents from PMNs contributes largely to tissue disruption.[1] However, the mechanisms underlying PMN recruitment and activation remain to be elucidated.

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine found to be a principal mediator in many inflammatory processes.[2] Recent evidence that administration of TNF-α in animals elicits acute lung injury reminiscent of that observed in human endotoxic shock and that antibodies against TNF-α prevent tissue injury during endotoxic shock, suggests a central role of TNF-α in LPS-induced lung injury.[3,4] Indeed, the intratracheal administration of LPS into rats induces TNF-α mRNA expression in total lung tissue[5] as well as TNF-α bioactivity in the bronchoalveolar lavage (BAL) fluid and in unfractionated BAL cell lysates.[6,7] However, the cellular source of TNF-α in the tissue of LPS-induced acute lung injury still remains largely unclear. Whereas alveolar macrophages (AMs) have long been considered a predominant, if not exclusive, source of TNF-α in the lung,[2,5,8] recent *in vitro* data have shown that PMNs are also capable of TNF-α expression in response to LPS.[9–12] Thus, we sought in the present study to investigate the kinetic

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expression and cellular source of TNF-α mRNA and protein localization in the rat lung *in vivo* after LPS challenge.

Materials and Methods

Probes and Antibodies

The murine TNF-α complementary (c)DNA probe was a 300-bp Pvu II fragment from pMuTNF-α obtained from Genentech, Inc. (San Francisco, CA).[13] The chicken actin cDNA probe was purchased from Oncor (Gaithersburg, MD). A 500-bp double stranded DNA ladder fragment (Bethesda Research Laboratories, Gaithersburg, MD) was used as a negative control probe for *in situ* hybridization (ISH).[14,15] For Northern blot analysis and ISH, probes were labeled with [α-^{32}P]dCTP and [^{35}S]-dCTP (New England Nuclear, Boston, MA), respectively, by random priming using an oligo-labeling kit (Pharmacia, Uppsala, Sweden). Rabbit anti-murine TNF-α was kindly provided by Dr. Steven L. Kunkel (Department of Pathology, University of Michigan, Ann Arbor, MI). This antiserum shows high titer activity (1:25,000), specificity for TNF-α, and high cross-reactivity with rat TNF-α.[16] Normal nonimmunized rabbit serum (DAKO A/S, Denmark) was used as a negative control for immunohistochemical (IH) staining.

Animals and Cell Separation

Sprague-Dawley rats weighing 220 to 240 g (Charles River Laboratories, Ottawa, Canada) were anesthetized with ether, and 0.3 ml of sterile saline (control) or saline containing 6 μg of LPS was intratracheally instilled (055:B5 *E. coli*, Sigma Chemical Co., St. Louis, MO). At 0.5, 1, 3, 6, 12, 24, 48, and 72 hours, animals were sacrificed. Some rats were subjected to BAL as previously described.[17] Differential cell counting was performed with Diff-Quik-stained cytospins from BAL cells. In some experiments, the AM fraction was separated from PMNs in pooled BAL fluids of six LPS-treated rats by a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden).[18] On average, AM-enriched fractions contained 90% AMs and 10% PMNs, whereas PMN-enriched fractions contained 97% PMNs and 3% AMs. Rat blood PMNs as controls were isolated by a Ficoll-Paque gradient.[18] Total cellular RNA was extracted from each of these purified cell fractions. In separate experiments, the unperfused left lung of rats was snap-frozen in liquid nitrogen for total tissue RNA extraction, and the right lung was fixed by perfusion with 4% paraformaldehyde for ISH and IH.

RNA Extraction and Northern Blot Analysis

Total tissue or cellular RNA extraction and Northern blot analysis were performed as previously described.[17] Briefly, total RNAs were size-separated by a 1% agarose formaldehyde gel. After blotting to a nylon membrane, the blot was hybridized with radiolabeled TNF-α and actin cDNA probes sequentially, stringency washed, and autoradiographed for 1 to 2 days.

In Situ *Hybridization*

ISH was performed as previously described except that the emulsion-coated slides were only autoradiographed for 8 days.[14,15] The specificity of the signal detected by ISH was shown by using several controls: control (saline) lung tissues showed little or no silver grain accumulation when hybridized with TNF-α probe; there were few or no signals when tissues were hybridized with the negative control probe that was similar to the TNF-α cDNA probe in size; and pretreatment of slides with RNAse-A (100 μg/ml in 2× standard saline citrate for 60 minutes) abolished the signal.

Immunohistochemistry

IH was performed using a HISTOSTAIN-SP kit (Zymed Laboratories Inc., San Francisco, CA). Briefly, after dewaxing through xylene and ethanol, slides were placed in 0.5% hydrogen peroxide in absolute methanol for 30 minutes to block endogenous peroxidase. The slides were next treated with 5% normal goat serum followed by exposure to an 1:100 dilution of rabbit anti-murine TNF-α serum or nonimmune rabbit serum in 2.5% normal goat serum for 60 minutes. After washing, the slides were overlaid with 1:1 diluted biotinylated goat anti-rabbit immunoglobulin G for 10 minutes and were then treated with streptavidin-peroxidase conjugate for 5 minutes, followed by exposure to a substrate-chromogen mixture for 15 minutes to allow color development. Counterstaining was performed with a hematoxylin solution. A red-colored deposit identified the specific antigen. All reactions described above were performed at room temperature. The preincubation of anti-murine TNF-α serum with an excess of recombinant murine TNF-α protein abolished the reaction, indicating the specificity of the staining.

Results

LPS-Induced PMN Infiltration in Lung Tissue

To characterize the model, differential cell counts were determined on cytospins made from BAL cells. As shown in Figure 1, saline alone did not induce a significant increase in PMN number. In contrast, LPS induced a marked PMN influx, which significantly rose at 3 hours, peaked between 6 and 24 hours, and declined to near basal levels by 72 hours. Cells other than PMNs were mainly AMs and a small percentage, 0.5 to 4%, of lymphocytes and/or monocytes. These findings are similar to those reported by Ulich et al.[5] and Pauwels et al.[19]

LPS-Induced TNF-α mRNA Expression by Northern Blot Analysis

TNF-α mRNA expression was first examined in total RNA preparations from whole lung tissues. Figure 2A shows that whereas saline induced little message for TNF-α in tissues, LPS strikingly induced TNF-α mRNA expression by as early as 30 minutes, which peaked at about 1 hour, remained at high levels at 3 and 6 hours, dropped by 12 to 24 hours, and became undetectable by 48 hours after LPS challenge.

Furthermore, cells in pooled BAL fluids recovered from rats at 6 and 12 hours after LPS exposure were fractionated, and similar amounts of total RNA (10 μg) were recovered from the purified AMs (6 to 7 × 10^6) and PMNs (35 to 38 × 10^6) and analyzed for TNF-α mRNA. Figure 2B shows the autoradiograph and quantified expression of TNF-α mRNA after normalization to actin mRNA as assessed by densitom-

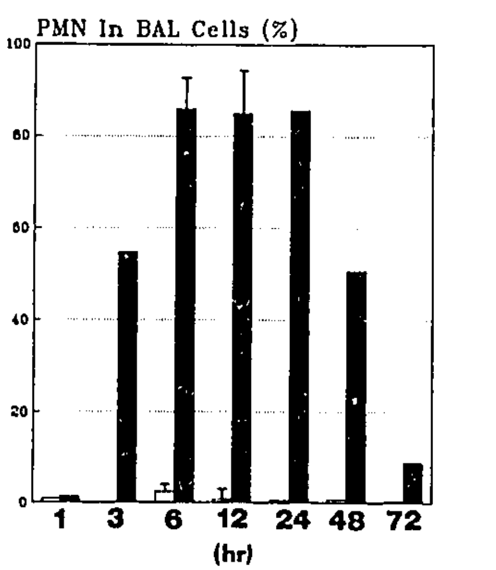

Figure 1. *Kinetics of percentage PMNs in BAL cells collected from rats at different times after instilled with 0.3 ml of saline (open bar) or saline + LPS (filled bar). Differential cell counts were determined with two Diff-Quik stained cytospins for each time point. Results for 6 and 12 hours represent mean ± SD of six rats.*

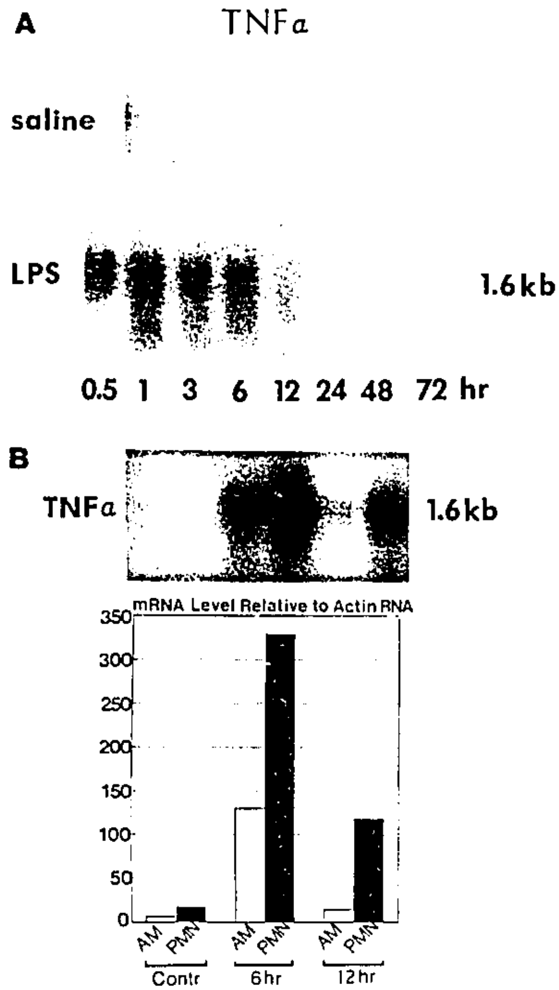

Figure 2. A: *Kinetics of TNF-α mRNA expression in total lung tissues of rats intratracheally instilled with 0.3 ml of saline or saline + LPS (6 μg/rat). Equal loading of total RNA (25 μg/lane) was confirmed by monitoring 28s and 18s ribosomal RNA.* B: *TNF-α mRNA levels in AM and PMN fractions of BAL cells from six rats challenged with LPS for 6 or 12 hours. In the top autoradiograph of Northern hybridization, from left,* lanes 1 *and* 2: *AMs and peripheral blood* PMNs *from saline-treated rats at 6 hours (control). These cells were all subjected to Ficoll-Paque fractionation before RNA extraction.* Lanes 3 *and* 4: *BAL AMs and BAL PMNs purified by Ficoll-Paque gradient from BAL fluids of LPS-treated rats at 6 hours.* Lanes 5 *and* 6: *BAL AMs and BAL PMNs from LPS-treated rats at 12 hours. The bottom graph represents densitometric measurement of TNF-α mRNA relative to actin mRNA, expressed as arbitrary densitometric units.*

etry. Both AMs and PMNs obtained at 6 hours expressed a significant signal for TNF-α that decreased dramatically at 12 hours, particularly in AMs. PMNs demonstrated about 3 and 8 times more TNF-α mRNA, relative to actin mRNA, than AMs at 6 and 12 hours, respectively.

