

**Monocyte Adherence to Fibronectin:
Role of CD11/CD18 Integrins
and Relationship to Other Monocyte Functions**

Dr. Caroline A. Owen

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Doctor of Philosophy**

**Department of Medicine
University of Birmingham
Birmingham, UK
B15 2TT
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Synopsis

Regulated adherence of monocytes to extracellular matrix macromolecules is a prerequisite for their accumulation at sites of pulmonary infection and inflammation. To begin to assess the pathobiological importance of alterations in monocyte adherence to extracellular matrix in inflammatory lung diseases, the adherence properties of monocytes from patients with an inflammatory lung disease (bronchiectasis) and healthy subjects to a representative matrix component (fibronectin) were compared. Spontaneous adherence of monocytes from the control subjects was 20 to **25%**, whereas that of the patients' cells was 2 to 3-fold higher and correlated with the severity of airway inflammation. Endotoxin (LPS) and cytokines from areas of airway disease are likely to be responsible for the observed monocyte activation since: 1) LPS was detected in plasma from all of the patients but none of the control subjects; and 2) LPS and cytokines produced dose-related increases in the adherence of normal monocytes in vitro. Monocyte adherence to fibronectin was substantially mediated by CD11/CD18 integrins, via both RGD-dependent and RGD-independent mechanisms. These data indicate that signals arising from foci of pulmonary inflammation are likely determinants of the accumulation of monocytes in the lungs of patients with chronic inflammatory lung diseases.

There was a striking relationship between the adherence properties of monocytes and functions that are of biological importance at sites of inflammation. Spontaneously adherent monocytes had an "inflammatory effector" phenotype, non-adherent cells had an "immune modulatory" phenotype and monocytes that could be stimulated to adhere by LPS (LPS-adherent cells) had an intermediate phenotype. In addition, only the adherent monocyte subpopulations were replete with HLE and these cells contained a substantial (10 to 11-fold) molar excess of HLE compared with the physiological inhibitor of this enzyme (**α_1 -antitrypsin**). Maturation in vitro increased the accumulation of **α_1 -antitrypsin** by all of the monocyte subpopulations. In contrast, pro-inflammatory mediators up-regulated **α_1 -antitrypsin** accumulation by only the spontaneously adherent cells, probably by translational or post-translational mechanisms.

In conclusion, these data indicate that monocytes are heterogeneous in their ability to accumulate at sites of infection and inflammation. In addition, the capacity of monocytes to adhere to fibronectin is related to monocyte functions that are of biological importance at sites of ~~infection~~ and inflammation. Furthermore, LPS released from foci of infection, may induce the accumulation of monocytes with an inflammatory effector phenotype, and may thereby promote resolution of tissue infection. Alternatively, LPS may promote the recruitment of monocytes with capacity to contribute to HLE-mediated tissue injury.

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Table of Contents

	<u>Contents</u>	Page
	List of Contents	i
	List of Figures	iii
	List of Tables	vi
	List of Abbreviations	vii
<u>Chapter 1</u>	Introduction	1
1.1.	Introduction	2
1.2.	Historical Aspects of the Mononuclear Phagocyte System	3
1.3.	Ontogeny of Mononuclear Phagocytes	4
1.4.	Morphology, Metabolism and Surface Receptors	4
1.5.	Secretory Products of Mononuclear Phagocytes	8
1.6.	Activation of Mononuclear Phagocytes	8
1.7.	Functions of Mononuclear Phagocytes	12
1.8.	Monocyte Heterogeneity	21
1.9.	Adherence Properties of Monocytes	35
1.10.	Potential Role of Mononuclear Phagocytes in the Pathogenesis of Chronic Inflammatory Lung Diseases	42
1.11.	Objectives of the Thesis	52
<u>Chapter 2</u>	Methods	53
2.1	Cell Isolation	54
2.2.	Recovery of Monocytes	58
2.3.	Assessment of Monocyte Purity	59
2.4.	Limulus Amoebocyte Lysate Assay	61
2.5.	Monocyte Adherence Assay	65

2.6.	Comparison of the Adherence Properties of Monocytes from Healthy Subjects and Patients with Bronchiectasis	68
2.7.	Mechanisms Involved in Monocyte Adherence to Fibronectin	70
2.8.	Monocyte Phenotype Evaluations	71
2.9.	Expression of Human Leukocyte Elastase and Alpha₁-Antitrypsin by Monocytes	79
2.10.	Dot-Blot Analysis of Alpha ₁ - Antitrypsin Gene Expression by Monocytes	88
2.11.	Statistical Analysis	105
Chapter 3	Results	106
3.1.	Optimisation of the Monocyte Isolation Procedure	107
3.2.	Monocyte Adherence Assay	114
3.3.	Adherence Properties of Monocytes from Healthy Subjects	121
3.4.	Comparison of the Adherence Properties of Monocytes from Healthy Subjects and Patients with Bronchiectasis	123
3.5.	Plasma Endotoxin Concentrations	123
3.6.	Effect of Pro-Inflammatory Mediators on Monocyte Adherence	127
3.7.	Mechanisms Involved in Monocyte adherence to Fibronectin	135
3.8.	Monocyte Phenotype Evaluations	141
3.9.	Expression of HLE and Alpha₁-Antitrypsin by Monocytes	152
3.10.	Expression of the Alpha ₁ - Antitrypsin Gene Expression by Monocytes	170
Chapter 4	Disenssion	178
4.1.	Overview	179
4.2.	Monocyte Isolation	180
4.3.	Monocyte Adherence Assay	184
4.4.	Comparison of the Adherence Properties of Monocytes from Healthy Subjects and Patients with Bronchiectasis	189

4.5.	Mechanisms Involved in Monocyte Adherence to Fibronectin	195
4.6.	Monocyte Phenotypes Evaluations	198
4.7.	Potential Role of Monocytes in the Pathogenesis of Chronic Inflammatory Lung Diseases	205
4.8.	Hypotheses for Monocyte Heterogeneity	220
4.9.	Conclusions and Implications	222
4.10.	Further Studies	224

Appendices

Appendix 1	Materials and Suppliers	228
Appendix 2	Demographic and Spirometric Data for Patients with Bronchiectasis	232
Appendix 3	Comparison of Monocyte Adherence to Fibronectin and Endothelial cells	233
Appendix 4	Adherence Properties and Superoxide Anion Release by CCE-Isolated Monocytes	235
Appendix 5	Publications	236

List of References	237
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List of Figures

Figure 1	Standard Curve for Limulus Amoebocyte Lysate Assay	64
Figure 2	Separation of the Monocyte Subpopulations	72
Figure 3	Phagocytosis of Latex beads by Monocytes	74
Figure 4	Standard Curve for the Sedmak Protein Assay	78
Figure 5	Standard curve for the HLE Indirect Binding ELISA	84
Figure 6	Standard Curve for the Alpha ₁ -antitrypsin Direct Binding ELISA	87
Figure 7	Plasmid Analyses	97
Figure 8	Monocyte Yields	109
Figure 9	Fate of Monocytes not Recovered by Nycodenz	109
Figure 10	Monocyte Purity: Modified Wright's and Non-Specific Esterase Staining	112

Figure 11	Monocyte Purity: Immunohistochemistry	113
Figure 12	Monocyte Adherence to Fibronectin	115
Figure 13	Specificity of Monocyte Adherence to Fibronectin	115
Figure 14	Monocyte Adherence To Fibronectin: Effect of Different Medium Additives	117
Figure 15	Monocyte Adherence to Fibronectin: Effect of Monocyte Number	117
Figure 16	Monocyte Adherence to Fibronectin: Comparison of Different Monocyte Isolation Methods	120
Figure 17	Adherence of Monocytes from Healthy Subjects and Patients with Bronchiectasis	124
Figure 18	Plasma Endotoxin Activity: Healthy Subjects and Patients with Bronchiectasis	126
Figure 19	Monocyte Activation in Bronchiectasis: Relationship to Plasma Endotoxin Activity	126
Figure 20	Effect of LPS on Monocyte Adherence: Healthy Subjects	128
Figure 21	Effect of LPS from Different Bacterial Species on Monocyte Adherence	128
Figure 22	Effect of Incubation Time on Monocyte Adherence to Fibronectin	130
Figure 23	Effect of LPS on Monocyte Adherence: Patients with Bronchiectasis	131
Figure 24	Effect of Recombinant TNF on Monocyte Adherence	133
Figure 25	Effect of Recombinant Cytokines on Monocyte Adherence	133
Figure 26	Effect of LPS and Cytokines on Monocyte Adherence	134
Figure 27 a	Effect of Anti-CD18 Antibody on Monocyte Adherence: Healthy Subjects	136
Figure 27 b	Specificity of Anti-CD18 Inhibition: Healthy Subjects	136
Figure 28 a	Effect of RGDS Peptide upon Monocyte Adherence: Healthy Subjects	137
Figure 28 b	Specificity of Inhibition by RGDS Peptide: Healthy Subjects	137
Figure 29	Additive Effects of Anti-CD18 and RGDS on Monocyte Adherence: Healthy Subjects	139
Figure 30 a	Effect of Anti-CD18 and RGDS on Monocyte Adherence: Patients with Bronchiectasis	140
Figure 30 b	Specificity of Inhibition by Anti-CD18 and RGDS: Patients with Bronchiectasis	140
Figure 31	Phagocytic Activity of Monocyte Subpopulations	143
Figure 32	Superoxide Anion Production by Monocyte Subpopulations	144

Figure 33	Peroxidase Content of Monocyte Subpopulations	146
Figure 34	HLA-DR Antigen Expression by Monocytes	148
Figure 35	Effect of Incubation Temperature on HLA-DR Antigen Expression by Monocytes	149
Figure 36	HLA-DR Antigen Expression by Monocyte Subpopulations	149
Figure 37	Total Protein Content of Monocyte Subpopulations	151
Figure 38	HLE Content of Monocyte Subpopulations	154
Figure 39	HLE Content of Monocytes: Comparison of Monocyte Isolation Procedures	154
Figure 40	Alpha_1 -Antitrypsin Content of Monocyte Subpopulations	158
Figure 41	Effect of Culture and LPS on Alpha_1 -Antitrypsin Accumulation: by Spontaneously Adherent and Non-Spontaneously Adherent Monocytes	164
Figure 42	Effect of Culture and LPS on Alpha_1 -Antitrypsin Accumulation: by LPS-Adherent and Non-Adherent Monocytes	165
Figure 43	Effect of incubation Time on Alpha_1 -Antitrypsin Accumulation	167
Figure 44	Effect of Cytokines on Alpha_1 -Antitrypsin Accumulation by Monocytes	168
Figure 45	Effect of GM-CSF on Alpha_1 -Antitrypsin Accumulation: Spontaneously Adherent Monocytes	169
Figure 46	Reproducibility of the Dot-Blot Technique	172
Figure 47	Specificity of the Dot-Blot Technique	172
Figure 48	Quantification of Alpha_1 -Antitrypsin mRNA in Monocytes	173
Figure 49	Quantification of β -actin mRNA in Monocytes	174
Figure 50	Steady State Alpha_1 -Antitrypsin mRNA Concentrations in Monocytes	176
Figure 51	Effect of LPS on Steady State Alpha_1 -Antitrypsin mRNA Levels in Monocytes	176
Figure 52	Steady State Levels of Alpha_1 -Antitrypsin mRNA in Monocytes: Effect of Cytokines	177

List of Tables

Table 1	Types of Macrophages and their Prominent Functions	7
Table 2	Secretory Products of Mononuclear Phagocytes	9
Table 3	Characteristics of Activated Mononuclear Phagocytes	11
Table 4	Heterogeneity of Human Mononuclear Phagocytes	27
Table 5 a	Donor Variability in Monocyte Adherence: Spontaneous Adherence	122
Table 5 b	Donor Variability in Monocyte Adherence: LPS-Stimulated Adherence	122
Table 6 a	HLE Content of Monocyte Populations: Between-Donor Variability	156
Table 6 b	HLE Content of Monocyte Populations: Within-Donor Variability	156
Table 7 a	Alpha ₁ -antitrypsin Content of Monocyte Populations: Between-Donor Variability	160
Table 7 b	Alpha ₁ -antitrypsin Content of Monocyte Populations: Within-Donor Variability	160
Table 8 a	HLE and Alpha ₁ -Antitrypsin Content of Monocyte Populations	161
Table 8 b	Molar Ratio of HLE and Alpha ₁ -Antitrypsin in Monocyte Populations	161
Table 9	Comparison of Monocyte Adherence to Fibronectin and HUVEC Monolayers	234

List of Abbreviations

ADCC	antibody dependent cellular cytotoxicity
APAAP	alkaline phosphatase - anti-alkaline phosphatase antibody complex
BamH 1	Bacillus Amyloliquefaciens H restriction enzyme
BSA	bovine serum albumin
C	complement
CaCl₂	calcium chloride
CCE	countercurrent centrifugai elutriation
cDNA	complementary DNA
Cl⁻	chloride ion
CO₂	carbon dioxide
CR	complement receptor
CS-1	connecting segment-1
CsCl	caesium chloride
C.V.	coefficient of variation
dATP	deoxyribose adenine triphosphate
dCTP	deoxyribase cytosine triphosphate
dPm	disintegrations per minute
dGTP	deoxyribose guanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxyribose thymine triphosphate
EDTA	ethylenediamine tetracetic acid
E.coli	Escherichia coli
EcoR1	restriction enzyme from the R strain of E. coli
ELAM	endothelial leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbant assay
Fab	fragment antigen binding of immunoglobulin
Fc	fragment crystalline of immunoglobulin
FcR	Fc receptor
FCS	fetal calf serum
f-MLP	N-formyl-methionyl-leucyl-phenylalanine
GAM-FITC	goat anti-mouse conjugated to fluorescein-isothiocyanate

GM-CSF	granulocyte macrophage-colony stimulating factor
GMP 140	granule membrane protein 140
Ig	immunoglobulin
HBSS	Hank's balanced salt solution
Hind III	restriction enzyme from Haemophilus influenzae
<u>II. influenzae</u>	Haemophilus influenzae
HLA	human histocompatibility leukocyte antigen
HLE	human leukocyte elastase
H₂O₂	hydrogen peroxide
HRP	horse radish peroxidase
HSA	human serum albumin
H₂SO₄	sulphuric acid
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN	interferon gamma
IL	interleukin
Ka	association rate constant
LAL	limulus amoebocyte lysate
LECAM-1 (LAM-1)	leukocyte-endothelial cell adhesion molecule-1
LFA-1	lymphocyte function associated antigen
LPS	lipopolysaccharide
LTB-4	leukotriene B-4
M-CSF	macrophage-colony stimulating factor
MES	2-N-morpholinoethane sulphonic acid
MgCl₂	magnesium chloride
MHC	major histocompatibility complex
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MSAPN	N-methoxysuccinyl-alanyl-alanyl-prolyl-valine -nitroanalide
NaCl	sodium chloride
NADH	E-nicotinamide adenine dinucleotide, reduced form
NADPH	B-nicotinamide adenine dinucleotide phosphate, reduced form
NaN₃	sodium azide
5'NT	5' nucleotidase

O_2^-	superoxide anion
HOCl	hypochlorous acid
OH	hydroxyl radical
<u>P. aeruginosa</u>	Pseudomonas aeruginosa
PBS	phosphate buffered saline (0.15 mol/L NaCl, 1.5 mmol/L NaH_2PO_4 , 8.5 mmol/L Na_2HPO_4 ; pH 7.4)
PDGF	platelet derived growth factor
PGE₂	prostaglandin E₂
PHA	phytohaemagglutinin
PI	phagocytic index
PMA	phorbol myristate acetate
polyI:polyC	polyriboinosinic:polyribocytidylic acid
PPD	purified protein derivative of tuberculin
PPE	porcine pancreatic enzyme
PWM	pokeweed mitogen
RAM	rabbit anti-mouse immunoglobulin
RGD	Arg-Gly-Asp
RGDS	Arg-Gly-Asp-Ser
RGES	Arg-Gly -Glu-Ser
RIA	radioimmunoassay
RPMI 1640 medium	Roswell Park Memorial Institute 1640 medium
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSC buffer	sodium chloride-sodium citrate buffer
TBE buffer	tris borate ethylenediamine tetracetic acid
TBS	tris buffered saline
TCA	trichloroacetic acid
TE buffer	tris-ethylene diamine tetracetic acid buffer
TFB	transformation buffer
TGF- β	transforming growth factor-6
TNF	tumour necrosis factor- α
VLA antigen	very late activation antigen
VCAM-1	vascular cell adhesion molecule

Chapter 1

Introduction

1.1 Introduction

Monocytes and macrophages are a family of phagocytic cells that are widely distributed throughout the body and are generally referred to as the mononuclear phagocyte system because of their common origin, and similar morphology and function. Mononuclear phagocytes have been known since the time of Ellie Metchnikoff (1845-1916) mainly for their property of phagocytosis which enables them to eliminate pathogens and other unwanted materials. However, recent research has demonstrated clearly that these cells play a pivotal role in processes as fundamental as inflammation, the induction and regulation of specific immune responses and tissue remodelling and repair. There are several basic properties of this cell line that enable it to participate in such varied reactions. First, mononuclear phagocytes are highly mobile and have the capacity to adhere to various biological Substrates, functions that enable them to be recruited into sites of inflammation. Second, these cells secrete a large number of soluble mediators that modulate the function of many different types of cells. Third, mononuclear phagocytes ingest and degrade various materials including senescent cells and tissue debris. Finally, mononuclear phagocytes can be activated by the external environment which generally results in an increase in their functional properties.

it is clear that mononuclear phagocytes play a critical role in host defence; however, these cells may also injure the host while exercising their defensive role. For example, these cells have been shown to contribute to tissue damage by releasing proteolytic enzymes (80,409), toxic oxygen metabolites (79,89), pro-fibrotic cytokines (291,415) and other mediators (382,445). Moreover, mononuclear phagocytes have been implicated in the pathogenesis of several inflammatory and degenerative diseases including chronic obstructive pulmonary disease (80,223,333), pulmonary fibrosis (17,382,415), rheumatoid arthritis (65), systemic lupus erythematosus (17), sarcoidosis (17), and atherosclerosis (58,189,387).

To understand the pathogenesis of these diseases, it is necessary to identify the factors that regulate mononuclear phagocyte recruitment and function at sites of inflammation. Recruitment of monocytes from the vasculature involves directional migration of monocytes in response to chemoattractant factors released from inflamed sites, and reversible adherence of monocytes to endothelial cells. The next barriers that monocytes encounter, as they migrate into sites of inflammation, are the extracellular matrix components of the basement membrane and the subendothelial space. It is clear that regulated and reversible adherence of monocytes to extracellular matrix macromolecules is also a prerequisite for the accumulation of these cells at sites of tissue

inflammation (172,423); however, little is known about the biological and pathological factors that regulate monocyte adherence to extracellular matrix, or the mechanisms involved.

To begin to address the pathobiological importance of alterations in monocyte adherence to extracellular matrix in inflammatory lung diseases, the adherence properties of monocytes from healthy donors and patients with an inflammatory lung disease (bronchiectasis) to a representative extracellular matrix component (fibronectin) were compared. In addition, the effects upon monocyte adherence of various signals (LPS and cytokines) that are likely to be released at sites of infection and inflammation were assessed. The role of **CD11/CD18** integrins in mediating monocyte adherence to fibronectin was investigated. Finally, the possibility was **tested** that there is a relationship between the adherence properties of monocytes and phenotypes that relate to important biological and pathological activities at sites of infection and inflammation.

The introductory section of this thesis describes the historical aspects of mononuclear phagocytes, their ontogeny, morphology and functions. The literature on the adherence properties of mononuclear phagocytes is summarised. Finally, the potential role of mononuclear phagocytes in the pathogenesis of a common chronic inflammatory **disease** of the lungs (pulmonary emphysema) is discussed.

1.2. Historical Aspects of the Mononuclear Phagocyte System

Macrophages were first described in **1882** by **Elie** Metchnikoff in Messina, Italy (306). Metchnikoff observed large cells phagocytosing tissue debris in starfish larvae, and subsequently named these cells macrophages, a **term** derived from the Greek for "big eaters". Schilling, in **1912**, was the first to identify the monocyte clearly in the peripheral blood (222). However, it was not until **1925** that the relationship between monocytes and macrophages was established when Lewis (274) demonstrated that cultured monocytes **transformed** into macrophages, epithelioid cells and multinucleate giant **cells** (274). This relationship was supported further in **1939** by the experiments of Ebert and Florey (149). These investigators used a rabbit ear chamber model to show that monocytes containing phagocytosed carbon particles migrated from the blood into connective tissue and transformed into tissue macrophages. More recent research using tritiated thymidine and chimeric studies have confirmed these observations and revealed the bone marrow origin of mononuclear Phagocytes, and their secretory and immune regulatory functions (217,457).

1.3. Ontogeny of Mononuclear Phagocytes

The mononuclear phagocyte cell line originates in the bone marrow as the granulocyte-monocyte colony forming unit (CFU-GM) which is a common committed **progenitor** cell for the granulocyte and monocyte-macrophage pathways. The **progenitor** cell is induced to differentiate into a monoblast by glycoprotein hormones called colony stimulating factors (416). Monoblasts differentiate into promonocytes, the first morphologically identifiable cell in the series (458), and promonocytes differentiate into monocytes. The process of differentiation from the committed stem cell to the mature monocyte takes about ~~six~~ days, and the cells go through three to four cell divisions between the monoblast and monocyte stage. The mature monocyte is released from the bone marrow into the circulation within 24 hours. Kinetic studies in man have shown that monocytes have a circulating half life of about three days (491). In contrast to neutrophils, humans do not have a pool of marginating monocytes (236).

There is no evidence that monocytes are predestined for any particular tissue once they leave the bone marrow; rather, migration of monocytes into tissues appears to be a random phenomenon in the absence of inflammation (236). Once in the tissues, monocytes do not re-enter the circulation; rather, they undergo transformation into tissue macrophages without further cell division (458). The terminal stage of differentiation in the mononuclear phagocyte line is believed to be the multinucleated giant cell which is found characteristically in granulomatous inflammatory diseases such as tuberculosis, sarcoidosis and Crohn's disease (113).

Precise data on the average life-span of tissue macrophages are not available. However, an estimate of the life span of human macrophages has been provided by the studies of Thomas and coworkers (439). These investigators demonstrated that in patients receiving bone marrow transplants for aplastic anaemia or leukaemia, host macrophages gradually disappeared from their tissues and were replaced by donor macrophages by approximately three months after transplantation.

1.4 Morphology, Metabolism and Surface Receptors

MonoMasts

There are no special features that allow monoblasts to be recognised. Monoblasts are assumed to be the immediate precursors of promonocytes, but they are indistinguishable from myeloblasts.

Promonocytes

Promonocytes represent 3% of marrow cells. They are round cells, 12 to 18 μM in diameter, with an indented or irregularly shaped nucleus. The cytoplasm contains a conspicuous Golgi complex but relatively few cisternae of the endoplasmic reticulum. The first type of cytoplasmic granule (peroxidase positive or primary lysosome) appears in the early promonocyte stage. Peroxidase positive granules contain peroxidase, lysosomal enzymes, acid phosphatase and aryl sulphatase (267). The second type of cytoplasmic granule (peroxidase negative) appears in more mature promonocytes and monocytes (332). Promonocytes are facultative anaerobes; that is, they obtain their metabolic energy from glycolysis. Promonocytes are actively dividing cells and they are capable of phagocytosis and adherence to glass surfaces (267). These cells contain non-specific esterases in addition to the lysosomal enzymes listed above, and they have surface receptors for the Fc component of IgG and complement component C3 (213).

Monocytes

Monocytes represent 3 to 8% of peripheral blood leukocytes. These cells measure 12 to 15 μM in diameter and possess a characteristic kidney-shaped nucleus. The cytoplasm contains a well-developed Golgi apparatus, numerous lysosomal granules and microtubules, and actin-containing filaments which are cross-linked by actin-binding protein and myosin. Their metabolism is similar to that of promonocytes. Monocytes are slowly motile, exhibit phagocytic activity and have a strong tendency to adhere and spread on glass surfaces (267). In contrast to promonocytes, monocytes do not undergo cell division. Monocytes give a positive reaction for non-specific esterases that is inhibited by sodium fluoride (502). These cells contain peroxidase, acid phosphatase, lysozyme, aryl sulphatase, β glucuronic acid and other hydrolytic enzymes. Monocytes express **HLA-DR** antigens on their surface (302,421) along with receptors for the Fc component of IgG, complement component C3 (213) and insulin (402).

Macrophages

Macrophages measure 20 to 80 μM in diameter and contain a large vacuolated nucleus often with prominent nucleoli. Their cytoplasm contains a large well-developed Golgi apparatus, abundant rough endoplasmic reticulum and ribosomes, large mitochondria, microtubules, microfilaments, numerous lysosomes rich in hydrolytic enzymes and a sub-plasmalemmal actin-myosin system. The transition from monocyte to macrophage is associated with increases in: 1) the number of lysosomes and mitochondria; 2) the activity of

mitochondrial enzymes and the rate of cellular respiration; **3**) phagocytic activity; **4**) protein synthesis; and **5**) the capacity to interact with lymphocytes (**267**). In contrast, maturation of mononuclear phagocytes is associated with a reduction in peroxidase content and mature macrophages possess little or no peroxidase activity (**458**).

Macrophages are also facultative anaerobes, with the notable exception of the pulmonary alveolar macrophage (see below). Macrophages are highly motile and have marked phagocytic activity. In contrast to monocytes, macrophages have been shown to proliferate in response to certain stimuli in vitro (**136,267**). Macrophages express receptors for the Fc component of IgG (**134,455**), C3 (**180**) and insulin (**30**). Both monocytes and macrophages express receptors for IgE which is relevant to host immunity to various parasites (**133,303**). Some macrophages express **HLA-DR** antigens and can function as antigen presenting cells for lymphocytes (section **1.7.2**). The expression of **HLA-DR** antigen varies with the type of macrophage; only **15%** of peritoneal macrophages express **HLA-DR** antigen compared with 50% of spleen and thymus macrophages (**37,118**).

Macrophages are widely distributed throughout the body but are particularly prominent in the spleen, lymph nodes, liver (Kupffer cells), peritoneum, skin (Langerhans cells) and pulmonary alveoli. Macrophages resident in different tissues have widely differing morphological and functional properties (table **1**). It has been postulated that the profile of local stimuli, to which macrophages are exposed in a particular tissue, influences their composition and metabolism, and thereby accounts for their diversity of form and function (**112**).

Pulmonary alveolar macrophages

One type of macrophage of particular interest is the pulmonary alveolar macrophage. These cells reside at an air-tissue interface within the alveolus with direct exposure to inhaled microbes and environmental toxins. As a result of this aerobic environment, these cells have developed a predominantly aerobic metabolism (**267**). In comparison with other types of macrophages, pulmonary alveolar macrophages have a greater number of mitochondria and lysosomes, and an increased content of mitochondrial and lysosomal enzymes. In addition, their respiratory rate that is greater than that of any other mammalian phagocyte (**267**). These features are consistent with the prominent antimicrobial function of the pulmonary alveolar macrophage.

Table 1

Types of Macrophages and their Prominent Functions

<u>Tissue</u>	<u>Important Functions</u>
Spleen	Removal of aged and damaged erythrocytes, leukocytes and platelets from the circulation (267) Antigen presentation to lymphocytes (267)
Lymph nodes	Antigen presentation to lymphocytes (267)
Bone marrow (nurse cells)	Highly phagocytic cells Synthesis of iron binding proteins (143) Important source of iron for developing normoblasts (111)
Liver (Kupffer cells)	Removal of microbes, endotoxins, antigens, immune complexes, colloid suspensions, chylomicrons and aged erythrocytes from the circulation (121)
Bone (osteoclasts)	Bone resorption during bone remodelling (267)
Brain (microglial cells)	Function unknown
Lung alveoli (pulmonary alveolar macrophages)	Removal of inhaled microbes and inert particles Antigen presentation to lymphocytes Clearance of surfactant from the alveoli (331)
Pleura and peritoneum	Little known May function as "roller bearings" to facilitate the movements of parietal and visceral membranes (7)
Epidermis (Langerhans cells)	Antigen presentation to lymphocytes (1782) Important roles in cell-mediated immune responses (eg delayed contact hypersensitivity) and contact sensitivity (417)

1.5. Secretory Products of Mononuclear Phagocytes

While early studies of mononuclear phagocyte function focussed predominantly on the phagocytic activity of these cells, research **in** the last two decades has highlighted the importance **of** their secretory function. Monocytes and macrophages release an enormous number of biologically active substances into their local milieu including proteins, lipids, oxygen and nucleotide metabolites. These products are important both in the local environment in mediating inflammation, tissue repair, antimicrobial, tumouricidal and immune regulatory functions, and also in systemic responses such as the acute phase response. Some **of** these factors are listed in table 2.

1.6. Activation of Mononuclear Phagocytes

Activation of mononuclear phagocytes is defined as the morphological, biochemical and functional changes that the cells undergo in expressing enhanced antimicrobial, immune regulatory and tumouricidal function compared with the baseline values of resident and unstimulated cells **(116,339)**. Over 50 structural and functional alterations occur in activated macrophages, some of which are shown in table 3.

Activation of mononuclear phagocytes can be induced by many factors including lymphocyte mediators **(128,379)**, bacterial products **(32,141)**, allergens **(339)**, phagocytosed particles **(370)**, immune complexes **(465)**, complement components **(400)**, viruses **(380)**, IgE **(133)** and phorbol esters **(207)**. However, only lymphokines **can** be considered the ultimate activators of mononuclear phagocytes since they induce the full spectrum of changes associated with mononuclear phagocyte activation including increased antimicrobial activity **(116)**.

The changes that mononuclear phagocytes undergo during activation include increased capacity for adherence and spreading on a substratum **(370)** and an increase in **the** number **of** phagolysosomes and endocytic vesicles **(207)**. The secretion of enzymes is markedly increased, particularly that **of** the neutral proteinases including plasminogen activator **(456)**, collagenase **(483)** and elastase **(482)**. in contrast, the secretion of lysozyme is not altered by activation of mononuclear phagocytes **(116)**. There is also an increase in the Phagocytic activity **of** mononuclear phagocytes which is related to an increase in the number and avidity of Fc receptors **(48)**. Although complement-coated erythrocytes bind equally well to resident and activated mononuclear phagocytes, they are ingested only by cells that have been activated **(116)**.

Table 2**Secretory Products of Mononuclear Phagocytes****A. Enzymes**

Lysozyme (178)

Neutral proteinases:

plasminogen activator (456)
 elastase (482)
 collagenase (483)
 angiotensin convertase (418)

Acid hydrolases (325):

proteinases:

esterases
 lipases
 ribonucleases
 phosphatases
 glycosidases
 cathepsins

Deaminase:

arginase (262)**B. Polypeptide Hormones****interleukin-1 α** and -1 β (288)

interleukin-6 (335)

interleukin-8 (435)

tumour necrosis factor— (42)

interferon- α (168)

platelet derived growth factor (313)

transforming growth factor-6 (131)

fibroblast activating factors (142)

fibroblast growth factors (51)

insulin-like activity (166)

erythropoietin (376)

granulocyte-macrophage colony

stimulating factor (356)

granulocyte colony stimulating
factor (244)**D. Oxygen Metabolites**

superoxide anion (147)

hydrogen peroxide (328)

hydroxyl radical (474)

hypohalous acids (252)

D. Inhibitors of enzymes and cytokines

Anti-proteinases:

 α_1 -antitrypsin (312) **α_2 -macroglobulin** (210) **α_1 -antichymotrypsin** (39)

plasminogen activator inhibitor (99)

collagenase inhibitor (478)

tissue inhibitor of metalloproteinases (76)

plasmin inhibitors (255)

phospholipase inhibitors:

lipomodulin (52)

IL-1 inhibitors (378)**E. Complement Components**

(38,173,325,485)

Classical pathway:C1, C4, C2, C3, **C5**

Alternative pathway:

factor B

factor D

properdin

Inhibitors:

C3b inhibitor

F. Coagulation factors

Intrinsic pathway:

IX, X, V, prothrombin (345)

Extrinsic pathway:

VII (98)

plasminogen activator (456)

plasminogen activator inhibitors (456)

G. Binding Proteins

fibronectin (374)

thrombospondin (221)

transferrin (191)

transcobalamin **II** (373)**H. Bioactive Lipids**leukotrienes **B₄**, C, D and E (163275)prostaglandins **E₂** and **F_{2 α}** and

thromboxane (348.403)

Activation of mononuclear phagocytes is associated with a reduction in the expression of the plasma membrane-associated ectoenzymes 5' nucleotidase **(151)** and adenosine monophosphatase **(243)**. The latter changes may be due to the increased pinocytosis and membrane internalisation that is associated with cellular **activation (481)**.

Glucocorticosteroids inhibit macrophage activation by rendering macrophages unresponsive to lymphokines (295). Steroids also inhibit neutral proteinase secretion. However, it is not clear whether this is the result of a direct action of steroids on the macrophage or because steroids inhibit lymphocytes that produce mediators of macrophage function **(480)**. Steroids also inhibit mononuclear phagocyte maturation (440) and the **accumulation** of these cells at sites of inflammation **(338)**.

Table 3

Characteristics of Activated Mononuclear Phagocytes

A. Morphological

Increases in:

cell size (267)
adhesiveness and spreading (370)
cytoplasmic granules (207)
ruffling of plasma membrane (236)
pseudopod formation (236)

B. Biochemical

Increases in:

respiratory burst (236)
glucose metabolism (116)
adenyl cyclase activity (267)
calcium ion influx (267)

Decreases in plasma membrane:

5' nucleotidase (151)
adenosine monophosphatase (243)

C. Secretory

increased secretion of

plasminogen activator (456)
elastase (482)
collagenase (483)
complement components (236)
acid hydrolases (236)
arghase (236)
fibronectin (236)
interleukin-1 (420)
interleukin-6 (201)
tumour necrosis factor- α (493)

Secretion not changed:

lysosome (116)

D. Functional (236,267)

Increases in:

pinocytosis
phagocytosis
intracellular microbicidal activity
chemotaxis
antigen presentation
tumouricidal activity

E. Receptor Expression

Increases in:

Fc receptors (48)
CR1 and CR3 (386)
CD11/CD18 antigens (130)
IL-2 receptors (454)
MHC class II antigens' (433,469)

Decreases in:

MHC class II antigens' (277,422,427)

- The effect of cellular activation on MHC class II antigen expression by mononuclear phagocytes depends upon the nature of the stimulus. Interferon- γ , interleukin-4, granulocyte-macrophage colony stimulating factor and granulocyte colony stimulating factor induce the expression of MHC class II antigen by mononuclear phagocytes. In contrast, prostaglandin E, and endotoxin inhibit class II MHC antigen expression by mononuclear phagocytes.

1.7. Functions of Mononuclear Phagocytes

1.7.1. Phagocytic and Antimicrobial Functions

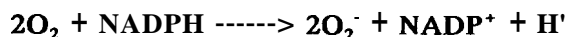
The mononuclear phagocyte plays a prominent role in the host defense against many microbes by using its well-developed phagocytic function to assist neutrophils in the ingestion and killing of various species of bacteria and fungi. Moreover, mononuclear phagocytes are the first line of defense against facultative and obligate intracellular parasites such as *Mycobacteria*, *Legionella*, *Listeria*, *Brucella*, *Chlamydia*, *Toxoplasma* and *Leishmania* spp (267). Mononuclear phagocytes also eliminate viruses and virus-infected cells (112).

The process of phagocytosis consists of three phases: attachment; ingestion; and killing (267). During the initial attachment phase, the mononuclear phagocyte binds to the microbe by non-specific surface receptors. However, attachment is enhanced greatly if the microorganism has been opsonised by immunoglobulin or by C3b (which is generated by activation of the complement system) since mononuclear phagocytes possess surface receptors that specifically recognise the Fc component of Ig and C3b. Following attachment, the ingestion phase occurs by the extension of pseudopods which enclose the microorganism in a membranous vacuole. Contraction of the cell's actin filaments results in the internalisation of the vacuole to form a phagosome. The killing phase involves both oxygen-dependent and oxygen-independent mechanisms (170). Oxygen-dependent mechanisms, which are discussed below, involve the production and intracellular release of reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radicals (21). Oxygen-independent mechanisms, which are probably less important, involve fusion of the primary lysosomes with the phagosome, followed by the discharge of lysosome, acid hydrolases and complement components into the phagolysosome which has been formed (170). As a result of this fusion step, the phagolysosome is acidified and the acid hydrolases initiate the digestion phase. The energy required for phagocytosis by mononuclear phagocytes is derived from anaerobic glycolysis, with the notable exception of the alveolar macrophage which relies heavily on aerobic glycolysis.

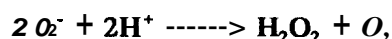
Oxygen-dependent microbial killing: The respiratory burst of mononuclear phagocytes

When mononuclear phagocytes are triggered by a phagocytic or pharmacological stimulus, they rapidly consume a large amount of oxygen and metabolise large quantities of glucose via the hexose monophosphate shunt (22). The purpose of this respiratory burst is to generate a number of reactive oxygen metabolites with the potential to inhibit the growth of, or to kill microorganisms and neoplastic cells. These products of oxygen include superoxide anion, hydrogen peroxide, hydroxyl radicals and oxidised halogens.

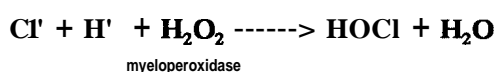
The respiratory burst results from activation of the NADPH oxidase respiratory complex which is a series of electron transporting molecules (26,388). The overall reaction is to transfer electrons from the high energy state of glucose to oxygen. The electrons from glucose are initially incorporated into reduced β -nicotinamide adenine dinucleotide phosphate (NADPH). The electrons are then transferred down an electron transport chain of redox molecules, resulting in the one electron reduction of oxygen (O_2) to form superoxide anion (O_2^-), (347):



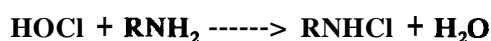
Most of this superoxide anion dismutates rapidly to produce oxygen and hydrogen peroxide (H_2O_2), (383):



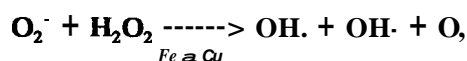
At the same time, glucose is metabolised through the hexose monophosphate shunt to regenerate the NADPH that has been consumed by the superoxide anion-forming system. Superoxide anion and hydrogen peroxide are not used directly by the cells for microbial killing, since hydrogen peroxide is only weakly microbicidal and superoxide anion is completely innocuous (22). Rather, these products are used to produce the true microbicidal oxidants of phagocytes which are divided into two classes: oxidised halogens and oxidising radicals. The production of oxidised halogens starts with the myeloperoxidase-catalysed oxidation of a halide ion by hydrogen peroxide to form hypochlorous acid (250). For example, chloride ion (Cl^-) is oxidised to hypochlorous acid ($HOCl$):



Hypochlorous acid, the prototype of the oxidised halogens, is an extremely powerful microbicidal agent. It is also the precursor of the chloramines ($RNHCl$), another group of microbicidal oxidised halogens, which are formed by the reaction between hypochlorous acid and ammonia or amines (RNH_2), (475):



Oxidising radicals, the other class of microbicidal oxidants, are formed in reactions involving superoxide anion. The hydroxyl radical ($OH\cdot$) is made by the Haber-Weiss reaction which is a reaction between superoxide anion and hydrogen peroxide catalysed by metal ions (23):



Enzymes involved in the respiratory burst

Two of the enzymes involved in the generation of reactive oxygen metabolites are studied in this thesis: the NADPH oxidase system and myeloperoxidase.

1. The NADPH oxidase system

In 1964 a membrane-associated pyridine nucleotide oxidase was described, which was thought to be the enzyme responsible for the respiratory burst by catalysing the production of superoxide anion from oxygen, using NADPH as the electron donor (389). However, in recent years it has become clear that the "NADPH oxidase" is a series of electron transporting proteins rather than a single enzyme.

The terminal oxygen-binding component of the oxidase system is cytochrome b-245 which is a heterodimer composed of a 23 kD α chain and a heavily glycosylated 76 to 92 kD β chain (405). The cytochrome is located in the plasmalemma of mononuclear phagocytes and becomes incorporated into the wall of the phagocytic vesicle as it is formed by an invagination of this membrane (405,407). The function of a redox molecule is largely dependent upon its midpoint potential (at which it is balanced between oxidation and reduction) and this governs its bioenergetic stature in the hierarchy of components in an electron transport chain. At 245 mV this cytochrome has the lowest midpoint potential of any mammalian cytochrome b, which provides it with the capacity to reduce oxygen directly to form superoxide anion (495). However, cytochrome b-245 is not the only component of the electron transport chain since cytochromes are not able to receive electrons directly from NADPH (406). There is evidence to suggest that: 1) a 65 to 67 kD membrane-associated flavoprotein binds to and links NADPH with cytochrome b-245 (238); and 2) the cofactor is flavin adenine dinucleotide (251,388). In addition, two cytosolic proteins (a 47 kD phosphoprotein and a 65 kD protein) may be involved in the electron transport chain since both proteins translocate into the membrane upon activation of the oxidase system (72,198). Moreover, Babior and coworkers (24) have identified at least 10 other proteins that may be involved in the electron transport chain.

One unifying theory of the electron transport chain has been proposed by Segal (406). He proposed that upon activation of the cell, the 65 kD cytosolic protein moves into the membrane and functions either as the proximal NADPH-binding flavoprotein, or intercalates into the chain distal to the 65 to 67 kD membrane protein with these characteristics. Activation of the cell also results in phosphorylation of the 47 kD cytosolic protein and movement of this protein into the membrane where it could attach to a binding site on cytochrome

b-245. The 47 **kD** protein may be an electron-transporting molecule or could play a structural or regulatory role in the activation of the chain. Electrons are thus transported across the membrane from the NADPH-binding protein, which projects into the cytosol, to cytochrome b-245 which projects into the lumen of the phagocytic vacuole where oxygen is reduced to form superoxide anion.

2. Myeloperoxidase

Monocytes contain large amounts of myeloperoxidase (MPO) which accounts for about 5% of their total cellular protein content (405). MPO is located within the lysosomal granules of promonocytes and monocytes. Following phagocytosis of microorganisms, lysosomal granules ~~fuse~~ with the phagocytic vesicle and discharge their content of enzymes, including MPO, into the phagolysosome. As described above, MPO catalyses the oxidation of halide ions by hydrogen peroxide to form hypohalous acids. Although these potent oxidising and halogenating agents are toxic to microorganisms, it is unclear whether this is the major physiological pathway. It has been postulated that the chief function of MPO may be to degrade hydrogen peroxide to water by a ~~caialase-like~~ activity (405).

MPO does not appear to be essential for ^{microbiocidal} activity since hereditary deficiency of this enzyme is usually associated with good health. In addition, the transition from monocytes to macrophages is accompanied by almost a complete loss of granule-associated MPO, but there is no concomitant loss of antimicrobial function. However, this may be because MPO is part of a very efficient and broad spectrum killing mechanism and organisms that are particularly susceptible to oxidised halogens have not yet evolved into human pathogens. It is also possible that macrophages obtain some MPO from monocytes or neutrophils either directly or following phagocytosis of damaged cells (306).