Localization of TNF-α mRNA in LPS-Challenged Lung Tissue by ISH

TNF-α mRNA expression was further examined in tissue sections by ISH. Only a minimal amount of positive reaction was found in lung tissues of saline-treated rats at any given time point. In contrast, strong positivity was detected in lung tissues from LPS-treated rats between 0.5 to 12 hours. The signal diminished significantly by 24 and 72 hours. The major type of cells positive for TNF-α mRNA was PMNs, and most of these positive cells were located in the parenchyma near bronchioles and small blood vessels. The majority of intravascular PMNs were noticeably negative. This internally distinct distribution of positive reactions illustrates the specificity of the signal. Representative results at 6 hours are shown in Figure 3, A to D.

Localization of TNF-α Protein in LPS-Challenged Lung Tissue by IH

To detect TNF-α protein in the tissue, lung sections were analyzed by IH. Only a minimal number of AMs were found positive for TNF-α antigen in tissues from saline-treated animals. At 1 hour after LPS exposure, a significant number of AMs were found positive for TNF-α, whereas most PMNs infiltrating the vessels were negative. By 3 hours, positive reactions were seen not only in the scattered AMs but also in PMNs infiltrating the vascular and bronchial walls. From 6 to 12 hours, PMNs seemed to be the predominant cell type stained. Indeed, over 90% of all intraalveolar positive cells were PMNs, and approximately 50% of all infiltrating PMNs were positive for TNF-α. The proportion of PMNs localizing TNF-α decreased by 24 hours. Only faint staining was seen in the tissue at 48 and 72 hours. Figure 3, E to H, shows representative TNF-α immunochemical staining in lung tissues obtained at 6 hours.

Discussion

Historically, PMNs have been viewed as terminally differentiated cells incapable of active RNA and protein synthesis. Recent *in vitro* data, however, have called attention to their potential upon stimulation to express a range of cytokines including TNF-α (reviewed in ref. 20). In support of these *in vitro* observations, our study provides the first *in vivo* evidence that PMNs act as a significant source of TNF-α in the tissue during acute lung inflammation elicited by LPS (6 μg/rat). The significant capability of PMNs to express TNF-α mRNA was shown not only in PMNs from BAL by Northern analysis but also in PMNs present in the tissue by ISH. Concomitant with the enhanced gene expression was TNF-α protein expression by PMNs in the tissue as detected by IH.

Our data show that TNF-α mRNA in total lungs increased markedly as early as 30 minutes after LPS exposure, peaked at about 1 hour, remained at high levels at 3 and 6 hours, and decreased between 12 and 24 hours. Thus, the marked expression of TNF-α mRNA preceded the peak PMN influx into the tissue that occurred between 6 and 24 hours, suggesting a temporal correlation between TNF-α expression and PMN infiltration, possibly in part due to release of neutrophil chemotactic cytokines such as IL-8 by stromal cells upon stimulation by TNF.[21]

The major cellular sources of TNF-α in the lung include AMs and PMNs. Our observation that AMs significantly expressed TNF-α product at early times, before the massive PMN infiltration (6 to 24 hours) after LPS challenge, suggests that AMs might serve as an important trigger in the ensuing PMN response, partly through TNF-α production. However, as this inflammatory process proceeded, characterized by increasing numbers of PMNs in the tissue, PMNs also became a potent source of TNF-α. Our data by Northern blot analysis show that at 6 hours after LPS stimulation, PMNs purified from pooled BAL fluids expressed a greater amount, relative to actin mRNA, of TNF-α mRNA than AMs. By 12 hours, whereas it dropped markedly in AM population, TNF-α mRNA expression still remained at a relatively high level in PMNs. Furthermore, by ISH, we localized TNF-α mRNA chiefly to PMNs, mostly those located within the tissue near vessels and bronchioles, suggesting that these locations might represent where PMNs became most activated. The fact that the majority of AMs in the tissue were negative by ISH likely reflects an overall lower sensitivity of this technique as compared to Northern blot analysis. Consistent with PMN expression of TNF-α mRNA, PMNs in the tissue localized TNF-α protein as shown by IH, and this protein expression emerged markedly in PMNs by 3 hours and became predominant between 6 and 12 hours after

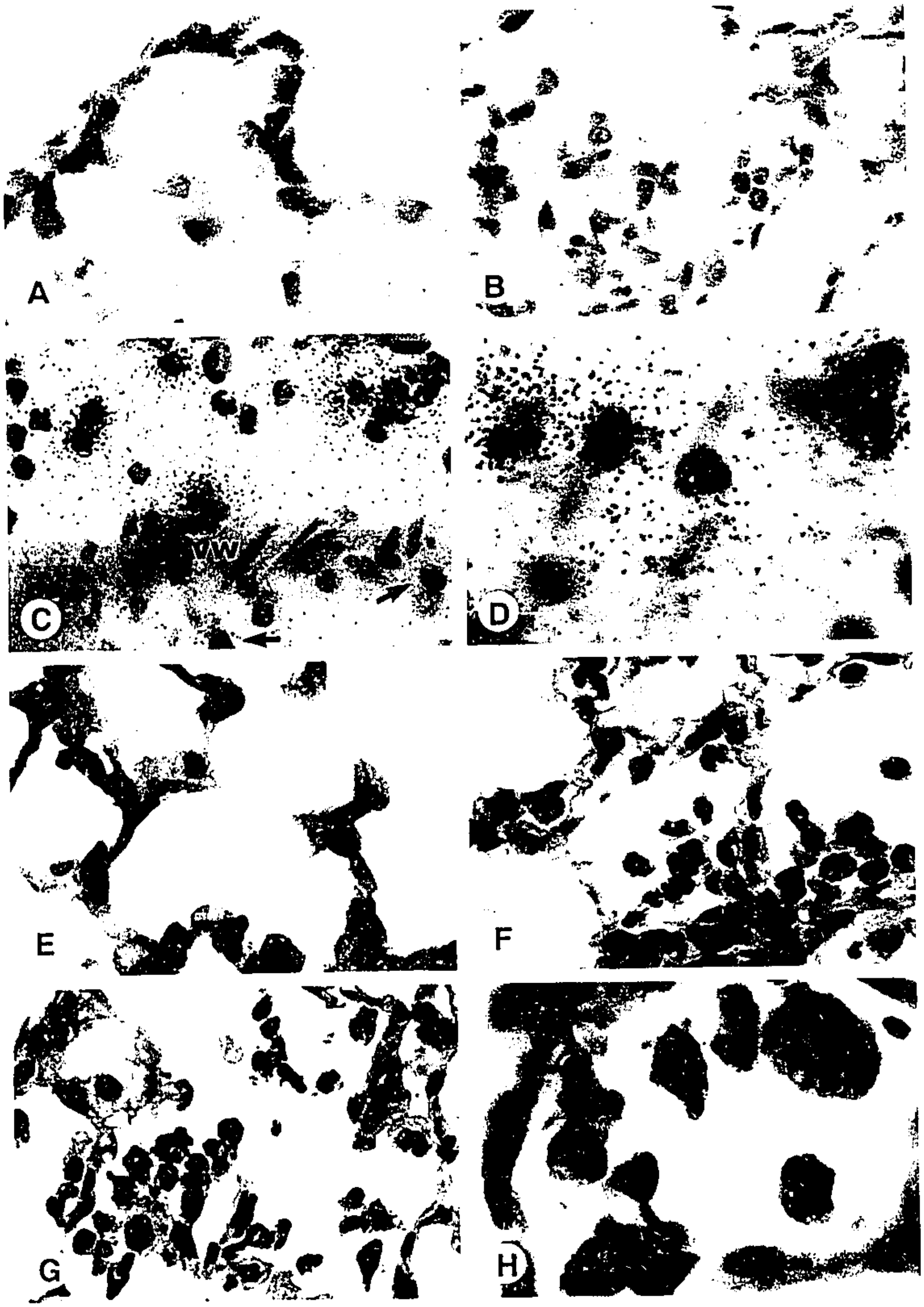

Figure 3. *Localization of TNF-α mRNA at 6 hours by in situ hybridization in saline-treated lung* (A)*, LPS-treated lung hybridized with a negative control DNA probe* (B)*, and LPS-treated lung hybridized with specific TNF-α cDNA probe* (C *and* D)*. Arrows indicate some of intravascular PMNs negative for TNF-α mRNA. VW, vascular wall. Immunochemical localization of TNF-α protein at 6 hours in saline-treated lung stained with anti-mTNF-α* (E)*, LPS-treated lung stained with nonimmune rabbit serum* (F)*, and intraalveolar cells in LPS-treated lung stained with anti-mTNF-α* (G *and* H)*. Slides for in situ hybridization and immunochemistry were counterstained with H & E and hematoxylin, respectively. (Magnification:* A *to* C, E *to* G: 250×; D *and* H: 600×.)

LPS challenge. In support of these findings, using a cytotoxicity bioassay for TNF, we detected significantly increased amounts of TNF protein in BAL fluids, on average, 400 pg/ml at 1 hour and 200 pg/ml BAL at 6 hours after LPS challenge. All of these data suggest that not only TNF-α mRNA expression but also bioactive protein release were enhanced, and that AMs at early times (1 to 3 hours) and PMNs at later times (6 to 12 hours) seemed to be significant sources for TNF-α expression.

TNF-α has been shown to release interleukin-8 (IL-8) from a variety of tissue cells, enhance adhesion of PMNs to endothelium, and induce PMN degranulation as well as oxygen radical release.[2] In addition, TNF-α stimulates PMNs to express IL-8,[22] IL-6,[23] and IL-1β.[24] Thus, TNF-α may play a pivotal role in recruiting and activating PMNs. PMNs, upon emigrating into the lung, become the most numerous and widely distributed inflammatory cell and an even greater source of TNF-α by themselves. PMN-derived TNF-α may exert self-amplifying effects on its own accumulation and function including the release of other cytokines such as IL-8. In the context of acute inflammation, the short life span of PMNs conforms in fact to the nature of this type of inflammatory response, thus rendering cytokine release by PMNs a rapid disappearance upon removal of the insult from the tissue. In addition, PMNs may also have the advantage over resident macrophages like AMs in that the continuous influx of PMNs provides a pool of the newly arrived PMNs for LPS stimulation, whereas the resident macrophage may become refractory after the initial exposure to LPS. This may explain why PMNs exhibited a more sustained level of TNF-α mRNA than AMs between 6 and 12 hours.