Other phagocytic functions of mononuclear phagocytes

Another important phagocytic function of mononuclear phagocytes is to remove damaged or aged cells from the circulation. Macrophages lining the sinusoids of the spleen have a particularly important role in ingesting senescent erythrocytes which are removed from the circulation at a rate of 0.9% per day (267). Erythrocytes circulating through the spleen pass through fenestrations in the basement membrane between sinus endothelial cell and collect in the splenic sinuses. Successful passage through ~~this~~ filtration system requires normal deformability of the erythrocyte. Cells ~~with~~ abnormal deformability, such as aged cells and cells from

patients with hereditary spherocytosis, are unable to pass through and these cells are removed by macrophages. Macrophages **also** recognise changes in the **erythrocyte** surface membrane that are induced by immunoglobulin coating chemical injury, trauma, parasitic inclusions and the ageing process (111,292,369). The destruction of erythrocytes occurs mainly by osmotic lysis, fragmentation and erythrophagocytosis (472). The globin moiety **of** the phagocytosed erythrocyte is then converted to amino acids and bilirubin by the enzyme haem oxygenase. **A** similar phenomenon occurs in the liver and bone marrow.

Mononuclear phagocytes also remove aged platelets, megakaryocyte nuclei and aged or damaged leukocytes from the circulation (111). In addition, macrophages recognise and ingest intact senescent neutrophils at sites of inflammation, and may thereby limit the degree of tissue injury and promote resolution of the inflammatory response (397). Other phagocytic functions of mononuclear phagocytes include the removal of inorganic particles that are inhaled into the lungs such as elements of cigarette smoke and dust particles. In addition, these cells ingest tissue debris which is an important step in the normal debridement of wounds (236). Macrophages have been identified early in fetal development. It is thought that these cells remove the tissue debris that is generated as one maturing tissue replaces another in the process of tissue remodelling during embryogenesis (112,236).

1.7.2 Immunological Functions

A second well-defined function of mononuclear phagocytes is their interaction with lymphocytes in the immune response. **This** interaction is essential for the development of cellular and humoral immunocompetence and **has** two major components (267). The first component involves the induction of a response to antigen, and **is** called the *afferent* or *inductive* limb of the immune response. The *efferent* or *effector* limb of the immune **response** includes the expression of cell-mediated immunity, and the secretion of mediators with immune regulatory functions such as interleukin-1 (IL-1), tumour necrosis factor- α (**TNF**) and interferon- γ (IFN).

The afferent limb

The role of mononuclear phagocytes in the induction of specific immune responses is complex and involves both B and T lymphocytes. B lymphocytes and certain **T** lymphocytes, in particular T-helper (T_H) cells, are unable to recognise and bind to free antigen. For recognition to occur, antigen must be expressed along with **MHC** class II products on the surface of cells called antigen presenting cells (454). Although several classes of

MHC type II antigens have been identified including HLA-DP, HLA-DQ, HLA-DR and HLA-DS (176,355,454,488), HLA-DR antigens have been studied the most thoroughly.

A proportion of mononuclear phagocytes express HLA-DR antigens and, therefore, act as antigen presenting cells for lymphocytes along with the non-phagocytic dendritic cells in blood, lymph and other tissues, and Langerhans cells in the skin (454). Foreign proteins are internalised by mononuclear phagocytes and undergo intracellular processing (11,506) which is thought to alter the protein so that it acquires an affinity for an HLA-DR antigen (20,69). The processed protein is then displayed on the cell surface along with the HLA-DR antigen. The T cell antigen receptor recognises and binds to this complex which, in turn, induces IL-1 production by the mononuclear phagocyte (264). The antigen-HLA-DR antigen complex, together with IL-1, stimulate the T cell to express interleukin-2 (IL-2) receptors and release IL-2 (280,454). The binding of IL-2 to its receptor stimulates the proliferation of T4 cells and initiates a cascade of activation steps resulting in:

- 1) the release of lymphokines from T4 cells which activate macrophages to **kill** intracellular microorganisms;
- 2) activation of cytotoxic T (T8) lymphocytes;
- 3) activation of B lymphocytes by T4 cells to produce antibody; and
- 4) the generation of an inflammatory reaction (454).

The effluent limb

Macrophage involvement in the effector limb of the immune response includes increased microbial **killing** tumouricidal activity, the delayed hypersensitivity reaction of tuberculosis and granulomatous hypersensitivity, and the rejection of allograft organs (267). To achieve optimal killing or inactivation of pathogenic organisms, macrophages must be activated. This activation is induced by lymphokines which are released by activated T lymphocytes such as migration inhibition factor, macrophage activation factor and a mononuclear phagocyte chemotactic factor (379,399). During this effector stage, macrophages exhibit increased phagocytosis and microbial **killing**, and secrete a wide variety of biologically active molecules that influence both limbs of the immune response (384).

The participation of mononuclear phagocytes in antibody-dependent cell-mediated cytotoxic events is thought to be important in the **killing** of tumour cells and in immunity to protozoa and nematodes. Mononuclear Phagocytes bind to antibody-coated tumour cells and parasites such as *Schistosomes*, *Leishmania*, *Trypanosoma*

and *Plasmodium* spp by their Fc receptors for IgG (286,324). This is followed by the secretion of enzymes and reactive oxygen metabolites which ultimately causes the mononuclear phagocyte to become cytotoxic for the target cells (326).

1.7.3. Inflammation

The acute phase response is a systemic inflammatory reaction to infection or injury. It is characterised by fever, tachycardia, shock and changes in the concentration of some circulating proteins such as C reactive protein and fibrinogen. At least three monocyte product are thought to be important in this response: IL-1, TNF, and interleukin-6 (IL-6), (137,445).

IL-1 is thought to mediate fever by stimulating the release of prostaglandin E in the hypothalamus which resets the hypothalamic thermoregulatory center (40,137). IL-1 also mediates the accelerated catabolism of muscle protein and the negative nitrogen balance that are associated with acute infection and inflammation (18,420).

TNF regulates the synthesis of acute phase proteins (127) and is also an endogenous pyrogen (138). In addition, TNF is thought to be responsible, in part, for the disseminated intravascular coagulation and multi-system failure seen in endotoxic **shock**. This hypothesis is supported by two observations. First, plasma TNF concentrations correlate with the degree of shock in patients with meningococcal septicaemia (464). Second, antibody to TNF limits experimental endotoxic **shock** (446).

IL-6 has been implicated recently in mediating the acute phase response since serum concentrations of IL-6 correlate with C-reactive protein levels and fever in patients with severe burns (335). Peripheral blood monocytes are the main source of IL-6 which is the **main** inducer of acute phase protein production in cultured hepatocytes (95).

In addition to their systemic effects in inflammation, IL-1 and TNF also induce local effects at inflammatory foci including: i) the recruitment of leukocytes into sites of inflammation; 2) the modulation of multiple functions of neutrophils such as degranulation and the generation of reactive oxygen metabolites; and 3) the proliferation of fibroblasts and increased synthesis of collagen by these cells (420,493).

1.7.4. Tumouricidal Function

Mononuclear phagocytes infiltrate tumours (150,494) and are probably one of the main host defenses against tumours that arise spontaneously (199). Mononuclear phagocytes use both antibody-independent and antibody-dependent mechanisms to **kill** tumour cells.

Antibody-independent mechanisms

Activated macrophages are non-specifically cytotoxic to tumour cell lines in vitro (2). The nature of the tumour antigen to which the macrophage binds is not known, but tumour recognition triggers a cytotoxic response that is a non-phagocytic and contact-mediated event. A later step in the process involves the secretion of effector substances such as neutral proteinases, arginase, thymidine, components of complement, hydrogen peroxide and other toxic oxygen metabolites (5,326). Mononuclear phagocytes also release TNF which produces haemorrhagic necrosis of murine tumour cells (94) and is an important mediator of the cytotoxicity of human monocytes for tumour cells in vitro (162).

Antibody-dependent mechanisms

Mononuclear phagocytes also **kill** tumour cells by an antibody-dependent cytolytic mechanism (286,324). Tumours are recognised by specific antibodies that bind to target cell antigen (324). Mononuclear phagocytes bind to these antibodies by their Fc receptors and **this** stimulates the release of reactive oxygen metabolites resulting in tumour cell lysis (326).

1.7.5. Wound **healing**, tissue repair and remodelling

Mononuclear phagocytes debride wounds by releasing proteinases such as collagenase and elastase, and by ingesting tissue debris (482,483). Monocytes and macrophages also participate in tissue remodelling and **repair** by releasing substances that stimulate fibroblast proliferation and neovascularisation (271,272). Osteoclasts, which are thought to belong to mononuclear phagocyte system, participate in the remodelling of bone (298,326).

1.7.6. Haemopoiesis

Part of the acute response to infection and injury is a peripheral blood leukocytosis. Mononuclear phagocytes play an important role in inducing increased leukopoiesis in response to these stresses by secreting

haemopoietic growth factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF), (416). These colony stimulating factors stimulate the formation **a d** growth of colonies of granulocytes and monocytes from committed progenitor cells (305,458). Mononuclear phagocytes also stimulate haemopoiesis indirectly by releasing IL-1 and TNF which induce T lymphocytes to produce haemopoietic growth factors such as GM-CSF and interleukin-3 (IL-3), (416).

Mononuclear phagocytes play a role in erythropoiesis. The role of the mononuclear phagocyte in iron metabolism is well established. When aged erythrocytes are removed from the circulation by mononuclear phagocytes, the iron component of haemoglobin is recycled. Most of the iron is transported back to the bone marrow by the iron transport protein transferrin, some of which is synthesised by macrophages (143). In **addition**, bone marrow macrophages **serve** as a source of iron for developing normoblasts (111). Finally, macrophages produce erythropoietin, a glycoprotein hormone that stimulates erythropoiesis (376).

1.7.7. Blood Coagulation

The host response to infections, tumours or injury may be to activate the coagulation system within the circulation resulting in disseminated intravascular coagulation. Mononuclear phagocytes activate the blood coagulation process in response to bacterial endotoxin, immune complexes, complement components and other stimuli by synthesising and expressing tissue thromboplastin, which is the most potent activator of the extrinsic coagulation pathway (460,461). Moreover, it has been shown that mononuclear phagocytes from patients with meningococcal septicaemia (346) and tumours of the lung (152) and breast (19) synthesise increased amounts of tissue thromboplastin in vitro when compared with cells from healthy subjects. In addition, monocyte products such as IL-1 and **TNF induce** the production of tissue thromboplastin by endothelial cells, and thereby potentiate the thrombogenic state (45,329). Mononuclear phagocytes also secrete plasminogen activator (456), plasmin inhibitor (326), prostaglandins (**263**), thromboxane (403) and platelet activating factor (**85**), all of which have an important role in the coagulation cascade, and in fibrin formation and degradation.

1.8 Monocyte Heterogeneity

In contrast to tissue macrophages, peripheral blood monocytes were initially considered to be a homogeneous population of cells. In recent years it has become increasingly clear that while monocytes share certain morphological and functional characteristics, they are by no means uniform in this respect. A large number of studies have documented the nature and extent of monocyte heterogeneity, and have suggested mechanisms by which this heterogeneity may have arisen.

Freshly-isolated monocytes have been shown to be heterogeneous with respect to physical properties such as size and density, the expression of surface receptors and various functional properties. Such differences have been exploited to separate subpopulations of monocytes. The following account summarises the literature on subpopulations of monocytes based on differences in: 1) size and volume; 2) density; 3) surface protein expression; 4) function; and 5) enzyme content.

1.8.1. Separation of Monocyte Subpopulations Based on Differences in Cell Size or Volume

Both counter current centrifugal elutration (CCE) and density gradient centrifugation have been employed to separate monocyte subpopulations on the basis of differences in cell size. Two major subpopulations have been identified: "small" and "large" monocytes with mean cell diameters of 7 to 8 μM and 8 to 10 μM , respectively (9,16,108).

Antimicrobial functions

"Large" monocytes have been shown to possess greater antimicrobial function when compared with "small" monocytes. For example, "large" monocytes are more responsive to chemotactic stimuli than "small" monocytes (110). In addition, "large" cells are more phagocytic with respect to ingestion of carbonyl iron (16), opsonised sheep erythrocytes (218) and latex beads (108) compared with "small" monocytes. The differences in phagocytic activity between "small" and "large" monocytes may reflect differences in Fc receptor expression. Since it has been reported that "large" monocytes are predominantly Fc receptor positive, whereas "small" monocytes are predominantly Fc receptor negative (337). However, other investigators failed to confirm the latter finding (9,299,401). Differences in the generation of oxidants by "small" and "large" monocytes have also been reported. "Large" monocytes produce 1.5-fold more superoxide anion in response to opsonised zymozan (503) and release up to 6-fold more hydrogen peroxide under basal conditions when compared with "small cells"

(452). Moreover, "large" cells produce more hydrogen peroxide in response to LPS and IFN than "small" monocytes (452). "Large" monocytes have also been shown to contain 1.4 to 2-fold more peroxidase activity (9,503) and a greater number of peroxidase positive granules (9) than "small" monocytes.

Biochemical markers

Differences in biochemical markers have been described. "Small" monocytes have a lower acid phosphatase content (10), lower peroxidase activity (10,503) and a lower total protein content (10) than "large" cells. However, Akiyama and coworkers demonstrated that "small" and "large" monocytes express similar amounts of 5' nucleotidase (9) and alkaline phosphodiesterase (10) which are markers of monocyte activation.

Cytokine production

Differences in cytokine release by "large" and "small" monocytes have been described. Akiyama and coworkers (10) demonstrated that when cultured under basal conditions, "small" and "large" cells produce similar amounts of IL-1, IFN, TNF and prostaglandin **E₂ (PGE₂)**, but "small" cells produce slightly more colony stimulating factor (CSF) than "large" cells. These investigators also reported that when monocytes are stimulated with **polyriboinosinic:polyribocytidylic acid (polyI:polyC)**, "small" cells release 3-fold more IFN and 30% more CSF, but 2-fold less **PGE₂** when compared with "large" cells (10). The pattern of IL-1 production by stimulated monocytes depends upon the nature of the stimulus. Akiyama and coworkers (10) reported that polyI:polyC stimulates the "small" cells to produce 4-fold more IL-1 than the "large" cells. in contrast, LPS induces similar increases in IL-1 production by "small" and "large" cells, and GM-CSF stimulates greater IL-1 production by "large" cells when compared with "small" monocytes (218).

Immune effector functions

"Small" and "large" monocyte subpopulations also differ with respect to several immune effector functions. Norris *et al.* (337) demonstrated that only "large" monocytes lyse erythrocyte target cells in antibody-dependent cellular cytotoxicity (ADCC) assays. Akiyama *et al.* (10) confirmed that "large" monocytes exhibit greater ADCC than "small" cells and demonstrated that "large" cells are more effective than "small" cells at mediating mitogen-induced T lymphocyte proliferation. The latter investigators also showed that a greater proportion of "large" cells express CD11b/CD18 surface antigen and the receptor for poke weed mitogen (PWM) compared with **"small"** cells but no differences in HLA-DR antigen expression by these monocyte subsets were detected. in contrast, ~~Ha~~ *et al.* (158) reported that "small" cells express fewer HLA-D and HLA-

DR molecules than "large" cells. ~~Esa~~ and coworkers also demonstrated that: 1) "small" cells are more efficient at presenting soluble and particulate antigens to lymphocytes than "large" cells; and 2) lymphocytes activated with antigen-pulsed "large" cells exhibit more suppressor cell activity than those activated with antigen-pulsed "small" cells.

Tumouricidal function

Differences in tumouricidal activity between "large" and "small" monocytes have been reported. **Normann** and coworkers (336) found that only "small" monocytes express native tumouricidal activity. Moreover, "small" cells **can** be activated by lymphokines and LPS for tumour cell killing whereas "large" cells **are** resistant to activation for this function. In contrast, Inamura et al. (218) did not detect any differences between "large" and "small" monocytes in cytotoxicity against allogeneic tumour cells when the monocytes were cultured under either basal conditions, or in the presence of LPS or GM-CSF.

1.8.2. Separation of Monocyte Subpopulations Based on Differences in Density

The technique of sedimentation of monocytes through discontinuous gradients of bovine or human serum albumin (246,357,501) or Percoll (153) has been widely used to separate monocytes subpopulations on the basis of differences in their density. Two major subpopulations have been identified using these techniques: "low" and "high" density cells.

Morphology

Morphological differences between the "low" and "high" density subsets have been reported. Monocytes within the "high" density fraction possess mainly round or ovoid nuclei and have few, if any, membrane pseudopodia (64). In contrast, monocytes in the "low" density fraction possess folded or indented nuclei and have numerous membrane pseudopodia (64). It has been postulated that these morphological differences represent differences in cytoplasmic or membrane fluidity between the subpopulations (64,144).

Biochemical markers

"High" and "low" density monocyte subpopulations differ with respect to several biochemical markers. It has been reported that the "high" density cells express high non-specific esterase activity, high 5' nucleotidase activity, high peroxidase activity and low acid phosphatase activity, whereas the "low" density cells express low non-specific esterase activity, low 5' nucleotidase activity, low peroxidase activity and high acid phosphatase

activity (164,357).

Receptor expression

Differences in the expression of surface receptors have been reported. "High" density cells express greater amounts of Fc and C3 receptors compared with "low" density cells (401).

production of soluble mediators

Differences in the synthesis of soluble mediators by "low" and "high" density cells have been demonstrated. A thymocyte IL-1-like activity is produced in larger amounts by "high" density monocytes when compared with "low" density cells (154,246). However, there is controversy regarding PGE₂ production by the monocyte subpopulations since PGE₂ production by the "high" density cells has been reported to be equal to (165), greater than (153,246) and lower than (357,501) that of the "low" density cells.

Immune effector functions

Density-defined subpopulations of monocytes also differ with respect to several immune effector functions. Schreiber and coworkers (401) reported that "high" density monocytes have a greater capacity to support PWM-induced B-cell differentiation compared with "low" density cells (401). However, Figdor et al. (164) demonstrated that "high" density monocytes are less capable of inducing the proliferation of lymphocytes in mixed leukocyte cultures than "low" density cells and this difference is not due to differences in HLA-DR antigen expression between the monocyte subsets. Figdor and coworkers (164) also reported that "high" density monocytes are more active with respect to ADCC than "low" density cells.

Khansari and coworkers (246) reported that the functional activities of the two subpopulations are mutually antagonistic. These investigators demonstrated that "high" density cells enhance the production of immunoglobulins by B lymphocytes in response to PWM. In contrast, the "low" density subset not only lack this activity but actively suppress the background response that is generated in the absence of added monocytes, and the response that is produced in cultures containing "high" density cells. Khansari and coworkers postulated that this effect is due in part to high basal production of PGE₂ by the "low" density subset.

Relationship between size and density-defined subpopulations of monocytes

In view of the relationship between cell size and density, Dougherty et *al.* (144) postulated that there is a relationship between the size and density-defined subpopulations of monocytes discussed above. These investigators proposed that: 1) the "small" monocytes are the same subpopulation as the "high" density cells and the "large" monocytes are identical to the "low" density cells; and 2) there are two major subpopulations of monocytes, "small, high" density cells and "large, low" density cells. However, this hypothesis has not yet been tested.

The properties of the size and density-defined monocyte subpopulations are summarised in table 4. While there is general agreement about the various biochemical and functional characteristics of the two monocyte subpopulations, there is some disagreement between different laboratories as to their precise phenotype (table 4). In particular, discrepant results have been reported for the following monocyte functions:

- 1) immune effector functions such as HLA-DR antigen expression (10,158,164) and the capacity to induce lymphocyte proliferative responses (9,164,246,401);
- 2) tumouricidal activity (218,336,501);
- 3) the release of biologically active mediators such as IL 1 (10,154,246) and PGE₂ (153,165,357); and
- 4) Fc receptor expression (9,299,337,401).

A number of different procedures were used to isolate monocytes for the studies listed above including CCE, density gradient centrifugation and adherence to tissue culture plastic. The variety of monocyte isolation procedures used may account for the discrepant results reported by these investigators. This possibility is supported by the following observations.

- 1) The initial population of monocytes from which the subpopulations were derived may differ *since* the yield of monocytes is always less than 100% and varies considerably with the isolation procedure used (146,219,442).
- 2) Some gradient media, such as ficoll-Hypaque, are frequently contaminated with endotoxin (190). Endotoxin has been shown to affect many aspects of monocytes function including HLA-DR antigen expression (504), and the production of TNF (43,106), PGE₂ (153) and IL-1 (185,245).
- 3) The temperature at which monocytes are incubated during isolation varies considerably with the method used. For example, monocytes are incubated at room temperature throughout the CCE method

whereas the density gradient centrifugation method involves an adherence step which is conducted at **37°C**. incubation temperature has been shown to affect the expression of several surface receptors by monocytes including **CR3**, **CR1** and **HLA-DR** antigen (**146,160,421**).

4) The number of contaminating lymphocytes and natural killer cells in the monocyte preparations varies with the isolation procedure used (**164,258**) and this may contribute to differences in immune effector and tumouricidal functions of the monocyte subpopulations in different studies.

Table 4**Functional Heterogeneity of Size and Density Defined Monocyte Subpopulations**

Function	<u>small/high density</u> monocytes	<u>large/low density</u> monocytes	References
A. Biochemical Markers			
non-specific esterase activity	high	low	Figdor <i>etal.</i> (164)
acid phosphatase activity	low	high	Pick <i>et al.</i> (357)
total protein content	low	high	Akiyama <i>etal.</i> (10)
5' nucleotidase activity	equal	high	Akiyama <i>etal.</i> (9)
	higher	lower	Pick <i>et al.</i> (357)
alkaline phosphodiesterase	equal	equal	Akiyama <i>etal.</i> (10)
B. Antimicrobial Functions			
chemotactic activity	lower	higher	Arensen <i>etal.</i> (16)
phagocytosis	lower	higher	Arensen <i>etal.</i> (16)
	lower	higher	Inamura <i>etal.</i> (218)
	equal	higher	Chiu <i>etal.</i> (108)
Superoxide production	equal	equal	Akiyama <i>etal.</i> (9)
hydrogen peroxide production	lower	higher	Yasaka <i>etal.</i> (503)
peroxidase activity	lower	higher	Turpin <i>etal.</i> (452)
	lower	higher	Yasaka <i>etal.</i> (503)
	higher	higher	Akiyama <i>etal.</i> (9)
	higher	lower	Figdor <i>etal.</i> (164)
C. Immune Effector Functions			
ADCC	inactive	active	Norris <i>etal.</i> (337)
	lower activity	higher activity	Akiyama <i>etal.</i> (9)
	higher activity	lower activity	Figdor <i>etal.</i> (164)
	equal	equal	McCarley <i>etal.</i> (299)
induction of lymphocyte proliferation	lower capacity	higher capacity	Figdor <i>etal.</i> (164)
capacity to support PWM-induced B cell differentiation	greater	lower	Schreiber <i>etal.</i> (401)
effect on PWM-induced Ig production by B cells	enhancement	suppression	Khansari <i>etal.</i> (246)
effect on mitogen-induced T cell proliferative responses	lower	higher	Akiyama <i>etal.</i> (9)
antigen presentation	more efficient	less efficient	Figdor <i>etal.</i> (158)
D. Tumouricidal activity			
	present	absent	Normann <i>etal.</i> (336)
	higher	lower	Wykoff <i>etal.</i> (501)
	equal	equal	Inamura <i>etal.</i> (218)

Table 4 (continued)

Function	small/high density monocytes	large/low density monocytes	References
E. Production of Soluble Mediators			
<u>IL-1 production</u>			
- basal	equal higher	equal lower	Akiyama etal. (10) Khansari etal. (246)
- polyI:polyC stimulated	4-fold higher than large cells		Akiyama etal. (10)
- GM-CSF stimulated	lower	higher	Inamura etal. (218)
- LPS	equal higher	equal lower	Inamura etal. (218) Elias etal. (154)
<u>IFN production:</u>			
- basal	equal	equal	Akiyama etal. (10)
- polyI:polyC stimulated	3-fold higher than large cells		Akiyama etal. (10)
<u>CSF production:</u>			
- basal	slightly higher than large cells		Akiyama etal. (10)
- polyI: polyC stimulated	30% higher than large cells		Akiyama etal. (10)
<u>basal PGE₂ production</u>			
-basal	equal equal	equal equal	Akiyama etal. (10) Figdor etal. (165)
	5-6 fold greater than low density cells		Khansari etal. (246)
	small amounts	substantial amounts	Picker etal. (357)
	4-10 fold lower than low density cells		Wykoff etal. (501)
- LPS stimulated	substantially greater	minimal amounts	Elias etal. (153)
- polyI:polyC stimulated	2-fold lower than large cells		Akiyama etal. (10)
F. Receptor Expression			
Fc receptor expression	negative equal equal higher	positive equal equal lower	Norris etal. (337) McCarley et al. (299) Akiyama etal. (9) Schreiber etal. (401)
CR3 receptor expression	higher	lower	Schreiber etal. (401)
HLA-DR antigen expression	equal equal lower	equal equal higher	Figdor etal. (164) Akiyama etal. (9) Esa etal. (158)
PWM receptor	lower	higher	Akiyama etal. (9)

1.8.3. Separation of Monocyte Subpopulations Based on Differences in Surface Receptors

Monocytes have been shown to be heterogeneous with respect to a large number of surface molecules including: 1) Fc receptors for IgG (342,437,505) and IgE (303); 2) receptors for complement (212,488); 3) class II MHC antigens (175,176,340,488); and 4) receptors for peanut agglutinin (186), PWM (9) and CD4 (436). Several research groups have exploited these differences to isolate monocyte subsets that are functionally distinct.

Fc receptor expression

Differences in the expression of Fc receptor for IgG have been used to isolate Fc receptor positive (**FcR⁺**) and negative (**FcR⁻**) monocyte subsets which differ in their immune regulatory functions. It has been reported that only **FcR⁻** cells enhance PWM-induced immunoglobulin secretion (505). In addition, several laboratories have demonstrated that only **FcR⁻** cells are highly active at presenting antigen to T lymphocytes and stimulating both autologous and allogeneic mixed lymphocyte reactions (342,437,505). In contrast, only **FcR⁺** monocytes suppress mitogen- and antigen-induced lymphocyte proliferation effectively (342,437,505). Szabo and coworkers (437) postulated that the immunosuppressive activity of the **FcR⁺** population is related to the fact that this subpopulation is the major source of **PGE₂**, a potent immunosuppressive monokine.

Differences in tumouricidal activity between the FcR subpopulations have also been identified. Zembala *et al.* (505) demonstrated that only the **FcR⁺ cells** are strongly cytostatic against tumor **cells in vitro**. In addition, the **FcR⁺ cells** are the major TNF-producing population of monocytes (437).

Complement receptors

Whisler and coworkers (487) demonstrated that 70% of monocytes express receptors for complement (**CR⁺**) and these cells are larger and express higher numbers of FcR than cells that are devoid of complement receptors (**CR⁻**). These investigators also demonstrated that the **CR⁺ cells** secrete 4-fold and 12-fold more IL-1 than **CR⁻** cells when cultured under basal and stimulated conditions, respectively. In addition, **CR⁺** monocytes support the formation of Staphylococcal protein A-stimulated human B cell colonies more effectively than **CR⁻** cells.

Class II MHC antigens

Monocyte subpopulations differing in class II MHC antigens have been described. On average, 75 to 91% of freshly-isolated monocytes express HLA-DR antigens, 32% express HLA-DQ, 15% express HLA-DP

antigens and 25 to 50% express **HLA-DS** molecules (175,488). Whisler et al. (488) reported that the **HLA-DR** positive (**HLA-DR⁺**) monocytes are 2 to 5-fold more effective than **HLA-DR** negative (**HLA-DR⁻**) cells in promoting B cell proliferative responses in cultures pulsed with *Staphylococcal* protein A. In addition, these investigators reported that there is a relationship between **HLA-DS** expression and accessory cell function; **HLA-DS** positive (**HLA-DS⁺**) cells, which are predominantly **HLA-DR⁺**, are less effective than **HLA-DS** negative (**HLA-DS⁻**) in promoting B cell proliferative responses. These differences could not be attributed to differences in the release of **IL-1** by the various monocyte subsets. However, Gonwa and coworkers (176) demonstrated that: 1) only **HLA-DS⁺** cells present antigen to T lymphocytes; and 2) **HLA-DS⁺** monocytes are more effective at stimulating autologous mixed lymphocyte reaction than **HLA-DS⁻** cells (176). In contrast to Whisler and coworkers (488), Gonwa et al. did not detect any differences in **HLA-DR** antigen expression by the **HLA-DS⁺** and **HLA-DS⁻** monocyte subsets.

CD4 expression

Szabo et al. (436) demonstrated there is a relationship between the expression of **CD4**, a surface antigen of unknown function, and an immune regulatory function of monocytes (436). Monocytes that are devoid of cell surface **CD4** (**CD4⁻**) are less effective at presenting antigen to lymphocytes than cells that express **CD4** (**CD4⁺**). Szabo and coworkers also reported that the **CD4⁻** monocytes produce more **PGE₂** than the **CD4⁺** subset. However, that is unlikely to be the primary cause of the reduced antigen presenting capacity of the **CD4⁻** cells, since the addition of a cyclo-oxygenase inhibitor to the **CD4⁻** monocytes did not restore their capacity to present antigen to that of the **CD4⁺** subset. These investigators also demonstrated that the **CD4⁺** subset produce more **TNF** than the **CD4⁻** cells; however, there were no differences in the production of other monokines that influence the immune regulatory function of monocytes such as **IL-1** and plasminogen activator.

1.8.4. Separation of Monocyte Subpopulations Based on Differences in Function

Monocyte subpopulations have been isolated on the basis of differences in their adherence properties. Treves and coworkers (450) demonstrated that monocytes are heterogeneous in their capacity to adhere to plastic. These investigators identified a subpopulation of monocytes that adheres tightly to plastic, and an additional, small subpopulation of monocytes that is loosely adherent to plastic. The loosely-adherent subpopulation secrete greater amounts of **IL-1** and **PGE₂** when cultured under basal conditions when compared

with the tightly-adherent monocytes. However, following maturation in vitro for 72 h, the loosely-adherent cells lose their capacity to produce more **IL-1** than the tightly-adherent cells. Moreover, **LPS** stimulates both subpopulations to produce similar amounts of **IL-1**. These data suggest that the loosely-adherent cells are partially activated either in vivo or during their isolation in vitro. Alternatively, the loosely and tightly adherent monocyte subpopulations may be different maturational stages of mononuclear phagocytes.

Chen et al. (107) demonstrated that exposure of mononuclear cells to **LPS** results in a 3-fold increase in the yield of monocytes isolated by adherence to serum-coated plastic. These investigators postulated that **LPS** promotes the recovery of weakly-adherent monocytes. The weakly-adherent cells, which are isolated by conducting the adherence assay in the presence of **LPS**, exhibit enhanced cytolytic activity compared with tightly-adherent monocytes that are exposed to **LPS** following the adherence assay. This difference could not be attributed to natural killer cell contamination of the weakly-adherent monocytes. Chen and coworkers postulated that weakly-adherent monocytes possess an intrinsically greater cytolytic potential than tightly-adherent cells. However, it is also possible that exposure of mononuclear cells to **LPS** during the monocyte isolation procedure induces the production of lymphokines that stimulate monocyte cytolytic activity.

1.8.5. Subpopulations of Monocytes Based on Differences in Enzyme Content

Subpopulations of monocytes that differ in their content of proteinases have been identified. Campbell and coworkers (81) demonstrated that about 20% of monocytes express marked but localised proteolytic activity against fibronectin, even in the presence of proteinase inhibitors. This proteolytic activity is due to the high content of serine proteinases (human leukocyte elastase and cathepsin G) within this subpopulation of monocytes. Karg et al. (242) showed that monocytes are heterogeneous in their content of serine proteinases and peroxidase by combined immunogold localisation and enzyme histochemistry. These investigators reported that about 60% of monocytes are completely unstained for **HLE**, cathepsin G and peroxidase. A minor subpopulation of monocytes (20%) stain intensely for these enzymes and the remainder of the cells vary greatly in staining intensity.

1.8.6. Theories on the Origins of Mononuclear Phagocyte Heterogeneity

Two theories have been proposed to explain the morphological, biochemical and functional

heterogeneity of human mononuclear phagocytes. The first and most widely accepted theory proposes that the heterogeneity results from differences in the states of activation or maturational stages of a single, highly dynamic cell lineage (144,146,449). The second theory proposes that an unspecified number of distinct monocyte lineages account for the impressive functional diversity of these cells (67,177,487).

There is a considerable amount of evidence to support the former theory. First, most of the biochemical and functional differences observed between the two major monocyte subpopulations are quantitative rather than qualitative. Second, many of the characteristics that have been shown to be expressed heterogeneously by monocytes are also modulated by cellular activation and/or maturation. For example, the small, dense, FcR⁺ subpopulation have features that are characteristic of cells at an early stage along the mononuclear phagocyte differentiation pathway. In contrast, most of the functions expressed by the large, low density, FcR⁺ population are generally associated with more mature cells. Third, there are rapid increases in the proportion of small monocytes in the peripheral blood following extensive cytopheresis (9), as well as conditions associated with a monocytosis including parturition (64), psoriasis, eczema and mycosis fungoides (307). These observations suggest that the small, low density cells are less mature than the large, high density cells.

Small monocytes have been shown to develop some of the characteristics of the larger cells when induced to differentiate *in vitro*. This is perhaps the most convincing evidence to support the maturation theory. For example, Turpin et al. (452) demonstrated that although freshly-isolated small monocytes produce substantially lower quantities of hydrogen peroxide than freshly-isolated large cells, culture of small monocytes for 72 h increases their production of hydrogen peroxide to that of freshly-isolated large monocytes (452). However, some of the characteristics that are associated with the large cells are also associated with immaturity. In particular, peroxidase activity, which is progressively lost as monocytes mature into macrophages, is present in greater amounts in large monocytes compared with small cells (9,503). In addition, differentiation of monocytes *in vitro* decreases their capacity to act as accessory cells in the generation of lymphocyte proliferative responses (287) and increases their cytolytic activity against tumour cells compared with freshly-isolated cells (241,318). However, the large, low density cells (which may represent mature monocytes) exhibit greater accessory cell function and lower tumouricidal activity than the small, high density monocytes (which may represent immature cells), (table 4). Therefore, it is unlikely that the two monocyte subpopulations simply reflect different maturational stages of the monocytes.

The large, low density cells possess many features of cells that have been activated including increased phagocytic activity, superoxide production, ADCC and the capacity to present antigen when compared with the small, high density cells (table 4). Therefore, it is possible that the large, low density cells represent a subpopulation of monocytes that have been activated in vivo or during their isolation and culture in vitro. However, some of the characteristics that are associated with the small, high density cells are also associated with mononuclear phagocyte activation (including enhanced tumouricidal activity and increased cytokine production compared with the large-low density cells). Moreover, the two monocyte subpopulations express similar amounts of 5' nucleotidase (9) and alkaline phosphodiesterase (10) which are two markers of monocyte activation. Therefore, it is unlikely that the monocyte subpopulations simply reflect differences in the state of activation of the cells.

Although there is no good evidence to date from human studies to support the existence of multiple distinct monocyte lineages, data from animal studies have shown that mononuclear phagocytes that are derived from individual bone marrow precursors retain certain distinctive characteristics during differentiation in vitro. For example, Bursucker et al. (67) demonstrated that bone marrow precursor cells from normal mice develop into two well-defined types of mononuclear phagocyte colonies with respect to 5' nucleotidase (5'NT) activity. This enzyme was found to be a stable marker for resident and inflammatory macrophages which express high and low enzyme activity, respectively. Twenty percent of the colonies are small colonies in which all of the cells express high 5'NT activity (high activity colonies), and 70% are large colonies in which all of the cells express low 5'NT activity (low activity colonies). The remaining 10% are mixed colonies in which all of the cells express low 5'NT activity apart from the cells located at the periphery of the colonies which express high activity. Since cells that express high and low enzyme activity are predominantly confined to discrete, homogeneous colonies that differ in phenotype, Bursucker and coworkers postulated that both may arise from distinct bone marrow precursor cells and that macrophage precursor cells in the bone marrow exhibit an inherent heterogeneity (67,68).

Further studies by these investigators demonstrated that the induction of an acute or chronic inflammatory process in mice results in a 7 to 8-fold reduction in the proportion of bone marrow-derived mononuclear phagocyte colonies that expressed high 5'NT activity (67,68). In addition, the proportion of peritoneal macrophages expressing high 5'NT activity is reduced by 17 and 60-fold in mice with acute and

chronic inflammatory processes, respectively. Furthermore, bone marrow-derived mononuclear phagocytes of the mice bearing inflammation are more actively phagocytic and have greater tumouricidal activity ~~than~~ cells ~~from~~ normal mice. Bursucker and coworkers postulated that during inflammation, there is a preferential expansion of certain subsets of macrophage precursors, giving rise to mononuclear phagocytes that express distinct functional characteristics (67,68).

Other investigators have also demonstrated heterogeneity of bone marrow precursor cells in animal models. Subpopulations of precursor cells that differ in density also vary in their sensitivity to colony stimulating factors (70), hydroxyurea (70), and haemolysate (492), indicating that there are differential requirements for the proliferation of different precursor cells. Moreover, Walker and coworkers (466) identified separate and distinct murine bone marrow precursors for mononuclear phagocytes bearing I region-associated (Ia) determinants (Ia⁺) and those lacking Ia (Ia⁻). However, Calamai et al. (71) failed to confirm the existence of distinct murine mononuclear phagocyte precursors for Ia⁺ phenotypes (71). Moreover, Bar-Eli and coworkers (31) demonstrated that a single progenitor cell *can* develop the metabolic characteristics of either peritoneal or alveolar murine macrophages and that such changes are reversible.

The ~~situation~~ regarding heterogeneity of human macrophage precursors is even less clear since few studies have been performed. Moreover, caution should be exercised in extrapolating data from animal models to the human ~~situation~~. However, two studies have demonstrated heterogeneity among human mononuclear phagocyte precursors. Morstyn et al. (315) reported that different colony stimulating factors vary ~~in~~ their **capacity** to support the survival and proliferation of macrophage progenitor cells. Johnson and coworkers (233) identified subpopulations ~~of~~ human macrophage precursor cells on the basis of differences in density. These subpopulations also differ in their sensitivity ~~to~~ colony stimulating factors, indicating that there are differences ~~in~~ their proliferative states. Finally, it has been proposed that distinct subpopulations of human monocytes exist ~~on~~ the basis of differences in the expression of HLA-D/DQ (177) and complement receptors (487). However, other studies have failed to confirm these findings (14,160,165).

In conclusion, on the basis of available data, it would seem likely that most of the functional heterogeneity observed in mononuclear phagocytes is the result of differences in maturation stages and/or states ~~or~~ activation ~~of~~ a highly dynamic cell lineage. However, the existence of functionally distinct subsets of monocytes remains a possibility and warrants further investigation.

1.9. Adherence Properties of Monocytes

To participate in the host response to infection or inflammation, it is necessary for monocytes to be recruited from the vasculature. Emigration from the vasculature involves reversible adherence of monocytes to endothelial cells, a process that has been the focus of considerable recent interest. The next barriers that monocytes encounter, as they migrate towards a focus of infection or inflammation, are the subendothelial basement membrane and the extracellular matrix (189), both of which contain various adhesive glycoproteins **such** as collagens (259), fibronectin (391) vitronectin (193) and laminin (290). It is clear that adherence to extracellular matrix components must be exquisitely regulated (172,423); however, biological and pathological factors that regulate **this** process and the mechanisms involved have not yet been fully elucidated.

1.9.1 Adherence Receptors

Monocyte adherence to biologic substrates is mediated by a superfamily of adherence receptors. There are three subfamilies of adherence receptors: 1) integrins; 2) members of the immunoglobulin supergene family; and 3) selectins.

1.9.1.1 integrins

Integrins are a superfamily of transmembrane glycoprotein receptors that mediate both cell-extracellular ~~matrix~~ and cell-cell adherence events (392). Integrins are composed of an α and a β subunit. Their ligand-binding site is formed by sequences from both subunits, and their cytoplasmic domains form connections with the cytoskeleton. These properties enable integrins to serve as a ~~link~~ between the intracellular cytoplasm and the extracellular matrix (392). Eleven α subunits and six β subunits have been identified to date, and ~~these~~ subunits have been shown to be distinct by partial sequencing (392). The α and β subunits, in various combinations, have been shown to form 16 integrins but it is likely that more ~~will~~ be discovered. Each of the ~~six~~ β subunits is able to associate with multiple α subunits and six subfamilies of integrins, therefore, have been identified. However, it has become clear recently that a single α subunit ~~can~~ also associate with multiple β subunits.

The complement of different integrins that is expressed by cultured mammalian cell lines varies from ~~two~~ to ten (392). Some integrins are clearly cell type specific. For example, ~~gp~~ IIb/IIIa is expressed exclusively

by megakaryocytes and platelets (174), and the $\beta 2$ subfamily is expressed only by leukocytes (425). The expression of individual integrins appears to be regulated during development of vertebrate species by agents that affect growth and differentiation such as TGF- β (194). It is likely that the proper temporal expression of the correct complement of integrins enables cells to find their appropriate adhesive Substrates in the body.

The $\beta 1$ or very late activation antigen (VLA) subfamily of integrins The $\beta 1$ integrin subfamily includes receptors that bind to extracellular matrix components such as fibronectin, laminin and collagen. These receptors are expressed by leukocytes and many non-hemopoietic cell types (1%). Members of the $\beta 1$ family share a common $\beta 1$ chain which associates with six different α chains ($\alpha 1$ through $\alpha 6$), (197). The $\beta 1$ family has been designated the very late activation (VLA) antigens because two members, VLA-1 and VLA-2, appear on lymphocytes two to four weeks after antigen stimulation in vitro (1%). However, some VLA molecules are expressed under basal conditions by leukocytes, and their expression by non-hemopoietic cells does not require activation. Induction of VLA-1,-2, -3, and -5 expression after leukocytes cross the endothelial barrier may be of great importance in controlling leukocyte localisation in inflammation (425). Some members of the $\beta 1$ family, such as VLA-3 and VLA-5, recognise Arg-Gly-Asp (RGD) tripeptide sequences within their ligands, and synthetic RGD-containing peptides have been shown inhibit the adherence of cells that express these receptors to ~~their~~ ligands in vitro (63,359,390).

The $\beta 2$ subfamily or leukocyte integrins Members of the $\beta 2$ subfamily of integrins share the common $\beta 2$ subunit (CD18 antigen). The three members of this family, lymphocyte function associated antigen-1 (LFA-1 or CD11a/CD18), Mac-1 (CD11b/CD18) and p150,95 (CD11c/CD18), are involved in mediating the adherence of leukocytes to endothelial cells (216). LFA-1 is expressed on all cells of hemopoietic origin but Mac-1 and p150/95 are expressed only by myeloid cells (203). The ligands for LFA-1 include the intercellular adhesion molecules, ICAM-1 and ICAM-2, which are members of the immunoglobulin superfamily that are expressed by endothelial cells (425,426). The LFA-1 molecules of both monocytes and T lymphocytes are also essential for their initial interaction in the immune response (145).

Mac-1 also recognises ICAM-1 (425), but the endothelial determinant recognised by p150,95 has not yet been elucidated. Other known ligands for Mac-1 include fibrinogen (500) and factor X (12,13). In addition, Mac-1 binds to C3bi and is an important receptor for phagocytosis (36). However, a second binding site on Mac-1, detected by the binding of zymosan and un-opsonised bacteria, must be engaged before particle ingestion

occurs (385). Although Mac-1 binding to C3bi is dependent upon the recognition of RGD sequences in this ligand (498), other Mac-1-ligand interactions appear to be RGD-independent. P150,95 also binds to fibrinogen has specificity for C3bi and functions as a receptor for phagocytosis (319,425).

The most concrete demonstration of the important role of the leukocyte integrins comes from the rare hereditary condition, leukocyte adhesion deficiency (**LAD**). This condition is associated with recurrent and sometimes life-threatening bacterial and fungal infections (15). LAD is due to a defect in the synthesis of the common $\beta 2$ subunit resulting in defective expression of all three members of the **$\beta 2$** family on circulating leukocytes (15,249). Leukocytes from patients with LAD fail to adhere to and move through the endothelium into sites of tissue injury, and exhibit defective phagocytosis, immune recognition and other functions that depend upon cell adherence.

1.9.1.2. The Immunoglobulin Superfamily

In addition to the T-cell antigen receptor (CD3), and antigen-independent receptors such as CD2, this family includes three endothelial cell receptors that are involved in mediating leukocyte adherence to endothelium: 1) intercellular adhesion molecule-1 (ICAM-1); 2) intercellular adhesion molecule-2 (ICAM-2); **and** 3) vascular cell adhesion molecule-1 (VCAM-1), (392,425). ICAM-1 is a ligand for LFA-1 and Mac-1 (425) and ICAM-2 is a ligand for LFA-1 (289,426). VCAM-1 is the endothelial receptor for VLA-4 (155). ~~All~~ three receptors are expressed in low numbers on unstimulated endothelial cells. The expression of ICAM-1 by endothelial cells is increased **by** their exposure to IL-1, TNF, IFN and LPS in vitro, by a mechanism that **is** dependent on protein synthesis (361,362). Similarly, the expression of VCAM-1 by endothelial cells is increased **by** various cytokines in a *long lasting* fashion (**344**).

1.9.1.3. Selectins

The three members of this novel class of receptors are involved in leukocyte adherence during inflammation and coagulation (47,432). These receptors have structural homology to C-type lectins. Endothelial leukocyte adhesion molecule-1 (ELAM-1) is expressed transiently on endothelial cells two to eight hours after **stimulation** with IL-1 and other pro-inflammatory agents (360). This receptor mediates a leukocyte adhesion pathway that is distinct from that mediated by ICAMs and the leukocyte integrins (46,47,282). Leukocyte-

endothelial cell adhesion molecule-1 (LECAM-1, LAM-1 or Mel-14) is expressed by the majority of circulating leukocytes(182), but is rapidly shed by a proteolytic mechanism when cells are stimulated by several cytokines including GM-CSF, TNF, f-MLP, and LTB₄ (182,248). It has been postulated that LECAM-1 and ELAM-1 are involved in an early step in the binding of leukocytes to endothelium prior to trans-endothelial migration (425).

The third member of the family is granule membrane protein-140 (GMP-140, PADGEM or CD62). This receptor is expressed by platelets and endothelial cells after stimulation by products of the clotting cascade (425). GMP-140 plays a role in mediating the adhesion of neutrophils and monocytes to endothelial cells (15,266).

1.9.2. Regulation of Monocyte Adherence to Endothelial Cells

Human monocytes and monocytic tumour cell lines have been shown to adhere to endothelial cell lines in vitro including: 1) porcine (135) and bovine (467) aortic endothelial cell monolayers; 2) human vascular smooth muscle cell monolayers (139); 3) human microvascular endothelial cell monolayers (139); and 4) human umbilical vein endothelial cell (HUVEC) monolayers (86,97,139,156,486). The proportion of monocytes that adheres to HUVEC monolayers under basal conditions has been shown to vary from 20% (139) to 65% (365). Moreover, several mediators including IL-1, LPS, TNF, PDGF, C5a, f-MLP, LTB₄, GM-CSF and IL-3 up-regulate the adherence of monocytes to HUVEC monolayers in vitro (139,141,156,216,309,486).

Several CD18-dependent mechanisms appear to be involved in this process. First, TNF, PDGF, C5a, f-MLP and LTB₄ increase monocyte adherence to endothelial cells by stimulating the mobilisation of intracellular stores of preformed CD18 integrins from peroxidase negative granules of monocytes. This process results in a rapid increase in the surface expression of CD18 molecules by monocytes (309). Second, several cytokines and ADP stimulate large increases in CD18-dependent adherence to endothelial cells by inducing a conformational change in the receptor that increases its affinity for ligand, rather than by increasing receptor number (14,156). Third, some mediators act upon endothelial cells to up-regulate monocyte-endothelial cell binding. For example, LPS, IL-1 and TNF induce the expression of ICAM-1 (360) and ELAM-1 (46) on endothelial cells by a mechanism that is dependent upon synthesis of new proteins. Finally, some mediators, such as LPS and TNF, stimulate monocyte-endothelial interactions by inducing the expression of adherence

receptors on both the leukocyte and the endothelial cell (141,309).

It has become clear recently that receptors other than CD18 are also involved in mediating monocyte-endothelial interactions, since several investigators have demonstrated that anti-CD18 antibodies produce only partial inhibition of basal and stimulated adherence of monocytes to endothelial cells (86,261,467). Moreover, Carlos *et al.* (86) identified two CD18-independent mechanisms: 1) a CD18-independent, phorbol myristate acetate (PMA)-inhibitable mechanism mediates basal monocyte-endothelial cell interactions; and 2) a CD18-independent, PMA-insensitive mechanism is involved in mediating monocyte adherence to LPS-stimulated endothelial cells. However, the receptors involved in mediating CD18-independent monocyte-endothelial interactions were not investigated in this study. Other possible receptors involved in mediating monocyte adherence to endothelial cells include: 1) VLA-4 which binds to VCAM-1 molecules expressed by cytokine-activated endothelial cells (425); and 2) LECAM-1 and ELAM-1 which may be involved in an early step in the **binding** of monocytes to endothelium (425).

It is likely that there are mechanisms that act as negative regulators of adherence to ensure the uninterrupted circulation of monocytes. However, there is paucity of information on mediators that down-regulate the adherence process. Only one mediator has been shown to inhibit monocyte adherence. Elliott *et al.* (156) demonstrated that interleukin-4 (IL-4) inhibits basal adherence and IL-3 and GM-CSF-stimulated adherence of monocytes to endothelial cells and plastic. However, the mechanisms involved were not elucidated.

1.93. Monocyte Adherence to Fibronectin

Although it has been shown that mononuclear phagocytes and the promonocytic U937 cell line adhere to various matrix components including collagen, laminin and fibronectin in vitro (63,97,414,442), most is known about monocyte adherence to fibronectin.

Fibronectin is a ubiquitous adhesive glycoprotein and is a component of basement membranes and the extracellular matrix (316,366). It has a molecular weight of 440 kD and consists of dimers which are linked by disulphide bonds near the carboxy terminus. Fibronectin functions via several specialised intramolecular regions or domains that interact with other proteins including collagen, fibrin and heparin (215,316). In addition, various types of cells (including leukocytes and fibroblasts) bind to the small (11.5 kD) cell-binding domain which is located approximately three-quarters of the length of the molecule from the amino terminus. This domain

contains the RGD tripeptide sequence that cells recognise. by specific receptors (216,391).

in addition to being an integral part of **all** connective tissues, fibronectin is also exists as a freely soluble form in plasma at the substantial concentration of 0.3 **g/l**. A second major form is situated on cells surfaces and **is** loosely termed "cellular fibronectin". Cellular fibronectins are synthesised by fibroblasts, endothelial cells and mononuclear phagocytes. This cellular form is relatively insoluble at physiologic pH and becomes organised into striking fibrillar arrays on the cell surface and in the extracellular matrix. **In** addition to its adhesive functions, fibronectin modulates cytodifferentiation, cell migration and also functions as a non-immune opsonin for mononuclear phagocytes (316,366).

1.9.4. Regulation of Monocyte Adherence to Fibronectin

Several investigators have **shown** that monocytes adhere spontaneously to purified human fibronectin **in vitro** (63,256,390,412). However, only Brown *et al.* (63) quantified this interaction; these investigators demonstrated that 25% of monocytes adhere spontaneously to fibronectin-coated tissue culture plastic. Moreover, there is only one report in the literature which documents that monocyte adherence to fibronectin **can** be regulated. Roth *et al.* (390) showed that exposure of monocytes to LPS stimulates a 2-fold increase in **the** proportion of adherent cells. However, human monocytes have been shown to adhere spontaneously to **dishes** that have been coated with **serum** which contains fibronectin, and that this process is up-regulated by C5a, f-MLP, LPS and TNF (139,141).

The effects of mediators on the adherence properties of murine macrophages and the promonocytic U937 cell line have been studied. Shaw and Mercurio (414) demonstrated that under basal conditions thioglycoll-elicited murine peritoneal macrophages adhere avidly to fibronectin, but exhibit **minimal** adherence **to laminin** and type IV collagen. Moreover, exposure to LPS and IFN results in a dose-related increase in the adherence of murine macrophages to **laminin** and collagen type IV, but does not alter their adherence to fibronectin. Cavender and coworkers (97) showed recently that only 2 to 20% of U937 cells adhere spontaneously to fibronectin and that phorbol esters up-regulate this process substantially (**97**).

Monocytes have been shown to express three types of fibronectin receptors: VLA-3 (195,470), VLA-4 (471) and **VLA-5** (63,470). VLA-3 and VLA-5 both recognise the RGD sequence in the cell-binding domain of the fibronectin molecule (196,358). VLA-4 binds to the CS-1 domain of fibronectin by an RGD-independent

mechanisms (471). VLA-5 is thought to be the most important integrin that mediates monocyte adherence to fibronectin since specific anti-VLA-5 antibodies inhibit this process, whereas anti-VLA4 antibodies are ineffective(63,256). However, the role of VLA-3 in mediating adherence of monocytes to fibronectin has not yet been assessed and the possibility remains that other integrins play a role in this process.

VLA-5 is expressed on the surface of about 50% of freshly-isolated monocytes (256). Singer and coworkers (419) demonstrated that preformed VLA-5 is present in the peroxidase negative granules of monocytes along with $\beta 2$ integrins and receptors for laminin and vitronectin. In addition, these investigators **showed** that LPS stimulates the fusion of peroxidase negative granules with the plasmalemma, resulting in increased surface expression of VLA-5. However, the effect of this increased VLA-5 expression by monocytes **on** their adherence to fibronectin was not assessed in this study.

Integrins other than VLA-5 may also be involved in mediating monocyte adherence to fibronectin since specific anti-VLA **5** antibodies and RGD-containing peptides fail to inhibit monocyte adherence to fibronectin completely (256). Moreover, antibodies to the common $\beta 1$ chain of the VLA integrins also fail to inhibit monocyte adherence to fibronectin completely, and antibodies directed against $\beta 3$ integrins are ineffective (63,256). Together, these **data** indicate that receptors other than $\beta 1$ and **$\beta 3$** integrins are involved in mediating monocyte adherence to fibronectin. The integrins involved in mediating the adherence of stimulated monocytes have not yet **been** determined. Moreover, there is only one report in the literature on the adherence properties of monocytes from subjects with disease. Setiadi and coworkers (412) demonstrated that a greater proportion of monocytes from patients with diabetes adheres compared with cells from healthy subjects. Although the **mechanisms** were not investigated **in** this study, the expression of Mac-1 by diabetic monocytes was greater than that of *cells* from healthy subjects. These **data** suggest that $\beta 2$ integrins may also be involved in mediating monocyte adherence to fibronectin.

1.10. The Potential Role of Mononuclear Phagocytes in the Pathogenesis of Chronic Inflammatory Lung Diseases

There is substantial evidence to suggest that mononuclear phagocytes play a role in the pathogenesis of several inflammatory and degenerative conditions including pulmonary fibrosis (382,415), rheumatoid arthritis (65), sarcoidosis (17), systemic lupus erythematosus (17) and atherosclerosis (58,189,387). In addition, it has been postulated that mononuclear phagocytes may contribute to the destruction of lung parenchyma that is characteristic of one chronic inflammatory lung disease (emphysema), (80,223,333).

1.10.1. Pulmonary Emphysema

Pulmonary emphysema is the major determinant of airflow obstruction in chronic obstructive lung disease (187,321), and represents one of the most important causes of morbidity and mortality in the Western world. Pulmonary emphysema is characterised anatomically by destruction of lung parenchyma often involving the walls of the respiratory bronchioles (centrilobular emphysema), but extending to true alveoli in panlobular disease.

Historically, tissue destruction in emphysema was largely unexplained until three decades ago, when two independent laboratory observations provided compelling evidence that proteolytic destruction of connective tissue elements is the **signal** event in the pathogenesis of this condition. First, Laurell and Eriksson (157,268) identified an uncommon inherited deficiency of **serum alpha₁-antitrypsin** (an important inhibitor of serine proteinases), which is associated with early onset familial panlobular emphysema. Second, Gross et al. (183) demonstrated that intratracheal instillation of the plant protease, papain, produced anatomical derangements characteristic of emphysema in rats. The observation made by Gross and coworkers has since been confirmed both in the rat and other animals with proteinases other than papain, most notably porcine pancreatic elastase (230) and HLE (226,411). Although all of these proteinases exhibit broad substrate specificity, the capacity to degrade elastin in vitro is an absolute requirement for producing emphysema in experimental animals. Viewed together, these observations suggested that proteolytic mechanisms might be important in the pathogenesis of emphysema, and that a critical balance might exist between lung proteinases and their inhibitors. Thus, the "proteinase-antiproteinase" or "elastase-antielastase" hypothesis of emphysema was formulated. According to the proteinase-antiproteinase hypothesis, injury to elastic fibres in the lung parenchyma is a prerequisite for the

development of pulmonary emphysema (223,333). Moreover, the theory predicts that in the healthy lung, a **balance** occurs ~~in~~ which the function of the elastase inhibitors is equal to, or exceeds that of the enzymes. Destructive lung disease occurs when this balance is disturbed such that there is a functional excess of enzymes relative to their inhibitors. The enzymes, therefore, remain active ~~within~~ the lung, resulting in destruction of connective tissue.

Although the genetic model of α_1 -antitrypsin deficiency provides compelling evidence for the role of **elastases** in the pathogenesis of emphysema, this condition accounts for only **1 to 2%** of all cases of emphysema (276). Cigarette smoking is, by far, the most common cause of emphysema, and most patients with emphysema have normal **serum** levels of α_1 -antitrypsin. However, a disturbance in the balance between lung elastases and ~~their~~ inhibitors is still believed to play an important role in the pathogenesis of emphysema caused by cigarette smoking.

1.10.2. Human Leukocyte Elastase

Human leukocyte elastase (HLE) is widely believed to be the most important proteolytic enzyme involved in the pathogenesis of emphysema for several reasons:

- 1) α_1 -antitrypsin is the major physiologic inhibitor of HLE and inherited deficiency of α_1 -antitrypsin is associated with early onset familial panlobular emphysema (157,268);
- 2) HLE produces emphysema when instilled via the trachea into animals (226,411);
- 3) cigarette smokers, who are at **risk** of developing emphysema, have an increased proportion of neutrophils (historically assumed to be the source of HLE) in broncho-alveolar lavage (223) and alveolar septae (334); and
- 4) Damiano *et al.* (126) demonstrated that extracellular HLE is found exclusively ~~in~~ association with interstitial elastic fibres in emphysematous lungs obtained at surgery.

Structure HLE is a serine proteinase, that is, a serine residue at ~~position~~ **195** of the enzyme's primary amino acid sequence contributes a nucleophilic hydroxyl group to attack carbonyl carbons of target peptide **bonds** (223). Valine or alanine groups are preferred in the **P1 position** of the target protein (the amino acid contributing the **α -carbonyl** carbon to the peptide bond). The enzyme is single-chain polypeptide of molecular

weight **25 kD** and consists of **18 to 20%** carbohydrate residues (**343**). The enzyme is active at neutral pH and **has** a strongly basic iso-electric point (pH **10 to 11**). It is synthesised primarily in promyelocytes and stored in the azurophil granules of neutrophils which contain about **1 to 3 pg** per cell (**120,477**).

Function During the host inflammatory response, HLE is released from living cells during phagocytosis or following cell death, and may contribute to tissue damage by catalysing the hydrolysis of matrix macromolecules. A wide variety of important matrix components are susceptible to attack by the enzyme including: **1)** elastin (**225**); **2)** fibronectin (**301**); **3)** type **III** collagen, a major supporting component of lung connective tissue and blood vessels (**284**); **4)** type IV collagen, an important structural component of epithelial and endothelial basement membranes (**283**); and **5)** proteoglycan molecules in the connective tissue ground substance (**237**). In addition, HLE has been shown to hydrolyse several plasma proteins including immunoglobulins, clotting factors and complement components (**192,223**). Activation or inactivation of these plasma cascades may have important local and systemic consequences. **HLE** has also induces lymphocyte activation and platelet aggregation (**223**).

Although the spectrum of HLE activity presented above indicates that the enzyme is predominantly harmful, it is clear that the presence of such large amounts of the enzyme within a major host-defense cell such as the neutrophil must reflect an important physiologic role. It is likely that some of these actions (such as complement activation) are beneficial to the host. In addition, experimental data investigating the bactericidal **and** bacterial digesting functions of HLE suggest that HLE activity may be beneficial when restricted to the phagocytic vacuole (**223,341,375**). However, regulated extracellular release of HLE may also be beneficial to **the** host since it may enable the neutrophil to: **1)** traverse connective tissue barriers; **2)** dissolve infected tissues **to** dislodge sequestered microbes; and **3)** participate in abscess formation and tissue debridement at sites of infection and inflammation (**92,223,473**).

1.10.3. Alpha₁-Antitrypsin

Alpha₁-antitrypsin is the predominant inhibitor of serine proteinases in serum and is a major proteinase inhibitor in the lower respiratory tract. It is a member of a superfamily of serine proteinase inhibitors (serpins) **that** includes antithrombin **III**, ovalbumin, angiotensinogen, **α_2 -antiplasmin**, **α_1 -antichymotrypsin**, protein **C** inhibitor and **C1** esterase inhibitor (**341,448**). These proteins exhibit **30** to 40% primary structural homology

and have a number of residues around the active inhibitory site that are highly conserved. Each serpin is characterised by a specificity for inhibition of individual serine proteinases, although each serpin is also able to inhibit other serine proteinases less effectively (448).

Structure Alpha₁-antitrypsin is a small, polar glycoprotein of molecular weight **54 kD** that is able to **diffuse** readily through tissues. It consists of a single polypeptide chain consisting of **394** amino acids with three carbohydrate side chains attached at asparagine residues **46,83** and **247 (93)**. It is a highly ordered molecule with 30% of its structure in the form of **α -helices** and **40%** as **β -pleated sheets (92)**. The active site of the molecule is formed by a methionine residue at position **358** which is situated on a **16** residue exposed loop (92). **This** loop is under considerable tension and holds the molecule in a stressed conformation such that the **358-359** Met-Ser bond fits precisely into the active site pocket of the serine proteinase.

Function It is thought that the primary function of **α_1 -antitrypsin** is to protect tissues from proteolytic enzymes that are released from cells during the inflammatory response. Although **α_1 -antitrypsin** is synthesised by a number of cell types including mononuclear phagocytes (32,350), neutrophils (148) and Paneth cells in the gastrointestinal tract (122), hepatocytes are the major source of plasma **α_1 -antitrypsin** in humans (122). Approximately 2 g of **α_1 -antitrypsin** are synthesised by the liver per day and released into circulation, where this protein has a half life of six days. **Alpha₁-antitrypsin** is an acute phase protein, and the normal plasma concentration of **20 to 48 $\mu\text{mol/L}$ (61,123,448)** can increase 4-fold in response to inflammation or other stimuli such as oestrogen therapy (93).

The physiologic target for **α_1 -antitrypsin** is human leukocyte elastase (92,93). The association rate constant (K_a) for **α_1 -antitrypsin** has been estimated to be **$10^7 \text{ M}^{-1} \text{ S}^{-1}$ (34)**. **Alpha₁-antitrypsin** is also able to inhibit other serine proteinases, but less efficiently than HLE. A comparison of K_a 's for other serine proteinases demonstrates a decreasing order with **HLE > chymotrypsin > cathepsin G > anionic trypsin > plasmin > thrombin (34)**. **Alpha₁-antitrypsin** is a suicidal protein that forms a tight **1:1** non-covalent complex with its target proteinases. Complex formation renders the enzyme and inhibitor inactive, and the complex is subsequently removed from the circulation by the reticuloendothelial system. **Alpha₁-antitrypsin** can be inactivated by two mechanisms. First, the active site Met-Ser bond is susceptible to cleavage by neutrophil elastase (92,278). Following cleavage, the methionine and **serine** residues spring apart irreversibly and are found at opposite poles of the molecule, resulting in a more stable, relaxed but inactive molecular structure (28). In addition, other

proteinases including neutrophil (132) and macrophage (27,28) metalloproteinases, cathepsin L (232) and *Pseudomonas aeruginosa* elastase (311) can inactivate α_1 -antitrypsin by cleaving the protein at the active center bond or other sites on the exposed loop.

The second mechanism involves oxidative inactivation. Methionine contains a sulphur atom that is readily oxidised to sulfoxide (231), a derivative that is too large to fit into the active site of serine proteinases. Thus, oxidation of α_1 -antitrypsin renders the molecule 2000-fold less effective as an inhibitor of HLE (34,89,224,231,484). Cigarette smoke (87,91,114,224,367) and reactive oxygen metabolites generated by stimulated phagocytes (88,89,211) inactivate α_1 -antitrypsin by this mechanism *in vitro*, as discussed in 1.10.4. This process can be reversed by the enzyme methionine sulfoxide peptide reductase which is found in bacteria and neutrophils (90).

Gene structure The α_1 -antitrypsin is located on the long arm of chromosome 14 at position q31-31.2 (265). The gene is about 12.2 kb in length and there are two promoter regions; a promoter for mononuclear phagocytes is located about 2 kb upstream from the other, which is the hepatocyte promoter (349). The promoters are tissue specific, that is, the monocyte promoter does not function in hepatocytes and vice-versa. Trans-activating factors are required to activate the promoters in a *tissue* specific manner. The genomic sequence contains seven exons: IA, IB, IC, II, III, IV and V. Messenger RNA (mRNA) produced by hepatocytes contains exons IC, II, III, IV and V. Messenger RNA produced by mononuclear phagocyte differs from hepatocyte mRNA in that it contains additional exons. There are two distinct mononuclear phagocyte mRNA species (349); one species contains all seven exons, the other results from exclusion of exon IB. It has been postulated that exons IA and IB are transcribed but not translated into protein. Since untranslated mRNA reduces the efficiency of translation, it is likely that the additional exons influence the rate of translation (239). Although monocytes express only 1% of the amount of α_1 -antitrypsin-specific mRNA that is produced by hepatocytes (381) and do not contribute significantly to serum levels of α_1 -antitrypsin (260), mononuclear phagocytes may be an important local source of this protein in the vicinity of the cells at sites of inflammation.

1.10.4. Theories on the Pathogenesis of Emphysema

Cigarette smoking is thought lead to emphysema both by increasing the elastase burden and reducing the anti-elastase screen in the lower respiratory tract.

Increased Elastase Burden

Cigarette smoke has been shown to cause the accumulation of neutrophils within the lower airspaces, thus increasing the HLE burden of the lower respiratory tract. Broncho-alveolar lavage fluid from healthy smokers contains **10** times the normal number of neutrophils (**206**). Similarly, the lung septae of cigarette smoke-exposed humans contain twice the normal number of neutrophils, most of which are sequestered within the vascular space (**281**).

The recruitment of neutrophils into the lungs of cigarette smokers is thought to be mediated by several mechanisms. First, cigarette smoke stimulates alveolar macrophages to release factors that are chemotactic for neutrophils (**214,304**). Second, nicotine, a constituent of cigarette smoke, causes directed migration of neutrophils at concentrations similar to those found in the blood of cigarette smokers (**444**). Third, proteolytic fragments of collagen and elastin which are generated by proteinases released by recruited phagocytes, are chemotactic for leukocytes in vitro (**364,410**), and may thereby perpetuate the accumulation of neutrophils in the lung in vivo.

Reduced Anti-Elastase Screen

Although the lung of cigarette smokers have an increased burden of neutrophils in the lower respiratory tract compared with non-smokers, the concentration of α_1 -antitrypsin in the lungs of smokers should be sufficient to protect the alveolar walls. It has been postulated that oxidation of α_1 -antitrypsin occurs in cigarette smokers (**87,171,224**). Since oxidised α_1 -antitrypsin is a less effective inhibitor of HLE than the native protein, this would decrease the lower respiratory tract HLE inhibitory capacity, thus increasing the susceptibility of the lung to elastolytic destruction and the development of emphysema.

There are two mechanisms by which oxidative inactivation of α_1 -antitrypsin may occur in cigarette smokers. First, oxidants in cigarette smoke cause this directly in vitro (**91,114,224,367**). However, physiological concentrations of plasma proteins inhibit smoke-induced oxidation of α_1 -antitrypsin in vitro (**74**), indicating that ~~this~~ mechanism may not be important in vivo. Second, toxic oxygen radicals and myeloperoxidase released by activated neutrophils and alveolar macrophages also inactivate α_1 -antitrypsin in vitro (**88,89,105**). Since

phagocytes are present in increased numbers in smokers, these oxidants pose an additional oxidant stress to lung α_1 -antitrypsin.

Studies on the presence of inactivated α_1 -antitrypsin in broncho-alveolar lavage samples from smokers have yielded conflicting results (3,57,78,91,171,429-431). However, it is important to bear in mind that the extracellular matrix is located within the interstitium of the lung, which is separated from the airspaces by an alveolar epithelium. This epithelium is normally effective in restricting the passage of hydrophobic molecules (179). Although cigarette smoke may impair the integrity of this epithelium, the quantities and function of proteinases and anti-proteinases in broncho-alveolar lavage (which samples the alveolar lining fluid) may bear little resemblance to those in the interstitium. Better methods for sampling microenvironments within the lung are **needed** to resolve this controversial issue.

It is possible that oxidants released by lung phagocytes result in localised oxidative inactivation of α_1 -antitrypsin. It has been postulated that oxidants released by stimulated phagocytes create a zone of oxidised α_1 -antitrypsin around them (88,92). If this occurs, HLE released by neutrophils would thus be able to degrade connective tissue virtually unopposed. However, connective tissue breakdown by HLE would be limited to this microenvironment because oxygen radicals have a short half life and do not diffuse to any great extent. Outlying tissue would continue to be protected by native α_1 -antitrypsin.

1.10.5. Potential Role of Mononuclear Phagocytes in the Pathogenesis of Emphysema.

Most research into the pathogenesis of emphysema has focussed on the role of the neutrophil since the lungs of smokers contain increased numbers of neutrophils (223,334) which are assumed to be the source of HLE in tissues. However, the **most striking** change in the lung inflammatory **cell** population in human smokers is the massive increase in mononuclear phagocytes in broncho-alveolar lavage. Moreover, mononuclear phagocytes constitute the vast majority of the inflammatory cells in the respiratory bronchiolitis which is a universal **finding** in human cigarette smokers (334). This airway inflammation is located at precisely the site of the earliest structural lesions in centrilobular emphysema in smokers. Given the increase in mononuclear phagocytes at strategic locations in human cigarette smokers, it is possible that mononuclear phagocytes play an important role in the pathogenesis of emphysema. Several mechanisms may be involved:

1) Activated macrophages from the lungs of cigarette smokers release factors that are chemotactic for

neutrophils, and may thereby increase the elastase burden in the lower respiratory tract (214,304).

2) Reactive oxygen metabolites that are released by mononuclear phagocytes may contribute to the localised oxidation of α_1 -antitrypsin, and thereby reduce the anti-elastase screen (89,211).

3) Monocytes have been shown recently to contain HLE (81,167,408) and these cells also synthesise and secrete α_1 -antitrypsin (350,351). Thus, the balance between the release of HLE and α_1 -antitrypsin by monocytes, as these cells are recruited into the lung and pass through the interstitial space, might be an important determinant of lung elastin injury and resultant emphysema.

4) Mononuclear phagocytes synthesise and release other enzymes such as a metalloelastase and cathepsin L, both of which have been shown to cleave α_1 -antitrypsin (27,28,232) and damage connective tissue components such as elastin *in vitro* (294,409). Moreover, mononuclear phagocytes synthesise and secrete other inhibitors of elastases such as α_2 -macroglobulin (490) and tissue inhibitor of metalloproteinases (76). These inhibitors may contribute to the anti-elastase screen in the lower respiratory tract.

1.10.6. Mononuclear Phagocyte Elastases

Monocytes have been shown to contain HLE within peroxidase positive granules (81,242). In addition, HLE is expressed on the surface of monocytes (269,508). Although synthesis of HLE by mature monocytes has been suggested by the results of biosynthetic labelling and immunoprecipitation studies (32), more recent data indicate that monocytes do not contain measurable amounts of mRNA for HLE (438). These latter data indicate that synthesis of HLE has been completed prior to release of mature cells from the bone marrow.

Although monocytes contain only 3 to 7% of the amount of HLE contained in neutrophils, (81,167,408), they release a greater proportion in response to stimulation (81). HLE released by monocytes has been shown to degrade various matrix components including elastin and fibronectin *in vitro* (81,228,269). In particular, Campbell et al. (81) demonstrated that stimulation of monocytes with phorbol ester results in extensive HLE-mediated degradation of a fibronectin substrate. However, these investigators also showed that in the presence of α_1 -antitrypsin, only 20% of the cells express marked but localised proteolytic activity. These data indicate that a minor subpopulation of monocytes is rich in HLE and may participate in extracellular events formerly associated with neutrophil derived-HLE.

As monocytes mature into macrophages, HLE is progressively lost and is replaced by a macrophage elastase which is a metalloenzyme (223,396). The macrophage-specific enzyme also has elastin-degrading activity (409), but is secreted immediately into the extracellular space rather than being stored within the cell. Although alveolar macrophages are a less rich source of elastase than monocytes on a cellular basis, the metalloenzyme is not inhibited by α_1 -antitrypsin and may actually inactivate it by proteolytic cleavage (27,28). Moreover, alveolar macrophages degrade insoluble elastin in the presence of proteinase inhibitors *in vitro* (102,104,409), and macrophage-derived metalloelastases have been shown to play a prominent role in this process (409). These data suggest that the macrophage-derived metalloelastase may also be involved in the pathogenesis of emphysema.

Mononuclear phagocytes may also take up HLE that has been released by neutrophils since HLE has been shown to bind to monocyte and macrophage surface receptors, and is rapidly internalised into the phagolysosomes (73,82,84). The internalised HLE is released slowly by mononuclear phagocytes, and hypoxic injury to the cells accelerates the release of the enzyme (83). Therefore, mononuclear phagocytes may also participate in the pathogenesis of emphysema by serving as a vector for neutrophil elastase.

These data, suggest that mononuclear phagocyte-derived elastases may play a role in the pathogenesis of pulmonary emphysema. However, the mechanisms by which mononuclear phagocyte elastases mediate proteolysis of matrix components in the presence of proteinase inhibitors *in vivo* have not been elucidated.

1.10.7. Expression of α_1 -Antitrypsin by Mononuclear Phagocytes

Although mononuclear phagocytes have been shown to synthesise and secrete α_1 -antitrypsin (350,351), the absolute amounts of α_1 -antitrypsin produced by these cells has not been quantified previously. Rather, it has been assumed that mononuclear phagocytes produce small quantities of α_1 -antitrypsin, and these cells have generally been ignored as playing a relevant role in the normal physiology of α_1 -antitrypsin in the lower respiratory tract. However, this view may be incorrect for several reasons. First, alveolar macrophages are located directly at the site where α_1 -antitrypsin may play a major role as an inhibitor of HLE (334). Second, alveolar macrophages represent about 10% of cells of the alveolar structures and are constantly being renewed by the circulating pool of monocytes (53,119). Third, maturation of monocytes into macrophages is associated with a three-fold increase in the expression of the α_1 -antitrypsin gene (312).

The expression of α_1 -antitrypsin by mononuclear phagocytes can be modulated in vitro. Perlmutter and coworkers showed that **LPS (32)**, **HLE (354)** and **IL-6 (352)** increase the rate of synthesis of α_1 -antitrypsin by human monocytes. Moreover, these mediators up-regulate the synthesis of α_1 -antitrypsin by monocytes by different mechanisms. **IL-6** and **HLE** stimulate increases in steady-state α_1 -antitrypsin mRNA concentrations in monocytes (352,354). In contrast, **LPS** produces little or no change in monocyte steady-state α_1 -antitrypsin mRNA concentrations, indicating that translational or post-translational mechanisms are involved (32,352). However, the absolute amounts of α_1 -antitrypsin synthesised by mononuclear phagocytes under basal conditions or in response these stimuli have not been quantified. Moreover, the effects of other pro-inflammatory mediators on α_1 -antitrypsin gene expression have not been assessed. Finally, the relative amounts of **HLE** and α_1 -antitrypsin synthesised by mononuclear phagocytes have not been quantified.

1-11 OBJECTIVES OF THE THESIS

The aims of this thesis were 4-fold.

- 1) To compare the adherence of monocytes from healthy subjects and patients with an inflammatory lung disease (bronchiectasis) to a representative extracellular matrix component (fibronectin).
- 2) To investigate the physiological and pathological factors that regulate monocyte adherence to fibronectin and to assess the role of CD11/CD18 integrins in mediating this process.
- 3) To assess the relationship between the adherence properties of monocytes and other monocyte functions that are of biological importance at sites of infection and inflammation.
- 4) To compare the expression of **HLE** and **α_1 -antitrypsin** by adherent and non-adherent monocytes to assess whether there is a relationship between the adherence properties of monocytes and their potential to cause tissue damage at sites of infection and inflammation.

Chapter II

Methods

2.1 Cell Isolation

Monocytes were isolated by four methods: 1) the conventional ficoll-Hypaque density gradient method followed by an adherence step (59); 2) the Percoll density gradient method followed by an adherence step (229); 3) the Nycodenz osmotic and density gradient method (60); and **4)** countercurrent centrifugal elutriation (CCE), (395). Neutrophils were isolated by the Percoll density gradient technique. Platelets and lymphocytes were isolated by CCE. Venous blood was collected into lithium heparin tubes for the ficoll-Hypaque, Percoll and CCE isolation methods, and polypropylene tubes containing ethylenediamine tetracetic acid (EDTA, 1.3 g/L final concentration) for the Nycodenz method.

2.1.1. Ficoll-Hypaque Isolation of Monocytes

For comparison of the yields of monocytes isolated by the ficoll-Hypaque and Nycodenz methods, ficoll-Hypaque gradient medium was purchased from Pharmacia, Uppsala, Sweden. For all other experiments, ficoll-Hypaque medium was prepared by dissolving 90 g of ficoll in one litre of sterile distilled water and adding 120 **ml** of the ficoll solution to 34 **ml** of Hypaque and 16 **ml** of sterile distilled water. The ficoll-Hypaque solution was then filtered (**0.2 μ M** filter) and stored at 4°C. The specific gravity of the prepared ficoll-Hypaque solution was 1.077 g/d.

Blood was collected into lithium-heparin tubes and diluted with an **equal** volume of sterile 0.15 mol/L **NaCl** solution. The diluted blood (30 **ml**) was layered over ficoll-Hypaque (15 **ml**) and centrifuged at 900 g for 30 min at room temperature. The mononuclear cells were harvested and washed twice with 20 **ml** of sterile 0.15 mol/L NaCl solution. The cells were re-suspended at 2×10^6 cells/ml in supplemented RPMI 1640 medium (**RPMI** 1640 medium containing 10% [v/v] complement-depleted [by incubation at 37°C for 30 min] fetal **calf serum** [FCS], L-glutamine [2mmol/L] and gentamicin [20 **μ g/ml**]).

An adherence step was then performed to separate the monocytes, which are assumed to be adherent cells, from the lymphocytes which are assumed to be the non-adherent cells (4,372). Aliquots (1 ml) of the mononuclear cell suspension were dispensed into 35 x 10 mm tissue culture dishes that had been pre-coated with **0.5 ml** of purified human serum fibronectin (20 **μ g/ml** in sterile 0.15 mol/L NaCl solution) at 37°C for 2 h, and then washed **three** times with 2 **ml** of sterile 0.15 mol/L NaCl solution to remove unbound fibronectin. The dishes were incubated for 2 h at 37°C in a humidified atmosphere of 5% CO₂, and 95% au. The non-adherent

cells were removed by washing the dishes four times with 2 ml of sterile 0.15 mol/L NaCl solution. The adherent cells were detached by incubating the dishes with sterile 0.15 mol/L NaCl solution containing 5 mmol/L EDTA for 45 min at 4°C, followed by gentle scraping with a rubber policeman. The dishes were again washed three times with 2 ml of sterile 0.15 mol/L NaCl solution, the suspension of previously adherent cells was centrifuged (600 g for 5 min at 4°C) and the supernatant was discarded. The cells were re-suspended in 2 ml of supplemented RPMI medium, counted three times using a haemocytometer and the mean value was determined. Complete recovery of the adherent monocytes was confirmed by phase-contrast microscopy of the tissue culture dishes. Differential counts on cytocentrifuge preparations (Shandon 2, Shandon, Runcorn, Cheshire, U.K.) stained with modified Wright's stain confirmed that the cells were greater than 90% pure. The monocytes were 90 to 95% viable, as assessed by exclusion of trypan blue dye.

2.1.2 Percoll Density Gradient Method for Isolation of Monocytes and Neutrophils

Blood was collected into lithium-heparin tubes and diluted with an equal volume of sterile 0.15 mol/L NaCl solution.

Monocyte Isolation

The diluted blood (5 ml) was layered carefully onto the surface of 5 ml of a 54% [v/v] solution of Percoll in 0.15 mol/l NaCl solution (density 1.075 g/ml). The gradients were centrifuged at 200 g for 25 min at room temperature. The mononuclear cell layer, at the top of the gradient, was collected and the cells were washed twice with 20 ml of sterile 0.15 mol/L NaCl solution and centrifuged at 600 g for 5 min. The supernatant was discarded and the cells were re-suspended at 2×10^6 cells/ml in supplemented RPMI 1640 medium. The monocytes were separated from the lymphocytes by an adherence step, and the adherent monocytes were detached from the dishes and counted, as described above. Differential counts on cytocentrifuge preparations stained with modified Wright's stain confirmed that the cells were greater-than 90% pure. The cells were 90 to 95% viable, as assessed by exclusion of trypan blue dye.

Neutrophil Isolation

The diluted blood (5 ml) was layered carefully onto the surface of 2 ml of a 54% [v/v] solution of Percoll in 0.15 mol/l NaCl solution (density 1.075 g/ml) which had been layered onto 3 ml of 78% [v/v] Percoll in 0.15 mol/L NaCl solution (density 1.096g/ml). The gradients were centrifuged at 200 g for 25 min at room

temperature. The neutrophil layer, at the interface of the two Percoll solutions, was collected and the cells were washed twice in 20 ml of sterile 0.15 mol/L NaCl solution. The neutrophils were then re-suspended in supplemented RPMI 1640 medium and counted using a haemocytometer. Differential counts on cytocentrifuge preparations stained with modified Wright's stain confirmed that the neutrophils were greater than 95% pure (the contaminating cells were predominantly eosinophils). The cells were greater than 99% viable, as assessed by exclusion of trypan blue dye.

2.1.3. Nycodenz Isolation of Monocytes

Blood was collected into 50 ml polypropylene tubes containing EDTA (1.3 g/L final concentration) and dextran sedimentation was performed to remove most of the erythrocytes. Four millilitres of a solution of dextran T 500 (6% [w/v] in sterile 0.15 mol/L NaCl solution) were added to 40 ml of blood in each polypropylene tube and the tubes were left to stand at room temperature for 45 min. The leukocyte rich plasma supernatant was layered in 5 ml aliquots over 3 ml of Nycodenz and centrifuged at 600 g for 15 min at 4°C. Monocytes suspended in the Nycodenz layer were harvested and washed once with an equal volume of ice-cold sterile 0.15 mol/L NaCl solution containing 1.3 g/L EDTA and centrifuged (600 g for 5 min at 4°C). Contaminating platelets were removed, as required, by re-suspending the monocytes in 1 ml of sterile 0.15 mol/L NaCl solution containing 1.3 g/L EDTA, and layering the suspension over 3 ml of autologous plasma. The plasma gradients were centrifuged at 150 g for 10 min and the plasma containing the platelets was discarded. The monocyte pellet was washed once with 25 ml of ice-cold, sterile 0.15 mol/L NaCl solution and re-suspended in 5 ml of supplemented RPMI 1640 medium (see above). The cells were counted four times using a haemocytometer and the mean value was calculated. Differential counts on modified Wright's stained cytocentrifuge preparations confirmed that the monocytes were greater than 90% pure. The cells were greater than 99% viable, assessed by exclusion of trypan blue dye.

2.1.4. Countercurrent Centrifugal Elutriation (CCE) for the Isolation of Monocytes, Lymphocytes and platelets

Monocyte Isolation

Mononuclear cells were prepared by the ficoll-Hypaque method, as described previously. The cells were washed once with 50 ml of sterile 0.15 mol/L NaCl solution and centrifuged at 600 g for 5 min at 4°C. The cells were re-suspended in 5 ml of elutriation buffer (0.1% [w/v] human serum albumin, 0.15 mol/L NaCl, 2.7 mmol/L KCl, 2.3 mmol/L phosphate, 6 mmol/L glucose and 1 mmol/L EDTA; pH 7.4).

The elutriator rotor (JE-6B rotor with standard chamber, mounted in a Beckman J2-21 centrifuge; Beckman Instruments Inc., Palo Alto, California, U.S.A.) was assembled and sterilised with 150 ml of 70% [v/v] ethanol, followed by 150 ml of 6% [v/v] H_2O_2 . The rotor was then rinsed with 400 ml of sterile 0.15 mol/L NaCl solution and primed with 200 ml of elutriation buffer. The mononuclear cells, suspended in elutriation buffer, were pumped at a flow rate of 8 ml/min into the rotor at a constant 2470 rpm. The flow rate was then increased to 17.5 ml/min, and 150 ml were collected. This eluate contained platelets, erythrocytes, lymphocytes and a few monocytes. A pure monocyte population was then eluted from the chamber in the subsequent 150 ml fraction at a flow rate of 22 ml/min. The monocyte suspension was centrifuged for 5 min (600 g at 4°C), the cells were washed with 50 ml of sterile 0.15 mol/L NaCl solution and centrifuged for a further 5 min. The cells were re-suspended in supplemented RPMI 1640 medium at 10^6 cells/ml. Differential counts on Wright's stained cytocentrifuged preparations confirmed that the monocytes were greater than 92% pure; the remaining cells were predominantly lymphocytes. The cells were greater than 99% viable, as assessed by exclusion of trypan blue dye.