The demonstration of significant expression of TNF-α mRNA by PMNs in the lung is in line with a recent finding by Ulich et al by Northern blot analysis that PMNs from BAL contained more abundant mRNA for IL-1α/β than AMs at 6 hours after intratracheal injection of LPS.[25] In addition, our preliminary results show that PMNs from LPS-challenged lung tissues were also a dramatically greater source of the mRNA for macrophage inflammatory protein-2, a functionally murine equivalent of human IL-8 (not shown). Whereas all of these data have shown that PMNs outside the circulation and within the lung tissue can be a potent source of cytokines upon encounter with airway-derived LPS, a recent report by Williams et al shows that PMNs rather than mononuclear cells in the pulmonary vasculature were the major contributor to IL-1β message detected in total vascular leukocytes upon intravenous LPS infusion.[26] Taken together, all of these data suggest that PMNs may operate, particularly in acute inflammatory responses, as a prominent source of cytokines in the tissue, and the scrutiny of the relative *in vivo* contribution of PMNs versus monocytes/macrophages to the expression of a given cytokine is of important value, considering the presence of the vast number of infiltrating PMNs in acute inflammatory settings.

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Chapter six

LPS INDUCTION OF CYTOKINES IN THE AIRWAYS IN VIVO (2)

The following article entitled "Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor α, macrophage inflammatory protein-2, interleukin-1ß and interleukin-6, but not RANTES or transforming growth factor $ß_1$ mRNAs expression in acute lung inflammation" has been published in The American Journal of Respiratory Cell and Molecular Biology. It represents the second part of the study on the in vivo effect of LPS. Having documented that LPS evoked an early TNFα response preceding the peak neutrophil influx into the lung and that both alveolar macrophages and infiltrating neutrophils were sources of this proinflammatory cytokine (chapter five), the following paper examined the kinetics and sources of several other cytokines including MIP-2, IL-1ß, IL-6, RANTES and TGF$ß_1$ by means of Northern blot analysis and in situ hybridization.

This study was carried out and the paper was written by the author of the thesis. Among other co-authors, Drs. K.E. Driscoll and T.J. Schall provided plasmid DNA preparations for MIP-2 and RANTES cDNA purification; Dr. H. Kirpalani participated in in situ hybridization; Drs. M. Jordana and J. Gauldie provided supervision.

Cytokine Expression by Neutrophils and Macrophages *In Vivo*: Endotoxin Induces Tumor Necrosis Factor-α, Macrophage Inflammatory Protein-2, Interleukin-1β, and Interleukin-6 but Not RANTES or Transforming Growth Factor-β_1 mRNA Expression in Acute Lung Inflammation

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Using a rat model of acute lung inflammation induced by intratracheal instillation of lipopolysaccharide (LPS), we investigated the kinetics of mRNA expression and the potential cellular sources of tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-2 (MIP-2), interleukin (IL)-1β, IL-6, RANTES, and transforming growth factor-β_1 (TGF-β_1). By Northern blot analysis, TNF-α and MIP-2 mRNAs in total lung tissue increased markedly by 30 min and peaked by 1 h after LPS exposure, whereas expression of IL-1β and IL-6 was not detected until 1 h and peaked within 6 h. In contrast, neither RANTES nor TGF-β_1 mRNA was induced by LPS throughout 72 h, although a basal expression was detected in both saline- and LPS-treated lung tissues. At 1 h after LPS, the bronchoalveolar lavage (BAL) fluid contained about 98% alveolar macrophages (AM), whereas by 6 or 12 h, 88% of BAL cells were polymorphonuclear neutrophils (PMN). Upon extraction of total RNA after separation of AM from PMN in BAL, Northern analysis showed that at 1 h, AM expressed pronounced signals for TNF-α, MIP-2, IL-1β, and IL-6. At 6 and 12 h, however, while cytokine transcripts decreased in AM, PMN exhibited strong signals for these cytokines. A low basal noninducible signal for TGF-β_1 but not RANTES was detected in both AM and PMN. Finally, by *in situ* hybridization techniques, PMN in the lung tissue, particularly those located in the vicinity of the bronchiole and vasculature, were demonstrated to localize MIP-2 mRNA. Thus, our data provide strong evidence that LPS *in vivo* induces a selective cytokine response in the lung, and AM and PMN represent different temporal and cellular waves of cytokine expression during the course of acute inflammation.

Endotoxin (lipopolysaccharide; LPS) induces acute lung injury in endotoxic sepsis and Gram-negative pneumonia, two conditions marked by the activation of alveolar macrophages (AM) and massive tissue infiltration of neutrophils (PMN), often leading to the adult respiratory distress syndrome (1). The molecular mechanisms underlying LPS-induced lung inflammation still remain poorly understood. Although *in vitro* evidence suggests that cytokines from effector cells, primarily AM (2) and possibly PMN (3), play an important role, little is known about the kinetics of expression of cytokines and the relative contribution of AM and PMN to the presence of these mediators *in vivo*.

Tumor necrosis factor-α (TNF-α) and interleukin (IL)-1 are potent proinflammatory cytokines that trigger recruitment of inflammatory cells by inducing expression of adhesion molecules and chemokines such as IL-8 (3) and RANTES (4) within the tissue. Macrophage inflammatory protein-2 (MIP-2), a murine functional equivalent of human IL-8, is a potent chemoattractant for PMN (5), whereas RANTES is chemoattractive to T cells, monocytes, and eosinophils (6, 7). IL-6 has been well recognized for its effects on induction of the acute phase proteins (8) and may exert anti-inflammatory activities at local sites of inflammation (9). Transforming growth factor-β (TGF-β) family members are cytokines with diverse bioactivities including chemoattractive activity for monocytes and enhancement of tissue repair (10).

We have previously reported that intratracheal administration of LPS in rats induces a massive neutrophilic response and marked TNF-α expression in the lung (11). In the current study, we sought to explore the kinetics of expression and the potential cellular sources of neutrophil chemotactic

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Abbreviations: alveolar macrophages(s), AM; bronchoalveolar lavage, BAL; interleukin, IL; *in situ* hybridization, ISH; lipopolysaccharide, LPS; macrophage inflammatory protein-2, MIP-2; polymorphonuclear neutrophil(s), PMN; transforming growth factor-β, TGF-β; tumor necrosis factor-α, TNF-α.

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factor MIP-2 along with IL-1β, IL-6, RANTES, and TGF-β_1, in the LPS model of acute lung inflammation. Our results demonstrate for the first time that PMN are a significant source of MIP-2 message in the tissue, and that LPS elicits a selective cytokine profile of expression to which the resident AM and infiltrating PMN contribute differently in a temporal manner depending on the relative abundance of these cells in the tissue during the course of acute inflammation.

Materials and Methods

Animal Model

A rat model of LPS-induced acute lung inflammation has been previously established and characterized (11). Briefly, Sprague-Dawley rats weighing 220 to 240 g (Charles River Laboratories, Ottawa, Canada) were anesthetized with ether, and 0.3 ml of pyrogen-free saline (control) or LPS (055:B5 *Escherichia coli*, 25 μg/kg; Sigma Chemical Co., St. Louis, MO) was intratracheally instilled. The rats were killed at 0.5, 1, 3, 6, 12, 24, 48, and 72 h. Bronchoalveolar lavage (BAL) was performed in some rats. In separate experiments, the unperfused left lung was snap-frozen in liquid nitrogen for tissue RNA extraction, and the right lung was fixed by perfusion with 4% paraformaldehyde for *in situ* hybridization (ISH).

BAL PMN and AM Purification

BAL was performed as previously described (12). BAL fluids were collected and kept on ice. Differential cell counts from individual rats were determined on Diff-Quik-stained cytospin slides. The BAL fluids collected from several rats at 1, 6, or 12 h after exposure to LPS were then pooled, and the total cells were separated into AM and PMN fractions by a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). On average, the subsequent Ficoll-Paque gradient centrifugation resulted in an AM fraction containing 90% AM and 10% PMN and a PMN fraction containing 97% PMN and 3% AM. The contamination of mononuclear cells other than AM, if any, was marginal, ranging from 0 to 1.5%. As controls, AM and PMN were isolated from BAL and peripheral blood, respectively, of rats 6 h after saline exposure by gradient centrifugation. BAL from rats exposed to saline alone contained very few PMN at any given time (0 to 2%). Purified AM (6×10^6) and PMN (35×10^6) fractions were further subjected to total cellular RNA extraction.

RNA Preparation and Northern Blot Hybridization

The procedure for total tissue or cellular RNA extraction and Northern blot hybridization has been previously described in detail (13). Hybridization of Northern gels was performed at 55°C sequentially with rat MIP-2, IL-1β (12), IL-6 (12), murine RANTES (6), porcine TGF-β_1 (14), and chicken β-actin cDNA probes, radiolabeled with α-[^{32}P]dCTP by random priming (Pharmacia). A very high homology of 98% exists at the nucleotide level between murine and rat RANTES (T. J. Schall, personal communication). TGF-β_1 possesses the highest homology across different species of all known cytokines (2), and the cDNA probe for this cytokine has been extensively shown to cross-react between species (14, 15). The rat MIP-2 cDNA probe was a 450 bp Hind III to EcoRI fragment from pBluescript II KS +/−, provided by Dr. K. E. Driscoll. The murine RANTES cDNA probe was a 300 bp Hind III to EcoRI fragment from pCR 1000, provided by Dr. T. J. Schall. The β-actin probe (control) was purchased from Oncor (Gaithersburg, MD). The blots were stringently washed and exposed to Kodak XAR film. The intensity of messages was determined by densitometric quantification, relative to the "housekeeping" β-actin mRNA.