Lymphocyte Isolation

Mononuclear cells were isolated and pumped into the rotor at a flow rate of 8 ml/min, as described above. The flow rate was then increased to 14 ml/min, and 150 ml were collected. This eluate contained platelets, erythrocytes and a few lymphocytes. A pure population of lymphocytes was then eluted from the chamber in the subsequent 150 ml fraction at a flow rate of 16 ml/min. The cells were washed in 50 ml of sterile 0.15 mol/L NaCl solution and re-suspended in supplemented RPMI 1640 medium at 2×10^6 cells/ml. Differential counts on modified Wright's stained cytocentrifuge preparations confirmed that the lymphocytes were greater than 95% pure; the remaining cells were predominantly monocytes. The cells were greater than

99% viable, as assessed by exclusion of trypan blue dye.

platelet Isolation

The Nycodenz isolation procedure was performed, as described previously. Monocytes and contaminating platelets were harvested from the gradients and suspended in elutriation buffer. The cells were pumped into the rotor at a flow rate of 8 ml/min at a constant 2470 rpm and **200** ml of eluate were collected during this loading procedure. This eluate contained a pure population of platelets. The platelets were then washed twice in 50 ml of Hank's balanced salt solution (HBSS; pH 7.4) re-suspended in **25** ml of HBSS and counted using a haemocytometer. Differential counts on cytocentrifuge preparation stained with modified Wright's stain confirmed that the platelets were not contaminated with nucleated cells.

2.2 Recovery of Monocytes

The recovery of monocytes by the ficoll-Hypaque and Nycodenz methods was determined by comparison with starting material. Sixty-five millilitres of venous blood were drawn from ~~six~~ healthy volunteers. Monocytes were isolated from **30** ml of blood by the ficoll-Hypaque method and from **30 ml** of blood by the Nycodenz method. The remaining **5** ml of blood were collected into tubes containing EDTA (**1.3** g/L final concentration) and assessed for total leukocyte number using a coulter counter. In addition, the proportion of leukocytes that were monocytes was determined morphologically from a blood smear stained with modified Wright's stain and counted independently (300 cells/smear). From these two values the actual number of monocytes in the initial peripheral blood sample was obtained. This value was compared to the number of monocytes recovered by the ficoll-Hypaque and Nycodenz isolation procedures and the percentage recovery for each method was calculated.

The proportion of monocytes recovered by the conventional ficoll-Hypaque method was **36.5% (\pm SEM 7.3%)**, whereas 69.0% (\pm SEM 18.4%) of monocytes were recovered by the Nycodenz method (**n = 6**). In view of the greater recovery ($p < 0.025$) by the latter method, subsequent studies of monocyte function were performed on cells isolated by the Nycodenz method.

The Nycodenz isolation method failed to recover about **30%** of monocytes in the initial blood samples which may reflect adherence of cells to the isolation vessels. Alternatively, since monocytes are heterogeneous with respect to density (**16**), it is possible that the fraction of cells of the highest density becomes separated from

the remainder during centrifugation through Nycodenz and sediment to the bottom of the test-tube. To test the latter possibility, cells in the pellet from the Nycodenz gradient step were suspended in 2 **ml** of sterile 0.15 mol/L NaCl solution and subjected to Percoll density gradient centrifugation to separate the mononuclear cells from the neutrophils and residual erythrocytes. The cell suspension was layered onto the surface of 2 ml of a **54%** [v/v] Percoll solution in 0.15 mol/L NaCl (density 1.075 g/ml) and the gradients were centrifuged at 200 g for 25 **min** at room temperature. The mononuclear cells were harvested and washed in 20 ml of sterile 0.15 mol/L NaCl solution. Cytocentrifuge preparations of the mononuclear cells were stained for non-specific esterase activity, as described below.

2.3 Assessment of Monocyte Purity

To confirm that monocytes isolated by the Nycodenz method were greater than **90%** pure, smears of the cells were made on multi-test slides and allowed to dry overnight. The smears were then stained for: 1) non-specific esterase activity (502); and 2) monocyte and lymphocyte markers by immunohistochemistry (117).

2.3.1. Non-Specific Esterase Staining

A commercial kit (Sigma, Chemical Co., Poole, Dorset, U.K.) was used to stain smears of Nycodenz-isolated cells for non-specific esterase activity. The following reagents were provided in the kit and reconstituted, as described below.

Buffers and reagents

1. Citrate-acetone-methanol fixative

Eighteen millilitres of dilute citrate buffer (citrate concentrate diluted 1 in 10 with deionized water) were added to 27 **ml** of acetone and 5 ml of methanol.

2. Trizmal dilute buffer

Five millilitres of Trizmal concentrate were added to **45** ml of deionised water.

3. Alpha-naphthyl acetate solution

One capsule of a-naphthyl acetate was dissolved in 2 ml of ethylene glycol monomethyl ether.

4. Mayer's haematoxylin solution

Method

The slides were fixed in citrate-acetone-methanol fixative for 1 min at room temperature, washed thoroughly in deionised water and allowed to dry for 1 h. The Trizmal dilute buffer solution (50 ml) was prewarmed to 37°C, and one capsule of Fast Blue RR Salt was added with constant stirring until it was completely dissolved. Two millilitres of a-naphthyl acetate solution were added, the solution was stirred for 30 s and decanted into a Coplin jar. The slides were placed in the staining solution and incubated in a water bath at 37°C for 30 min, protected from light. The slides were removed from the staining solution, washed for 3 min in deionised water, counter-stained for 10 min in Mayer's hematoxylin solution and finally washed in tap water. The slides were air dried and examined microscopically. Two hundred cells were counted in successive high-magnification fields ($\times 400$) and the percentage of positive cells was determined.

2.3.2. Immunohistochemistry

Smears of Nycodenz-isolated cells were stained by the alkaline phosphatase anti-alkaline phosphatase method (117) for: 1) the p150/95 integrin which is expressed by monocytes and macrophages but not by lymphocytes (205); and 2) CD22 and CD3 which are pan B and pan T lymphocyte markers, respectively.

Buffers

1. Tris-buffered saline (TBS)

A solution of 0.15 mol/L NaCl and 0.5 mol/L Tris; pH 7.6

2. Substrate buffer

Naphthol As-Mx phosphate (20 mg) was dissolved in 2 ml of dimethyl formamide in a glass tube and added to 100 ml of 0.1 mol/L Tris HCl; pH 8.2. One hundred microlitres of 0.1 mol/L levamisole were added and the buffer was stored at 4°C.

3. Fast red Substrate

Fast red (5 mg) was dissolved in 5 ml of Substrate buffer.

4. Ammonia water

A solution of NH_3 (0.11 mol/L) in tap water.

Method

Smears of Nycodenz-isolated monocytes were fixed in acetone for 2 min at room temperature. The smears were incubated for 30 min at room temperature with normal rabbit serum (diluted 1/5 in Tris-buffered saline; pH 7.6, [TBS]). The smears were then incubated for 30 min at room temperature with a murine monoclonal antibody recognising p150/95 (diluted 1/50 in TBS), or with CD22 and CD3 together (diluted 1/20 and 1/50 in TBS, respectively). After washing with TBS, the smears were incubated for 30 min with a polyclonal rabbit antibody against murine immunoglobulin (RAM; diluted 1/25 in TBS). After further washing with TBS, the smears were incubated for 30 min with a complex of alkaline phosphatase and murine immunoglobulin to alkaline phosphatase (APAAP; diluted 1/50 in TBS). The smears were washed in TBS and the RAM and APAAP cycles were repeated. The complex was developed with the fast-red Substrate solution for 10 to 20 min. The smears were washed in TBS followed by tap water and counter-stained in Mayer's Hematoxylin solution. The smears were dipped twelve times in ammonia water, mounted in glycagel and viewed under oil immersion. Two hundred cells were counted in successive high-magnification fields (x 400) and the percentage of positive cells in each smear was determined. Murine ascites that did not contain immunoglobulin to human antigens was used instead of the primary antibody as a negative control.

2.4 Limulus Amoebocyte Lysate Assay

2.4.1. Measurement of Endotoxin Concentrations in Reagents and Media

Bacterial lipopolysaccharide (LPS) has been shown to affect many aspects of leukocyte function (188,190,451,468). Since one aim of these studies was to investigate the effects of LPS on monocyte adherence, care was taken to **minimise** LPS contamination during isolation and subsequent culture of the cells. To confirm **this**, all of the solutions used in the Nycodenz isolation and culture of monocytes were assayed for LPS using a commercial limulus amoebocyte lysate assay (220).

The limulus amoebocyte lysate assay was performed using an aseptic technique. The LPS standard curve and test samples were prepared in a laminar **flow** hood using disposable endotoxin free 12 x 75 mm glass test-tubes and sterile disposable plastic pipette tips.

Materials

The following reagents were provided with the commercial kit (Coatest Assay, KabiVitrum, Stockholm, Sweden) and reconstituted as follows:

1. Lyophilised endotoxin from *E. coli* 0111:B4

The endotoxin was dissolved in sterile endotoxin-free water to obtain a concentration of **1 ng/ml** and mixed vigorously for **3 min** using a Vortex mixer.

2. Limulus amoebocyte lysate (LAL)

Each vial of LAL was reconstituted immediately before use with 1.4 ml of sterile endotoxin-free water and swirled gently to prevent foaming. The solution was kept at room temperature for **15 min** to allow proper dissolution.

3. Substrate buffer (sterile endotoxin-free 0.5 mol/L Tris 500; pH 9.0).

4. **S-2423**, the chromogenic Substrate (Ac-Ile-Glu-Gly-Arg-pNAHCl)

The Substrate (9 mg) was reconstituted with **6.6 ml** of sterile endotoxin-free water to a concentration of **1.8 mmol/L** and stored at **4°C** until required. Prior to use in the assay, one volume of Substrate solution was added to one volume of Substrate buffer and incubated at **37°C**

Method

A standard curve was prepared by diluting the stock solution of LPS with endotoxin-free water to give final concentrations of 0, **10, 25, 50, 75** and **100 pg/ml** of LPS. The standards were mixed for **1 min** Using a Vortex mixer. One hundred microlitres of LPS standard or test sample were pipetted into test-tubes and incubated at **37°C** for **5 min**. An equal volume of LAL solution was added to each test tube, mixed, then incubated at **37°C** for **10 min**. Substrate buffer solution (**200 µl**) was added and the reagents were mixed. Following incubation for **3 min** at **37°C**, acetic acid (**200 µl** of a **20%** [v/v] solution) was added to each tube and mixed immediately to terminate the reaction. A sample blank was made by adding **200 µl** of substrate buffer to **100 µl** of water, **100 µl** of LAL and **200 µl** of acetic acid.

Two hundred microlitres of each standard, test sample or blank were transferred to the wells of a **96**-well plate. The absorbance values for the standard and test samples were measured (compared with the assay blank) at **405 nm**. A calibration curve was derived by linear regression analysis and the results for the test samples were obtained by interpolation. Figure 1 shows the standard curve for the limulus amoebocyte lysate

assay. The within-batch coefficient variation for the assay was 5.5% (n = 3). The lower limit of detection of the assay was 10 pg/ml of LPS.

2.4.2. Measurement of Endotoxin Activity in Plasma from Healthy Subjects and Patients with Bronchiectasis

The LPS concentrations in plasma samples from six healthy subjects and 22 patients with bronchiectasis, 10 producing mucoid sputum, six producing mucopurulent sputum and ~~six~~ producing purulent sputum (see 2.5.4) were measured using the limulus amoebocyte lysate assay. Ten millilitres of venous blood were drawn from each subject and collected into sterile lithium heparin tubes. The tubes were centrifuged immediately at 200 g for 10 min at 4°C. Two millilitres of platelet-rich plasma from each subject were transferred to sterile polypropylene tubes, diluted to 20 ml with an endotoxin-free 0.15 mol/L NaCl solution and heated to 75°C for 5 min to remove non-specific activators and inhibitors of the pro-coagulant enzyme (115). The samples were left at room temperature for 15 min then shaken vigorously prior to assay. The commercial limulus amoebocyte lysate kit was used to quantify LPS concentrations as described previously, with the following exceptions.

- 1) Plasma from ~~six~~ healthy donors was pooled and heat-inactivated, as described above. Two millilitres of the pooled plasma were added to 18 ml of endotoxin-free water and the diluted plasma was used as the diluent in the assay blank and the LPS standard samples.
- 2) The **limulus** amoebocyte lysate solution was incubated with the standards and samples for 25 min.
- 3) The Substrate was incubated with the sample and limulus amoebocyte lysate for 5 min.

Figure 1
Standard Curve for the Limulus Amoebocyte Lysate Assay

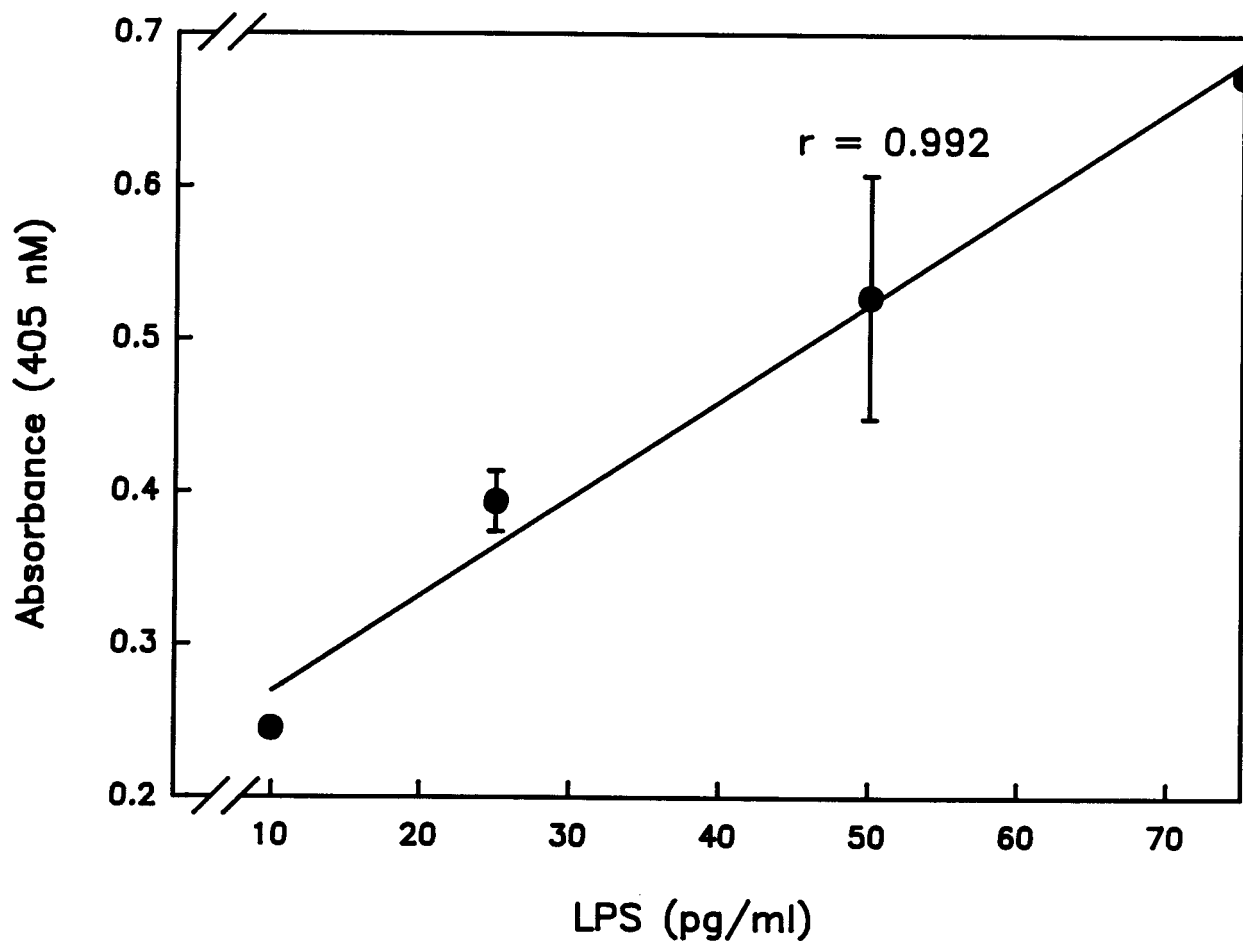


Figure 1 Figure shows the standard curve for the limulus amoebocyte lysate assay. The LPS standard samples were prepared from a stock solution of LPS from *E. coli* 0111:EM in sterile endotoxin-free water. Note the linear relationship between LPS concentration and absorbance measured at 405 nM ($r = 0.990$). The lower limit of detection of the assay was 10 pg/ml. Data represent mean values \pm SD; $n = 2$.

2.5 Monocyte Adherence Assay

2.5.1. Coating of Tissue Culture Dishes with Fibronectin

To determine the optimal coating concentration of fibronectin for monocyte adherence, tissue culture dishes were incubated with 0.5 ml of purified human serum fibronectin at varying concentrations (0 to 50 $\mu\text{g/ml}$ in sterile 0.15 mol/L NaCl solution) for 2 h at 37°C. The dishes were washed three times with 2 ml of sterile 0.15 mol/L NaCl solution to remove unbound fibronectin. A coating concentration of 20 $\mu\text{g/ml}$ of fibronectin was found to be optimal for monocyte adherence (see Results), and this concentration, therefore, was used in all subsequent experiments.

2.5.2. Adherence Assay

Monocytes were suspended at 10^6 cells/ml in supplemented RPMI 1640 medium (see above) and 1 ml aliquots were dispensed into dishes that had been pre-coated with fibronectin (see above). The dishes were incubated for 1 h at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Non-adherent monocytes were removed by washing the dishes three times with 2 ml of ice-cold, sterile 0.15 mol/L NaCl solution. The non-adherent cells were centrifuged at 600 g for 5 min at 4°C, re-suspended in 0.5 ml of supplemented RPMI 1640 medium and counted using a haemocytometer. The percentage of adherent cells was calculated from the initial number of cells and the number of non-adherent monocytes recovered.

2.5.3. Optimization of the Adherence Assay

2.5.3.1 Confirmation of the Specificity of Monocyte Adherence to Fibronectin

The specificity of monocyte adherence to fibronectin was assessed by comparing monocyte adherence to tissue culture dishes coated with fibronectin or 25% [w/v] human serum albumin (HSA). In addition, fibronectin-coated dishes were incubated for 30 min at 37°C with and without varying concentrations of rabbit anti-human fibronectin Fab₂ or non-immune rabbit Fab₂ (0.1, 1, 10 and 100 $\mu\text{g/ml}$ in HBSS; pH 7.4). The dishes were washed three times with 2 ml of HBSS to remove unbound Fab₂. Monocytes from three healthy donors were incubated in the dishes for 1 h and the proportion of adherent cells was determined, as described above.

2.5.3.2. Comparison of Different Sources of Fibronectin

To compare the adherence of monocytes to fibronectin obtained from different sources, tissue culture dishes were coated with: 1) purified human serum fibronectin (20 $\mu\text{g/ml}$), as described previously; 2) complement-depleted (by incubation at **56°C** for 30 min) autologous plasma; and 3) complement-depleted autologous serum. In each case, the tissue culture dishes were washed three times with 2 ml of sterile 0.15 mol/L NaCl solution to remove unbound protein. The proportion of adherent monocytes was then determined, as described previously. A greater proportion of monocytes adhered to tissue culture dishes that had been coated with purified human serum fibronectin compared with dishes coated with autologous serum or plastic (see Results). Purified human serum fibronectin, therefore, was used as the adherence substrate for monocytes in all subsequent assays.

2.5.3.3. Effect of Culture Medium Additives

The effect of culture medium additives on monocyte adherence to fibronectin was assessed. Monocyte adherence was tested in RPMI 1640 medium supplemented with: 1) 10% and 25% [v/v] complement-depleted (by incubation at **56°C** for 30 min) FCS; 2) 10% and 25% [v/v] complement-depleted autologous serum; and 3) 0.5% [w/v] human serum albumin (**HSA**). Similar proportions of monocytes suspended in medium containing 10% [v/v] FCS, 10% (v/v) autologous serum and 0.5% [w/v] **HSA** adhered to fibronectin (see Results). However, the proportion of adherent monocytes was reduced by 30% when the serum concentration was increased from 10% [v/v] to **25%** [v/v]. All subsequent studies, therefore, were performed on monocytes suspended in RPMI 1640 medium supplemented with 10% [v/v] **FCS**.

2.5.3.4. Effect of Monocyte Number

The effect of dispensing different numbers of monocytes into fibronectin-coated dishes on subsequent monocyte adherence was assessed. Varying numbers of monocytes suspended in 1 ml of medium (5×10^5 , 7.5×10^5 , 10^6 , 2×10^6 and 3×10^6) were incubated in fibronectin-coated dishes for 1 h at 37°C. The absolute number of adherent cells was calculated by subtracting the number of non-adherent cells from the initial number of cells dispensed into the dish. A linear relationship was observed between the number of cells added and the number of cells that adhered to fibronectin (see Results). Therefore, in all subsequent experiments 1×10^6 to

2×10^6 monocytes were added to fibronectin-coated tissue culture dishes.

2.5.3.5. Effect of Anti-Proteinases on Monocyte Adherence to Fibronectin

Monocytes contain proteinases that are capable of degrading fibronectin (81). To exclude the possibility that degradation of the fibronectin substrate by monocyte-derived proteinases limits monocyte binding, monocytes were incubated with and without α_1 -antitrypsin ($250 \mu\text{g/ml}$) and α_2 -macroglobulin ($50 \mu\text{g/ml}$) during the adherence assay. The proportion of adherent cells was determined, as described previously.

2.5.3.6. Reproducibility of the Adherence Assay

To assess the reproducibility of the adherence assay, 5×10^6 monocytes isolated from one subject were suspended in 5 ml of medium, 1 ml aliquots were dispensed into five fibronectin-coated dishes and the dishes were incubated for 1 h at 37°C . The percentage of monocytes adhering to each dish was determined, as described previously. The within-batch coefficient of variation (C.V.) for the assay was then calculated. To assess within- and between-donor variability in monocyte adherence to fibronectin, the adherence assay was performed on cells isolated from six subjects on *six* different occasions.

2.5.3.7. Comparison of the Adherence Properties of Monocytes Isolated by the Nycodenz Method and by Countercurrent Centrifugal Elutriation

To exclude the possibility that either the Nycodenz isolation procedure itself or the presence of contaminating platelets altered the adherence properties of Nycodenz-isolated monocytes, the adherence properties of monocytes isolated by Nycodenz (with and without the centrifugation through plasma step to remove platelets) were compared with those of cells isolated by countercurrent centrifugal elutriation (CCE). Ninety millilitres of venous blood were drawn from five healthy donors. Monocytes were isolated from 30 ml of blood by the standard Nycodenz method, from 30 ml of blood by the Nycodenz method followed by centrifugation **through** plasma, and from the remaining 30 ml of blood by CCE. The proportion of monocytes that adhered to fibronectin was then determined for **cells** isolated by the three methods.

2.6. Comparison of the Adherence Properties of Monocytes from Healthy subjects and Patients with Bronchiectasis

Subjects

Bronchiectasis was chosen as a model for an inflammatory disease state. Studies were undertaken on 37 patients with bronchiectasis. The initial diagnosis was based on clinical grounds and confirmed radiologically by bronchography. The patients were derived from a large bronchiectasis clinic and they were selected because they were clinically stable and had had no evidence of acute infective exacerbations (such as pyrexia, chest pain, haemoptysis, or increase in symptoms for at least 2 wk prior to study). Seventeen subjects were male and the age range was 36 to 75 yr (median = 63 yr). None of the patients were receiving antibiotics, steroids orally, or non steroidal anti-inflammatory drugs at the time of study. None of the patients had a recognised primary cause of bronchiectasis (such as ciliary dysfunction or hypogammaglobulinaemia). The patients were subdivided into three groups according to the usual nature of the sputum samples that they produce, as described previously (447). The patients included 13 who produced mucoid sputum (M) persistently, 13 who usually produced mucopurulent sputum (MP) and 11 who produced purulent sputum (P) consistently. The patient groups defined by this method have been shown to vary in the severity of lung inflammation, as assessed by protein transudation into the lung and elastolytic activity of the sputum (447). The demographic and spirometric data on the patients with bronchiectasis are summarised in Appendix 2.

A total of 16 healthy non-smoking subjects were recruited from hospital staff and studied as controls. None of these subjects had a history of lung or other chronic disease or a current infection and none were receiving drugs at the time of study. Nine subjects were male and the age range was 23 to 39 yr (median = 26 Yr).

2.6.1. Basal Adherence of Monocytes to Fibronectin

Monocytes were isolated from patients with bronchiectasis (six subjects from each patient group) and six healthy control subjects. The cells were re-suspended at $10^6/\text{ml}$ in supplemented RPMI 1640 medium and incubated under basal conditions for 1 h in fibronectin-coated dishes. The percentage of adherent monocytes was determined, as described previously.

2.6.2. Effect of Bacterial Lipopolysaccharide (LPS) and Recombinant Cytokines on Monocyte Adherence to Fibronectin

To assess the effect of LPS on monocyte adherence to fibronectin, monocytes from healthy subjects were incubated for 1 h at 37°C, both with and without LPS from *E. coli* 0111:B4 at concentrations ranging from 0.01 to 10 µg/ml. The percentage of adherent monocytes was calculated, as described previously. To compare the effects of LPS from different bacterial species on monocyte adherence to fibronectin, monocytes were incubated for 1 h at 37°C with and without LPS purified from *E. coli* 0111:B4 and from *H. influenzae* at concentrations ranging from 0.01 to 10 µg/ml.

To determine the reproducibility of LPS-stimulated adherence of monocytes to fibronectin, 10⁶ monocytes suspended in 1 ml of medium were dispensed into each of five fibronectin-coated dishes and incubated for 1 h at 37°C in the presence of 1 µg/ml of LPS from *E. coli* 0111:B4. The proportion of adherent monocytes was determined and the within-batch coefficient of variation was calculated. To assess within- and between-donor variability in LPS-stimulated adherence of monocytes to fibronectin, monocytes isolated from four healthy donors on four different occasions were incubated with 1 µg/ml of LPS from *E. coli* 0111:B4 during the adherence assay and the proportion of adherent cells was determined.

To investigate the effect of LPS on the adherence of monocytes from patients with bronchiectasis, cells from patients with bronchiectasis (six patients from each group) were incubated for 1 h at 37°C in fibronectin-coated dishes with and without LPS from *E. coli* 0111:B4 (1 µg/ml). The proportion of adherent cells was determined, as described previously.

To assess the effect of cytokines on monocyte adherence, cells from healthy subjects were incubated with and without the following recombinant cytokines during the adherence assay: 1) interleukin-1β (IL-1, 25 units/ml); 2) tumour necrosis factor-α (TNF, 10, 100, 1000 and 10000 units/ml); 3) interferon γ (IFN, 1000 units/ml); and 4) granulocyte-macrophage colony stimulating factor (GM-CSF, 50 units/ml). To determine whether LPS and cytokines produce additive effects on the adherence of monocytes to fibronectin, monocytes were incubated with and without combinations of cytokines together with the optimal concentration of LPS from *E. coli* 0111:B4: 1) TNF (1000 units/ml) and LPS (1 µg/ml); n = 6; and 2) TNF (1000 units/ml), IFN (1000 units/ml) and LPS (1 µg/ml); n = 6.

2.6.3. Effect of Incubation Time on Monocyte Adherence to Fibronectin

To assess the effect of incubation time on monocyte adherence to fibronectin, monocytes were incubated at 37°C with and without 1 µg/ml of LPS from *E. coli* 0111:B4 in fibronectin-coated dishes for 30 min, 1 h, 4 h and 24 h. The percentage of adherent cells was determined, as described previously.

2.7. Mechanisms Involved in Monocyte Adherence to Fibronectin

To determine whether the CD11/CD18 group of integrins plays a role in mediating basal and LPS-stimulated adherence of monocytes to fibronectin, monocytes from healthy subjects were incubated with anti-CD18, a murine monoclonal antibody (206 µg/ml, subclass immunoglobulin G1 [IgG1]) that reacts with the beta chain (CD18 antigen) common to the three members of the CD11/CD18 subfamily of integrins. In addition, to assess whether RGD-dependent integrins are involved in mediating LPS-stimulated adherence of monocytes to fibronectin, monocytes were also incubated with a synthetic Arg-Gly-AspSer (RGDS) tetrapeptide.

In preliminary experiments, monocytes (10⁶ cells suspended in 500 µl of supplemented RPMI 1640 medium) were pre-incubated with and without varying concentrations of the anti-CD18 antibody (0.4, 1.0, 4.0 and 10 µg/ml) for 15 min at 37°C in sterile polypropylene tubes. The cells were then transferred to fibronectin-coated tissue culture dishes and the dishes were incubated for 1 h at 37°C with and without LPS from *E. coli* 0111:B4 (1 µg/ml). The percentage of adherent monocytes was determined, as described previously. This protocol was repeated for the RGDS peptide (0.025, 0.05, 0.1, 1.0 and 2.0 mg/ml). For the anti-CD18 and RGDS inhibition studies, antibody buffer (RPMI 1640 medium dialysed against 0.05 mol/L Tris HCl containing 15 nmol/L NaN₃; pH 7.2) and medium alone, respectively, were added to the control wells. Anti-CD18 at 4 µg/ml and RGDS at 1 mg/ml were found to be the optimal concentrations for inhibition of monocyte adherence (see Results), and these concentrations, therefore, were used in subsequent experiments.

As further controls, monocytes were also incubated with Arg-Gly-Glu-Ser (RGES; 1 mg/ml) and the following murine monoclonal antibodies: 1) I3, which recognises a non-polymorphic HLA-DR antigen that is expressed by greater than 75% of monocytes (40 µg/ml, IgG2 subclass), (443); 2) MY7, which binds to a monocyte surface antigen (25 µg/ml, IgG1 subclass), (181); and 3) murine IgG, as a non-binding control antibody (50 µg/ml, IgG1 subclass).

The effect of the anti-CD18 antibody and the RGDS peptide together on monocyte adherence to

fibronectin was also assessed. Monocytes (with and without $1\text{ }\mu\text{g/ml}$ of LPS from *E. coli* 0111:B4) were incubated with anti-CD18 alone ($4\text{ }\mu\text{g/ml}$), RGDS alone (1 mg/ml) and with anti-CD18 and RGDS together ($4\text{ }\mu\text{g/ml}$ and 1 mg/ml, respectively) and the proportion of adherent cells was determined.

To assess the mechanisms involved in the adherence of monocytes from patients with bronchiectasis to fibronectin, monocytes from six patients with bronchiectasis (two patients from each subject group) were incubated with and without anti-CD18 alone ($4\text{ }\mu\text{g/ml}$), RGDS peptide alone (1 mg/ml) and anti-CD18 and RGDS together ($4\text{ }\mu\text{g/ml}$ and 1 mg/ml, respectively). The percentage of adherent cells was then determined.

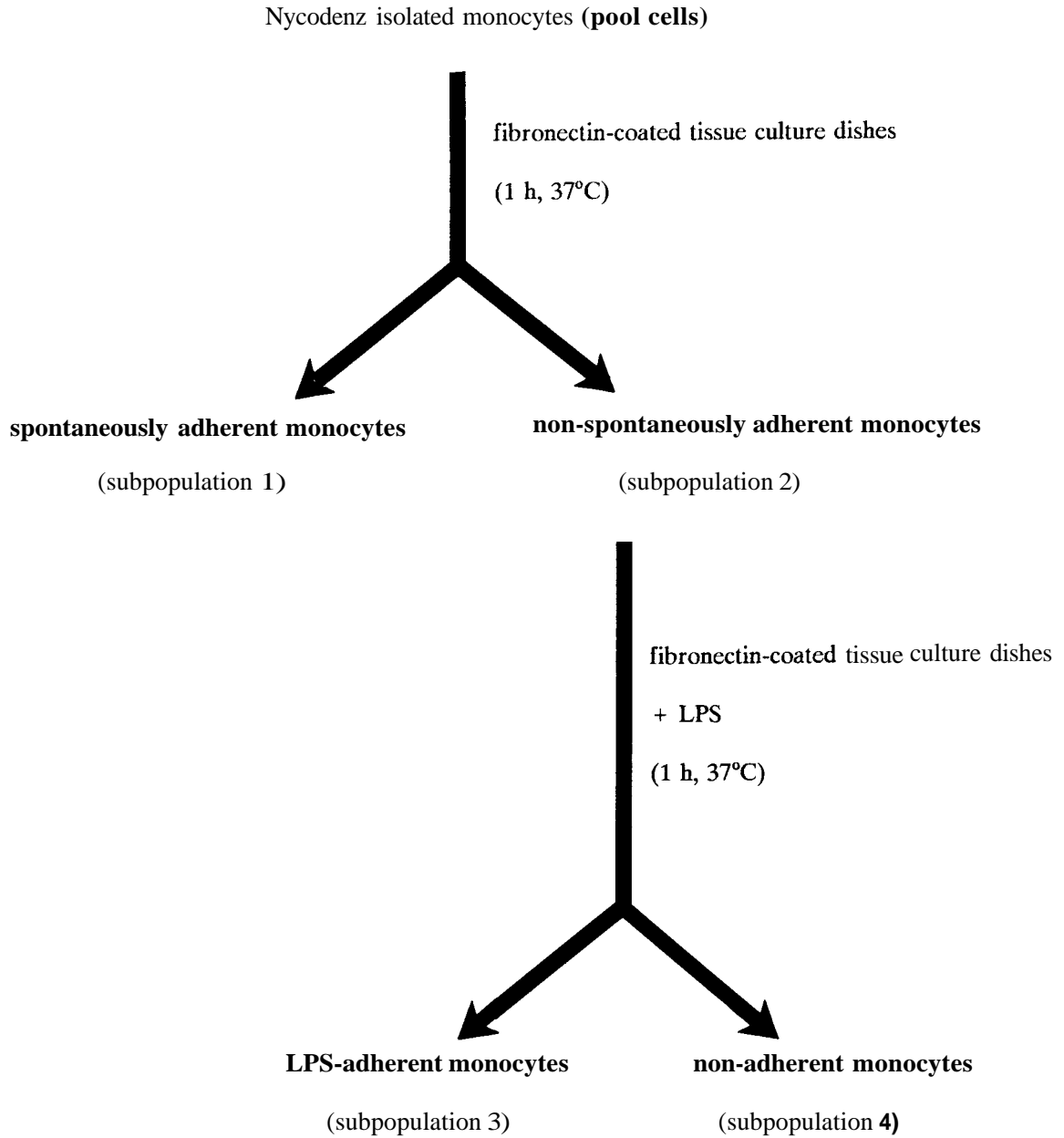
2.8. Monocyte Phenotype Evaluations

2.8.1. Separation of Monocyte Subpopulations

Further studies were conducted to test the possibility that various functional properties of monocytes are related to their capacity to adhere to fibronectin. Monocytes that adhered spontaneously to fibronectin (*spontaneously adherent monocytes*) were isolated by incubating pool monocytes (2×10^6 cells in 1 ml of supplemented RPMI 1640 medium) in fibronectin-coated dishes and removing the non-adherent cells after 1 h, as described previously. An aliquot (5×10^5 cells) of the monocytes that did not adhere spontaneously to fibronectin (*non-spontaneously adherent monocytes*) was retained. The remaining non-spontaneously adherent monocytes (10^6 cells) were re-suspended in 1 ml of supplemented RPMI 1640 medium and incubated in fibronectin-coated dishes for a further 60 min in the presence of LPS from *E. coli* 0111:B4 ($1\text{ }\mu\text{g/ml}$). cells that remained non-adherent (*non-adherent monocytes*) were separated from the cells that had been induced to adhere by LPS (*LPS-adherent monocytes*) by washing the dishes three times with 2 ml of sterile 0.15 mol/L NaCl solution. The non-adherent cells were re-suspended in supplemented RPMI 1640 medium and counted using a haemocytometer. The number of LPS-adherent monocytes was calculated from the number of cells added to the dish and the number of non-adherent monocytes that were recovered. The four monocyte subpopulations (spontaneously adherent, non-spontaneously adherent, LPS-adherent and non-adherent) were cultured in supplemented RPMI 1640 medium in separate tissue culture dishes for all subsequent assays. The separation of the monocyte subpopulations is shown in the flow diagram (figure 2).

Figure 2

Separation of the Monocyte Subpopulations



2.8.2. Confirmation of the Authenticity of the Monocyte Subpopulations

To confirm the authenticity of the monocyte subpopulations, the four subpopulations were separated as described above, and subjected to identical second adherence assays. The non-spontaneously adherent and non-adherent cells were transferred directly to fibronectin-coated dishes and incubated at 37°C for 1 h; the non-spontaneously adherent cells were cultured under basal conditions, and the non-adherent cells were cultured in the presence of 1 µg/ml of LPS from *E. coli* 0111:B4. The proportion of adherent cells was determined, as described previously.

The spontaneously adherent and LPS-adherent cells from the ~~initial~~ adherence assays were also subjected to second adherence assays. These cells were detached by incubating the dishes with sterile 0.15 mol/L NaCl solution containing 5 mmol/L EDTA for 45 min at 4°C, followed by gentle scraping with a rubber policeman. The cells were washed once in 20 ml of sterile 0.15 mol/L NaCl solution and re-suspended in supplemented RPMI 1640 medium. The cells were then incubated in fibronectin-coated dishes at 37°C for 1 h; the spontaneously adherent cells were cultured under basal conditions and the LPS-adherent cells were incubated in the presence of LPS from *E. coli* 0111:B4 (1 µg/ml). The proportion of adherent cells was calculated, as described previously.

2.8.3. Phagocytic activity

Phagocytic activities of the monocyte subpopulations were assessed by incubating the cells with 3 µM diameter latex beads for 40 min at 37°C (41,424). The ratio of beads to monocytes was 75:1. To control for possible effects of LPS on phagocytosis, spontaneously adherent cells were also pre-incubated with LPS from *E. coli* 0111:B4 (1 µg/ml) for 1 h before assessment of their phagocytic activity. Following the phagocytic assay, the adherent monocyte subpopulations were detached by gentle scraping with a rubber policeman. Cytocentrifuge preparations of the four monocyte subpopulations were stained with modified Wright's stain and viewed under oil immersion. Two hundred cells were counted independently in successive high-magnification fields. Both the mean number of beads associated with each cell, and the percentage of cells that were positive (associated with one or more beads) were determined. The phagocytic index was calculated as follows: Phagocytic index (PI) = percentage of positive cells X mean number of beads/cell. Figure 3 shows pool monocytes phagocytosing latex beads.

Figure 3

Phagocytosis of Latex Beads by Monocytes

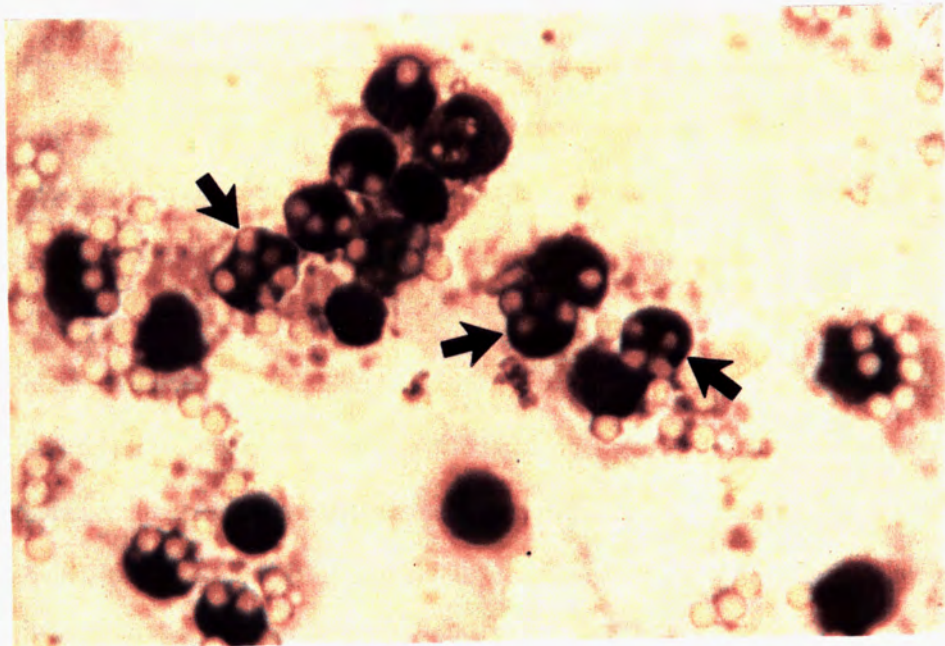


Figure 3 Photomicrograph shows a typical preparation of human monocytes phagocytosing latex beads of 3 μm diameter (arrows). Magnification is x 400.

2.8.4. Superoxide Anion Generation

Superoxide anion generation was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* (25). The monocyte subpopulations were incubated at 37°C in 35 x 10 mm tissue culture dishes in 1 ml of Dulbecco's buffer (phosphate buffered saline containing 1 mmol/L MgCl₂ and 1 mmol/L CaCl₂) with cytochrome *c* (1.25 mg/ml). Control cells were incubated in the presence of superoxide dismutase (50 µg/ml). Spontaneously adherent and LPS-adherent monocytes were adherent to fibronectin-coated dishes, and the non-adherent monocyte subpopulations were incubated in uncoated dishes during the assay. To control for possible effects of LPS on superoxide production by monocytes, spontaneously adherent cells were also pre-incubated for 1 h with LPS from *E. coli* 0111: B4 (1 µg/ml) prior to assay. After incubation for 1 h, the media samples were centrifuged for 5 min at 600 g at 4°C and the cell-free supernatants were transferred to plastic disposable cuvettes. Superoxide production was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* using an extinction coefficient of 21.1 x 10³ M⁻¹cm⁻¹ at a wavelength of 550 nm (459). The results were expressed as nmol of cytochrome *c* reduced/10⁶ cells/h. The within-batch coefficient of variation for the superoxide assay was 5.8% (n = 3).

2.8.5. Peroxidase Activity

The peroxidase activity of the monocyte subpopulations was measured as described previously (428). The monocyte subpopulations were separated as described above. Culture supernatants were harvested and the cells were washed once with 1 ml of a 0.15 mol/L NaCl solution. Extracts of the monocyte subpopulations were made at 2 x 10⁶ cells/ml in lysing buffer (phosphate buffered saline [PBS] containing 0.04% [v/v] Triton X-100 and 1 mol/L NaCl; pH 7.4). Extracts of the adherent monocyte subpopulations were prepared within the tissue culture dishes and examination of the dishes by phase-contrast microscopy confirmed that all of the cells had been lysed. To control for the possible effects of LPS on peroxidase activity, spontaneously adherent monocytes were also pre-incubated with LPS from *E. coli* 0111:B4 (1 µg/ml) for 1 h prior to assay. Extracts of platelets were also prepared and assayed for peroxidase activity since Nycodenz-isolated monocytes are contaminated variably with platelets.

Cell extract or culture supernatant (100 µl) was mixed with 900 µl of Substrate (0.01 mol/L phosphate buffer containing 0.017% [w/v] O-dianisidine [3,3'-dimethoxy benzidine] and 0.003% [v/v] H₂O₂; pH 6.0). The

change in absorbance was measured immediately in a spectrophotometer (Beckman DU-8; Beckman Instrument Co., Irvine, California, U.S.A.) at 460 nM, using 100 μ l of lysing buffer or supplemented RPMI 1640 medium and 900 μ l of substrate as the assay blank. The peroxidase activity was expressed as mU/ 10^6 cells, where one unit is the amount of enzyme decomposing 1 μ mol of peroxide per min. The within-batch coefficient of variation for the peroxidase assay was 5.6% (n = 5).

2.8.6. HLA-DR Antigen Expression by Monocyte Subpopulations

The indirect immunofluorescence technique was used to label monocytes with **I3**, which is a murine monoclonal antibody that binds to a non-polymorphic HLA-DR antigen expressed by greater than 75% of human monocytes (443). Since the binding of I3 by monocytes was analysed initially by epifluorescence microscopy, platelet-free monocyte preparations were necessary for these experiments and monocytes, therefore, were isolated by CCE. The monocyte subpopulations were separated and the cells were fixed for 5 min at 4°C in 1 ml of fixative (PBS containing 1% [w/v] paraformaldehyde, 0.5 mmol/L MgCl_2 and 1 mmol/L CaCl_2 ; pH 7.4). Pool monocytes and the non-adherent monocyte subpopulations were fixed in suspension. The monocyte subpopulations that were adherent to fibronectin-coated dishes were fixed within the dishes and then detached by gentle scraping with a rubber policeman. Cells (2×10^6) were suspended in 400 μ l of medium (HBSS containing 1% [w/v] HSA and 50 μ g/ml of goat IgG; pH 7.4) and incubated for 30 min at 4°C to block monocyte Fc receptors. I3 antibody or non-immune murine IgG (as a negative control) were added to the cell suspension both at a final concentration of 20 μ g/ml. The cells were incubated for a further 30 min at 4°C, then washed three times with 500 μ l of medium. The cells were re-suspended in 200 μ l of medium, goat antibody to mouse immunoglobulin conjugated to FITC was added (170 μ g/ml final concentration) and the cells were incubated at 4°C for 30 min. The cells were washed three times in 500 μ l of medium and re-suspended in 200 μ l of medium. The samples were analysed initially by phase-contrast and epifluorescence microscopy (Leitz Dialux 20, Ernst Leitz Westlar G.M.B.H.). The samples were analysed subsequently by flow cytometry (Ortho B.D., Mountain View, California, U.S.A.). Contaminating lymphocytes (5 to 8%) were separated from the monocytes by bit map gating on forward angle and 90° light scatter characteristics. Fluorescence data were collected on a three decade log scale. Mean log scales were converted to linear fluorescence with calibration curves obtained by analysing fluorescent microspheres with both log and linear amplifications.

2.8.7. Total Protein Content

The protein content of the monocyte subpopulations was quantified by the Sedmak method as described previously (404). Monocytes were isolated by CCE for these experiments since Nycodenz-isolated monocytes are contaminated variably with platelets and platelets contain protein (1). Extracts of the monocyte subpopulations were made at 2×10^6 cells/ml in lysing buffer (phosphate buffered saline [PBS] containing 1 mol/L NaCl and 0.04% [v/v] Triton X-100; pH 7.4). The cell extracts were frozen to -70°C until analysis. The Sedmak reagent was made by diluting 120 mg of Coomassie brilliant blue G250 dye in 200 ml of deionised water containing 1.9% [v/v] perchloric acid, and the solution was filtered prior to use. The protein standard was made by dissolving bovine serum albumin (BSA) in lysing buffer at a concentration of 1 mg/ml. Serial dilutions of the BSA standard were made in lysing buffer to give final concentrations of BSA ranging from 50 to 400 $\mu\text{g/ml}$. Fifty microlitres of the BSA standard or test samples were added to 1 ml of Sedmak reagent in a test tube and the absorbance of the solution was read in a spectrophotometer (Beckman DU-8; Beckman instrument Co., Irvine, California, U.S.A.) at a wavelength of 620 nm using 50 μl of lysing buffer added to 1 ml of Sedmak reagent as the assay blank. A calibration line was derived by linear regression analysis and the results for the test samples were obtained by interpolation. Figure 4 shows a typical standard curve for the Sedmak assay. The within- and between-batch coefficients of variation for the assay were 2.5% ($n = 6$) and 6.7% ($n = 5$), respectively.

Figure 4

Standard Curve for the Sedmak Protein Assay

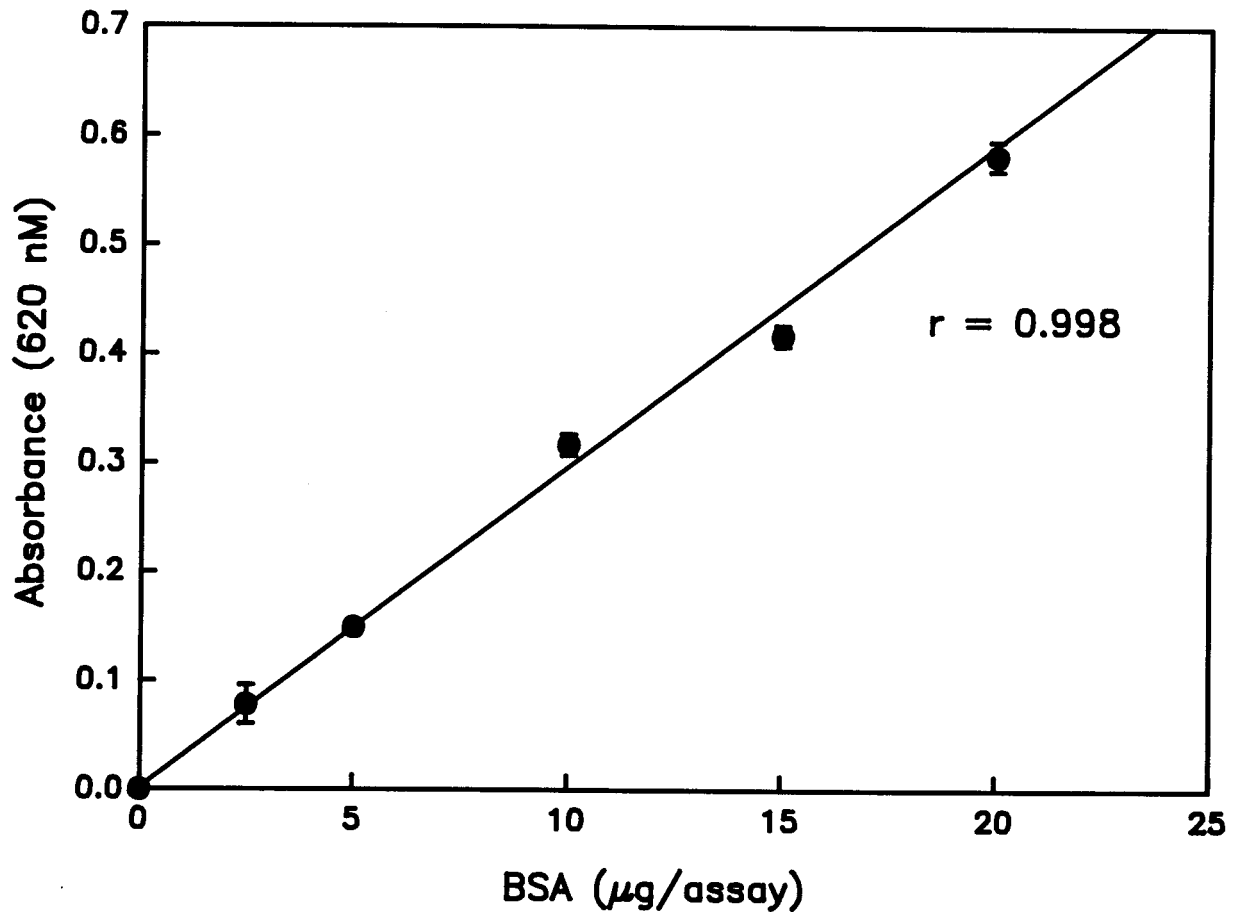


Figure 4 Figure shows a typical standard curve for the Sedmak assay which was used to measure the total protein content of monocyte extracts. The protein standard for the assay was prepared by making serial dilutions of a 1 mg/ml solution of bovine ~~serum~~ albumin (BSA) using the monocyte lysing buffer (PBS containing 1 mol/L NaCl and 0.04% [v/v] Triton X-100) as the diluent. Note the linear relationship between BSA concentration and absorbance over the range of 0 to 20 μg/assay of BSA ($r = 0.998$). Data represent mean \pm SEM; $n = 3$.

2.9. Expression of Human Leukocyte Elastase and Alpha₁-Antitrypsin by Monocytes

2.9.1. Preparation of Cell Extracts

Extracts of monocytes, neutrophils and platelets were prepared in lysing buffer (PBS containing 0.04% [v/v] Triton X-100 and 1.05 mol/L NaCl; pH 7.4). It has been shown previously that 0.04% [v/v] Triton X-100 in this salt concentration is adequate to extract HLE maximally from whole cells without producing anomalies in the immunoassays (81). **High** salt concentrations are necessary during the preparation and subsequent assay of **cell** extracts due to the relative insolubility of HLE in buffers of low ionic strength (453). Cells were washed with 10 ml of sterile 0.15 mol/L NaCl solution and cell extracts were prepared in lysing buffer. Extracts of monocytes and neutrophils were prepared at 2×10^6 cells/ml and extracts of platelets were prepared at 2×10^8 cells/ml in lysing buffer. Extracts of adherent monocytes were prepared while the cells were adherent to the tissue culture dishes and examination of the dishes by phase-contrast microscopy confirmed that all of the cells had been lysed. Cell extracts (and culture supernatants) were stored at -70°C until analysis.

2.9.2. HLE and Alpha₁-Antitrypsin Content of Monocyte Subpopulations

Monocytes were isolated by the Nycodenz method, extracts of the monocyte subpopulations were prepared (2.9.1.) and assayed for HLE content using an indirect binding ELISA (2.9.4.). To assess whether the Nycodenz isolation procedure influences the HLE content of monocytes, monocytes were isolated by both the Nycodenz method and CCE, extracts of pool, spontaneously adherent and non-spontaneously adherent cells were prepared and assayed for immunoreactive HLE. Extracts of platelets were also assayed for immunoreactive HLE since Nycodenz-isolated monocytes are contaminated variably with platelets. The HLE content of pool monocytes and neutrophils were also compared. Monocytes and neutrophils were prepared from three donors by CCE and Percoll density gradient centrifugation, respectively. **Cell** extracts were prepared and assayed for HLE. The results for these experiments were expressed as **ng** or pmol **HLE/10⁶ cells**.

To compare the **α₁-antitrypsin** content of the monocyte subpopulations it was necessary to isolate monocytes by CCE since Nycodenz-isolated monocytes are contaminated variably with platelets and preliminary experiments demonstrated that platelets contain **α₁-antitrypsin** (see Results). Extracts of the monocyte subpopulations were prepared (2.9.1) and assayed for **α₁-antitrypsin** using a direct binding ELISA (2.9.5.). The results were expressed as **ng** or pmol **α₁-antitrypsin/10⁶ cells**.

2.9.3. Accumulation of **Alpha₁-Antitrypsin** by Monocyte Subpopulations

2.9.3.1. Protocol for **Quantifying Alpha₁-Antitrypsin** Accumulation by Monocytes

A direct binding ELISA (2.9.5.) was used to quantify immunoreactive **α₁-antitrypsin** in monocyte extracts and culture supernatants. The amount of **α₁-antitrypsin** measured in monocyte extracts and culture supernatants using **this** technique represents the net balance between synthesis and catabolism of this protein by the cells. Therefore, the results were expressed as the amount of **α₁-antitrypsin** accumulated rather **than** synthesised by monocytes.

Monocytes were isolated by the Nycodenz method for these experiments. Although Nycodenz-isolated monocytes are contaminated with platelets and platelets were subsequently found to contain **α₁-antitrypsin** (see Results), it is unlikely that platelets have the capacity to synthesise **α₁-antitrypsin** de novo since: 1) platelets contain negligible amounts of DNA and RNA; and 2) any RNA that is present within platelets is in a metabolically inactive form (1). Therefore, to overcome the problem of platelet contamination, the experiments were designed as follows. Monocyte subpopulations were cultured in several tissue culture dishes (35x 10 mm) in **1 ml** of supplemented RPMI 1640 medium at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After incubation overnight, **cells** (i.e. monocytes and contaminating platelets) were harvested from one of the dishes (time = 0, control cells) and cell extracts were prepared, as described in 2.9.1. Cells in the remaining dishes were incubated in fresh medium (**1 ml**) for up to **48 h** with and without pro-inflammatory mediators. After incubation for **24 h**, cells and culture supernatants were harvested and cell extracts were prepared. Culture supernatants from **the** remaining dishes were harvested and the **cells** were cultured in fresh medium with and without pro-inflammatory mediators. After incubation for a further 24 h, the cells and culture supernatants were harvested and cell extracts were prepared.

The **α₁-antitrypsin** content of the cell extract and supernatant samples was quantified using the direct binding ELISA (2.9.5.), and all of these results were expressed as **ng α₁-antitrypsin/10⁶ cells**. To determine the total amount of **α₁-antitrypsin** accumulated by the monocytes, the values obtained for the cell extracts and culture supernatants for each time interval were added together and the value obtained for the time = 0, control cells (monocytes and their contaminating platelets) was subtracted from **this** number. The results were then expressed as: 1) percentage increase in **α₁-antitrypsin** accumulated by the monocytes (compared with the amount of **α₁-antitrypsin** contained within the control, time = 0 cells) when the monocyte subpopulations were isolated

from different donors; or 2) ng α_1 -antitrypsin accumulated/ 10^6 cells, when the monocyte subpopulations were isolated from the same donor.

2.9.3.2. Effect of Culture and Lipopolysaccharide on α_1 -Antitrypsin Accumulation by Monocytes

The effects of culture and **LPS** exposure on the accumulation of α_1 -antitrypsin by the monocyte subpopulations were assessed. To obtain a sufficient number of cells for study, a large volume of blood had to be drawn from the donors and the experiment, therefore, was conducted in two stages. Initially, spontaneously adherent and non-spontaneously adherent monocytes were separated from six healthy donors and incubated at 37°C in tissue culture dishes 1 ml of supplemented **RPMI** 1640 medium. After overnight culture, extracts of the time = 0, control cells were prepared and the remainder of the cells were incubated in fresh medium for 48 h with and without **LPS** from *E. coli* 0111:B4 (1 μ g/ml). Cells and culture supernatants were harvested at 24 h intervals and cell extracts were prepared as described in 2.9.3.1. This protocol was then repeated for **LPS**-adherent and non-adherent monocytes prepared from an additional five healthy donors. The amount of α_1 -antitrypsin that had accumulated in the cells and culture supernatants was quantified, as described in 2.9.3.1. The results were expressed as percentage increase in the amount of α_1 -antitrypsin accumulated by monocytes compared with the amount of α_1 -antitrypsin contained within the time = 0, control cells.

To assess the absolute amounts of α_1 -antitrypsin accumulated by spontaneously adherent and non-spontaneously adherent cells, both subpopulations were isolated from 15 healthy donors and incubated at 37°C in supplemented **RPMI** 1640 medium in tissue culture dishes. After overnight culture, extracts of the time = 0, control cells were prepared. The remaining cells were incubated in fresh medium with and without **LPS** from *E. coli* 0111:B4 (1 μ g/ml). After incubation for 24 h, the cells and culture supernatants were then harvested and cell extracts were prepared. The amount of α_1 -antitrypsin that had accumulated in the cells and culture supernatants was quantified as described in 2.9.3.1, and the results were expressed as ng α_1 -antitrypsin accumulated/ 10^6 cells.

2.9.3.3. Effect of Time on α_1 -Antitrypsin Accumulation by Spontaneously Adherent Monocytes

Spontaneously adherent monocytes from five healthy donors were incubated at 37°C in 1 ml of supplemented **RPMI** 1640 medium in tissue culture dishes. After overnight culture, extracts of time = 0, control

cells were prepared. The remaining cells were cultured in fresh medium for up to **24 h** with and without LPS from *E. coli* 0111:B4 (**1 µg/ml**). After culture for 6 h, 12 h, and 24 h, **cells** and culture supernatants were harvested and cell extracts were prepared. The amount of **α₁-antitrypsin** that had accumulated in the cells and culture supernatants was quantified, as described in 2.9.3.1. The results were then expressed as **ng α₁-antitrypsin accumulated/10⁶ cells**.

2.9.3.4. Effect of Recombinant Cytokines on Alpha₁-Antitrypsin Accumulation by Monocyte Subpopulations

Spontaneously adherent and non-spontaneously adherent monocytes from six healthy donors were incubated at **37°C** in tissue culture dishes in 1 ml of supplemented RPMI 1640 medium (**1 ml**). After overnight culture extracts of time = 0, control cells were prepared. The remaining cells were cultured in fresh medium with and without TNF (1000 units/ml), **TNF** (1000 units/ml) and GM-CSF (**10 to 75 units/ml**). After incubation for 24 h, **cells** and culture supernatants were harvested and **cell** extracts were prepared. The amount of **α₁-antitrypsin** that had accumulated in the cells and culture supernatants was quantified, as described in **2.9.3.1**. The results were then expressed as **ng α₁-antitrypsin accumulated/10⁶ cells**.

2.9.4. Indirect Binding ELISA for Human Leukocyte Elastase

The indirect binding ELISA for **HLE** was performed, as described previously (81).

Materials and Buffers

1. HLE purified from sputum, as described previously (**49**)
2. Polyclonal sheep anti-human HLE
3. Rabbit anti-sheep IgG conjugated to horse radish peroxidase.
4. PBS containing 0.6 mol/L NaCl; pH **7.4**
5. PBS containing 0.6 mol/L NaCl, 0.05% [v/v] Tween 20 and 1% [w/v] BSA.
6. Sodium citrate (0.05 mol/L); pH **4.5**

Method

HLE was reconstituted in PBS (containing 0.6 mol/L NaCl; pH **7.4**) and the protein content was determined spectrophotometrically (Beckman DU-8; **Beckman** Instrument Co., Irvine, California, U.S.A.) using

the extinction coefficient ($E_{280}^{1\%}$) of 9.85 (33). The HLE standard samples were made from a stock solution of 1 mg/ml of HLE in PBS containing 0.6 mol/L NaCl. Serial dilutions of the stock solution were made in PBS containing 0.05% [v/v] Tween 20 and 1% [w/v] BSA to obtain final concentrations of 160, 80, 40, 20, 10 and 5 ng/ml. The monocyte extracts (2×10^6 cells/ml in PBS containing 1.05 mol/L NaCl and 0.04% [v/v] Triton X-100) were diluted in an equal volume of PBS containing 0.15 mol/L NaCl, 0.05% [v/v] Tween 20 and 1% [w/v] BSA to adjust the salt concentration to 0.6 mol/L. An additional 1:3 dilution, for assay, was made in PBS containing 0.6 mol/L NaCl, 0.05% [v/v] Tween and 1% [w/v] BSA. Sixty-five microlitres of HLE standard, diluted cell extract or undiluted culture supernatant and 65 μ l of polyclonal sheep anti-human HLE (10 μ g/ml) were pipetted into polypropylene radioimmunoassay (RIA) vials and incubated overnight at 4°C.

Ninety-six well microtitre plates were coated with 200 μ l of polyclonal sheep anti-human HLE (5 μ g/ml in PBS containing 0.6 mol/L NaCl), and the plates were incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% [v/v] Tween 20 to remove unbound protein. The plates were then incubated for 1 h at room temperature with 200 μ l of PBS containing 0.05% [v/v] Tween 20 and 1% [w/v] BSA to block additional protein binding sites.

After washing the plate three times with PBS containing 0.05% [v/v] Tween 20, 100 μ l of the competitive mixtures were transferred to the antigen-coated plates and the plates were incubated at room temperature for 2 h. The plates were washed three times and incubated for 90 min at room temperature with 100 μ l of peroxidase-conjugated rabbit anti-sheep IgG. The plates were washed a further three times and developed using 100 μ l of 50 mmol/L sodium citrate buffer (pH 4.5) containing 0.001% [w/v] o-phenylenediamine and 0.03% [v/v] H_2O_2 . The plates were incubated at room temperature for 15 to 30 min to permit colour development, and the reaction was then terminated by the addition of 50 μ l of 4 mol/L H_2SO_4 . Absorbances were read with an automated microtitre plate reader (V-Max Kinetic Microplate Reader, Molecular Devices Corp., Palo Alto, California, U.S.A.). A calibration line was constructed by linear regression analysis, and the results for the test samples were obtained by interpolation. The results were expressed as ng HLE/ 10^6 monocytes. Figure 5 shows a typical standard curve for the indirect binding ELISA for HLE. This assay had sub-nanogram sensitivity and showed less than 0.2% cross-reactivity with cathepsin G and less than 0.1% cross-reactivity with myeloperoxidase (81). The within- and between-batch coefficients of variation for this assay were 5.6% (n = 6) and 14.2% (n = 3).

Figure 5

Standard Curve for the HLE Indirect Binding ELISA

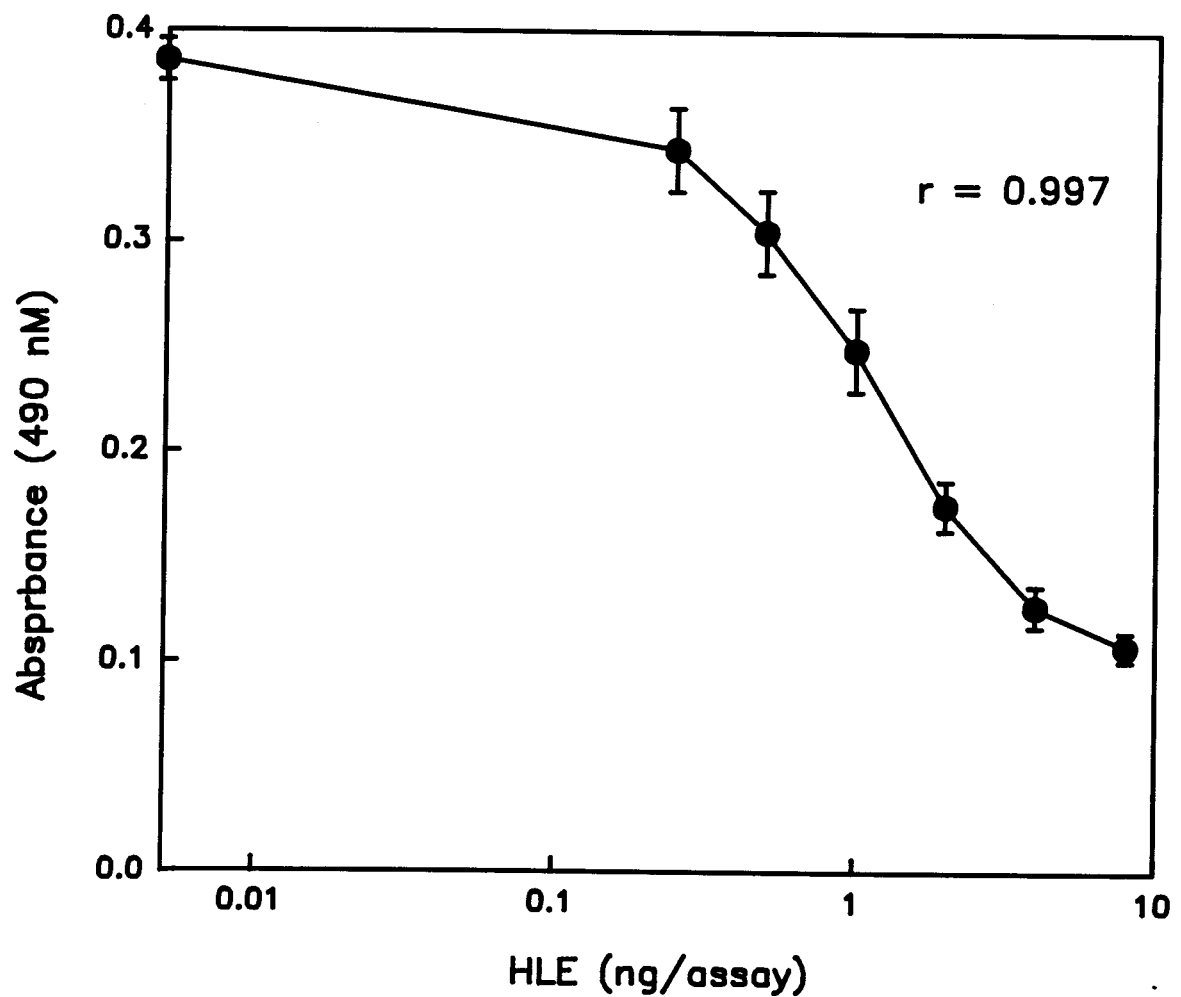


Figure 5 Figure shows a typical standard curve for the HLE indirect binding ELISA. The HLE standard samples were prepared from a stock solution of purified HLE dissolved in PBS containing 0.6 mol/L NaCl. Note the linear relationship between HLE concentration and absorbance at 490 nm over the range of 0.25 to 4.0 ng HLE/assay ($r = 0.997$). Data represent mean \pm SD; $n = 3$.

2.9.5. Alpha₁-Antitrypsin Direct Binding ELISA

Sheep anti-human α_1 -antitrypsin was conjugated to horse radish peroxidase, as described previously (323).

2.9.5.1. Conjugation of Sheep Anti-Human Alpha₁-Antitrypsin with Horse Radish Peroxidase

Buffers

1. Carbonate-bicarbonate buffer (0.01 mol/L NaHCO_3 adjusted to pH 9.5 with 0.01 mol/L Na_2CO_3)
2. **Sodium** acetate, 1 mmol/L; pH **4.4**
3. PBS; pH **7.4**
4. Glycerol 60% [v/v] **in** borate buffer, 0.1 mol/L; pH **7.4** with thiomersal 0.02% [w/v]
5. Sodium periodate, 0.1 mol/L
6. Sodium borohydride (**4** mg/ml)

Method

Horse radish peroxidase (HRP, 8 mg) was dissolved in 2 **ml** of distilled water and incubated at room temperature for 20 **min** with 400 μl of sodium periodate (0.1 mol/L). This solution was then dialysed against the 1 mmol/L acetate buffer for 24 h at **4°C**. At the same time, 18.3 mg of sheep anti-human α_1 -antitrypsin were dialysed against the carbonate-bicarbonate buffer (0.01 mol/L) for 24 h at **4°C**. The HRP and sheep anti-human α_1 -antitrypsin solutions were mixed and incubated at room temperature for 2 h. Fresh sodium borohydride (0.2 **ml**) was added to terminate the reaction. The conjugated antibody was dialysed against PBS for 24 h at **4°C**, prior to the addition of an **equal** volume of 60% [v/v] glycerol in 0.1 mol/L borate buffer with 0.02% [w/v] thiomersal. The conjugated antibody was titrated in the α_1 -antitrypsin ELISA (*see* below) to determine the optimal concentration.

2.9.5.2. Direct **Binding** ELISA for Alpha₁-Antitrypsin

This assay was performed as described previously (6).

Materials and Buffers

1. Polyclonal **sheep** anti-human α_1 -antitrypsin (12.5 mg/d)
2. Human α_1 -antitrypsin standard (2.04 mg/ml)

3. Carbonate-bicarbonate buffer (0.05 mol/L NaHCO_3 adjusted to pH 9.6 with 0.05 mol/L Na_2CO_3)
4. PBS containing 1% [v/v] haemaccel and 0.1% [v/v] Tween-20; [PBS/haemaccel/Tween]
5. Citrate-phosphate buffer (0.1 mol/L K_2PO_4 adjusted to pH 5.0 with 1 mol/L citric acid)
6. Citric acid 0.5 mol/L

Method

Microtitre plates (%-well) were coated with 200 μl of sheep anti-human α_1 -antitrypsin antibody diluted 1/2000 in carbonate-bicarbonate buffer. The plates were incubated overnight at 4°C and then washed three times with 200 μl of PBS/haemaccel/Tween to remove unbound protein.

The α_1 -antitrypsin standard samples were prepared by diluting the stock human α_1 -antitrypsin standard solution in PBS/haemaccel/Tween to give final concentrations ranging from 3.2 to 204 **ng/ml**. Two hundred microlitres of the α_1 -antitrypsin standard samples, test samples or assay blanks (PBS/haemaccel/Tween, lysing buffer and supplemented RPMI 1640 medium) were added to the wells and the plates were incubated for 2 h at room temperature covered with cling-film. The plates were then washed **three** times with PBS/haemaccel/Tween (200 μl /well). HRP-conjugated sheep anti-human α_1 -antitrypsin was diluted 1/100 in PBS/haemaccel/Tween and 200 μl were added to each well. The plates were covered with cling-film and incubated for 2 h at room temperature, then washed **three** times with PBS/haemaccel/Tween.

Ten milligrams of the Substrate (o-phenylenediamine, OPD) were dissolved **in** 25 **ml** of citrate-phosphate buffer, 20 μl of hydrogen peroxide were added and the solution was mixed using a Vortex mixer. The Substrate solution (200 μl) was added to each well, and the plates were incubated at room temperature for 10 to 30 min to allow colour development. The reaction was terminated with 0.5 mol/L citric acid (50 μl). The absorbance values of the standards and test samples were measured (compared with the assay blanks) at a wavelength of 492 nm. A calibration line was constructed by linear regression analysis and the sample results were obtained by interpolation. The results were expressed as: 1) **ng** or pmol α_1 -antitrypsin/ 10^6 cells (2.9.2); or 2) ng α_1 -antitrypsin accumulated/ 10^6 cells or percentage increase in the amount of α_1 -antitrypsin accumulated compared with the amount of α_1 -antitrypsin contained within time = 0, control cells (2.9.3).

Figure 6 shows a typical standard curve for the α_1 -antitrypsin direct binding ELISA. The within- and between-batch coefficients of variation for the assay were **4.7%** (**n** = 6) and 8.3% (**n** = 6), respectively.

Figure 6

Standard Curve for the Alpha₁-Antitrypsin Direct Binding ELISA

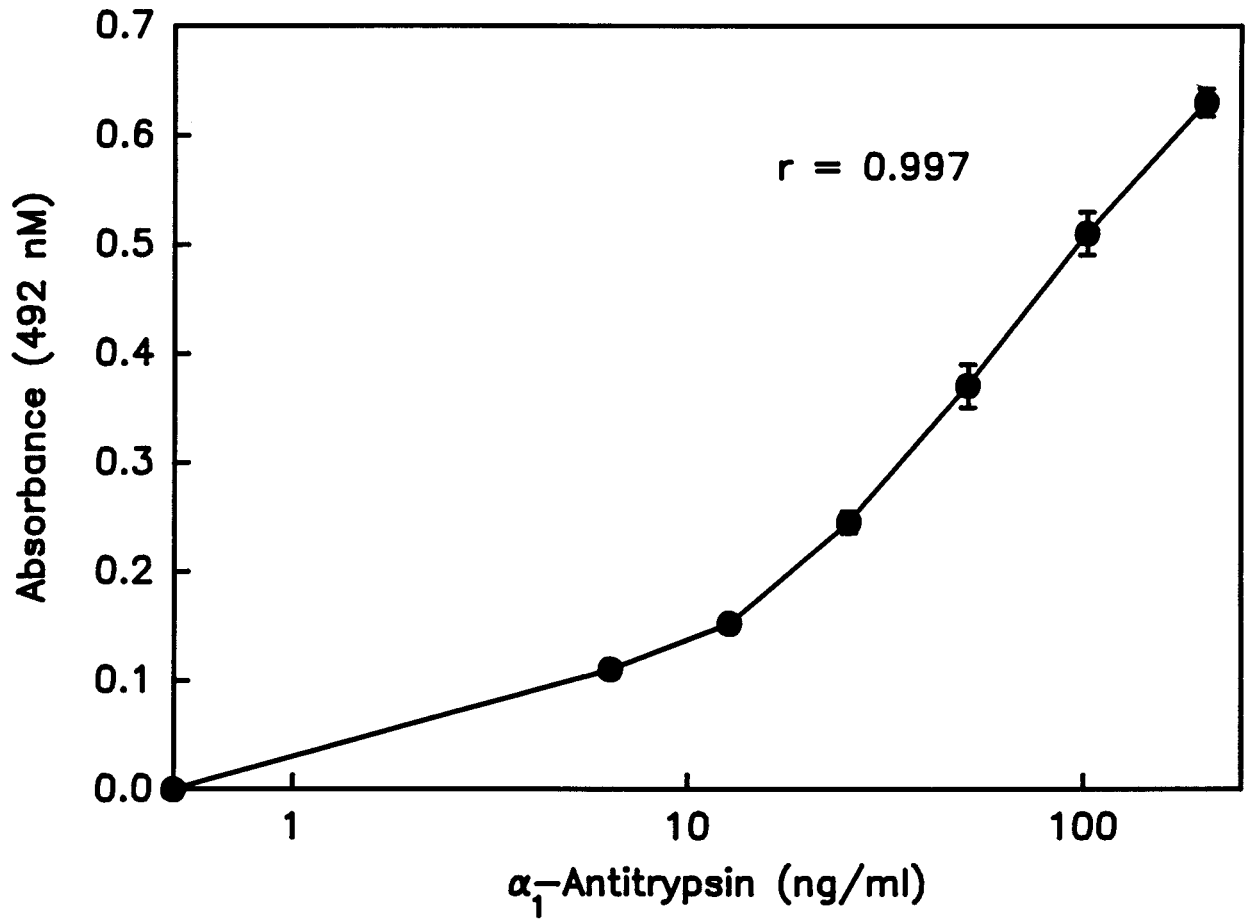


Figure 6 Figure shows a typical standard curve for the α_1 -antitrypsin direct binding ELISA.' The α_1 -antitrypsin standard samples were prepared from a stock solution of α_1 -antitrypsin (2.04 mg/ml). Note the linear relationship between α_1 -antitrypsin concentration and absorbance at 492 nM over the range of 12.75 to 204 ng α_1 -antitrypsin/ml ($r = 0.997$). Data are mean values \pm SEM; $n = 6$.

2.10. Dot-Blot Analysis of Alpha₁-Antitrypsin Gene Expression by Monocytes

Alpha₁-antitrypsin specific messenger RNA (mRNA) concentrations in monocytes were quantified by the dot-blot technique using a human genomic α_1 -antitrypsin DNA probe. A chimeric plasmid containing a 9.6-kb human DNA inserted into the **EcoR** 1 site of pBR332 (PAT 9.6) was provided by Dr N. Kalsheker, University of Cardiff, U.K. Digestion of pAT 9.6 with BamH 1 generated a 6.5-kb fragment (AT 6.5) that contained human α_1 -antitrypsin gene sequences. A human β -actin complementary DNA (cDNA) probe was used as an internal standard. The β -actin probe was a 1.9-kb cDNA in the BamH 1 site of plasmid PUC 9B (supplied by Dr T. Ley, Jewish Hospital, St Louis, Missouri, U.S.A.). Both probes were cloned in *E. coli* prior to dot-blot analysis.

2.10.1. General Purpose Molecular Biology Solutions and Reagents

1. TE buffer: 10 mmol/L Tris HCl and 1 mmol/l EDTA; pH 7.5

This solution was made up in distilled water with stock solutions of 1mol/L Tris HCl; pH 7.5, and 0.5 mol/L EDTA; pH 8.0.

2. Tris-saturated phenol

Solid phenol was redistilled at 180°C and stored at -20°C in aliquots until required. When needed, phenol (400 ml) was removed from the freezer, allowed to warm to room temperature and melted at 68°C. Hydroxyquinone (800 mg) was dissolved in the molten phenol and 500 ml of an aqueous solvent were added (50 mmol/L Tris HCl containing 10mmol/L EDTA and 10mmol/L NaCl; pH 8.0). The mixture was shaken and allowed to settle into layers. When the term "phenol" is used subsequently, this means Tris-saturated phenol.

3. Chloroform/isoamyl alcohol mixture

Chloroform (96 ml) was mixed with 4 ml of isoamyl alcohol. When the term "chloroform" is used subsequently, this means chloroform/isoamyl alcohol mixture.

2.10.2. Transformation of E. Coli

Media, Reagents and buffers

1. L.B. (Luria-Bertani) medium

reagents: bacto-tryptone (10 g)
 yeast extract (5 g)
 NaCl (10 g)

The above reagents were dissolved in 800 **ml** of distilled water and the pH was adjusted to 7.5 with a 10 mol/L NaOH solution. The volume was adjusted to 1 L and the medium was sterilised by autoclaving. For preparation of the L.B. plates, 1.5% [w/v] bacto agar was added to L.B. medium prior to autoclaving. For plates and media containing ampicillin, this was added as a stock solution (500 mg/ml of ampicillin in sterile water) to a final concentration of 50 $\mu\text{g/ml}$, after the autoclaved solution had cooled to 50°C. Agar plates were poured in 85 mm Petri dishes (30 **ml** per plate).

2. SOB medium

reagents: bacto-tryptone (**20** g)
 yeast extract (5 g)
 NaCl (**4 ml** of a 2.5 mol/L stock solution)
 KCl (2.5 **ml** of a 1 mol/L stock solution)

The above reagents were dissolved in 1 L of distilled water and the medium was autoclaved. Ten millilitres of a 2 mol/L stock solution of Mg^{2+} (1 mol/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 1 mol/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, filtered using a 0.2 μM filter) were added to make the medium 20 mmol/L in Mg^{2+} . The final pH was **6.8** to 7.0.

3. SOC medium

This medium was made by adding 20 mmol/L glucose to SOB medium.

4. Stock K-MES (0.5 mol/L)

MES (1.95 **g**) was dissolved in **16 ml** of distilled water and the pH was adjusted to 6.3 using a solution of potassium hydroxide. The volume was adjusted to 20 **ml**, and the solution was filtered (0.2 μM filter) and stored at -20°C.

5. TFB (transformation buffer)

Reagents: KCl (7.4 g)

CaCl₂ · H₂O (7.5 g)

K-MES 20 ml of a 0.5 mol/L stock (pH 6.3)

The reagents were dissolved in 1 L of distilled water. The solution was filtered (0.2 µm filter) and stored at 4°C.

Method

The transformation technique of Mandel and Higa (285) was employed using *E. coli* strain HB101, which was kindly provided by Dr K.Docherty, Dept of Medicine, University of Birmingham, U.K., as a fresh streak culture on L.B. agar. Five colonies, 2 to 3 mm in diameter, were picked off the plate using a flame-sterilised tungsten loop, and dispersed in 1 ml of SOB medium using a Vortex mixer. The dispersed cells were inoculated into 50 ml of SOB in an Erlenmeyer flask. The culture was incubated at 37°C on a plate shaker until it was mid-way through the logarithmic phase of growth and contained about 4×10^7 to 9×10^7 cells/ml.

The culture was collected into 50 ml polypropylene tubes and chilled on ice for 30 min. The cells were centrifuged (750 g for 15 min at 4°C), the supernatant was decanted and the tube was inverted briefly on paper towels to remove remaining fluid. The cells were re-suspended in 4 ml of TFB and 200 µl aliquots were pipetted into each of five polypropylene tubes (17 x 100 mm). Plasmid DNA (10 ng in a volume of 20 µl) was added to four of the tubes, and no plasmid DNA was added to a fifth tube which served as a control. The suspensions were mixed and incubated on ice for 30 min. The cells were heat shocked by placing the tubes in a 42°C water bath for 90 s, then chilled immediately by returning the tubes onto crushed ice.

SOC medium (800 µl) was added and the tubes were incubated at 37°C for 60 min with moderate agitation. A 10 µl aliquot of each culture was pipetted into a 100 µl pool of SOB medium on each of four L.B. ampicillin plates, and the plates were incubated overnight at 37°C. The control plate showed no growth. Two discrete colonies were picked from each of the other plates and used for a plasmid "mini-prep".

2.10.3. Plasmid Analysis

2.10.3.1. Plasmid mini-preparation (mini-prep)

Solutions **and** Buffers

1. Lysozyme stock solution

Lysozyme (500 mg) was dissolved in 10 **ml** of distilled water, dispensed into aliquots and stored at **-20°C**.

2. Mini-prep Solution A

Reagents: glucose (225 **μl** of a 20% [w/v] stock solution)
EDTA (100 **μl** of a 0.5 mol/L stock solution; pH 8.0)
Tris HCl (125 **μl** of a 1 mol/L stock solution; pH 8.0)
Lysozyme (400 **μl** of the stock solution)

The solution was made up immediately before use in distilled water to a final volume of 5 ml.

3. Mini-prep solution B

Reagents: potassium acetate (60 **ml** of a **5** mol/L stock solution)
glacial acetic acid (**115 ml**)
distilled water (**28.5 ml**)

The solutions were mixed together. The final pH of the solution was approximately **4.8**.

Method

To confirm that the bacterial colonies contained the plasmid, small-scale plasmid preparations ("mini-preps") were performed using a modification of the alkaline lysis method (394). Five millilitres of L.B. medium (containing 50 **μg/ml** of ampicillin) in a 10 ml polypropylene tube were inoculated ~~with~~ a single bacterial colony, and incubated overnight at 37°C with vigorous shaking. ~~An~~ aliquot (1.5 ml) of the culture was placed in an Eppendorf tube, centrifuged for **1 min** at room temperature in an Eppendorf centrifuge and the supernatant was removed. The remainder of the overnight culture was stored at **4°C**.

The bacterial pellet was re-suspended in 100 **μl** of ice-cold mini-prep solution A using a Vortex mixer, and incubated at room temperature for **5 min**. Two hundred microlitres of a freshly-prepared solution of **0.2** mol/L NaOH containing 1% [w/v] sodium dodecyl sulphate (SDS) were added and the Eppendorf tube was inverted several times. The tube was stored on ice for **5 min** and 150 **μl** of ice cold mini-prep solution B were

added. The Eppendorf tube was held in an inverted position and the contents were mixed for 10 s using a Vortex mixer. The tube was then held on ice for 5 min and centrifuged in an Eppendorf centrifuge for 5 min.