In Situ Hybridization

ISH was performed as previously described with some modifications (14, 16). Briefly, tissue sections (2 to 3 μm thick) mounted on 3-aminopropyl triethoxysilane-coated slides were deparaffinized in xylene and rehydrated in graded ethanol. After treatment with 0.25% acetic anhydride, 0.1 M glycine, and 2× SSC, slides were dehydrated through graded ethanol. Hybridization was performed at 42°C overnight in a buffer containing 0.2× SSC, 50% formamide, 0.5× Denhardt's solution, 10 mM dithiothreitol, 1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, and the MIP-2 cDNA probe or a 500 bp negative control DNA probe (prepared from a DNA ladder) radiolabeled with [^{35}S]dCTP (5×10^5 cpm/slide). Slides were then washed sequentially at 42°C in 50% formamide in 1× SSC for 30 min and in 0.2× SSC for 10 min twice, and at room temperature in 0.1× SSC for 10 min. For autoradiography, slides were coated with Kodak NTB-2 emulsion and autoradiographed for 8 days. After development and fixation, slides were counterstained with hematoxylin-eosin. In addition to the use of saline-treated tissues and a 500 bp DNA probe as controls, some slides were also pretreated with 100 μg/ml RNase A in 2× SSC at 37°C for 1 h before hybridization.

Results

Kinetic Expression of Cytokine mRNAs in Total Lung Tissue by Northern Blot Analysis

To determine cytokine profile and kinetics, total lung tissues of rats exposed to saline or LPS were analyzed for TNF-α, MIP-2, IL-1β, IL-6, RANTES, and TGF-β_1 messages by Northern blot analysis. As shown in Figure 1, saline alone did not induce significant signals for any cytokine. In contrast, the messages for MIP-2 and TNF-α rapidly increased in lung tissues as early as 30 min after exposure to LPS, peaked at about 1 h, remained at high levels at 3 and 6 h, and decreased between 12 and 24 h. The expression became undetectable by 48 h. The kinetics of LPS-provoked IL-1β and IL-6 expression differed from those of MIP-2 or TNF-α. The IL-1β mRNA was not markedly induced at 30 min and failed to reach a maximum until 6 h, falling off to basal levels by 48 h after LPS stimulation, whereas IL-6 expression was undetectable at 30 min but emerged and peaked by about 1 h, gradually declining back to control levels by 48 h. Both RANTES and TGF-β mRNAs were found noninducible by LPS over the period of observation, but a low basal expression was detected in both saline- and LPS-treated lung tissues.

Kinetic Expression of Cytokine mRNAs in BAL AM and PMN by Northern Blot Analysis

To investigate the potential cellular sources of MIP-2, as well as other cytokines in LPS-challenged lung tissues, BAL fluids were collected and pooled from lungs of six rats at 1,

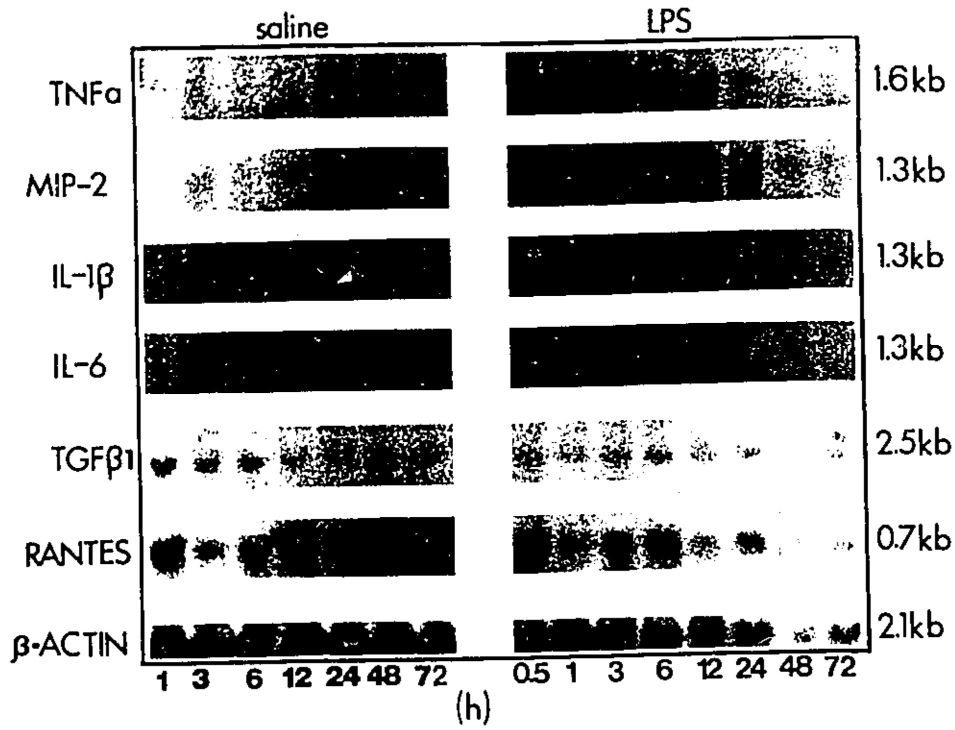

Figure 1. Kinetic expression of TNF-α (reference 11), MIP-2, IL-1β, IL-6, TGF-β_1, and RANTES mRNAs in total lung tissues of rats (one rat per time point) intratracheally instilled with 0.3 ml of saline or LPS (25 μg/kg). β-actin mRNA shows the input of total RNA samples.

6, and 12 h after exposure to LPS. Cells in BAL were fractionated into AM and PMN, from which total RNA was extracted. Similar quantities (10 to 12 μg) of RNA were recovered and analyzed for gene expression of various cytokines by Northern blot analysis. Figure 2A shows that while control AM or peripheral blood PMN displayed little message for cytokines, BAL cells obtained at 1 h after LPS exposure (containing 98% AM, Figure 3A) expressed strong signals for TNF-α, MIP-2, IL-1β, and IL-6. At 6 or 12 h after LPS, 13 to 22 × 10^6 of total cells were recovered from BAL of each rat and 85 to 90% of total BAL cells were PMN and 10 to 15% were AM (Figure 3B). At 6 h, both AM and PMN exhibited significant expression of mRNA for TNF-α, MIP-2, IL-1β, and IL-6, but PMN expressed several times more, relative to actin mRNA, than AM (Figures 2A and 2B). At 12 h, the signals decreased markedly in AM but were still sustained in PMN (Figures 2A and 2B). In contrast to LPS induction of TNF-α, MIP-2, IL-1β, and IL-6 mRNAs, a relatively consistent basal expression for TGF-β_1 was found in both AM and PMN. Meanwhile, RANTES mRNA was not significantly expressed in any of these cell populations (Figure 2A).

Localization of LPS-induced MIP-2 mRNA in Lung Tissue by ISH

To localize cytokine mRNA expression in LPS-challenged lung tissue, we chose to analyze MIP-2 mRNA expression in tissue sections by means of ISH because of its direct role in eliciting the neutrophilic response (5). Little positivity for MIP-2 mRNA was found in lung tissues of rats after saline exposure (not shown). In contrast, a significant positivity was present in lung tissues after LPS challenge, detectable as early as 30 min and markedly decreased by 48 h in PMN and AM. At 6 and 12 h, the majority of positive cells were PMN (> 90%), particularly those near or surrounding the bronchiole and vasculature. Neutrophils within the vasculature were, on the most part, negative. Hybridization with the negative control DNA probe similar to the MIP-2 cDNA probe in size resulted in little reaction, and pretreatment of tissue sections with RNase A abolished the signal. These data, in conjunction with the lack of a positive reaction in saline-treated tissues and the distinct distribution pattern of reaction, serve to demonstrate the specificity of the message. Figures 3C through G show representative results obtained at 6 and 12 h.

Discussion

In this study, we demonstrate that LPS triggers a distinct cytokine response in the lung, selectively inducing TNF-α, MIP-2, IL-1β, and IL-6 but not RANTES or TGF-β_1 mRNAs, and that the cellular sources for these cytokine signals include AM and PMN. At the time (1 h) when only a minimal PMN infiltration is present, AM appear to be the predominant source of the cytokines examined, whereas at later times (6 and 12 h) when PMN infiltration becomes maximal (88% PMN), PMN become a significant source of these cytokines.

To our knowledge, this is the first report to demonstrate that PMN can significantly express the message for a neutrophil chemoattractive factor, MIP-2, in the tissue during acute inflammation. Although at this point, we have not been able to provide direct evidence of MIP-2 protein expression by PMN, since there is no anti-rat MIP-2 reagent available yet, we have previously shown that PMN serve as a significant source for both TNF-α mRNA and protein in the same model of LPS-elicited acute lung inflammation (11), suggesting that those PMN positive for MIP-2 mRNA might also express the MIP-2 protein. Together, these observations pro-