The supernatant was transferred to a fresh Eppendorf tube, an equal volume of phenol/chloroform was added and the solution was mixed briefly using a Vortex mixer. The tube was centrifuged for 2 min and the supernatant was transferred to a fresh tube. Two volumes of absolute ethanol were added. The solution was mixed, allowed to stand at room temperature for 2 min and centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was discarded and the tube was inverted on a paper towel to remove residual fluid. Ethanol (1 ml of a 70% solution) was added, the solution was mixed using a Vortex mixer and centrifuged for 5 min. The supernatant was removed and the pellet was freeze-dried (Speedvac). TE buffer (pH 8.0, 50 µl) containing DNase-free pancreatic RNase (20 µg/ml) was added and the solution was mixed briefly. This solution was then used in a restriction digest reaction in order to confirm the identity of the plasmid.

A 10 µl aliquot of the solution was pipetted into a fresh Eppendorf tube, and 12 µl of restriction buffer and 1 µl of BamH 1 were added. The reaction mixture was incubated at 37°C for 6 h. The restriction digest was then analysed on an agarose mini-gel.

2.10.3.2. Agarose Gel Electrophoresis

Buffers

1 Tris-borate-EDTA (TBE) stock solution (5X).

Reagents: Tris base (54 g)
27.5 g of boric acid
EDTA (20 ml of a 0.5 mol/L solution; pH 8.0)

The salts were dissolved in 500 ml of distilled water. The EDTA solution was added and the volume was adjusted to 1 L with distilled water.

2 *Loading* buffer.

Reagents: SDS solution (500 µl of a 10% [w/v] solution)
bromophenol blue (50 µl of a 10% [w/v] solution)
sucrose (125 g)
EDTA (1 ml of 0.5 mol/L solution; pH 8.0)

Tris HCL (500 μ l of 1 mol/L solution [w/v]; pH 8.0)

The reagents were dissolved in 10 ml of distilled water and 1 ml aliquots were stored at 4°C.

Method

Electrophoresis of the restriction digest was performed using a submerged mini-gel electrophoresis apparatus (B.R.L., Uxbridge, Middlesex, U.K.). Five hundred millilitres of **1X** TBE solution were prepared from the stock TBE solution. Agarose (800 mg) was dissolved in 100 ml of **1X** TBE solution by boiling for 5 min. The solution was allowed to cool to **50°C**, 5 μ l of ethidium bromide solution (10 mg/ml) were added and the solution was mixed by stirring. The solution was poured into the gel-casting tray with a 10-well comb. The gel was cooled at 4°C until set. The comb was removed and the gel was placed in the electrophoresis apparatus. Ethidium bromide solution (**40 μ l**) was added to the remaining TBE solution, which was poured into the electrophoresis apparatus to submerge the gel.

The plasmid digest and Hind III-digested lambda DNA molecular weight markers (4 μ g) were each **mixed** with 6 μ l of loading buffer, and the volumes were made up to **36 ml** with distilled water. The molecular weight markers were heated to **65°C** in a water bath for 15 min. The gel was loaded with the restriction digest samples and the molecular weight markers, and electrophoresis was performed at 50 V, 25 mA, for 6 h. The gel was examined on an ultraviolet transilluminator (U.V.P. Inc., U.K.) to confirm: 1) complete digestion of the sample (an even smear of DNA); and 2) adequate separation of the molecular weight markers (the **560 base-pair** marker had moved approximately 10 cm from the origin).

2.10.3.3. Large Scale Plasmid preparation

Buffers

1. Tris-sucrose solution (TS)

Reagents: Tris HCl (5 ml of a 1 mol/L solution; pH 8.0)
sucrose (**25 g**)

This solution was made up to 100 ml in **distilled** water.

2. Tris-EDTA-Triton solution (TET)

Reagents: Tris HCl (**25 ml** of a 1 mol/L stock solution; pH 8.0)
EDTA (**6.62 μ l** of a 0.5 mol/L stock solution; pH 8.0)

Triton X-100 (200 μ l)

This solution was made up in distilled water to 100 ml.

Method

Aliquots (2 ml) of the culture from which the mini-prep sample was taken, were inoculated into two 20 ml polypropylene tubes containing 10 ml of L.B. medium with 50 μ g/ml of ampicillin. The tubes were incubated overnight with vigorous agitation at 37°C. Aliquots (5 ml) of this overnight culture were inoculated into four 2 L conical flasks containing 400 ml of L.B. medium containing 50 μ g/ml of ampicillin. The flasks were incubated with vigorous agitation overnight at 37°C. The culture fluid was poured into 50 ml polypropylene tubes and centrifuged at 4°C for 20 min at 5000 rpm (GSA rotor, Sorvall centrifuge) to pellet the bacteria. After centrifugation, the pellets were re-suspended in a total volume of 150 ml of cold, sterile TE buffer (10 mmol/L Tris and 1 mmol/L EDTA; pH 8.0) and re-centrifuged at 4°C for 20 min at 5000 rpm.

The pellets were re-suspended in a total volume of 16 ml of TS solution and held on ice for 5 min. The bacterial suspension was divided equally and transferred to two 30 ml polypropylene centrifugation tubes. Lysozyme (100 mg or 2 ml of the stock solution) was added to each tube, the tubes were held on ice for 20 min and the contents were then mixed gently by swirling. Four millilitres of 0.5 mol/L EDTA solution (pH 8.8) were added, the solution was mixed gently and held on ice for 5 min. TET solution (13.3 ml) was added, the tubes were mixed by inversion and held on ice for 15 min. The tubes were centrifuged at 19000 rpm for 60 min at 4°C (SS-34 rotor, Sorvall centrifuge). The supernatants from each tube were carefully transferred with a pasteur pipette into a 50 ml measuring cylinder, and the volume was made up to 31 ml with TE buffer. The two supernatants were transferred to two 100 ml conical flasks, CsCl (31 g) and 3.1 ml of ethidium bromide solution (10 mg/ml stock solution) were added to each flask, and the contents were stirred until the CsCl was dissolved. The contents of each flask were transferred to two 30 ml polypropylene ultracentrifugation tubes (Beckman-RIIC Ltd., High Wycombe, Buckinghamshire, U.K.) and the weights were matched to within 0.1 g. The tubes were centrifuged at 55,000 rpm for 16 h at 20°C (Ti 70 rotor, Beckman ultracentrifuge, Beckman-RIIC Ltd.).

After centrifugation, the tubes were examined under ultraviolet light. Two bands of DNA were observed; the upper band corresponded to linear bacterial DNA and the lower band corresponded to closed circular plasmid DNA. The band of plasmid DNA was removed by puncturing the sides of the tubes at a point

just below the plasmid DNA band, using an 18-gauge needle attached to a 5 ml syringe. The plasmid DNA was then removed by slowly withdrawing the plunger of the syringe, taking care not to contaminate the sample with bacterial DNA. The plasmid DNA samples were then combined.

Ethidium bromide was removed from the sample by extracting twice with water-saturated N-butanol. Dialysis tubing (10 cm length, 2 cm width) was prepared by boiling for 10 **min** in a solution of 2% [w/v] **NaHCO₃** containing 1 mmol/L EDTA. The dialysis tubing was then rinsed in distilled water, boiled for 10 min in 1 mmol/L EDTA and allowed to cool. The DNA solution was dialysed overnight against 2 L of TE buffer (pH 8.0) at room temperature to remove the CsCl. This step was repeated with 2 L of fresh TE (pH 8.0) for a further **4 h**.

RNA was removed from the plasmid DNA by centrifugation through 1 mol/L NaCl (394). The volume of the DNA solution was measured and 0.1 volumes of 3 mol/L sodium acetate (pH 5.2) were added. Two volumes of ethanol were added, the solution was mixed well and allowed to stand at -20°C for 2 h. The DNA was recovered by centrifugation. The ethanol was discarded and the DNA pellet was dried in a vacuum desiccator. The DNA was dissolved in 500 μ l of TE buffer (pH **8.0**), DNase-free RNase was added to a final concentration of 10 μ g/ml, and the solution was incubated for **1 h** at room temperature. A Beckman centrifuge tube (SW 50.1) containing **4 ml** of 1 mol/L NaCl in TE buffer was prepared. The RNase-treated plasmid DNA solution was layered on top of the NaCl solution, and the tube was centrifuged at 40,000 rpm for **6 h** at 20°C in a Beckman SW 50.1 rotor. The supernatant was discarded and the pellet of plasmid DNA was dissolved in **400 μ l** of TE buffer (pH 8.0).

The solution was transferred to an Eppendorf tube and an equal volume of phenol was added. The solution was mixed gently (Vortex mixer) and centrifuged for 5 min at room temperature in an Eppendorf centrifuge. The upper (aqueous) layer was pipetted into a new Eppendorf tube; care was taken to leave any particulate material at the interface. Water-saturated ether (**1 ml**) was added, the solution was mixed gently (Vortex mixer) and the sample was centrifuged at room temperature for **5 min**. The upper (ether) layer was discarded, and the sample was heated to 60°C for 10 **min** then cooled on ice.

The volume of the sample was measured, 1/10 of the volume of 3 mol/L sodium acetate (pH 5.2) was added and the sample was mixed by gently flicking the Eppendorf tube. Twice the new volume of ice-cold ethanol was added and the sample was mixed gently, as described above. The tube was held at -20°C for 60

min then centrifuged **in** an Eppendorf centrifuge at 4°C for 30 min. The supernatant was carefully removed and ice-cold 70% ethanol (500 μ l) was added without disturbing the pellet. The tube was held at -20°C for 15 min, centrifuged for 30 **min** at 4°C and the supernatant was removed without disturbing the pellet. The pellet was freeze-dried (Speedvac) and re-suspended in 400 μ l of TE buffer (pH 8.0). The pellet was allowed to dissolve completely at room temperature and the solution was divided into 10 μ l aliquots and stored at -70°C.

A 5 μ l aliquot of the sample was added to 1 ml of distilled water and mixed thoroughly. The absorbance was read at 260 nm and 280 nm in a spectrophotometer. Multiplication of the absorbance value at 260 nm by 50 gave the **DNA** concentration in μ g/ml, and the ratio of the absorbance at 260 nm to that at **280 nm** provided an estimate of the purity of the **DNA** sample.

To separate the probe insert from the plasmid **DNA**, a restriction digest of 2 μ g of the sample was performed, as described previously. Agarose gel electrophoresis was performed on the restriction digest using a 1% low melting point agarose gel stained with ethidium bromide (Figure 7). The gel was viewed under an ultraviolet transilluminator, and the band corresponding to the probe insert **DNA** was excised with a minimum amount of excess agarose. The yield of insert was estimated and distilled water was added to give a final **DNA** concentration of approximately 5 ng/ μ l. The sample was boiled for 10 **min** to melt the agarose and denature the **DNA**. The sample was cooled to 37°C for 30 **min** and stored in 10 μ l aliquots at -70°C.

2.10.4. Extraction and Purification of RNA

Steady-state concentrations of α_1 -antitrypsin and β -actin messenger **RNA (mRNA)** in human monocytes were measured by the cytoplasmic dot-blot method (489). **During** all procedures where **RNA** was being handled, care was taken **to** minimise **RNase** contamination of the samples. For example:

- 1) glassware was sealed with aluminium foil and baked at 200°C for 6 h prior **to** use;
 - 2) sterile disposable plastic tubes were used and plastic pipette tips were autoclaved;
 - 3) all chemicals were molecular biology grade and solutions were made with sterile **RNase-free** water;
- and
- 4) gloves were worn at all times.

Figure 7

Plasmid Analyses

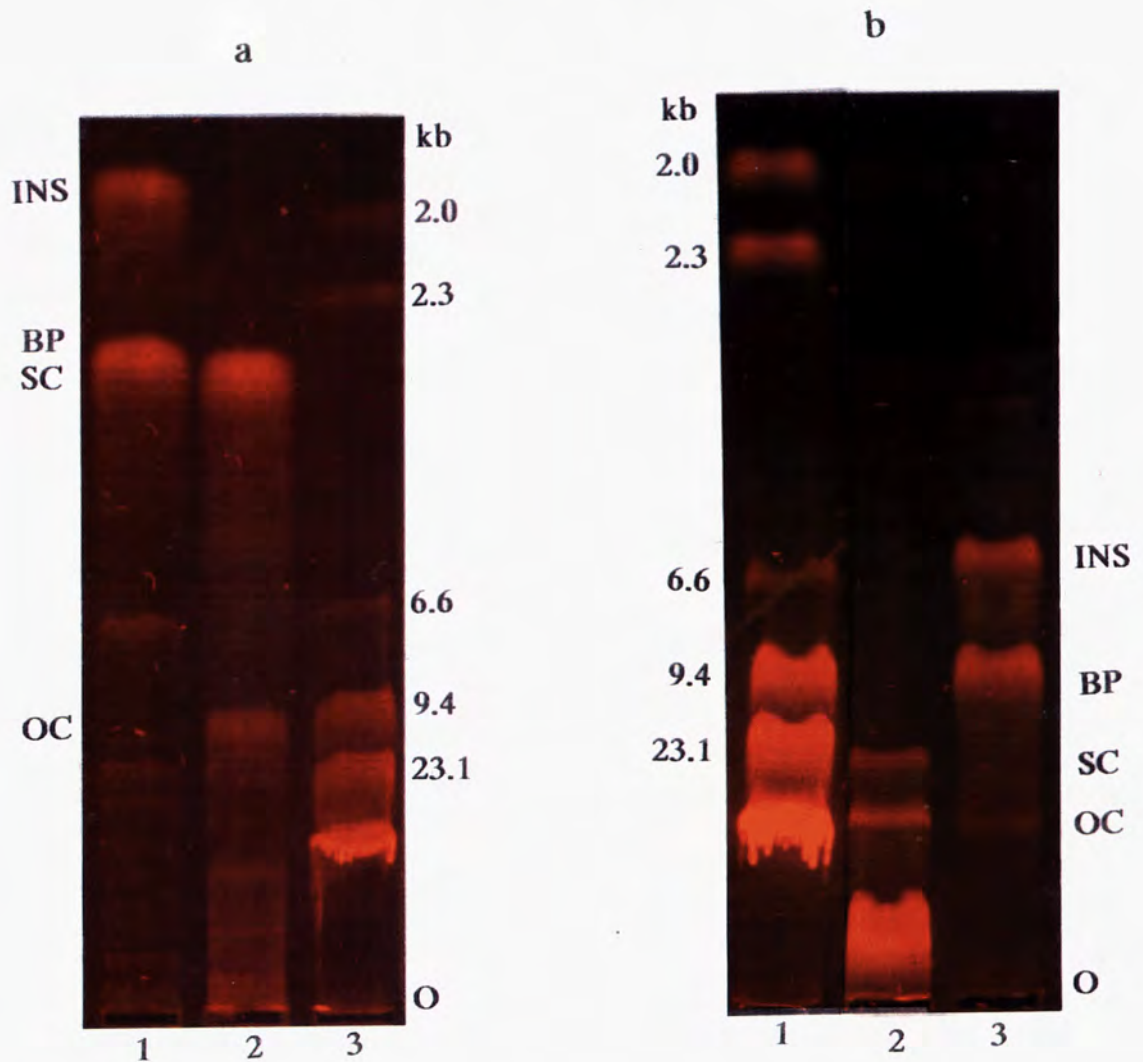


Figure 7 Photomicrographs of agarose gel electrophoresis of BamH 1 restriction digest of the PUC 9B and pAT 9.6 plasmids containing the β -actin and α_1 -antitrypsin DNA probes, respectively. The plasmids were cloned in *E. coli*, purified and subjected to restriction digestion with BamH 1 restriction enzyme. Agarose gel electrophoresis was performed on unrestricted plasmids, the restriction digests and Lambda DNA Hind III digest molecular weight markers (MWM), as a reference. The sizes of the MWM in kb are shown in the figure **a**. **Digest of PUC 9B containing the 1.9 kb β -actin cDNA probe** Lane 1, PUC 9B digest; Lane 2, undigested PUC 9B; and Lane 3, MWM. Note the 1.9 β -actin cDNA insert (INS) and bacterial plasmid (BP) bands in lane 1. **b. Digest of pAT 9.6 containing human α_1 -antitrypsin gene sequences** Lane 1, MWM; Lane 2, undigested pAT 9.6; and Lane 3, pAT 9.6 restriction digest. Note the 6.5 kb α_1 -antitrypsin DNA insert (INS) and bacterial plasmid (BP) bands in Lane 3. SC = supercoiled plasmid DNA; OC = open circular plasmid DNA; and O = origin.

2.10.4.1. Purification of RNA from Human Liver

To obtain a positive control sample for α_1 -antitrypsin mRNA, RNA was extracted and purified from normal human liver using the guanidinium/hot phenol method, as described previously (394).

Reagents *and buffers*

1. 4 mol/L guanidinium isothiocyanate mixture

reagents: guanidinium isothiocyanate (100 g)
 deionised water (100 ml)
 1 mol/L Tris HCl; pH 7.6 (10.6 ml)
 0.2 mol/L EDTA; pH 8.0 (600 μ l)
 20% [w/v] solution of sodium lauryl sarkosinate (21.2 ml)
 β -mercaptoethanol (2.1 ml)

The water and Tris HCl and EDTA solutions were added to 100 g of guanidinium isothiocyanate, and the solution was **stirred** overnight at room temperature. The solution was **stirred** while warmed to 60 to 70°C for 10 **min** to assist dissolution. Residual insoluble material was removed by centrifugation at 3000 g for 10 min at 20°C. The sodium lauryl sarkosinate and β mercaptoethanol were added to the supernatant, and the volume was adjusted to 212 **ml** with sterile water. The solution was stored at 4°C protected from light.

2. STE buffer

Reagents: 0.1 mol/L solution of sodium acetate; pH 5.2
 10 mmol/L Tris HCl; pH 7.4
 1 mmol/L EDTA

3. TSES buffer

Reagents: 0.1 mol/L Tris HCl; pH 7.4
 50 nmol/L NaCl
 10 mmol/L EDTA
 0.2% [w/v] solution of SDS

Method

Normal human liver was supplied by Dr. D.A. Adams, Liver Research Laboratories, Queen Elizabeth Hospital, Birmingham U.K. The 4 mol/L guanidinium mixture (10 ml) was added to 2 g of normal human liver in a plastic disposable centrifuge tube. The liver was homogenized in an omnimixer. The mixture was heated to 60°C in a water bath. While maintaining this temperature, the suspension was repeatedly drawn into, and expressed from a syringe fitted with an 18-gauge needle ~~until~~ the viscosity of the suspension was reduced by shearing of the liberated chromosomal DNA. ~~An equal~~ volume of phenol preheated to 60°C was added and the emulsion was passed through the syringe several times.

STE buffer (0.5 volumes) and an ~~equal~~ volume of chloroform were added. The solution was shaken vigorously while maintaining the temperature at 60°C. The solution was then cooled on ice and centrifuged at 2000 g for 10 ~~min~~ at 4°C. The aqueous phase was recovered and re-extracted with phenol/chloroform. The solution was centrifuged, the aqueous phase was recovered and re-extracted twice with chloroform. Two volumes of ethanol were added and the solution was stored at -20°C for 1 to 2 h. The RNA was recovered by centrifugation at 12,000 g for 20 min at 4°C, and the pellet was dissolved in 10 ml of TSES solution. Protease K was added (200 $\mu\text{g}/\text{ml}$) and the solution was incubated for 1 to 2 h at 37°C.

The solution was heated to 60°C, 0.5 volumes of phenol preheated to 60°C were added and the solution was mixed. Chloroform (0.5 volumes) was added and the solution was mixed vigorously at 60°C for 10 min. The solution was cooled on ice and centrifuged at 200 g for 10 ~~min~~ at 4°C. The solution was extracted once more with phenol/chloroform at 60°C, then extracted twice with chloroform at room temperature. The RNA was precipitated with absolute ethanol by centrifugation and the RNA pellet was rinsed with 70% [v/v] ethanol. The yield of RNA was approximately 5 mg (determined by dissolving the RNA in sterile, distilled water and measuring the absorbance of the solution at 260 ~~nM~~). The liver RNA sample was stored ~~in~~ aliquots at -70°C. For dot-blot analysis, the liver RNA sample was diluted in TE buffer (pH 7.5) and applied to Gene-screen TM membrane, as described in 2.10.6.

2.10.42. Purification of Cytosolic RNA from Monocytes, U937 Cells, Lymphocytes and Neutrophils

Cytosolic RNA was extracted and purified from monocytes, the U937 promonocytic cell line, neutrophils and lymphocytes, as described previously (489).

Buffers

1. Stock SSC (20X)

Reagents: NaCl (175.3 g)
 sodium citrate (88.2 g)

The salts were dissolved in 800 ml of distilled water. The pH was adjusted to 7.0 with dilute HCl and the volume was adjusted to 1 L.

2. Lysing buffer

Reagents: TE buffer; pH 7.5 (100 ml)
 Nonidet P40 (0.5 ml)

The solutions were mixed, and the buffer was filtered (0.2 μ M filter) and autoclaved.

Method

Cells were transferred to Eppendorf tubes (1.8 ml) and washed with 1.5 ml of an RNAase-free solution of 0.15 mol/L NaCl. Two hundred microlitres of TE buffer (pH 8.0) containing 0.5% [v/v] Nonidet P40 and ribonucleoside vanadyl complexes (20 mmol/L final concentration) were added and the cells were held on ice for 5 min. Nonidet P40 (5 μ l of a 5% [v/v] solution in RNAse-free water) was added, and the samples were held on ice for a further 2 min. The tubes were centrifuged in an Eppendorf centrifuge for 5 min to pellet the nuclei.

The supernatants were transferred to fresh Eppendorf tubes, 0.5 volumes of phenol and 0.5 volumes of chloroform were added, and the solution was mixed vigorously using a Vortex mixer. After centrifugation for 5 min in an Eppendorf centrifuge, the supernatants were transferred to fresh tubes and an equal volume of chloroform was added. The solution was mixed gently and centrifuged for a further 5 min. The supernatants were transferred to fresh tubes, and 1/10 of the volume of 3 mol/L sodium acetate (pH 6.0) and 2.5 volumes of absolute ethanol were added. The samples were stored at -70°C overnight to precipitate the RNA.

The samples were again centrifuged in an Eppendorf centrifuge for 20 min and the supernatant was removed. The RNA pellets were re-suspended in 100 μ l of TE (pH 8.0) and allowed to dissolve completely on ice. Sixty microlitres of 20X SSC and 40 μ l of formaldehyde were added and the samples were incubated at 60°C in a water bath for 15 min and then stored at -70°C until dot-blot analysis was performed (2.10.6.)

2.10.5. Radiolabelling of the DNA probes

The α_1 -antitrypsin and β -actin DNA probes were radiolabelled by the oligonucleotide primer method (161) using a commercial kit (Boehringer Mannheim U.K., Lewes, East Sussex, U.K.). For each labelling reaction, a 5 μ l aliquot of insert was removed from the freezer and allowed to warm to room temperature. The DNA was denatured by boiling in a water bath for 10 min and cooled on ice. The following reagents were mixed in an Eppendorf tube on ice:

- 1.5 μ l of denatured DNA
- 2.1 μ l each of dATP, dGTP and dTTP
- 3.2 μ l of reaction mixture
- 4. 5 μ l of (50 μ Ci) α^{32} P-dCTP
- 5.4 μ l of distilled water

Klenow enzyme (1 μ l) was added and the reagents were mixed by flicking the tube. The contents of the tube were brought to the bottom by brief centrifugation and the tube was incubated at 37°C for 30 min. The reaction was stopped by heating to 65°C for 10 min.

The percentage incorporation of α^{32} P-dCTP into the DNA was determined by trichloroacetic acid (TCA) precipitation. One microlitre of the reaction mixture was removed into an Eppendorf tube containing salmon sperm DNA (3 μ l of a 10 mg/ml solution) and 96 μ l of distilled water. The tube contents were mixed thoroughly and a 20 μ l aliquot was placed on a dry glass fibre filter disc (GF/C 2.5 cm, Whatman) which was placed in a scintillation vial containing 5 ml of scintillation fluid (Scintran Cocktail O). A further 20 μ l aliquot was placed in an Eppendorf tube containing salmon sperm DNA (3 μ l of a 10 mg/ml solution) with 500 μ l of distilled water, and the contents of the tube were mixed thoroughly. TCA (150 μ l of a 50% [w/v] solution) was added and the solution was mixed thoroughly. The tube was held on ice for 20 min and the sample was filtered through a 2.5 cm GF/C disc. The disc was flushed through with 50 ml of ice-cold 5% [w/v] TCA solution and 10 ml of ice-cold absolute ethanol. The disc was placed in a scintillation vial with 5 ml of scintillation fluid, and both discs were counted in a liquid scintillation counter (Beckman RIIC Ltd., High Wycombe, Buckinghamshire, U.K.). The percentage incorporation of α^{32} P into the DNA was calculated from the ratio of the counts obtained from the TCA-treated sample to the counts from the non-treated sample. Typical incorporation of α^{32} P into the DNA was approximately 60% (2×10^9 dpm/ μ g).

After successful labelling, non-incorporated deoxyribonucleotide triphosphates were removed by chromatography on a Sephadex column. Sephadex G-50 (10 g) was swollen with distilled water and poured into a **1 ml** syringe that had been plugged with glass wool. The column was equilibrated with STE buffer (10 mmol/L Tris HCl containing 150 mmol/L NaCl and 1 mmol/L EDTA; pH **8.0**), the syringe was placed inside a polypropylene test-tube and centrifuged at 500 g for **5 min**. This procedure was repeated three times, and then **50 µl** of STE were pipetted onto the column which was centrifuged at 500 g for **5 min**. The volume of the eluant was measured and, if it was approximately **50 µl**, the radiolabelled DNA mixture was made up to **50 µl** with STE, pipetted onto the column and the column was centrifuged again for **5 min**. The eluant containing the radiolabelled DNA was stored at -20°C ~~until~~ **used** and the column containing the unincorporated nucleotides was discarded.

2.10.6. Dot-Blot Analysis of Alpha₁-Antitrypsin and Beta-Actin mRNA

2.10.6.1. Method

Analysis of α_1 -antitrypsin and β -actin mRNA in human liver, human peripheral blood monocytes, neutrophils and lymphocytes, and the U937 promonocytic cell line was performed by the dot-blot technique, as described previously (**489**).

Reagents

1. Deionised formamide

Formamide (20 ml) was **mixed** with **1 g** of AG 501-X8 Mixed **Bed** Resin, stirred for **30 min** at room temperature and filtered to remove resin.

2. Denhardt's solution 100X

Reagents: BSA (2 g)
 ficoll (2 g)
 polyvinylpyrrolidone (2 g)

The reagents were dissolved in **100 ml** of distilled water and stored at -20°C in **5 ml** aliquots.

3. Denatured ~~salmon~~ sperm DNA

Salmon sperm DNA (10 mg/ml) was passed repeatedly through an 18-gauge needle to shear the DNA. The solution was boiled for 10 min prior **to** use.

4. Hybridisation buffer

50% deionised formamide, 10% [w/v] dextran sulphate, 10X Denhardt's solution, 0.05 mol/L Tris HCl (pH 7.5), 1.0 mol/L NaCl, 0.1% [w/v] sodium pyrophosphate, 1% [w/v] SDS and denatured salmon sperm DNA (100 μ l/ml).

Method

Gene-screen TM membrane was cut to the **size** of a dot-blot manifold (B.R.L., Bethesda, Maryland, U.S.A.) and soaked in 15X SSC buffer. The membrane was inserted into the manifold which was connected to a vacuum, and 100 μ l of each RNA sample was pipetted into separate wells. The samples were washed through with 100 μ l of 15X SSC. The membrane was removed from the manifold and placed dot side down on an ultraviolet transilluminator for 5 min to fix RNA onto the membrane.

The membrane was placed inside a plastic bag and 10 **ml** of hybridisation buffer were added. The bag was sealed and incubated overnight with constant agitation in a shaking water bath at 42°C. The membrane was probed with the β -actin cDNA probe. The β -actin probe was radiolabelled and denatured by boiling **in** a water bath for 10 **min** and cooled on ice. The probe was added to the bag which was resealed. The bag was incubated with constant agitation overnight at 42°C. The hybridisation fluid was then removed and the membrane was washed in a sandwich box as follows:

- 1) twice in 200 **ml** of 2X SSC at room temperature for 5 **min** with constant agitation;
- 2) twice in 200 **ml** of 2X SSC containing 1% [w/v] SDS at 65°C for **30 min** with constant agitation;
- 3) twice **in** 200 ml of 0.1X SSC at room temperature for 30 min with constant agitation.

The membrane was air-dried, wrapped in Saran wrap, subjected to autoradiography for 3 to 4 days at -70°C. The membrane was then stripped by washing in 200 ml of **0.1%** [w/v] SDS solution at 75°C for **3 h** with constant agitation. Following this, the membrane was exposed to the α_1 -**antitrypsin** probe using the above hybridisation protocol, with the exception that the second washing step was performed at 55°C instead of 65°C. The membrane was subjected to autoradiography for 7 to 10 days at -70°C. The autoradiograms were scanned With a laser densitometer (L.K.B., Milton Keynes, U.K.).

2.10.6.2. Reproducibility and Specificity of the Dot-Blot Technique

To assess the reproducibility of the dot-blot technique, RNA was extracted from 6×10^6 monocytes and

re-suspended in a final volume of 600 μ l of TE buffer. One hundred microlitre aliquots were pipetted into six Eppendorf tubes and the **RNA** was denatured, as described previously. The samples were applied to Gene-screen TM membrane, probed with radiolabelled α_1 -antitrypsin **DNA** probe, subjected to autoradiography and scanned with a laser densitometer (L.K.B., Milton Keynes, U.K.).

To confirm that the α_1 -antitrypsin and B actin **DNA** probes hybridised to **RNA** species rather than to any **DNA** that may have contaminated the samples, **RNA** samples purified from human liver, monocytes, lymphocytes, neutrophils and U937 cells were incubated with and without **RNAse**. **RNA** extracted from each cell type was re-suspended in 200 μ l of TE buffer and 100 μ l were pipetted into two Eppendorf tubes. **DNAse-free RNAse** was added to one tube at a final concentration of 10 μ g/ml, and the tubes were incubated at room temperature for 1 h. The samples were denatured as described previously, applied to Gene-screen TM membrane, probed with the β actin **cDNA** probe and subjected to autoradiography. The blot was stripped, reprobed with the α_1 -antitrypsin genomic probe and subjected to autoradiography.

2.10.6.3. Quantification of α_1 -Antitrypsin and Beta-Actin mRNA using the Dot-Blot Technique

To confirm that there is a correlation between the amount of **RNA** applied to the membrane and the autoradiogram signal, varying amounts of **RNA** were applied to Gene-screen TM membranes. **RNA** was extracted from 4×10^6 monocytes, re-suspended in 400 μ l of TE buffer and denatured as described previously. Serial dilutions of the **RNA** sample were made in TE buffer and **RNA** from the equivalent of 10^4 to 4×10^5 monocytes was then applied to two Gene-screen TM membranes. The blots were probed with the β -actin and α_1 -antitrypsin **DNA** probes, subjected to autoradiography and scanned with a laser densitometer (L.K.B., Milton Keynes, U.K.).

2.10.6.4. Comparison of Steady-State α_1 -Antitrypsin mRNA Levels in Monocyte Subpopulations

Spontaneously adherent and non-spontaneously adherent monocytes were isolated from 11 healthy donors and incubated in supplemented RPMI medium (1 ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After overnight incubation, fresh medium was added and the cells were cultured for a further 48 h under basal conditions. Cells were harvested at 24 h intervals and **RNA** was extracted, as described in 2.10.4.2. Dot-blot analysis of the **RNA** samples was performed using the β -actin **DNA** probe and the blots were

subjected to autoradiography for 3 to 4 days. The blots were then stripped, reprobed with the α_1 -antitrypsin DNA probe and subjected to autoradiography for 7 to 10 days. The autoradiograms were scanned with the laser densitometer (L.K.B., Milton Keynes, U.K.). The densitometry results for α_1 -antitrypsin mRNA were expressed as ratios to the values obtained for β -actin mRNA to correct for any variation in the yield of RNA.

2.10.6.5. Effect of LPS and Cytokines on Steady-State α_1 -Antitrypsin Concentrations in Monocytes

Spontaneously adherent and non-spontaneously adherent monocytes were isolated from eight healthy donors and incubated in supplemented RPMI medium (1 ml) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. After overnight incubation, fresh medium was added and the cells were incubated for a further 24 h with and without TNF (1000 units/ml), IFN (1000 units/ml), GM-CSF (50 units/ml) and LPS from E. coli 0111:B4 (1 µg/ml). The cells were harvested and RNA was extracted as described in 2.10.4.2. Dot-blot analysis of the RNA samples was performed using the β -actin and α_1 -antitrypsin DNA probes, the blots were subjected to autoradiography and the autoradiograms were scanned with the laser densitometer, as described above. The densitometry results for α_1 -antitrypsin mRNA were expressed as ratios to the values obtained for β -actin mRNA to correct for any variation in the yield of RNA.

2.11. Statistical Analysis

All data were expressed as mean \pm SEM or mean \pm SD. Results were compared using the Wilcoxon test for paired samples and the Wilcoxon rank sign test for non-parametric data. P values less than 0.05 were considered significant.

Chapter 3

Results

Aims of the Study

Mononuclear phagocytes play a critical role in the host response to pulmonary infection and inflammation. To participate in these responses, monocytes must be recruited into the lung. It is clear that regulated and reversible adherence of monocytes to extracellular matrix components is a prerequisite for their recruitment into sites of inflammation (172,423). However, the pathological and physiological factors that regulate monocyte adherence and the mechanisms involved have not been fully elucidated.

To begin to address the pathobiological importance of alterations in monocyte adherence to extracellular matrix in inflammatory lung diseases, the adherence properties of monocytes from healthy donors and from patients with an inflammatory lung disease (bronchiectasis) to a representative extracellular matrix component (fibronectin) were compared. In addition, the effects upon monocyte adherence of various signals (LPS and cytokines) that are likely to be released at sites of infection and inflammation were assessed. The role of CD11/CD18 integrins in mediating monocyte adherence to fibronectin was investigated. Finally, the possibility was tested that there is a relationship between the adherence properties of monocytes and phenotypes that relate to important biological or pathological activities at sites of infection and inflammation.

3.1. Optimisation of the Monocyte Isolation Procedure

Preliminary studies were undertaken to optimise the monocyte isolation procedure. Although counter current centrifugal elutriation (CCE) is widely regarded as the gold standard method for monocyte isolation, the equipment that is required for this technique is expensive and not widely available. Therefore, most investigators isolate monocytes by the ficoll-Hypaque density gradient method. The ficoll-Hypaque technique involves centrifugation of blood through ficoll-Hypaque gradient medium to isolate mononuclear cells (59). An adherence step is then performed to separate the monocytes, which are assumed to be adherent cells, from the lymphocytes which are assumed to be the non-adherent cells (4,372).

The ficoll-Hypaque method was used to isolate monocytes for preliminary experiments. The yields of mononuclear cells following the ficoll-Hypaque density gradient step were always close to the expected yields, as determined by total and differential white blood cells counts (approximately 2.5×10^6 mononuclear cells per millilitre of whole blood). However, consistently poor yields of monocytes were obtained following the adherence step. Typically, 10^5 monocytes were isolated per millilitre of whole blood which represented only

about 30% of the potential yield of monocytes. Similar results were obtained when mononuclear cells were isolated by the Percoll density gradient technique (229) instead of the ficoll-Hypaque method. One possible explanation for these findings is that only a proportion of monocytes have the capacity to adhere. The ficoll-Hypaque method, therefore, may select a minor and possibly unrepresentative subpopulation of cells for study. This hypothesis is supported by the findings of Chen et al. (107) that: 1) the yield of adherent monocytes purified by density gradient centrifugation is increased 3-fold by conducting the adherence step in the presence of bacterial endotoxin; and 2) 5 to 25% of non-adherent mononuclear cells are monocytes. To test the hypothesis that the ficoll-Hypaque method recovers only a minor subpopulation of monocytes, the average yield of monocytes isolated from six healthy donors by the conventional ficoll-Hypaque method (with an adherence step) was compared with that isolated by Nycodenz osmotic and density gradient centrifugation, an alternative method that does not involve an adherence step (60).

3.1.1. Comparison of The Ficoll-Hypaque And Nycodenz Isolation Methods

The average yield of mononuclear cells following ficoll-Hypaque density gradient centrifugation was $2.46 (\pm \text{SEM } 0.04) \times 10^6$ cells per millilitre of blood, which represented 89.2% ($\pm \text{SEM } 5.7\%$) of the potential yield of mononuclear cells, as determined by total and differential white blood cell counts. The mononuclear cells were then subjected to an adherence step, detached from the tissue culture dishes, as described in Methods, and counted. The yield of adherent monocytes was $1.32 (\pm \text{SEM } 0.46) \times 10^5$ cells per millilitre of whole blood, which represented only 36.5% ($\pm \text{SEM } 7.3\%$) of the potential yield of monocytes (figure 8). The adherent monocytes were 90 to 95% viable, as assessed by exclusion of trypan blue dye, and greater than 90% pure, as assessed by modified Wright's staining of cytocentrifuge preparations. Among the mononuclear cells that did not adhere during the adherence step, greater than 99% excluded trypan blue dye indicating that they were viable.

The yield of monocytes from the Nycodenz method was substantially greater ($p < 0.025$) than that of the ficoll-Hypaque method. On average, the Nycodenz method recovered $2.37 (\pm \text{SEM } 0\%) \times 10^5$ cells per millilitre of whole blood which represented 69.0% ($\pm \text{SEM } 18.4\%$) of the potential yield of monocytes (figure 8). The Nycodenz-isolated monocytes were greater than 99% viable, as assessed by exclusion of trypan blue dye, and greater than 90% pure, as assessed by modified Wright's staining of cytocentrifuge preparations. Since

Figure 8
Monocyte Yields

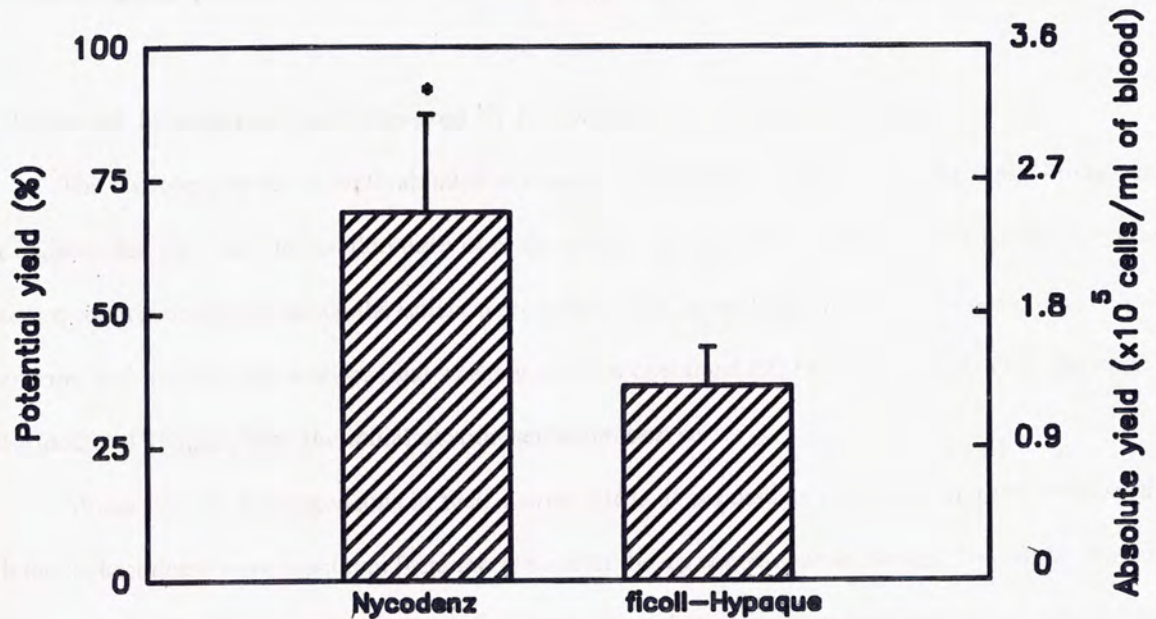


Figure 8 Monocytes were isolated from healthy donors by the ficoll-Hypaque and Nycodenz methods. Cells were counted to determine the absolute yield and the percentage of the potential yield was calculated from the total and differential white blood cell counts. Error bars represent SEM; $n = 6$ donors. Asterisk indicates $p < 0.025$.

Figure 9
Fate of Monocytes not Recovered by Nycodenz.

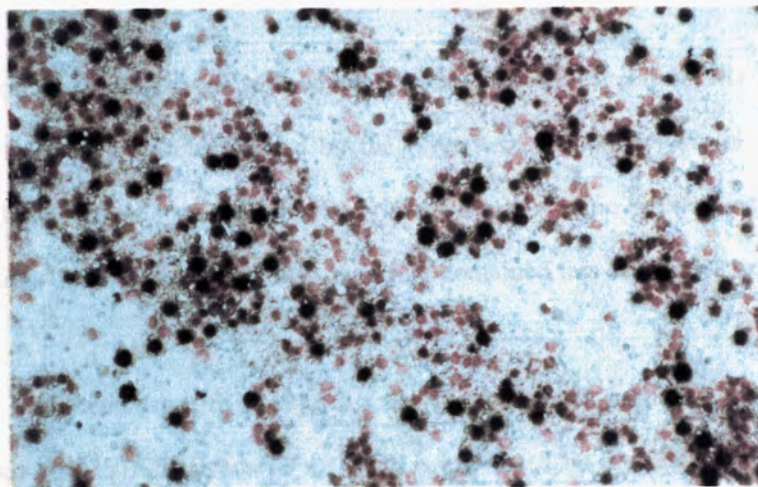


Figure 9 Cells remaining in the pellets following Nycodenz gradient centrifugation were subjected to density gradient centrifugation to separate the mononuclear cells from the erythrocytes and neutrophils. The photomicrograph shows a cytocentrifuge preparation of the mononuclear cells stained for non-specific esterase activity. Note the presence of monocytes (black granulation) in this preparation.

a greater proportion of the potential yield of monocytes was recovered by the Nycodenz method, this method was used to isolate monocytes for subsequent experiments.

3.1.2. Fate of Monocytes Not Recovered by Nycodenz

The Nycodenz isolation method failed to recover about 30% of monocytes in the initial blood samples. It is unlikely that this was due to adherence of monocytes to the isolation vessels since measures were taken to minimise adherence of cells during the isolation procedure. For example, all of the solutions used in the isolation procedure, with the exception of the final washing solution contained EDTA. In addition, all of the steps were performed at 4°C apart from the initial dextran sedimentation step.

Monocytes are heterogeneous in their density (16). It is possible, therefore, that the fraction of cells with the highest density are separated from the remainder during centrifugation through Nycodenz. To test this possibility, the cells remaining in the pellet following the Nycodenz separation were subjected to Percoll density gradient centrifugation to separate the mononuclear cells from the erythrocytes and neutrophils. A cytocentrifuge preparation of these mononuclear cells, stained for non-specific esterase, confirmed that a proportion of monocytes sediment through the Nycodenz gradient during centrifugation (figure 9). Therefore, it is likely that at least some of monocytes that were not recovered by the Nycodenz method were lost because of their physical Characteristics rather than their adherence properties.