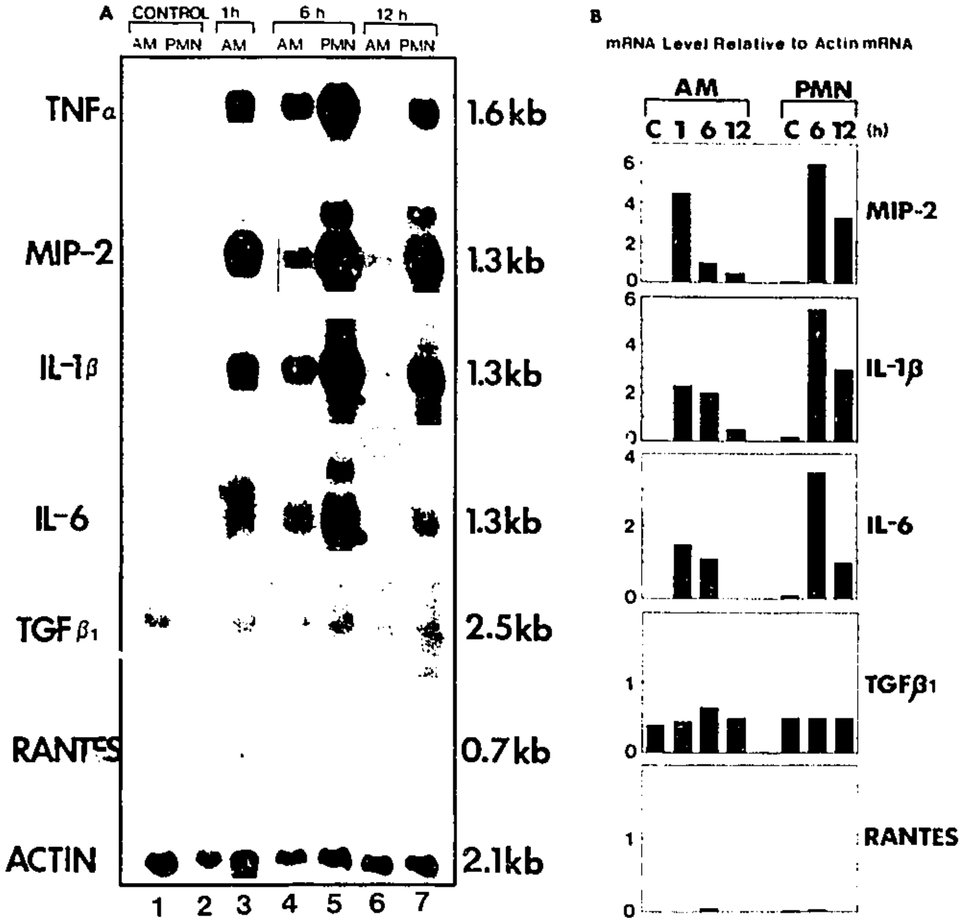

Figure 2. A. Kinetic expression of TNF-α (in part from reference 11), MIP-2, IL-1β, IL-6, TGF-β_1, and RANTES mRNAs in fractionated AM and PMN populations of BAL cells from rats exposed to LPS (25 μg/kg). Lane 1: control AM from BAL of saline-treated rats at 6 h (C); lane 2: control PMN from peripheral blood of saline-treated rats at 6 h (C); lane 3: AM from BAL of LPS-treated rats at 1 h; lane 4: AM from BAL of LPS-treated rats at 6 h; lane 5: PMN from BAL of LPS-treated rats at 6 h; lane 6: AM from BAL of LPS-treated rats at 12 h; lane 7: PMN from BAL of LPS-treated rats at 12 h. B. Densitometric quantification of messages in panel A. The TNF-α mRNA levels are similar to those of MIP-2 (not shown). The intensity of messages is expressed as arbitrary densitometric units relative to β-actin mRNA.

vide *in vivo* evidence to support the notion (3) that PMN can no longer be regarded as a terminally differentiated cell incapable of active RNA and protein synthesis, but in contrast, because of their constant migration into and wide distribution within the tissue in large numbers, PMN may actively regulate inflammatory processes via *de novo* cytokine expression. The actual contribution of PMN versus AM to cytokine expression depends on the relative abundance of these cellular components during the course of acute inflammation. Our data indicate that at the time prior to the marked PMN influx, AM appear to play a crucial role in initiating neutrophilic response via expression of TNF-α, IL-1, and MIP-2. Thereafter, the infiltrating PMN became the prominent source of these cytokines, likely in response to TNF-α and LPS (17), suggesting an important direct auto-recruiting and auto-activating mechanism.

Both MIP-2 and RANTES are members of a closely related chemokine superfamily. By the relative positioning of the first two conserved cysteine residues, MIP-2 falls into "C-X-C" subfamily and RANTES into "C-C" subfamily, chemoattractive to distinct inflammatory cell types (7, 18). A variety of cells seem to express these two chemokines. For instance, MIP-2 mRNA was induced in rat AM by LPS and in whole trachea tissues and total BAL cells after *in vivo* exposure to LPS (19) or mineral dusts (20), whereas RANTES mRNA was constitutively expressed by cultured rat T cells or monocytic cells (6). Our results showing that MIP-2 but not RANTES mRNA was induced by LPS in lung tissues and in BAL AM and PMN, as shown by both Northern analysis and ISH, illustrate a selective induction of chemokine family members by LPS *in vivo*. It suggests that the regulation of these chemokines is under tight control, perhaps dependent on the nature of stimulus, and what is subsequently "turned on" defines the type of infiltrating inflammatory cells in the tissue. In addition, the early marked induction of MIP-2 (0.5 to 6 h) prior to maximal PMN influx (6 to 24 h) suggests a role for this chemokine in directly recruiting PMN into the tissue. The fact that saline- or LPS-treated rat lung tissues, but not BAL AM or PMN, constitutively expressed the message for RANTES implies that RANTES may possess other as yet unidentified physiologic function(s) and can be expressed by cells other than AM or PMN in the lung.

In addition to MIP-2, our data also show an induction of IL-1β mRNA by LPS in total lung tissu s and in isolated AM and PMN from BAL. The ability of M in BAL fluids of LPS-challenged lung to express IL-1 ..anscripts has been reported previously by Ulich and colleagues (21). We further demonstrate here that expression of IL-1β mRNA by the lung tissue does not significantly increase or peak until 1 and 6 h, respectively. This is in contrast to TNF-α (11) and MIP-2 expression, which increased strikingly and peaked at 30 min and 1 h, respectively, well preceding the later marked influx of PMN seen at 3 h. This suggests that IL-1 may play a less important role in initiating neutrophil response in this model,

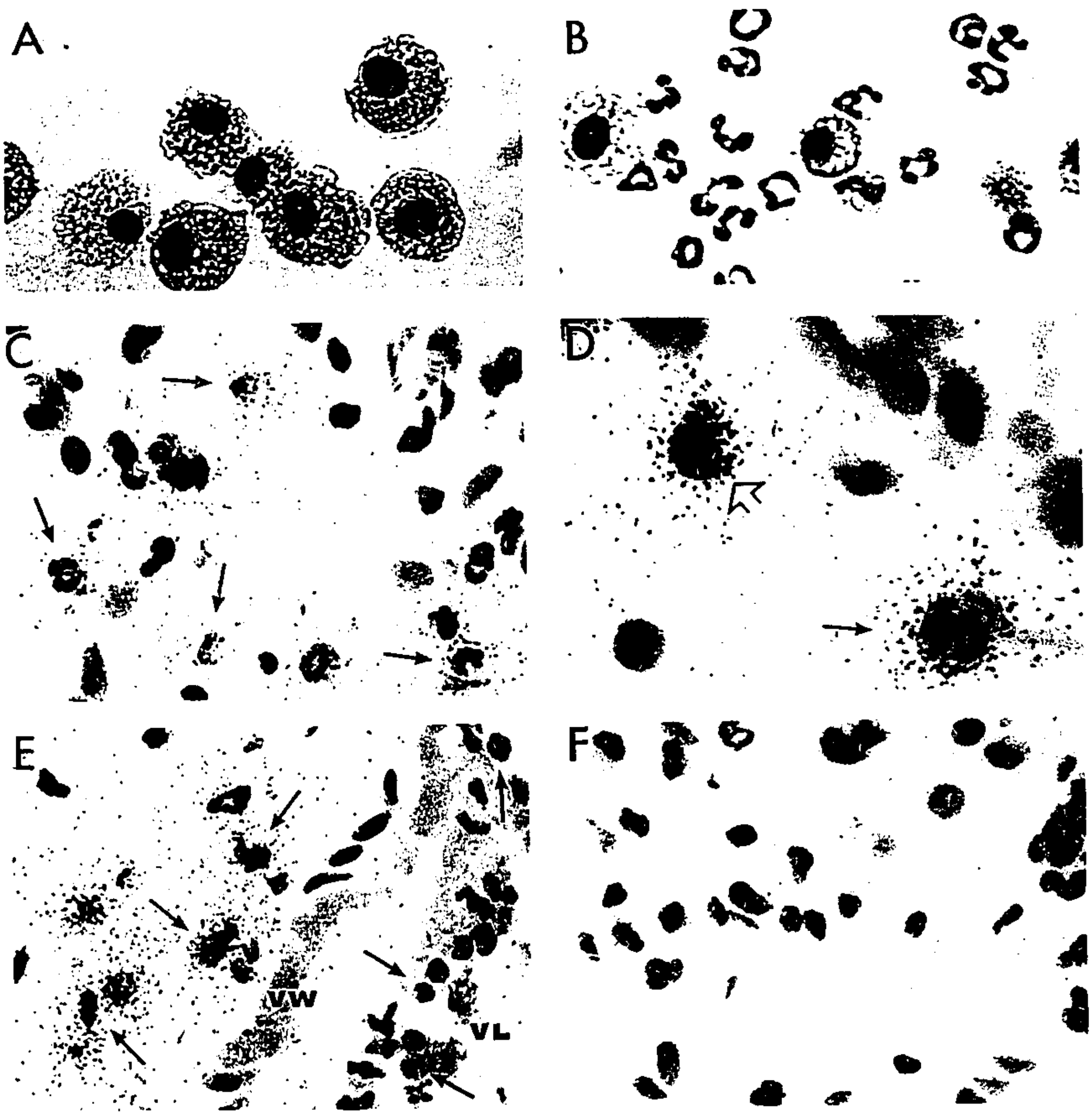

Figure 3. Diff-Quik-stained BAL cells of LPS-treated rats at 1 h (A) and 6 h (B) and localization of MIP-2 mRNA in LPS-treated rat lung tissues at 6 h (C, D) and 12 h (E) by *in situ* hybridization. C through E: hybridized with MIP-2 cDNA probe (*open arrow*: macrophage; *solid arrows*: some of neutrophils). VW: vascular wall; VL: vascular lumen. Note intravascular negative neutrophils. F: as a control, section C pretreated with RNase A and then hybridized with MIP-2 cDNA probe; G: as a control, section C hybridized with the negative control DNA probe. (C through G were counterstained with hematoxylin-eosin; magnification: A through C and E through G: ×250; D: ×640.)

in support of the findings from other models of lung inflammation that TNF-α appears to play a significant primary role in the initiation of the response (22–24).

The *in vivo* IL-6 mRNA expression by PMN at tissue sites has not been previously reported. Here, we demonstrate a remarkable expression of IL-6 mRNA by PMN in BAL of rats exposed to LPS, in support of the recent finding that the circulating PMN of septic mice immunolocalized IL-6 (25). Although IL-6 has long been known for its effect on the induction of acute phase proteins during acute inflammation, its role at the local site of acute inflammation remains speculative. However, recent *in vitro* data suggest that IL-6 may act as an antiinflammatory molecule, perhaps by inhibiting expression of proinflammatory cytokines such as TNF-α (26) and enhancing neutrophil apoptosis (27). Furthermore, administration of IL-6 was shown to reduce the number of PMN in BAL of rats after intratracheal instillation of LPS (28). Hence, in our model, IL-6 may be involved in the self-limiting process of the response. Nonetheless, we cannot rule out the possibility that other inhibitory cytokines such as IL-4 or IL-10 are also involved. Interestingly, the other cytokine, TGF-β_1, also an anti-inflammatory molecule (10), was not significantly induced in either total lung tissue or purified AM and PMN in response to *in vivo* LPS challenge. This differs from the elevated expression of TGF-β_1 mRNA seen in lungs exposed to bleomycin (23) or cadmium chloride (29) or in AM within the lung tissue undergoing idiopathic pulmonary fibrosis (30). This demonstrates a temporal disassociation of TNF-α and TGF-β_1 mRNA induction, in contrast to the concurrent induction of TNF-α and TGF-β mRNAs observed in animal models of lung fibrosis (23, 29). It is notable that in the model shown here LPS only triggers a self-limiting inflammatory response without inducing a fibrotic response. Thus, it appears that the type of cytokines induced may dictate the type of tissue response and the early expression of TNF-α and perhaps other proinflammatory cytokines does not always result in TGF-β induction and subsequent matrix deposition. The destiny of a given inflammatory reaction may involve a puzzling interaction of multiple pro- and anti-inflammatory mediators, depending upon the nature of the primary insult.