3.1.3. Confirmation of the Purity of Monocytes Isolated by the Nycodenz Method

To assess the purity of cells isolated by the Nycodenz method, smears of monocytes were stained routinely with modified Wright's stain (figure 10 a) and for non-specific esterase activity (figure 10 b). The monocyte preparations were greater than ~~92%~~ pure and contained less than 0.5% neutrophils, as determined by these methods.

Smears of monocytes from five subjects were also stained by immunohistochemistry with a murine monoclonal antibody to p150/95 (figure 11 a) which is expressed by the majority of monocytes but not by lymphocytes (205). To determine the percentage of contaminating lymphocytes, duplicate smears were stained with a murine monoclonal antibodies to **CD22** and CD3, which are pan B and pan T lymphocyte markers, respectively (figure 11 b). Non-hmune murine ascites was used as a negative control (figure 11 c). The

percentage of positive cells was determined for each staining procedure. On average, 91% (\pm SEM 1%) of the cells stained for p150/95, 7% (\pm SEM 1%) of the cells stained for the lymphocyte markers, and less than 0.5% of the cells showed non-specific staining with the non-immune murine ascites. These results confirmed that: 1) the monocytes preparations were greater than 90% pure; and 2) the remaining nucleated cells were predominantly lymphocytes.

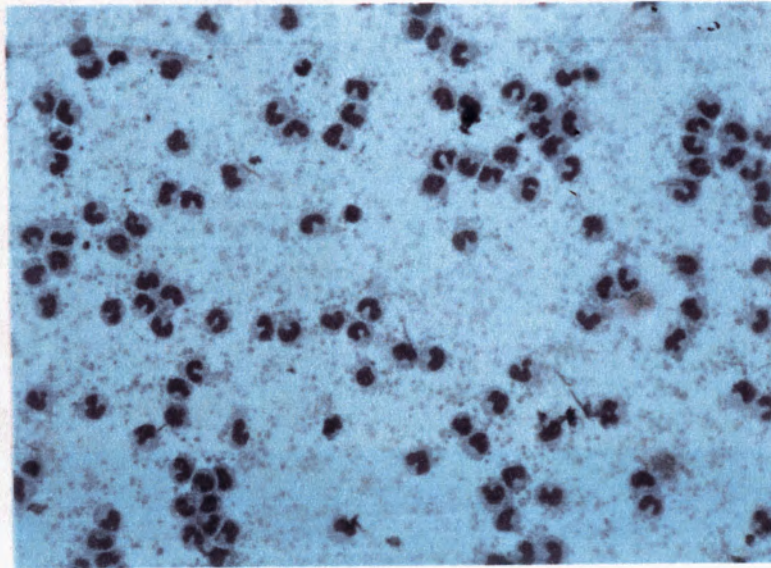
Monocytes isolated by the Nycodenz method were always contaminated with platelets, as illustrated by figures 10 and 11. However, there was considerable donor-to-donor variability in the extent of platelet contamination of the monocyte preparations ranging from about 20 to 200 platelets per monocyte. This platelet contamination could be reduced to 5 to 20 platelets per monocyte by centrifuging the cells through autologous plasma. However, this step was associated with a 25 to 30% reduction in monocyte yield and, therefore, was not performed on a routine basis.

3.1.4. Assessment of Endotoxin Contamination of Nycodenz Reagents and Culture Media

Bacterial lipopolysaccharide (LPS) has been shown to affect many aspects of leukocyte function (188,190,451,468). Since one aim of these studies was to investigate the effects of LPS on monocyte adherence, care was taken to minimise LPS contamination during isolation and subsequent culture of the cells. In addition, all of the solutions used in the Nycodenz isolation and culture of monocytes were assayed for LPS using a commercial limulus amoebocyte lysate kit, as described in Methods. The results confirmed that all of the solutions used in the Nycodenz isolation procedure and the culture media contained endotoxin concentrations that were less than the lower limit of detection of the assay (i.e. less than 10 pg/ml of LPS).

Figure 10
Monocyte Purity
Modified Wright's and Non-specific Esterase Staining

a.



b.

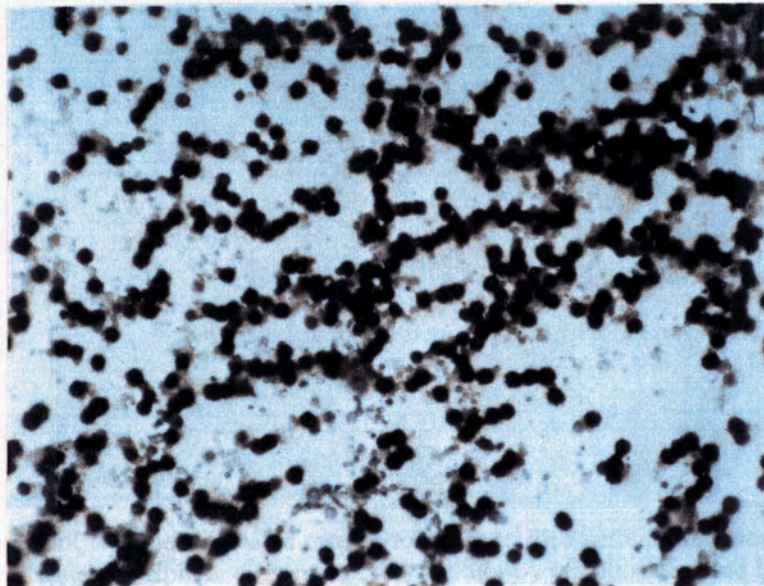


Figure 10 Photomicrographs of cytocentrifuge preparations of monocytes isolated by the Nycodenz method. The cytocentrifuge preparations were stained: a. with modified Wright's stain; and b. for non-specific esterase activity. Note that the monocyte preparations were greater than 92% pure.

Figure 11
Monocyte Purity
Immunohistochemistry

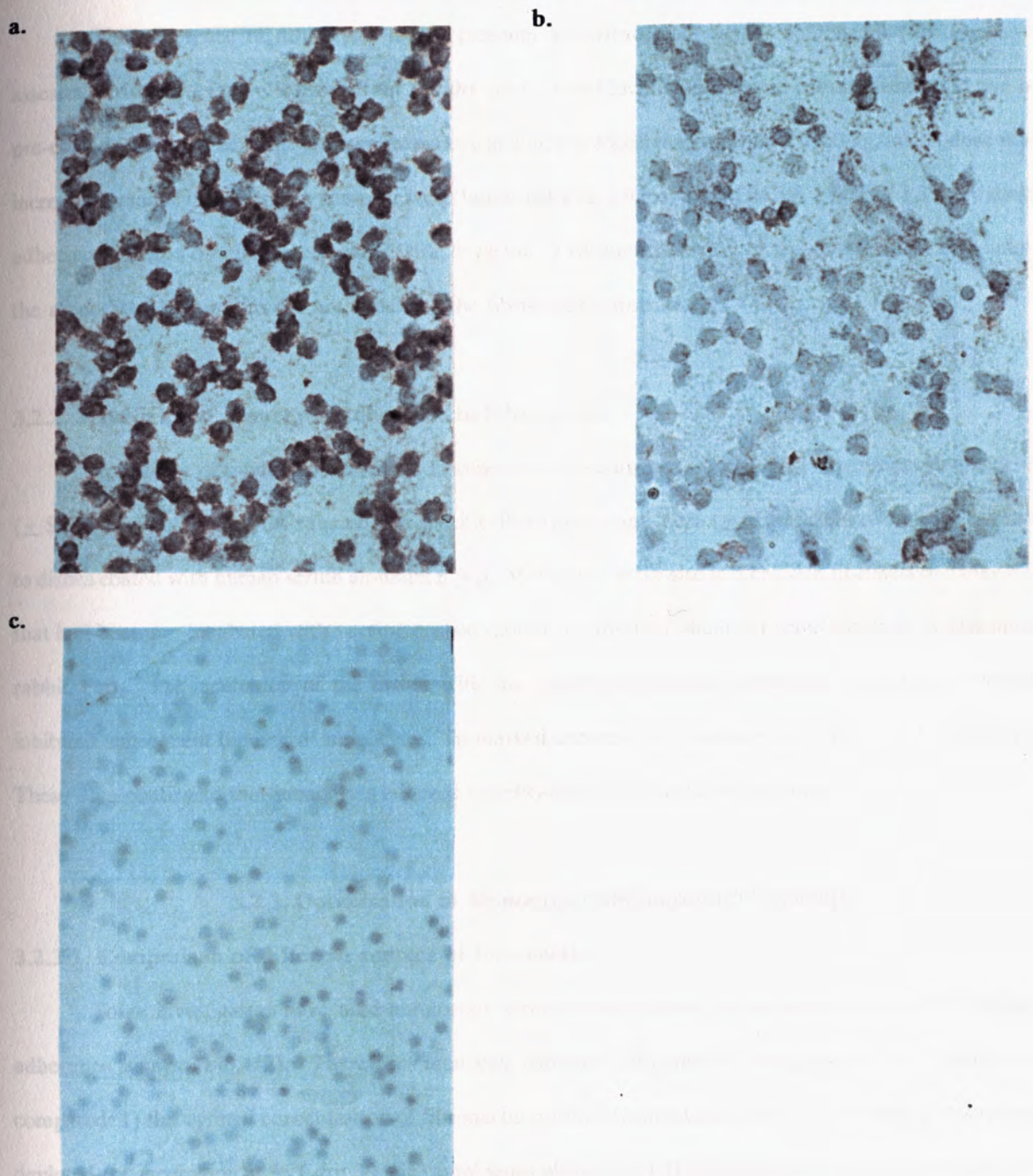


Figure 11 Smears of Nycodenz-isolated monocytes were stained with murine monoclonal antibodies to: **a.** the p150/95 integrin which is expressed by monocytes but not by lymphocytes; and **b.** CD22 and CD3 which are pan B and pan T lymphocyte markers, respectively. **c.** Monocytes were also incubated with non-immune mouse ascites as a negative control. Staining was accomplished by the alkaline phosphatase anti-alkaline phosphatase method. Note that greater than 90% of the cells are monocytes and less than 10% are lymphocytes.

3.2. Monocyte Adherence Assay

3.2.1. Adherence of Monocytes to Fibronectin-Coated Plastic

The adherence of monocytes to a representative extracellular matrix component (fibronectin) was assessed. Monocytes were isolated from healthy subjects and incubated in tissue culture dishes that had been pre-coated with purified human serum fibronectin at concentrations ranging from 0 to 50 $\mu\text{g/ml}$. A dose-related increase in monocyte adherence was observed (figure 12) with a maximum of 24.5% (\pm SEM 1.3%) of the cells adhering to dishes that had been coated with 20 $\mu\text{g/ml}$ of fibronectin. Most of the platelets that contaminated the monocyte preparations did not adhere to the fibronectin Substrate.

3.2.2. Specificity of Monocyte Adherence to Fibronectin

Specificity of monocyte binding to fibronectin was confirmed by a control experiment in which 4.7% (\pm SEM 0.4%) of monocytes adhered non-specifically to plastic and 2.0% (\pm SEM 0.2%) of monocytes adhered to dishes coated with human serum albumin; $n = 3$. Monocytes were also incubated in fibronectin-coated dishes that had been pre-incubated with varying concentrations of rabbit anti-human fibronectin Fab₂ or non-immune rabbit Fab₂. Pre-incubation of the dishes with the rabbit anti-human fibronectin Fab₂ almost completely inhibited subsequent binding of monocytes. in marked contrast, non-immune Fab₂ had no effect (figure 13). These data confirmed that monocytes adhered specifically to fibronectin in the assay.

3.2.3. Optimisation of Monocyte Adherence to Fibronectin

3.2.3.1. Comparison of different sources of fibronectin

Some investigators have used autologous serum or autologous plasma as a source of fibronectin for adherence assays (140,412). Therefore, monocyte adherence to the following sources of fibronectin was compared: 1) the optimal concentration of fibronectin purified from human serum (20 $\mu\text{g/ml}$); 2) complement-depleted (by incubation at 56°C for 30 min) autologous plasma; and 3) complement-depleted autologous serum. A greater proportion of monocytes adhered to dishes coated with fibronectin (22.7% \pm SEM 1.9%) than to dishes coated with complement-depleted autologous plasma (14.2% \pm SEM 1.9%) or serum (12.7% \pm SEM 0.9%); $n = 3$ donors. Therefore, fibronectin purified from human serum was used as the adherence Substrate in all subsequent assays.

Figure 12
Monocyte Adherence To Fibronectin

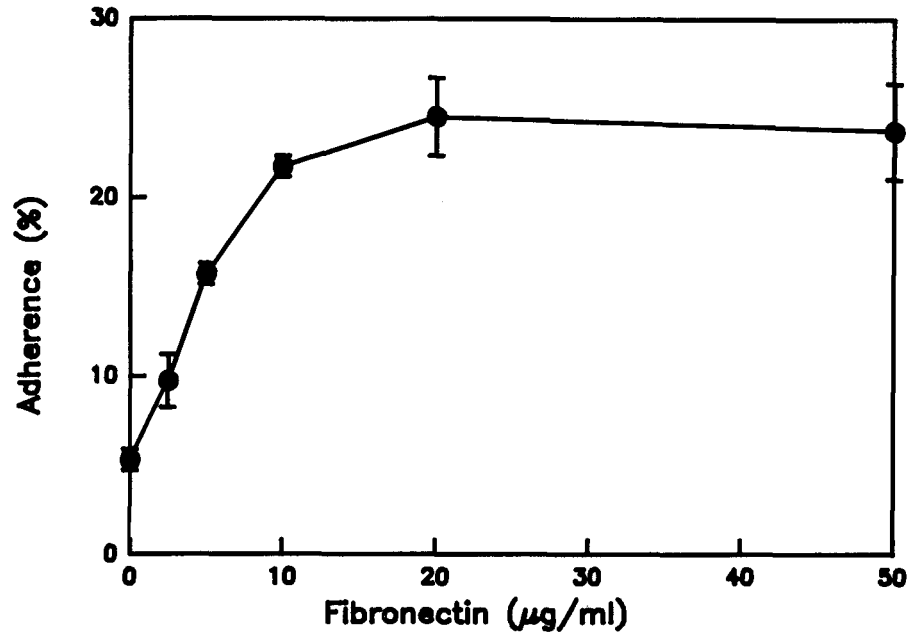


Figure 12 Monocytes were incubated for 1 h at 37°C in dishes that had been coated with varying concentrations of fibronectin and the percentage of adherent cells was determined. Error bars represent SEM; $n = 3$ donors.

Figure 13
Specificity of Monocyte Adherence to Fibronectin

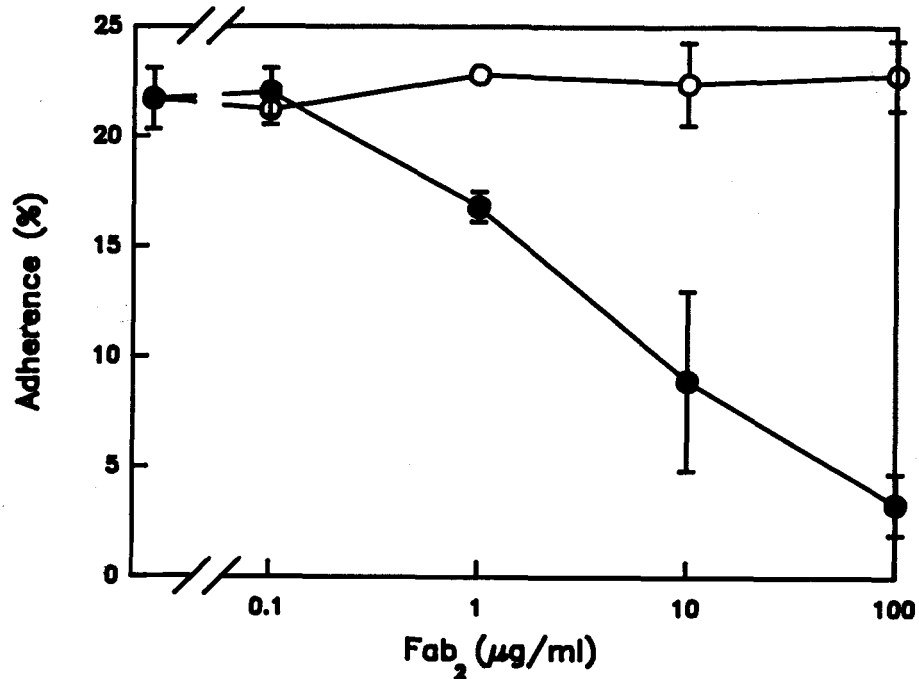


Figure 13 Fibronectin-coated dishes were incubated for 1 h with varying concentrations of rabbit anti-human fibronectin Fab_2 (solid circles) or non-immune rabbit Fab_2 (open circles). Monocytes were incubated for 1 h in the dishes and the percentage of adherent cells was determined. Error bars represent SEM; $n = 3$ donors.

33.3.2. Effect of Different Culture Medium Additives

Although adherence assays are most commonly conducted in the presence of **10%** [v/v] complement-depleted fetal ~~cal~~**f** serum (**FCS**), some investigators use alternative culture medium additives including: **1)** complement-depleted human serum (**35,44,322**); **2)** human serum albumin (HSA), (**54,261,419**); or **3)** concentrations of serum as high as **20** to **25%** [v/v], (**107,219,390,398**). Therefore, the adherence properties of cells suspended medium containing the following additives were compared: **1)** **10%** and **25%** [v/v] complement-depleted **FCS**; **2)** **10%** and **25%** [v/v] complement-depleted autologous serum; and **3)** **0.5%** [w/v] HSA.

A similar proportion of monocytes adhered ~~in~~ the presence of **10%** [v/v] **FCS**, **10%** [v/v] autologous ~~serum~~ and **0.5%** [w/v] HSA (figure **14**). However, increasing the concentration of **FCS** from **10%** [v/v] to **25%** [v/v] resulted ~~in~~ a **30%** reduction ~~in~~ the proportion of adherent cells. A similar reduction ~~in~~ the proportion of adherent monocytes was observed when the concentration of autologous serum was increased from **10%** [v/v] to **25%** [v/v]. Among the monocytes that did not adhere in the presence of **25%** [v/v] serum, greater than **99%** excluded trypan blue dye indicating that they were viable. All subsequent adherence assays were conducted on monocytes suspended in medium supplemented with **10%** [v/v] **FCS**.

3233. Effect of Monocyte Number

The effect of dispensing different numbers of monocytes into fibronectin-coated dishes on monocyte adherence was assessed. Varying numbers of monocytes (**5 x 10⁵** to **3 x 10⁶** cells) were incubated in fibronectin-coated dishes for **1** h and the absolute number of adherent cells was determined. A correlation was observed between the number of monocytes dispensed into the dishes and the number of adherent ~~cells~~, $r = 0.998$ (figure **15**). The percentage of adherent cells was about **20%** for all of the monocyte concentrations tested. Visual assessment of the monocyte preparations was performed by phase-contrast microscopy and confirmed: **1)** the integrity of the underlying monocytes since the cells in all of the dishes adhered in ~~an~~ evenly distributed and non-aggregated pattern; and **2)** that only a small proportion of the platelets that contaminated the monocytes adhered to the fibronectin Substrate. These data indicate that the addition of up to **3 x 10⁶** monocytes to fibronectin-coated dishes does not alter either the proportion of adherent cells or the appearance of the cells that are adherent to the dishes. In subsequent assays **10⁶** to **2 x 10⁶** monocytes were added to fibronectin-coated dishes.

Figure 14
Monocyte Adherence To Fibronectin: Effect of Culture Medium Additives

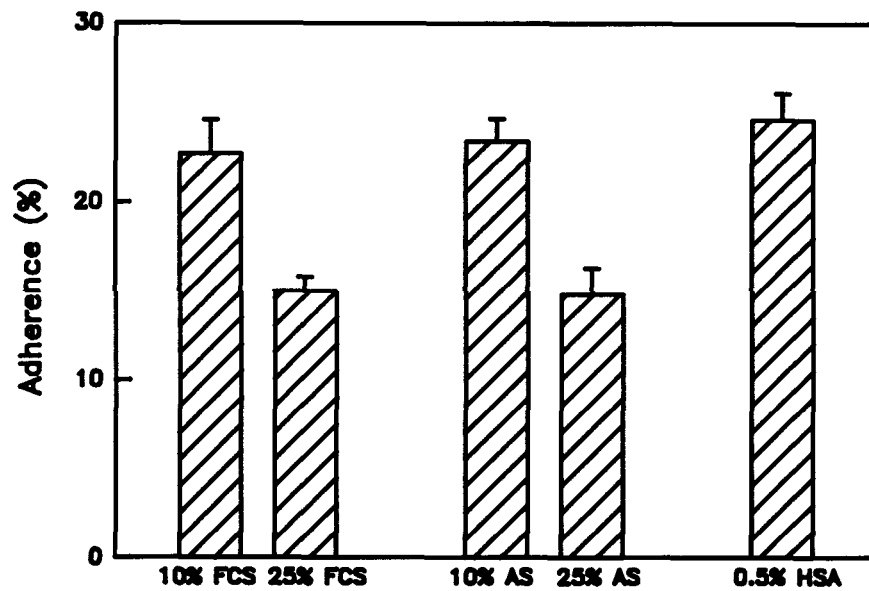


Figure 14 Monocytes were suspended in medium supplemented with: 1) 10% [v/v] FCS; 2) 25% [v/v] FCS; 3) 10% [v/v] autologous serum (AS); 4) 25% [v/v] AS; or 5) 0.5% [w/v] human serum albumin (HSA). Error bars represent SEM; n = 3 donors.

Figure 15
Monocyte Adherence to Fibronectin: Effect of Monocyte Number

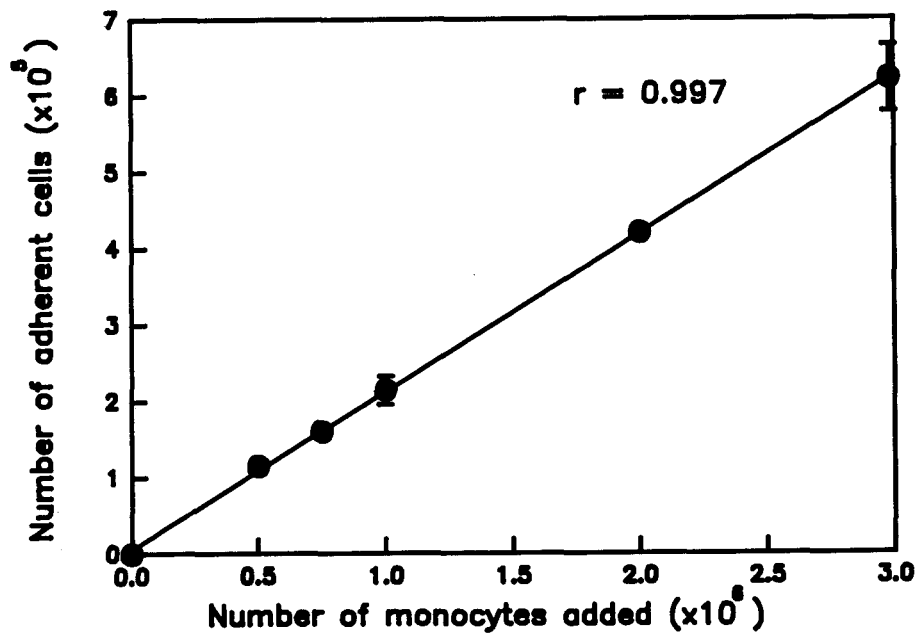


Figure 15 Varying numbers of monocytes were suspended in 1 ml of medium and incubated for 1 h at 37°C in fibronectin-coated dishes. The absolute number of adherent cells was determined. Error bars are SEM; n = 3 donors.

3.2.3.4. Effect of Proteinase Inhibitors on Monocyte Adherence

Monocytes contain proteinases that are capable of degrading fibronectin (81). To exclude the possibility that monocyte-derived proteinases degrade the fibronectin Substrate, and thereby limit cell binding during the adherence assay, monocytes were incubated in fibronectin-coated dishes with and without α_1 -antitrypsin and α_2 -macroglobulin. The proportions of monocytes that adhered to fibronectin in the presence of α_1 -antitrypsin alone ($23.8\% \pm \text{SEM } 1.4\%$) and in the presence of α_1 -antitrypsin and α_2 -macroglobulin together ($24.7\% \pm \text{SEM } 0.6\%$) were similar to the proportion of cells that adhered in the absence of proteinase inhibitors ($21.7\% \pm \text{SEM } 1.9\%$); $n = 3$. These data indicate that any degradation of the fibronectin Substrate that occurs during the assay does not limit monocyte binding to fibronectin.

3.2.3.5. Reproducibility of the Adherence Assay

To assess the reproducibility of the assay, 10^6 monocytes were incubated in five fibronectin-coated dishes for 1 h at 37°C , the proportion of adherent monocytes was determined and the within-batch coefficient of variation (C.V.) was calculated. The within-batch C.V. for the assay was 3.7%. Monocytes were then isolated from the same donor on six different occasions and the proportion of adherent cells was determined. The proportion of adherent monocytes ranged from 17 to 21% and the C.V. for monocyte adherence was 8.4% (donor B, table 5 a). These data indicate that the adherence assay is reproducible.

3.2.4. Comparison of the Adherence Properties of Monocytes Isolated by the Nycodenz Method and by Countercurrent Centrifugal Elutriation

Three potential problems were identified in using the Nycodenz method to isolate monocytes for assessment of their adherence properties. First, Nycodenz-isolated monocytes are always contaminated with platelets and one platelet product (platelet derived growth factor) has been shown increase the adherence of monocytes to endothelial cells (309). Therefore, it is possible that either the contaminating platelets or their products may affect monocyte adherence to fibronectin. Second, it is possible that the adherence properties of the cells recovered by the Nycodenz method are not representative of all monocytes since the Nycodenz procedure fails to recover about 30% of the potential yield of monocytes. Third, the adherence properties of monocytes isolated by the Nycodenz method may reflect a functional alteration in the cells induced by the

Nycodenz isolation procedure. To investigate these possibilities, the adherence properties of monocytes isolated from four healthy donors by the standard Nycodenz procedure were compared with those of cells isolated by: 1) Nycodenz method followed by centrifuging the cells through autologous plasma to remove contaminating platelets; and 2) CCE, the gold standard method for isolating monocytes, which provides almost 100% of the potential yield of monocytes (personal communication; Professor E.J. Campbell, University of Utah, Salt Lake City, Utah, U.S.A.).

The proportion of monocytes that adhered spontaneously to fibronectin was similar for cells isolated by the Nycodenz method and by the Nycodenz method followed by centrifuging the cells through plasma (figure 16). These data indicated that neither the contaminating platelets nor their products affect the adherence of Nycodenz-isolated monocytes to fibronectin. In addition, the adherence properties of monocytes isolated by Nycodenz were similar to those of cells isolated by CCE, indicating that: 1) the Nycodenz isolation procedure ~~per se~~ does not alter the adherence properties of monocytes; and 2) the adherence properties of monocytes recovered by the Nycodenz method are representative of all monocytes.

Figure 16

Monocyte Adherence to Fibronectin
Comparison of Different Monocyte Isolation Methods

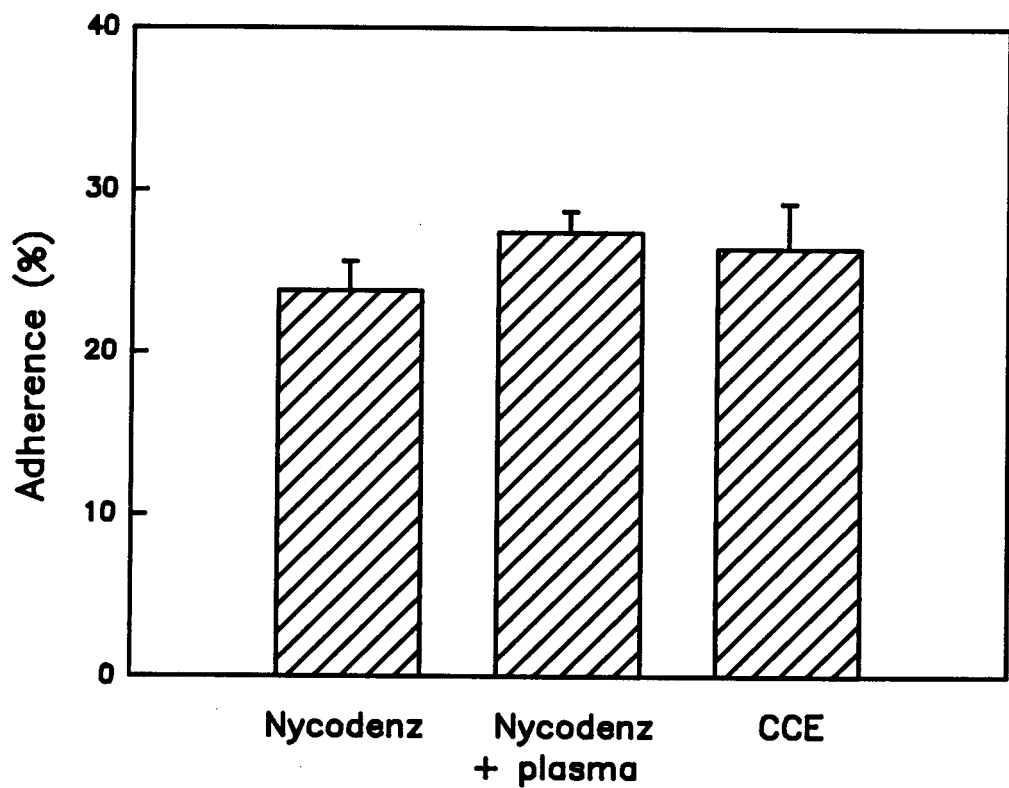


Figure 16 Monocytes were isolated by the standard Nycodenz method (Nycodenz), the Nycodenz method and removal of platelets by centrifuging the cells at low speed through plasma (Nycodenz + plasma) and by countercurrent centrifugal elutriation (CCE). Monocytes were incubated in fibronectin-coated dishes for 1 h at 37°C. Error bars represent SEM; n = 4 donors.

3.3. Adherence Properties of Monocytes from Healthy Subjects

3.3.1. Spontaneous Adherence of Monocytes to Fibronectin

To assess the adherence properties of monocytes from healthy subjects, cells were isolated from six healthy donors on six different occasions and the proportion of monocytes that adhered spontaneously to fibronectin under optimal assay conditions was determined (i.e. 10^6 monocytes suspended in medium supplemented with 10% [v/v] FCS were dispensed into tissue culture dishes that had been pre-coated with **20 $\mu\text{g/ml}$** of fibronectin). Only a minor subpopulation (17 to 28%) of monocytes from these subjects adhered to fibronectin under basal conditions. Moreover, there was minimal day-to-day variability in the mean proportion of spontaneously adherent monocytes from the six donors since this proportion only varied between 20.0% and 22.8% when cells were isolated on six different occasions (table 5 a). There was also minimal within-donor variability in monocyte adherence since the coefficient of variation for monocyte adherence when the cells were isolated from the same donor on different occasions ranged from 6.7% to 10.3% (table 5 a). However, between-donor variability in monocyte adherence to fibronectin was observed since the proportion of adherent cells from two donors (A and F) was consistently greater than that of the other donors.

3.3.2. Comparison of Monocyte Adherence to Fibronectin and to Endothelial Cells

To be recruited from the vasculature into tissues, monocytes must first adhere to endothelial cells. Therefore, to assess whether the capacity of monocytes to adhere to fibronectin is related to their capacity to be recruited into tissues, the adherence of monocytes from healthy subjects to fibronectin and to human umbilical vein endothelial cell (HUVEC) monolayers was compared. Preliminary results, presented in Appendix 3, showed that a similar proportion of monocytes adhered to fibronectin and HUVEC monolayers. These data support the hypothesis that the capacity of monocytes to adhere to fibronectin in vitro is related to their potential to be recruited from the vasculature into tissues in vivo.

Table 5 a**Donor Variability in Monocyte Adherence: Spontaneous Adherence**

	Monocyte Donor								
Occasion #	A	B	C	D	E	F	Mean	S.D.	C.V.
1	28%	18%	20%	22%	23%	26%	22.8%	3.7%	16.3%
2	26%	20%	22%	18%	23%	23%	22.0%	2.7%	12.5%
3	25%	20%	19%	20%	21%	27%	22.0%	3.2%	14.7%
4	25%	18%	22%	23%	23%	24%	22.5%	2.4%	10.8%
5	23%	21%	23%	18%	23%	25%	22.2%	2.4%	10.8%
6	24%	17%	20%	21%	18%	20%	20.0%	2.4%	12.2%
Mean	25.2%	19.0%	21.0%	20.3%	21.8%	24.2%			
S.D.	1.7%	1.6%	1.6%	2.1%	2.5%	2.5%			
C.V.	6.7%	8.4%	7.6%	10.3%	9.2%	10.3%			

Monocytes were isolated from **six** different donors (A to F) on six different occasions (1 to 6). The proportion of cells that adhered spontaneously to fibronectin was determined and the coefficient of variation (C.V.) for monocyte adherence was calculated.

Table 5 b**Donor Variability in Monocyte Adherence: LPS-Stimulated Adherence**

	Monocyte Donor						
Occasion #	A	B	C	D	Mean	S.D.	C.V.
1	55%	60%	52%	61%	57.0%	4.2%	7.4%
2	56%	66%	61%	67%	62.5%	5.1%	8.2%
3	59%	62%	61%	66%	62.0%	2.9%	4.7%
4	63%	62%	62%	66%	63.2%	1.9%	3.0%
Mean	58.2%	63.3%	59.0%	65.0%			
S.D.	3.6%	2.3%	4.7%	2.7%			
C.V.	6.2%	3.6%	8.0%	4.2%			

Monocytes were isolated from four different donors (A to D) on four different occasions (1 to 4). The proportion of cells that adhered in the presence of LPS from *E. coli* 0111:B4 (1µg/ml) was determined and the coefficient of variation (C.V.) for monocyte adherence was calculated.

3.4. Comparison of the Adherence Properties of Monocytes from Healthy Subjects and Patients with Bronchiectasis

To investigate whether the adherence of monocytes to the extracellular matrix is increased in inflammation, the adherence properties of monocytes from healthy subjects to fibronectin were compared with those of cells from patients with an inflammatory lung disease (bronchiectasis). The patients with bronchiectasis were subdivided into three groups according to sputum characteristics (patients producing mucoid, mucopurulent and purulent sputum, respectively) which are related to the severity of airway inflammation in bronchiectasis (200). Under basal conditions, **20%** of monocytes from healthy subjects adhered to fibronectin-coated plastic within **1 h** (figure 17). A greater proportion of monocytes from all three patient groups with bronchiectasis adhered spontaneously to fibronectin compared with monocytes from healthy subjects. In addition, there was a relationship between monocyte adherence and the degree of airway inflammation as assessed by sputum characteristics. The proportion of adherent monocytes was greater in patients producing purulent sputum (**65% \pm SEM 5%**) compared with cells from patients producing mucopurulent sputum (**50% \pm SEM 3%**) which was similar to that of patients producing mucoid sputum (**40% \pm SEM 8%**).

3.5. Plasma Endotoxin Concentrations

The possibility that bacterial endotoxin (**LPS**) is one in vivo mediator of the increased adherence of monocytes obtained from patients with bronchiectasis was tested since the lungs of these patients are colonised frequently with gram negative bacteria which are the source of **LPS**. Moreover, **LPS** has been shown to enhance the accumulation of leukocytes at sites of inflammation in vivo (140,308) and to increase the adherence of monocytes to endothelial cells in vitro (486).

Plasma samples from healthy subjects and patients with bronchiectasis were assayed for endotoxin activity. The plasma endotoxin levels of the healthy subjects (figure 18) were all below the lower limit of detection of the assay (**10 pg/ml**). In marked contrast, endotoxin activity was detected in all of the patient samples. A wide range in plasma concentrations was observed (**25 to 990 pg/ml**) which related to the clinical features of the patient populations. The average concentration in 10 patients who produced mucoid sputum regularly was **76.6 (\pm SEM 10.8) pg/ml**. This was similar to the concentrations in plasma from six patients who produced mucopurulent sputum (**682 \pm SEM 21.8 pg/ml**), but both groups were lower ($p < 0.01$) than the

Figure 17

Adherence of Monocytes from Healthy subjects and Patients With Bronchiectasis

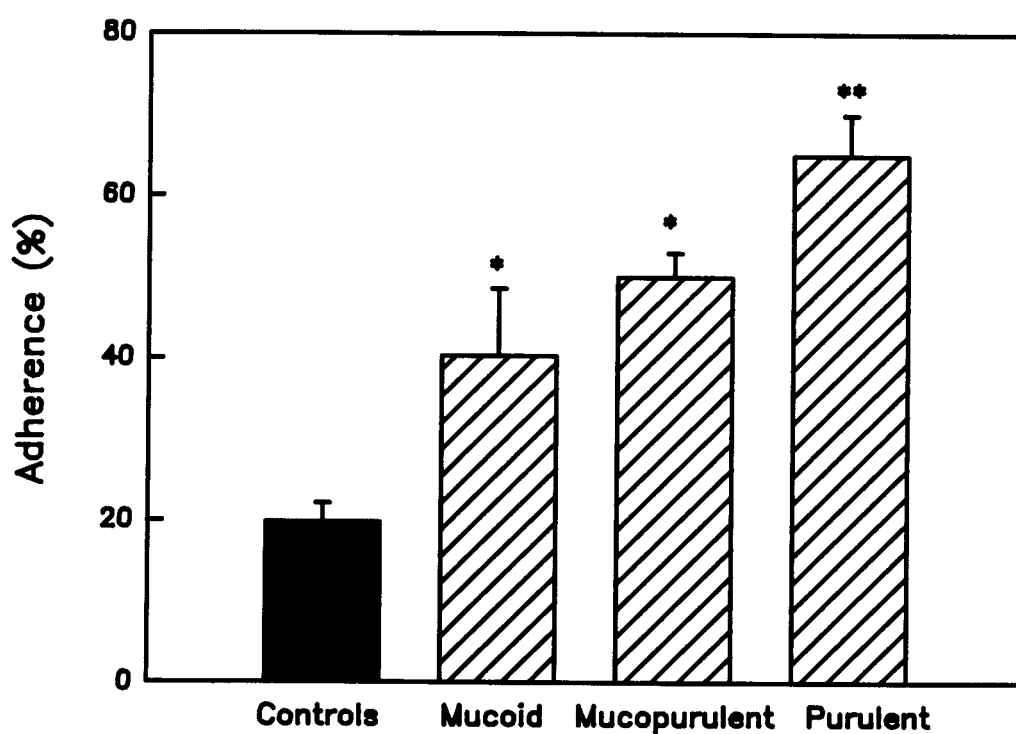


Figure 17 Monocytes from control subjects and patients with bronchiectasis producing mucoïd, mucopurulent and purulent sputum were incubated for 1 h at 37°C in fibronectin-coated dishes. Error bars represent SEM; n = 6 donors for each group. Asterisk indicates $p < 0.025$ compared with cells from healthy subjects; **, $p < 0.025$ compared with patients producing mucoïd and mucopurulent sputum

patients who produced purulent sputum (**714.8 ± SEM 88.4** pg/ml).

To test the relationship between plasma endotoxin activity and monocyte adherence further, both were measured **in** a subset of the control subjects and patients with bronchiectasis (figure 19). A striking correlation **was** observed between plasma endotoxin activity and monocyte adherence when only patient data were considered (plasma endotoxin activity was less than the lower limit of detection of the assay in all control subjects); the correlation coefficient was 0.83.

Figure 18
Plasma Endotoxin Activity: Healthy Subjects and Patients with Bronchiectasis

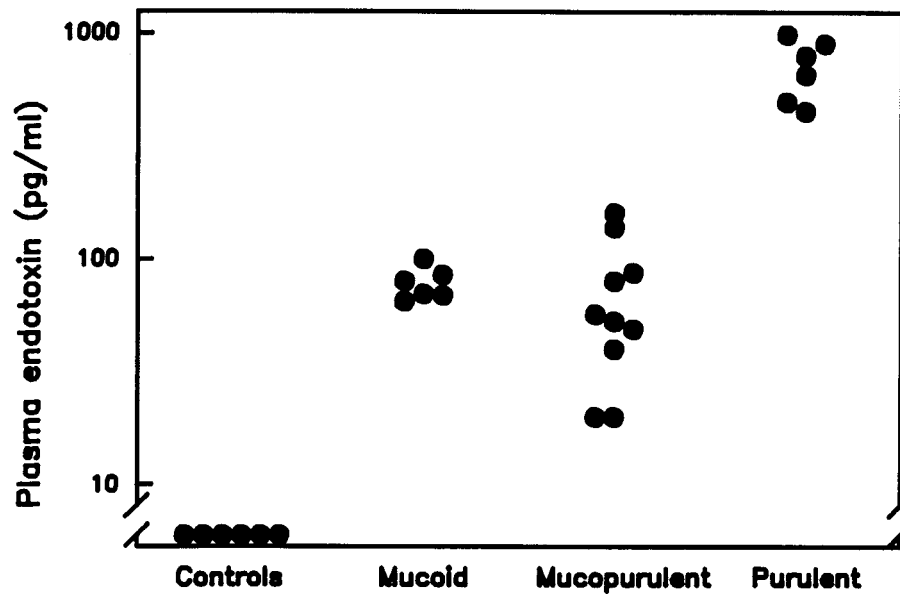


Figure 18 Plasma samples from control subjects and the patient groups detailed in figure 17, were assayed for endotoxin activity using a commercial limulus amoebocyte lysate assay, as described in Methods.

Figure 19
Monocyte Activation in Bronchiectasis: Relationship to Plasma Endotoxin Activity

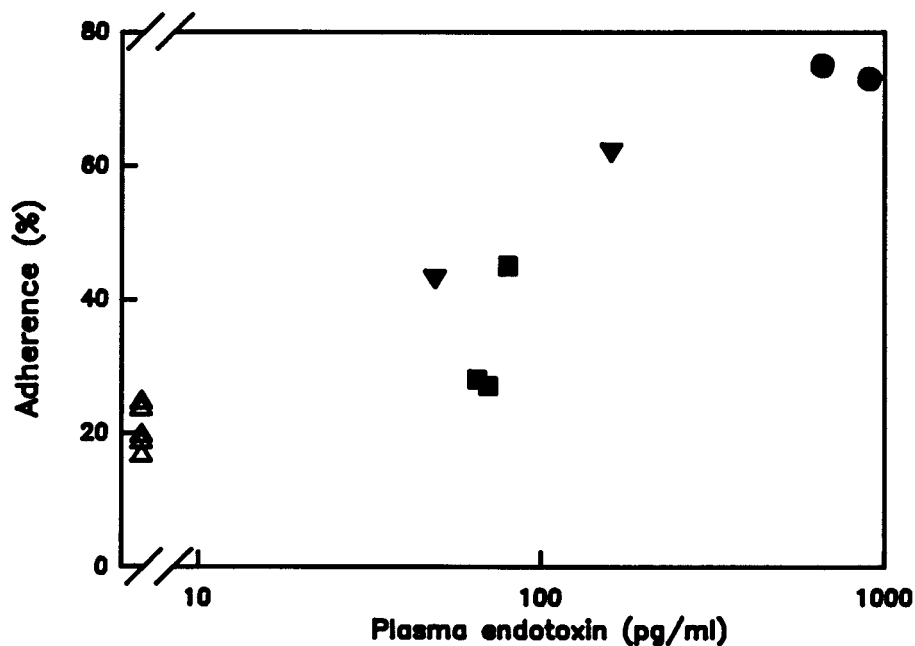


Figure 19 Plasma endotoxin activity and monocyte adherence were measured in patients with bronchiectasis producing mucoid (solid squares), mucopurulent (solid triangles) and purulent sputum (solid circles). The plasma endotoxin activity of five control subjects (open triangles), shown as a reference were all below the lower limit of detection of the assay (10 pg/ml).