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Chapter seven

SUMMARY

LPS is a major component of the outer membrane of gram-negative bacteria, responsible for a number of critical conditions in the airways, including gram-negative pneumonia and ARDS associated with gram-negative sepsis. These conditions are characterized by the overwhelming influx of PMNs into the airway tissue. The molecular mechanisms underlying this process remain to be fully understood. While cytokines have been recently implicated in LPS-induced inflammatory response in the airways, the cellullar sources, kinetics and regulation of cytokine expression remain to be fully clarified. A series of studies, described in the preceding chapters, were carried out to examine the in vitro effect of LPS on cytokine expression by the alveolar macrophage, a powerful resident defense cell in the airways, and by the fibroblast, the largest structural cell population in the airways. Furthermore, an extensive investigation was conducted to investigate the effect of LPS on cytokine response in the airways in vivo using a rat model.

The data from these studies demonstrate that while in vitro freshly isolated unstimulated alveolar macrophages do not express detectable amounts of cytokine mRNAs, LPS can induce these cells to produce cytokines including IL-1ß and IL-6. This finding supports the notion that cells do not express detectable amounts of cytokines, especially proinflammatory cytokines, under normal circumstances, and that the synthesis of cytokines represents a cellular response to perturbation. The data further show that the cytokine response of alveolar macrophages to LPS can be modulated by the nature of extracellular substrates. Macrophages maintained on fibroblast-derived ECM appear to retain optimal responsiveness to LPS over a longer period of time in culture compared to those cultured on plastic or a collagen membrane. This implies that qualitative or quantitative alterations in matrix components may influence the cellular responses to exogenous stimuli in vivo. Damage to the ECM and changes in the ratio of ECM components during the course of inflammatory responses in the airways have been well documented. A number of elements including proteinases, proteinase inhibitors and matrix-producing cells such as fibroblasts participate in ECM regulation (Campbell et al, 1987). The mechanisms by which the ECM regulates cellular responses are far from clear, but presumably they involve a second messenger response initiated by adhesion receptor and ligand interaction, or mimicry of adhesion receptor ligation to cytokine receptor ligation, changes in cell shape which may activate strech-sensitive ion channels, or reorganization of cytoskeleton (Nathan and Sporn, 1991). In this regard, alveolar macrophages cultured on fibroblast-derived ECM spread to a much greater degree than those on plastic as revealed by scanning electron microscopy (Appendix one).

Activation of monocytes/macrophages may be affected not only by insoluble ECM components but also by soluble mediators produced by structural cells such as fibroblasts. This notion is supported by another series of studies presented in this thesis. It was found that airway fibroblast- or epithelial cell-derived GM-CSF and M-CSF promoted survival and differentiation, but not proliferation, of monocytes, thus demonstrating a mechanism by which airway structural cells may interact with monocytes/macrophages in the tissue during the response to LPS in vivo. In contrast to the inability of CSFs to enhance the replication of peripheral blood monocytes, these cytokines increased both survival and proliferation of alveolar macrophages. As shown in Appendices 2, 3 and 4, airway fibroblast-derived conditioned medium (FCM) enhanced both survival and proliferation of alveolar macrophages as shown by a DNA synthesis assay, and this FCM-induced proliferation could be completely abrogated by pre-incubation of FCM with antibody against M-CSF. This evidence illustrates a cell type-restricted response mediated by airway structural cell-derived CSFs. Moreover, this response may be augmented during the inflammatory response due to increased production of CSFs by tissue structural cells induced by proinflammatory cytokines such as IL-1, and downregulated through the inhibitory effect of exogenous steroids on cytokine expression by these cells. In this respect, Appendix 5 demonstrates the ability of IL-1 to enhance fibroblast expression of GM-CSF and IL-8, that specifically act on inflammatory cells, and the ability of the synthetic steroid budesonide to markedly suppress this cytokine upregulation.

While alveolar macrophages may act as an important cellular component in the lower airway, capable of releasing cytokines in direct response to LPS, the data presented here also show that fibroblasts from certain anatomic sites within the airways, can also be a direct target for LPS stimulation. Indeed, fibroblasts derived from the upper airway, in sharp contrast to those from the lower airway, can release significant amounts of cytokines in response not only to IL-1 but also to LPS. These data demonstrate a universal, i.e. regardless of the site of origin, responsiveness of airway fibroblasts to IL-1 stimulation, and a site-specific responsiveness to LPS stimulation. The ability of airway fibroblasts from all sites to respond to IL-1 suggests that these cells may play a role in the amplification of the inflammatory response. The ability of the upper airway fibroblasts to respond to LPS suggests a role for these cells in the initiation of inflammation and merits particular consideration. Similar to nasal fibroblasts, skin fibroblasts were also found capable of responding to LPS (Appendix six). This suggests a role for fibroblasts from those sites with closer proximity to the external environment, hence with greater direct exposure to LPS, in the initiation of inflammation. Neonatal lung fibroblasts, similar to adult lower airway fibroblasts, responded to IL-1 but not to LPS (Appendix seven), suggesting that unresponsiveness to LPS already existed during the developmental stage. The mechanism underlying LPS responsiveness of upper airway fibroblasts remains unclear but likely involves LPS binding sites or tissue-specific nuclear responsive elements for LPS stimulation. An LPS responsive element has been identified in the promoter region of certain cytokine genes in monocytic cells (Mackman et al, 1991). The cytokine response of nasal fibroblasts to LPS was also found to be serum-dependent (Appendix eight), supporting previous observations that cells that do not possess the membrane-bound CD14 molecules require soluble CD14 and LBP in serum in order to respond to LPS (Frey et al., 1992; Haziot et al., 1993b).

Consistent with our in vitro observations, alveolar macrophages were confirmed to be a potent responder to LPS challenge in vivo. These cells appear to account largely for the dramatically enhanced expression of TNFα and MIP-2 in total lung tissue seen at 30 min and 1 h after LPS administration, respectively. These events precede the marked migration of PMNs into the lung. Peak induction of cytokines by LPS in vivo tends to take place earlier compared to in vitro. Cells express maximal levels of mRNA for cytokines generally at 4-6 h after stimulation in vitro.

When the inflammatory response is maximal, as shown by an increase in numbers of both total BAL cells (Appendix nine) and neutrophils, neutrophils were surprisingly found to be the prominent source of the same set of the cytokines detected earlier in alveolar macrophages. This neutrophil-mediated cytokine response reached its peak when the cytokine expression in alveolar macrophages started to decline, demonstrating different kinetics of cytokine expression between alveolar macrophages and neutrophils. This finding provides in vivo evidence to support recent in vitro data demonstrating the ability of neutrophils to express cytokines (Lloyd and Oppenheim, 1992). It also strengthens the concept that, with respect to cytokine production, PMNs migrate into the tissue and operate as a powerful addition to the resident defence forces (Reynolds, 1992).

In this experimental model, there was no significant expression of cytokines detected in airway fibroblasts or other structural cell types. On the one hand, this finding is not unexpected since our in vitro studies show that lower airway fibroblasts are not the primary target for direct LPS stimulation. On the other hand, these cells were expected to express in vivo "second wave" cytokines in response to pro-inflammatory cytokines such as TNFα and IL-1 released from inflammatory cells. However, it cannot be ruled out that failure to show a significant number of fibroblasts or epithelial cells actively expressing cytokines is due to low sensitivity of the techniques used in these studies.

The profile of cytokines induced by LPS in the airways is of particular interest. LPS upregulated expression of TNFα, MIP-2, IL-1ß and IL-6 but not RANTES or TGFβ_1 mRNAs. This selective induction of cytokines is possibly determined by the nature of the causative agent. The cytokine profile induced then defines the type of tissue responses. LPS results in an inflammatory response marked by massive neutrophilic infiltration, which is self-limited. Compelling evidence indicates that TNFα, IL-1 and MIP-2 all directly or indirectly contribute to the accumulation of PMNs whereas RANTES is chemoattractive to distinctive types of inflammatory cells including monocytes, T lymphocytes and eosinophils (Schall et al, 1992; Kameyoshi et al, 1992), and $TGFß_1$ plays a major role in tissue remodelling processes (Kelley, 1993). The Induction of $TGFß_1$ has been mainly observed in chronic inflammatory conditions associated with fibrosis (Piguet et al, 1989; Brokelmann et al, 1991; Ohno et al, 1993). In addition to its selectivity, the LPS-induced cytokine response in the airways was transient. This transient nature, perhaps in conjunction with the selective expression of proinflammatory cytokines but not growth factors and the induction of potentially inhibitory cytokines such as IL-6, is likely to determine the self-limiting nature of the tissue response.

Taken together, LPS has potent effects on the induction of a wide spectrum of cytokines in the airways. In vitro, this molecule can directly induce a number of cytokines in alveolar macrophages and upper airway-derived fibroblasts. The effector activities of alveolar macrophages may be modulated by both soluble cytokines and insoluble ECM components elaborated by structural fibroblasts. In vivo, both resident alveolar macrophages and recruited neutrophils represent major sources of cytokine production in response to LPS challenge. The selective and transient features of the cytokine response likely account for the self-limited neutrophilic response elicited by a single LPS challenge. These observations provide insights into the molecular mechanisms underlying LPS-induced lung injury and hold implications in developing potential interventions. In addition, this in vivo model of LPS-induced acute lung inflammation may be useful for exploring the regulatory mechanisms of the response using recombniant anti-inflammatory cytokines or transgene expression techniques, anti-cytokine antibodies or anti-sense oligonucleotides, and soluble cytokine receptors or receptor antagonists.

Appendix One

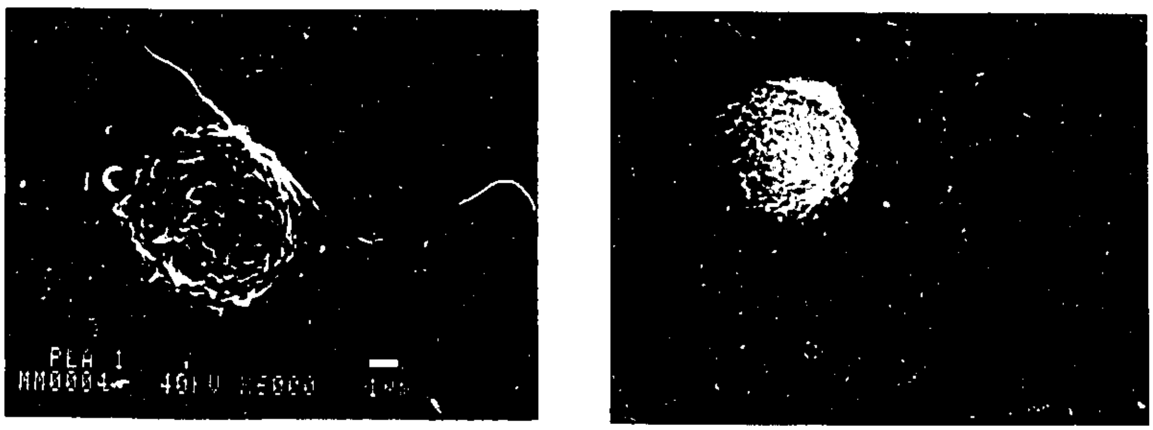

Figure 3. Scanning electron microphotographs showing the effect of upper airway fibroblast-derived ECM on the spreading of rat alveolar macrophages in culture. Alveolar macrophages were cultured on plastic (PLA) or extracellular matrix-coated plastic (ECM) for 6 days and then processed for scanning electron microscopic observation. These photographs demonstrate that macrophages cultured on ECM spread to a greater degree than those cultured on plastic.

Appendix Two

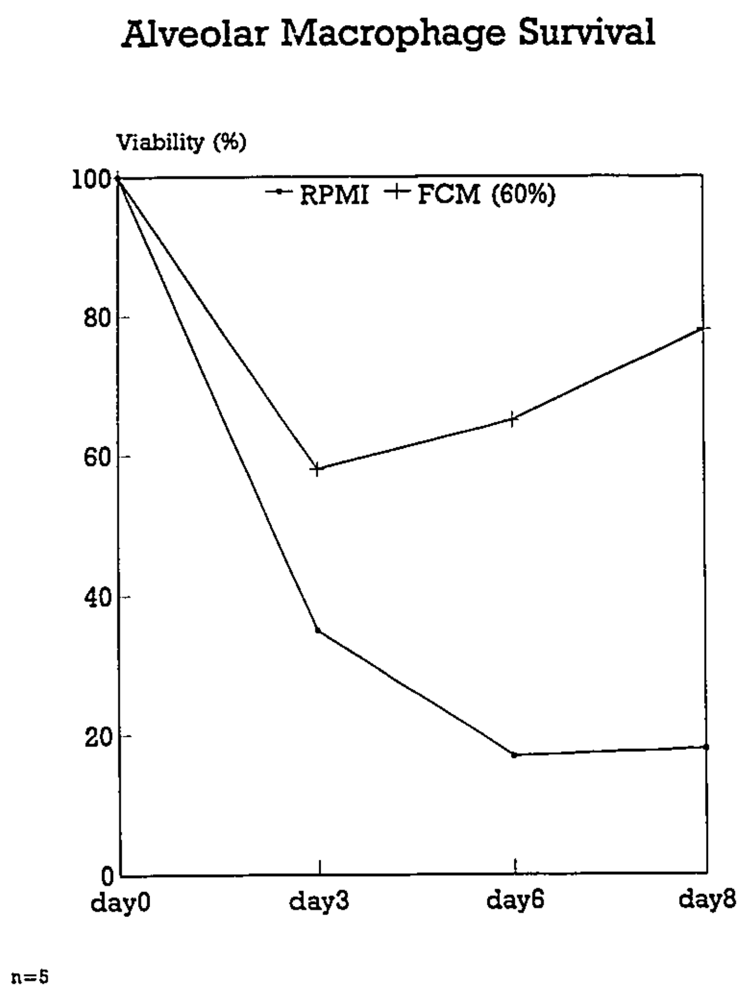

Figure 4. Enhanced survival of rat alveolar macrophages by fibroblast-derived conditioned medium. Alveolar macrophages were cultured for different periods of time with RPMI medium alone or with 60% of human upper airway fibroblast-derived conditioned medium (FCM) in RPMI, and the viability was then assessed. A striking survival-enhancing activity was observed with FCM. The data are expressed as average percentages from five independent experiments (n = 5).

Appendix Three

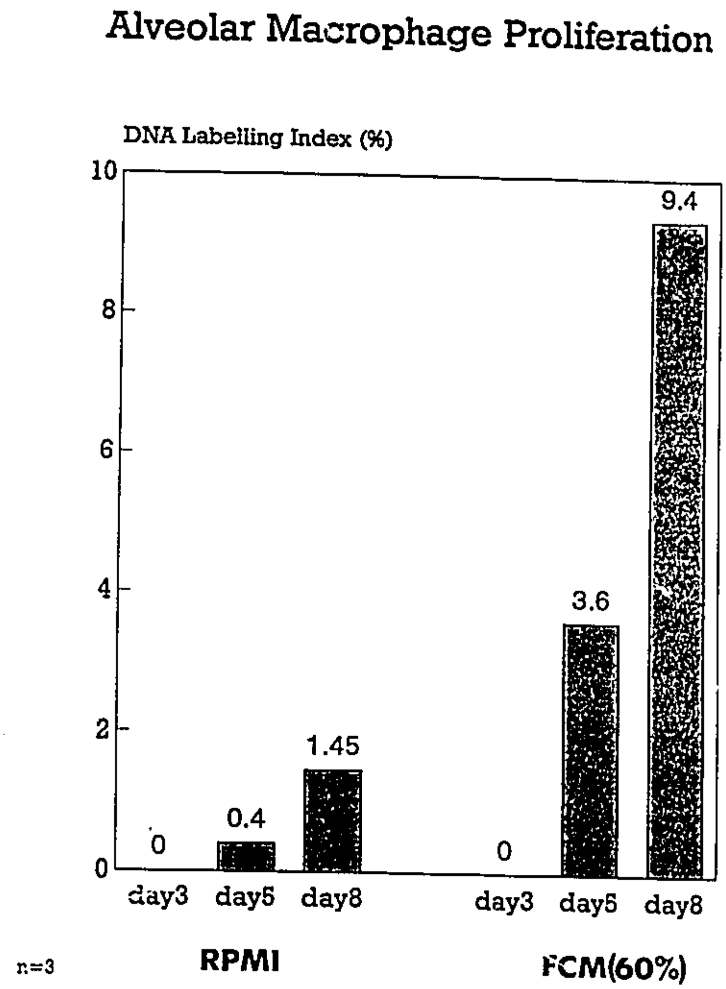

Figure 5. Enhanced proliferation of rat alveolar macrophages by fibroblast-derived conditioned medium. Alveolar macrophages were cultured for different periods of time with or without 60% of human upper airway fibroblast-derived conditioned medium (FCM). Autoradiography was utilized to measure DNA synthesis. Tritiated thymidine (2.5 μCi/ml) was pulsed for the last 20 hours of culture and in situ autoradiography then performed. Results are expressed as the percentage of cells which have taken up tritiated thymidine (labelling index). It was noted that while DNA synthesis was not increased until day 5, enhanced survival was observed as early as day 3, indicating that FCM promoted both cell survival and proliferation.

Appendix Four

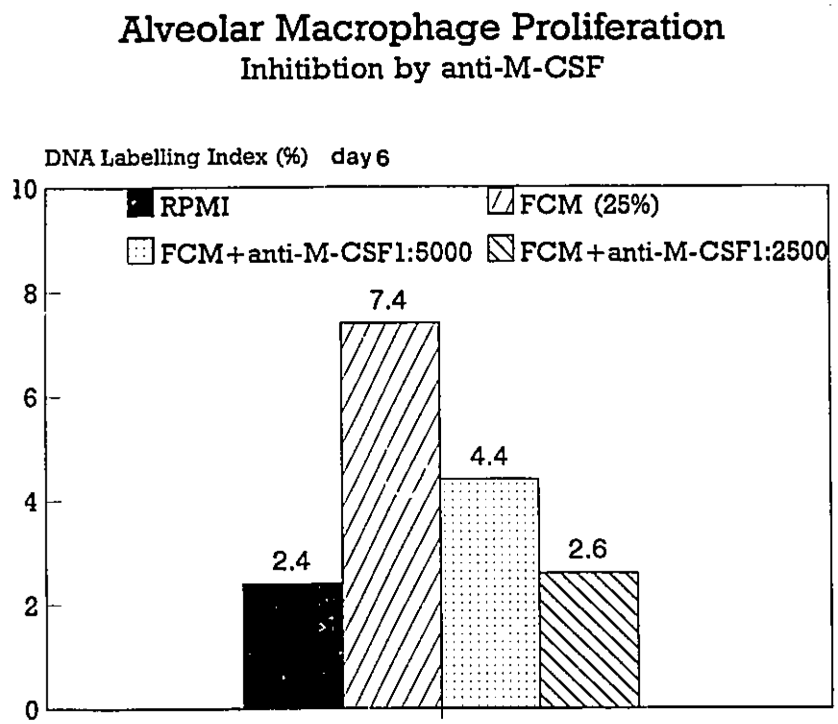

Figure 6. Inhibition of enhanced alveolar macrophage proliferation by anti-M-CSF. The rat alveolar macrophages were cultured for 6 days with RPMI medium alone, 25% of human upper airway fibroblast-derived conditioned medium (FCM), or 25% of FCM pretreated with 1:5000 or 1:2500 rabbit anti-human macrophage colony-stimulating factor (M-CSF) serum. DNA synthesis was assayed by autoradiography. Tritiated thymidine (2.5 μCi/ml) was pulsed for the last 20 hours of culture. Results are expressed as described in Appendix Three. The data indicate that M-CSF was the cytokine in FCM responsible for the increased proliferation of alveolar macrophages.

Appendix Five

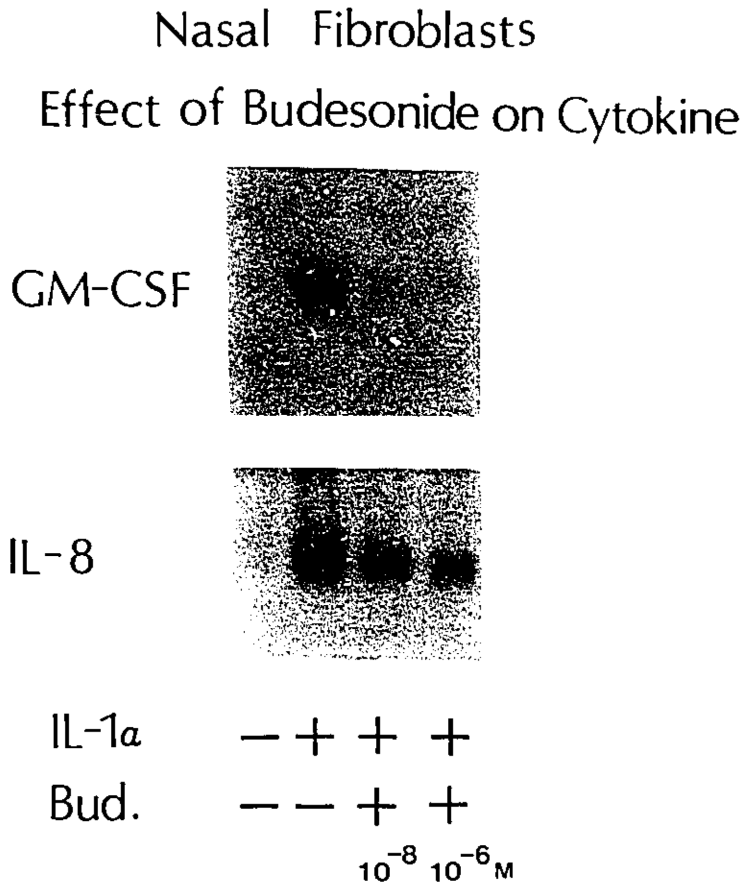

Figure 7. Induction of cytokine mRNAs by IL-1α and inhibition by budesonide in the upper airway fibroblasts. Human nasal fibroblasts were cultured with or without IL-1α (5 ng/ml) and the synthetic steroid budesonide (10^{-8} or 10^{-6} M) for 24 hours. Expression of mRNAs for granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) was analyzed by Northern blot hybridization.

Appendix Six

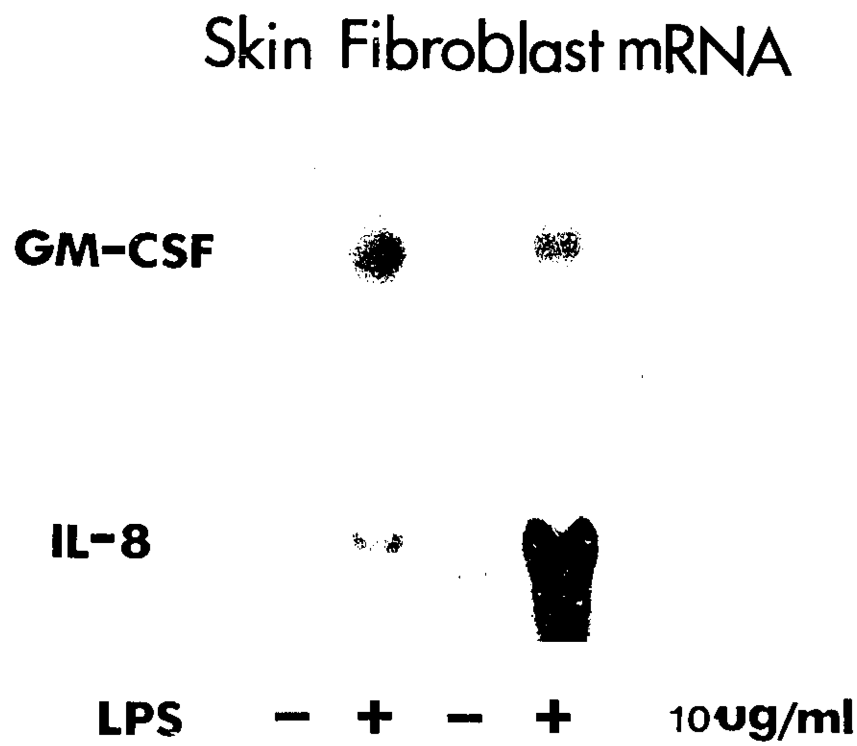

Figure 8. Induction of cytokine mRNAs by LPS in skin fibroblasts. Two human primary fibroblast lines were cultured with or without LPS (10 μg/ml) for 24 hours. Expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) mRNAs was assessed by Northern blot hybridization. Skin fibroblasts responded, albeit to a lesser degree compared to nasal fibroblasts, to LPS, in contrast to the unresponsiveness of lung fibroblasts.

Appendix Seven

H. IL-8 mRNA IN NEONATAL FIBROBLAST LINES

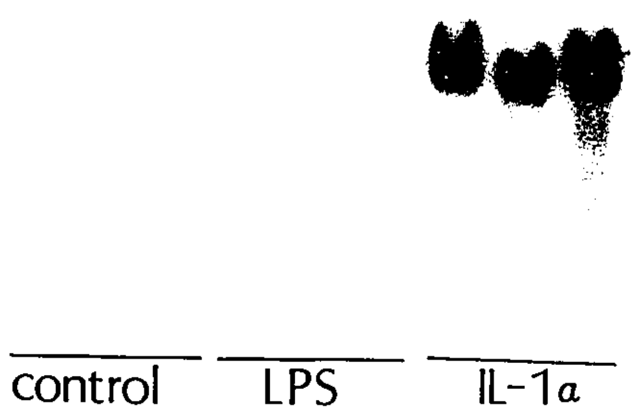

Figure 9. Responsiveness to IL-1 and unresponsiveness to LPS of neonatal lung fibroblasts. Three human neonatal lung fibroblast lines were cultured with or without LPS (10 μg/ml) or IL-1α (5 ng/ml) for 24 hours. Expression of interleukin-8 (IL-8) mRNA was examined by Northern blot analysis. Similar to the human adult lung fibroblasts, the neonatal lung fibroblasts responded to IL-1 but not LPS.

Appendix Eight

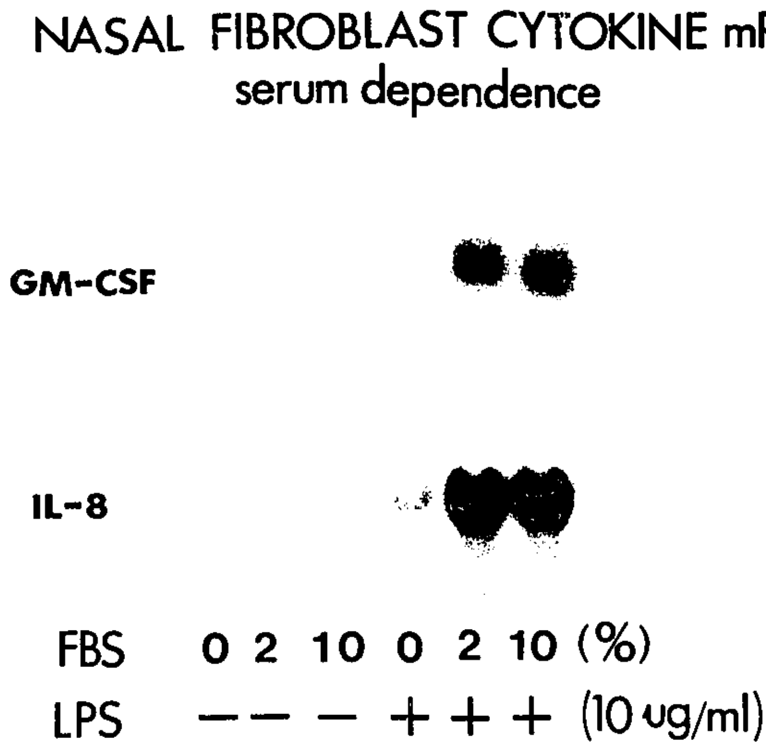

Figure 10. Serum dependence of the cytokine response to LPS of nasal fibroblasts. Human nasal fibroblasts were cultured in the presence of different concentrations of fetal bovine serum (FBS) with or without LPS for 24 hours. Expression of mRNAs for granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) was assessed by Northern blot analysis. The optimal cytokine response required the presence of a minimal amount of FBS.

Appendix Nine

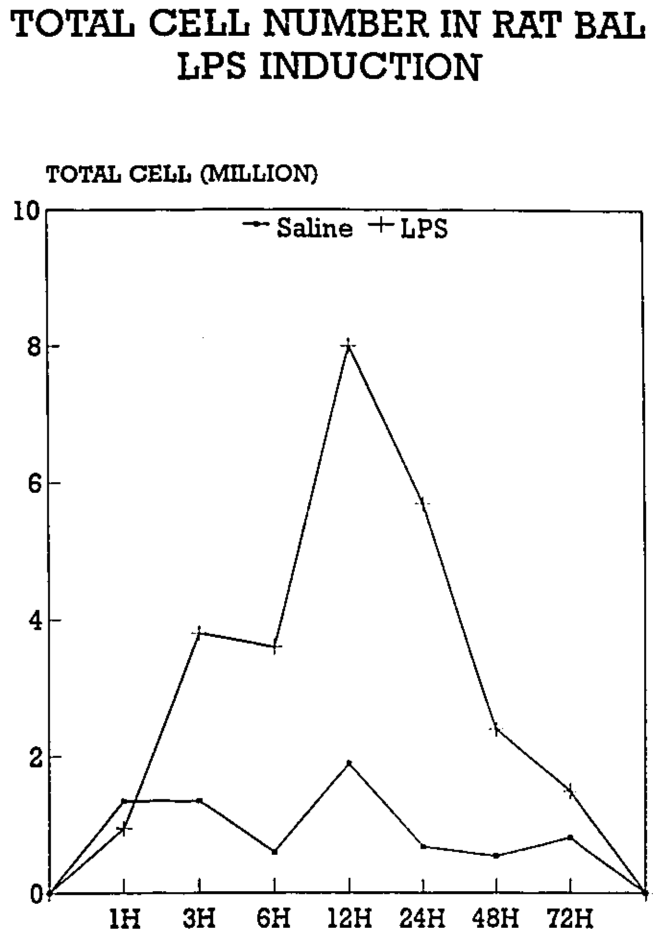

Figure 11. Increase of the total cell number in broncho-alveolar lavage (BAL) fluids of rats challenged by intratracheal administration of LPS. Saline or saline containing LPS (6 μg/ml) was intratracheally instilled. The total cell number in BAL was examined at various times after LPS administration. Saline had very little effect whereas LPS markedly increased the total cell numbers in BAL. The peak increase occurred around 12 hours. This was consistent with the peak increase in the percentage of neutrophils in total BAL cells.

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