3.6. Effect of Pro-Inflammatory Mediators on Monocyte Adherence

3.6.1. Effect of LPS on the Adherence of Monocytes from Healthy Subjects

To explore the potential role of endotoxin in vivo in increasing monocyte adherence in the patients with bronchiectasis, monocytes from healthy subjects were incubated with LPS from *E. coli* 0111:B4 at concentrations ranging from 0.01 to 10 $\mu\text{g/ml}$. Exposure of monocytes from healthy subjects to LPS resulted in a dose-related increase in monocyte adherence at all concentrations tested, with a maximum of 56.4% of monocytes adhering in the presence of 1 $\mu\text{g/ml}$ of LPS (figure 20). This was similar to the proportion of monocytes from patients producing purulent sputum that adhered spontaneously to fibronectin (figure 17). Visual assessment of the monocytes by phase-contrast microscopy confirmed the integrity of the underlying monocytes which adhered in an evenly distributed and non-aggregated pattern.

To compare the effects of LPS purified from different bacterial species on monocyte adherence to fibronectin, cells were incubated with LPS from *E. coli* 0111:B4 and *H. influenzae* at concentrations ranging from 0.10 to 10 $\mu\text{g/ml}$. LPS from both bacterial species produced dose-related increases in monocyte adherence to fibronectin, with a maximum of about 65% of cells adhering in the presence of 1 $\mu\text{g/ml}$ of LPS (figure 21). However, at sub-optimal concentrations of LPS, monocytes were more responsive to the effects of LPS from *H. influenzae* compared with LPS from *E. coli*. For example, similar proportions of monocytes were adherent in the presence of 10 ng/ml of LPS from *H. influenzae* (53.4%) and 100 ng/ml of LPS from *E. coli* (55.4%).

3.6.2. Reproducibility of the Effect of LPS on Monocyte Adherence

The within-batch coefficient of variation (C.V.) for the monocyte adherence assay conducted in the presence of LPS from *E. coli* 0111:B4 was 3.8% ($n = 5$), indicating that LPS-adherence assay is reproducible. To assess donor variability in LPS-stimulated adherence of monocytes to fibronectin, monocytes were isolated from four healthy donors on four different occasions and the proportion of cells that adhered in the presence of 1 $\mu\text{g/ml}$ of LPS from *E. coli* 0111:B4 was determined. There was minimal day-to-day variability in the mean proportion of monocytes from the four donors that adhered in the presence of LPS since this proportion only varied between 57.0% and 63.2% when cells were isolated on different occasions (table 5 b). In addition, there was minimal within-donor variability in the effect of LPS on monocyte adherence since the C.V. for monocyte adherence in the presence of LPS ranged from 3.6 to 8.0% when cells were isolated from the same

Figure 20

Effect of LPS on Monocyte Adherence: Healthy Subjects

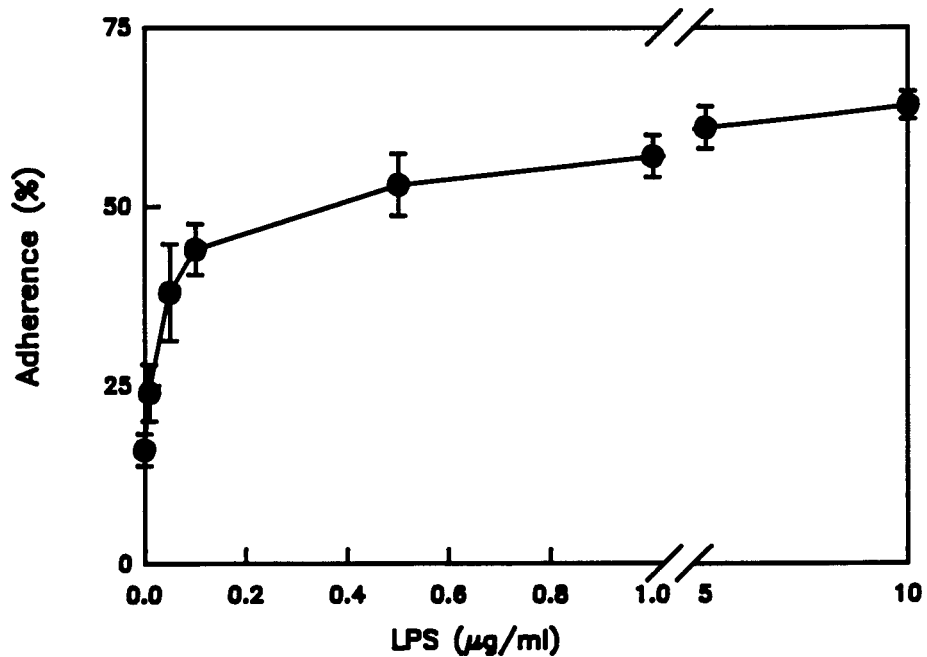


Figure 20 Monocytes from healthy subjects were incubated for 1 h in fibronectin-coated dishes with varying concentrations of LPS from *E. coli* 0111:B4 and the percentage of adherent cells was determined. Error bars represent SEM; n = 6 donors.

Figure 21

Effect of LPS from Different Bacterial Species on Monocyte adherence

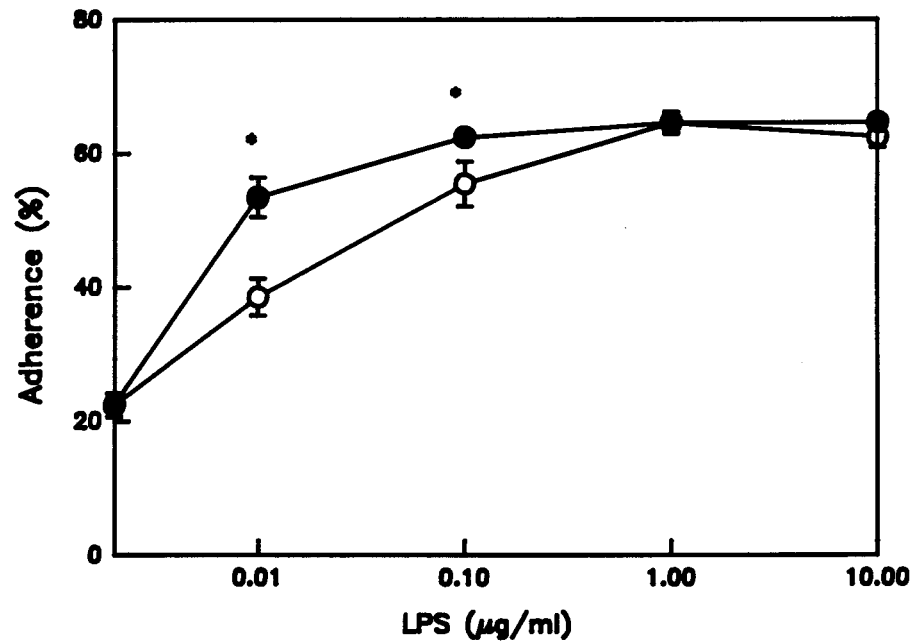


Figure 21 Monocytes from were incubated for 1 h with LPS from *E. coli* 0111:B4 (open circles) and LPS from *H. influenzae* (solid circles) at varying concentrations (0 to 10 µg/ml). Error bars are SEM; n = 5 donors, *, indicates p < 0.05 compared with cells incubated with the same concentration of LPS from *E. coli* 0111:B4

donor **on** different occasions. However, between-donor variability in the responsiveness of monocytes to **LPS** was observed since the proportion of monocytes from one donor (donor D) that adhered in the presence of **LPS** was consistently higher **than** that of the other donors (table 5 b).

3.6.3. Effect of Incubation Time on Monocyte Adherence to Fibronectin

To assess the effect of incubation time **on** monocyte adherence to fibronectin, cells from healthy subjects were incubated with and without **LPS** from *E. coli* 0111:B4 ($1\ \mu\text{g/ml}$) for 30 **min** to 24 h. Both spontaneous and LPS-stimulated adherence were maximal by 30 min and remained at that level for the entire 24 h incubation **period** (figure 22). These results indicate that the standard incubation time used in the current studies (1 h) would result in maximal monocyte adherence for each assay.

3.6.4. Effect of LPS on the Adherence Properties of Monocytes from Patients with Bronchiectasis

Monocytes from patients with bronchiectasis were also incubated with and without **LPS** (0.1 and $1\ \mu\text{g/ml}$). Exposure of monocytes from patients with bronchiectasis to $0.1\ \mu\text{g/ml}$ of **LPS** resulted in an increase in the adherence of monocytes from **all** of the patient groups (figure 23). Moreover, **LPS** at $0.1\ \mu\text{g/ml}$ increased the proportion of adherent monocytes from the mucoid and mucopurulent groups to a level similar to that observed for the purulent patient group under basal conditions. However, in the presence of $1\ \mu\text{g/ml}$ there was a further increase in the proportion of adherent cells from the mucoid patient group only. These data suggest that monocytes from patients with bronchiectasis have already been partially activated by exposure to endotoxin in vivo. However, in the presence of optimal concentrations of **LPS**, a greater proportion **of** monocytes from the mucopurulent and purulent patient groups adhered to fibronectin when compared with that of **LPS-exposed** cells from healthy subjects. The latter observation suggested that other factors may be involved in up-regulating the adherence of monocytes from these patients with intense airway inflammation:

3.6.5. Effect of Recombinant Cytokines on Monocyte Adherence

Cytokines are released from activated host cells at sites of inflammation. Moreover, several cytokines have been detected in plasma from patients with bronchiectasis due to cystic fibrosis (434). **To** test the possibility that cytokines also activate monocytes from patients with bronchiectasis in vivo, resulting in increased

Figure 22

Effect of Incubation Time on Monocyte Adherence to Fibronectin

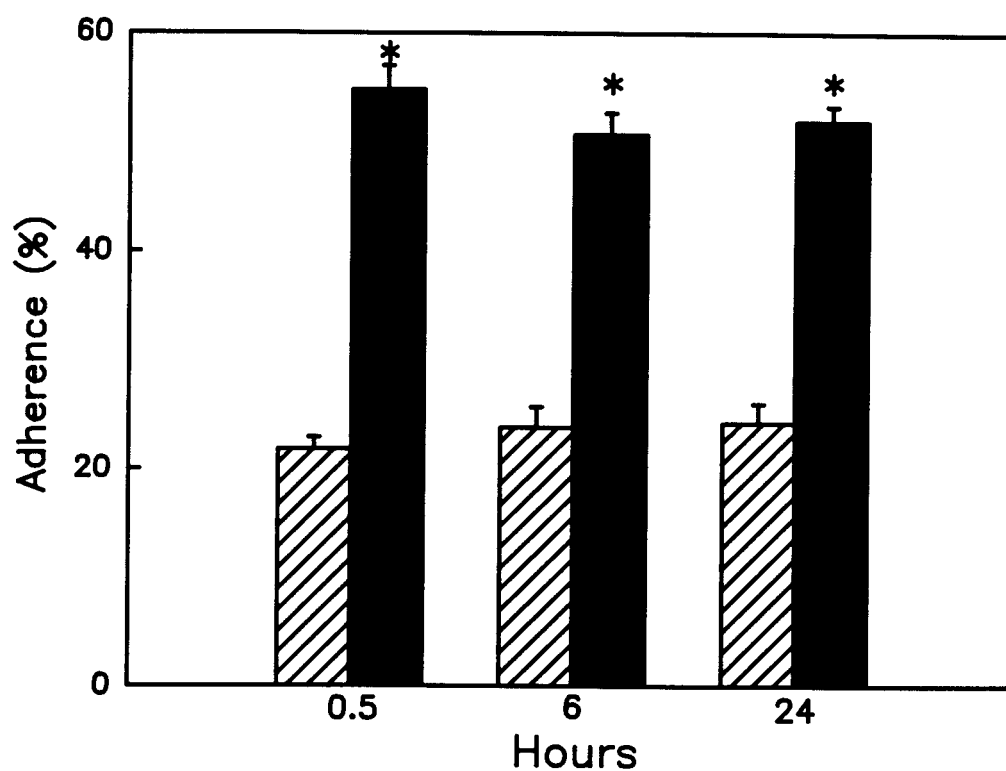


Figure 22 Monocytes were incubated at 37°C for 30 min, 6 h and 24 h in fibronectin-coated dishes. Cells were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS from *E. coli* 0111:B4 (solid bars). Error bars represent SEM; n = 6 donors. Asterisk indicates p < 0.025 compared with unstimulated cells.

Figure 23

Effect of LPS on Monocyte Adherence to Fibronectin
Patients with Bronchiectasis

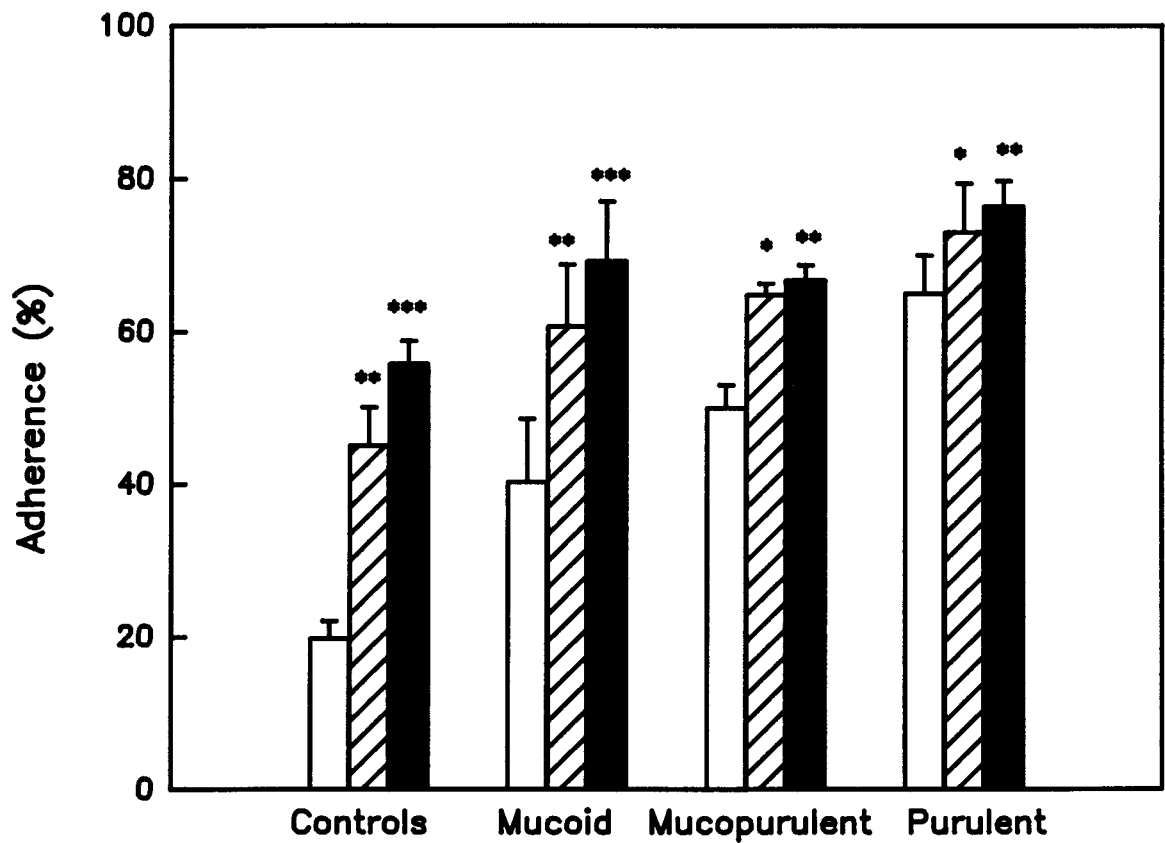


Figure 23 Monocytes from control subjects and the patient groups detailed in figure 17 were incubated for 1h at 37°C in fibronectin-coated dishes. Cells were either unstimulated (open bars), or were stimulated with 0.1 µg/ml or 1 µg/ml of LPS from *E. coli* 0111:B4 (cross-hatched and solid bars, respectively). Error bars represent SEM; n = 6 donors in each group. Asterisk indicates p < 0.05; **, p < 0.025 compared with unstimulated cells; ***, p < 0.025 compared with cells stimulated with 0.1 µg/ml of LPS.

adherence of their monocytes to fibronectin in vitro, cells from healthy subjects were incubated with recombinant cytokines during the adherence assay. In an initial experiment, monocytes were incubated with varying concentrations of TNF (10 to 10,000 units/ml). Exposure to TNF produced a dose-related increase in monocyte adherence to fibronectin with a maximum of 56.0% (\pm SEM 1.2%) of cells adhering in the presence of 1000 units/ml (figure 24).

Monocytes were then incubated with several recombinant cytokines during the adherence assay. Exposure of monocytes to IFN, GM-CSF and TNF increased the proportion of adherent monocytes compared with unstimulated cells (figure 25). In contrast, exposure of monocytes to IL-1 had no effect on monocyte adherence.

3.6.6. Effect of LPS and Recombinant Cytokines on Monocyte Adherence

The effect of LPS (1 μ g/ml) on monocyte adherence to fibronectin was compared with that of two of the cytokines (IFN and TNF). The proportion of monocytes from healthy subjects that adhered to fibronectin in the presence of LPS alone (58.3% \pm SEM 2.0%) was greater than the proportion that adhered in the presence of IFN alone (38.6% \pm SEM 1.3%) or TNF alone (46.8% \pm SEM 1.9%; $n = 6$, figure 26 b).

Monocytes from healthy subjects were also incubated with optimal concentration of LPS and cytokines together during the adherence assay. When the optimal concentration of TNF was added with the optimal concentration of LPS, an additive effect on monocyte adherence was observed (figure 26 a) compared with cells incubated in the presence TNF alone (46.7% \pm SEM 0.9%) or LPS alone (59.8% \pm SEM 1.9%) since LPS and TNF together increased the proportion of adherent monocytes to 70.7% (\pm SEM 1.2%); $n = 6$. However, there was no further increase in the proportion of monocytes when two cytokines (TNF and IFN) were added with the optimal concentration of LPS during the adherence assay (71.6% \pm SEM 2.3%), figure 26 b. Among the cells that failed to adhere in the presence of LPS and cytokines, greater than 99% excluded trypan blue dye indicating that they were viable. These data demonstrated that cytokines and LPS produce additive effects on monocyte adherence to fibronectin. Moreover, the results suggest that: 1) LPS and cytokines up-regulate monocyte adherence to fibronectin by different mechanisms; and 2) TNF and IFN up-regulate monocyte adherence by similar mechanisms.

Figure 24
Effect of Recombinant TNF on Monocyte Adherence

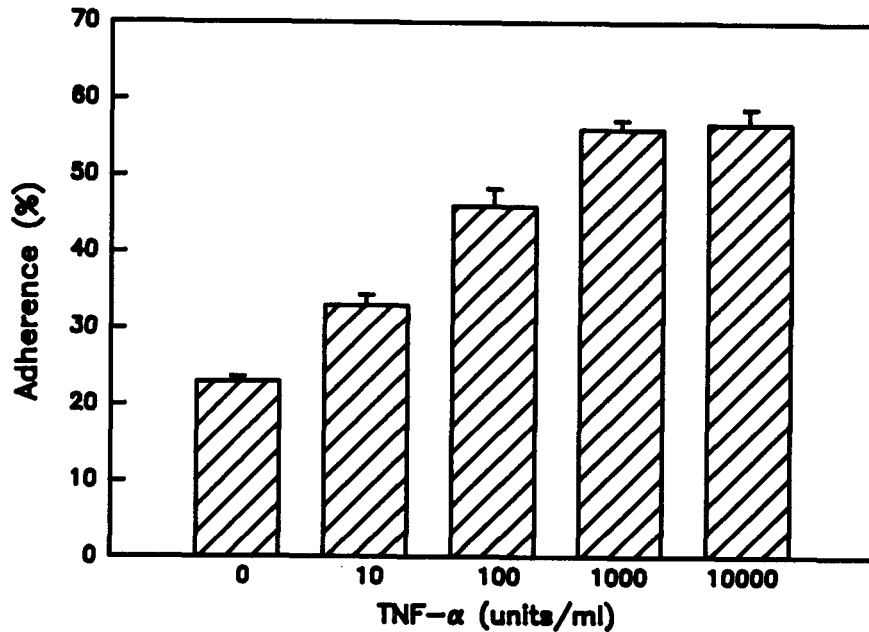


Figure 24 Monocytes were incubated in fibronectin-coated dishes for 1 h in the presence of varying concentrations of TNF and the percentage of adherent cells was determined. Error bars represent SEM; n = 3 donors.

Figure 25
Effect of Recombinant Cytokines on Monocyte Adherence

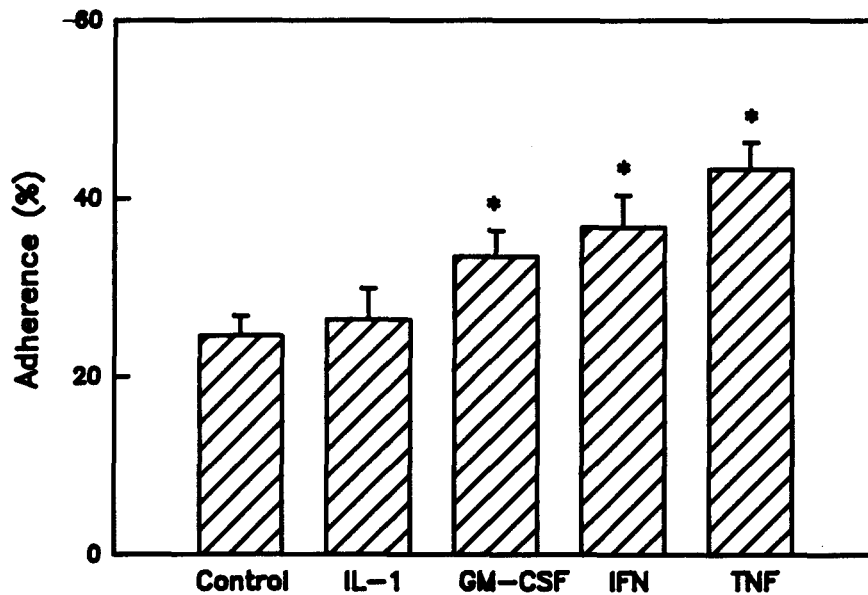
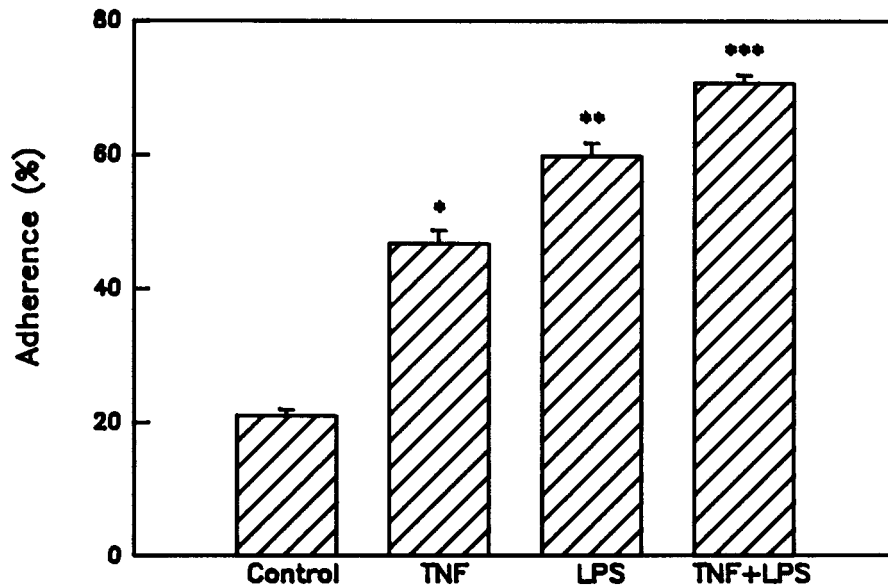


Figure 25 Monocytes were incubated for 1 h in fibronectin-coated dishes. Cells were unstimulated (control) or were stimulated with IL-1 (25 units/ml), GM-CSF (50 units/ml), IFN (1000 units/ml) and TNF (1000 units/ml). Error bars represent SEM; n = 5 donors. Asterisk indicates $p < 0.05$ compared with control cells.

Figure 26
Effect of LPS and Cytokines on Monocyte Adherence

a.



b.

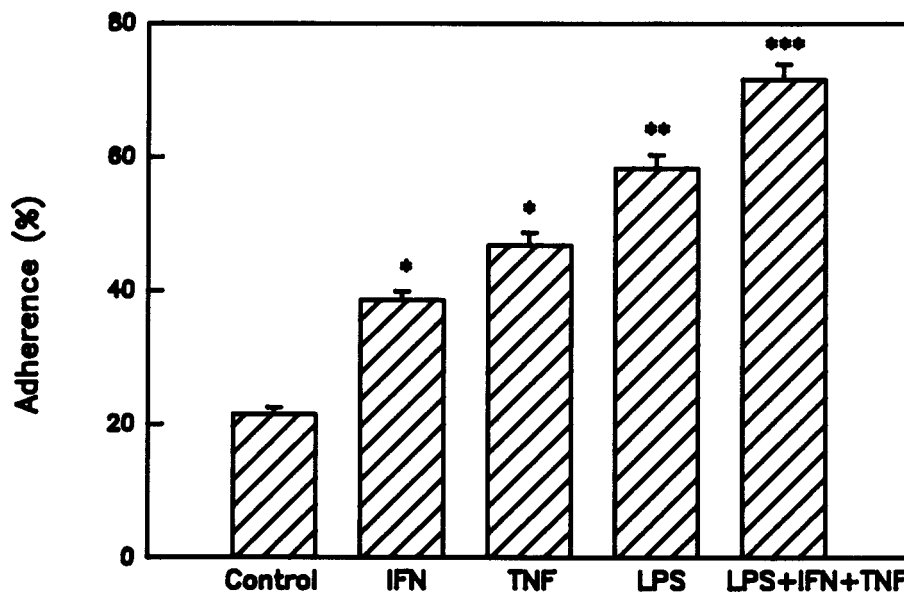


Figure 26 Monocytes were incubated at 37°C for 1 h in fibronectin-coated dishes. Cells were unstimulated (control) or stimulated with cytokines and LPS from *E. coli* 0111:B4. **a.** Cells were stimulated with **TNF** (1000 units/ml), **LPS** (1 µg/ml) or with **TNF** and **LPS** together (**TNF + LPS**); n = 6 donors. Asterisk and ** indicate p < 0.025 compared with unstimulated cells, ***, p < 0.025 compared with cells incubated with TNF alone or LPS alone. **b.** Cells were stimulated with IFN (1000 units/ml), TNF (1000 unit/ml), **LPS** (1 µg/ml) or with the cytokines and LPS together (**IFN + TNF + LPS**); n = 6 donors. Asterisk indicates p < 0.025 compared with unstimulated cells; **, p < 0.025 compared with cells incubated with IFN and TNF, ***, p < 0.025 compared with all other groups. Error bars represent SEM.

3.7. Mechanisms Involved in Monocyte Adherence to Fibronectin

3.7.1. Adherence of Monocytes from Healthy Subjects to Fibronectin

The role of CD11\CD18 integrins in mediating both basal and LPS-stimulated monocyte adherence to fibronectin was investigated since: 1) it had been demonstrated that a specific anti-VLA-5 antibody produces only **40%** inhibition of spontaneous adherence of monocytes to fibronectin (**256**), indicating that other integrins may be involved; and 2) LPS up-regulated monocyte adherence to fibronectin in the current study and LPS has been shown previously to increase the expression of CD11/CD18 integrins *in vitro* (419).

Unstimulated and LPS-stimulated monocytes from healthy subjects were incubated with an anti-CD18 murine monoclonal antibody during the adherence assay. The antibody produced a dose-dependent inhibition of monocyte adherence to fibronectin (figure 27 a). Maximal inhibition of basal and LPS-stimulated adherence was observed at a concentration of **4 µg/ml** of anti-CD18 (57% and **69%** inhibition of basal and LPS-stimulated monocyte adherence, respectively).

To exclude **the** possibility that the anti-CD18 antibody inhibited monocyte adherence by non-specific mechanisms, monocytes were incubated with two murine monoclonal antibodies (**I3** and **MY7**), both of which bind to monocyte surface antigens that are irrelevant **to** the adherence process. Neither antibody inhibited basal or LPS-stimulated adherence of monocytes to fibronectin (figure 27 b). When monocytes were incubated with non-binding antibodies **of** the same immunoglobulin subclass as the CD18 antibody (non-immune murine IgG1), no inhibition of monocyte adherence was observed (figure 27 b).

To test the possibility that recognition of RGD sequences is involved in the adherence of monocytes to fibronectin, monocytes were incubated with a synthetic RGDS tetrapeptide prior to and during the adherence assay. The RGDS peptide produced a dose-dependent inhibition of both basal and LPS-stimulated adherence of monocytes to fibronectin (figure 28 a). Maximal inhibition of both basal and LPS-stimulated adherence was observed at an RGDS concentration of **1 mg/ml** (72% and **67%** inhibition of control adherence, respectively). In contrast, a control tetrapeptide (RGES) did not inhibit either basal or LPS-stimulated adherence of monocytes to fibronectin (figure 28 **b**). These data indicate that recognition of RGD sequences is important **in** mediating both basal and LPS-stimulated adherence of monocytes to fibronectin.

Since some CD11\CD18 adherence events are RGD-dependent (**498**), monocytes were incubated with optimal concentrations of anti-CD18 and RGDS to determine whether an additive effect on inhibition of

Figure 27 a
Effect of Anti-CD18 Antibody on Monocyte Adherence: Healthy Subjects

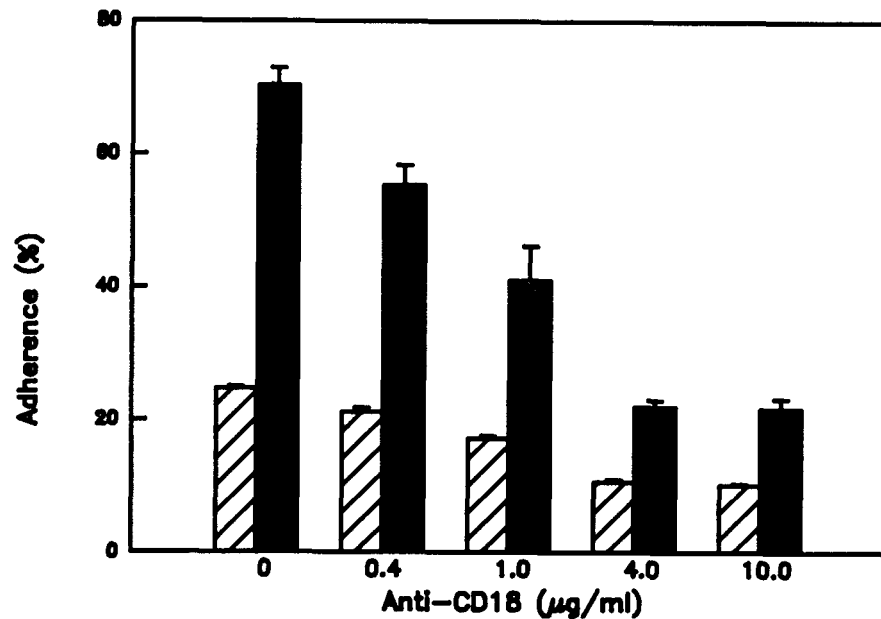


Figure 27 a Monocytes were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS from *E. coli* 0111:B4 (solid bars). Cells were incubated for 1 h with varying concentrations of anti-CD18 in fibronectin-coated dishes and the percentage of adherent cells was determined. Error bars are SEM; n = 3 donors.

Figure 27 b
Specificity of Anti-CD18 Inhibition: Healthy Subjects

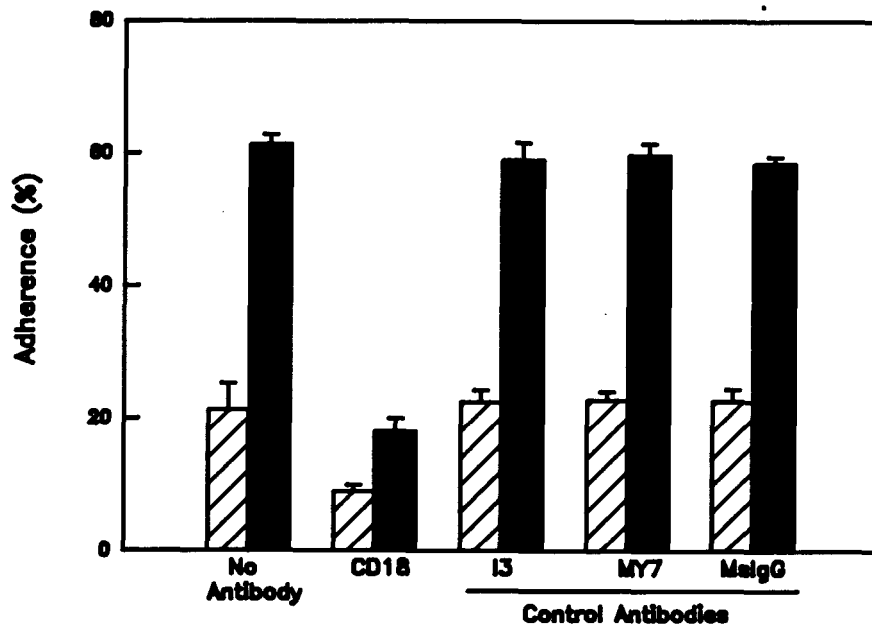


Figure 27 b Monocytes were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS (solid bars). Cells were incubated in fibronectin-coated dishes for 1 h with and without anti-CD18 (4 µg/ml), and the following control antibodies: I3 (40 µg/ml) and MY7 (25 µg/ml), antibodies that bind to the monocyte surface; and non-immune murine IgG1 (MsIgG, 50 µg/ml) as a non-binding control. Error bars are SEM, n = 3 donors.

Figure 28 a
Effect of RGDS Peptide on Monocyte Adherence: Healthy Subjects

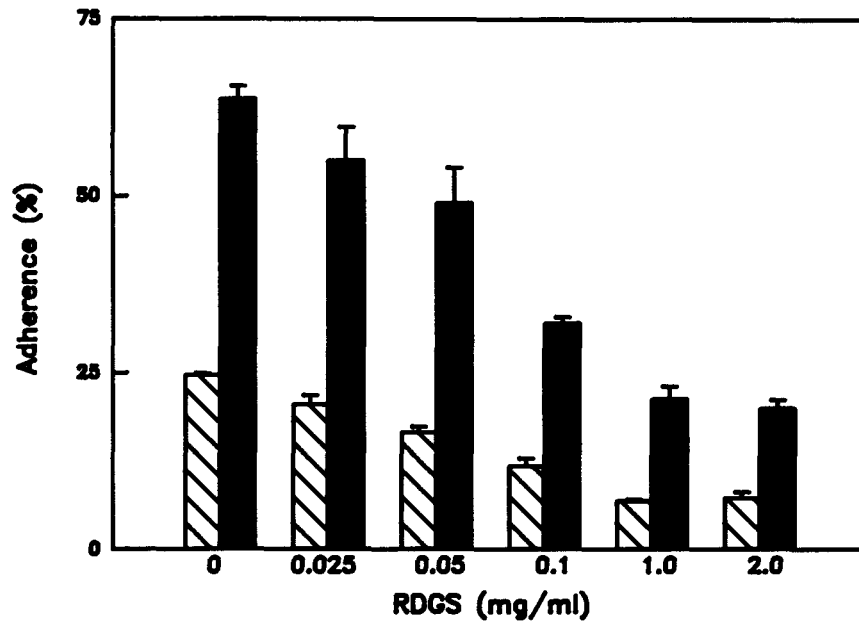


Figure 28 a Cells were unstimulated (cross-hatched bars) or stimulated with $1\text{ }\mu\text{g/ml}$ of LPS from *E. coli* 0111:B4 (solid bars). Monocytes were incubated for 1 h with varying concentrations of RGDS peptide in fibronectin-coated dishes. Error bars represent SEM; $n = 3$ donors.

Figure 28 b
Specificity of Inhibition by RGDS Peptide: Healthy Subjects

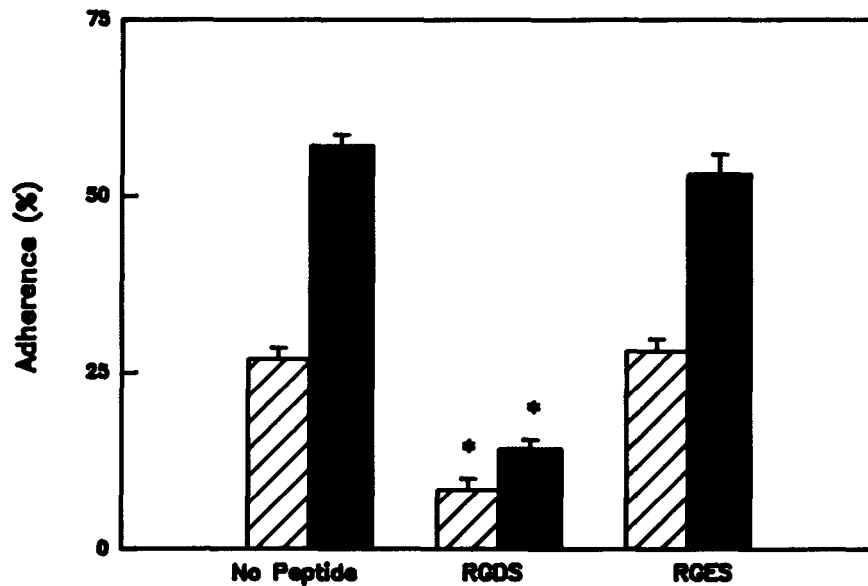


Figure 28 b Monocytes were unstimulated (cross-hatched bars) or stimulated with $1\text{ }\mu\text{g/ml}$ of LPS (solid bars). Cells were incubated with and without RGDS (1 mg/ml) or a control RGES peptide (1 mg/ml). Error bars represent SEM; $n = 6$ donors. Asterisk indicates $p < 0.05$ compared with cells incubated without peptide.

monocyte adherence could be achieved. The antibody and peptide together produced greater inhibition of both basal and LPS-stimulated adherence than either alone (figure 29).

3.7.2. Mechanisms Involved in the Adherence of Monocytes from Patients with Bronchiectasis to Fibronectin

To investigate the mechanisms involved in the adherence of monocytes from patients with bronchiectasis to fibronectin, monocytes from six patients with bronchiectasis (two from each patient group, as defined by their sputum characteristics) were incubated with optimal concentrations of RGDS peptide and the anti-CD18 monoclonal antibody (figure 30 a). On average, 50% of the patients' monocytes adhered spontaneously to fibronectin. The RGDS peptide and the anti-CD18 antibody both produced substantial but incomplete inhibition of monocyte adherence. The RGDS produced greater inhibition (68%) of monocyte adherence than did the anti-CD18 antibody (53%). In addition, the peptide and antibody together produced greater inhibition of monocyte adherence (78%) than either alone. In contrast, the control peptide (RGES) and a control antibody did not affect monocyte adherence (figure 30 b).

Figure 29
Additive Effects of Anti-CD18 and RGDS on Monocyte Adherence
Healthy Subjects

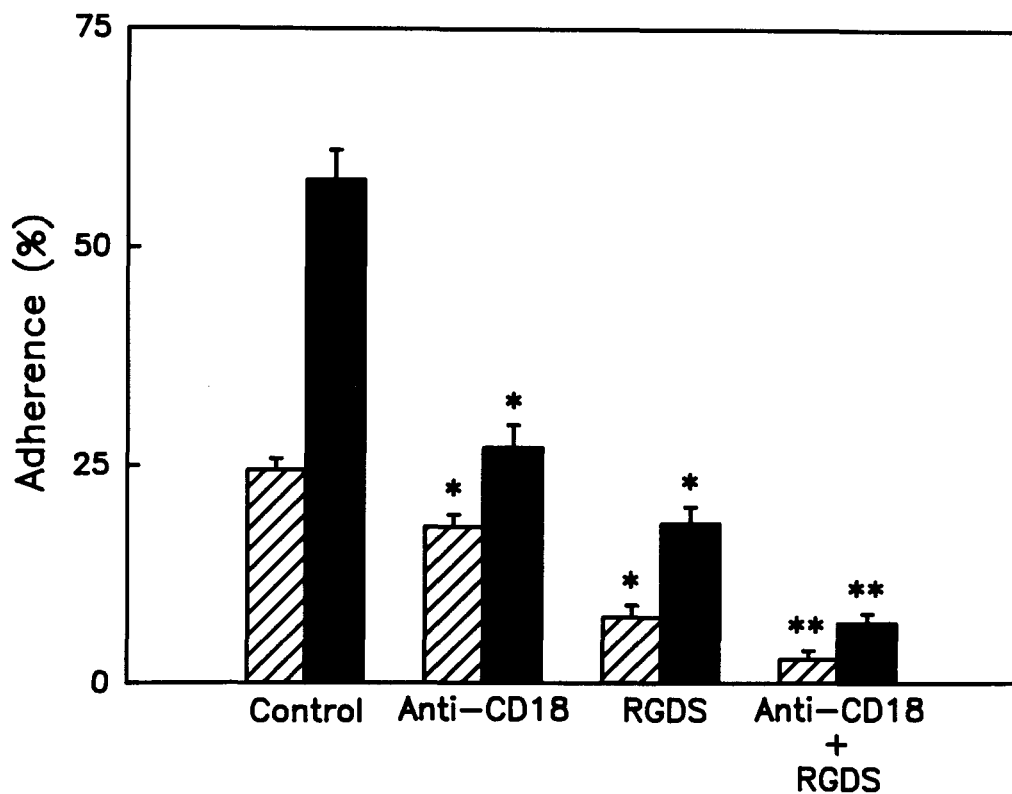


Figure 29 Monocytes were incubated for 1 h at 37°C with and without **anti-CD18** antibody alone (**CD18**; 4 µg/ml), **RGDS** peptide alone (1 mg/ml) or the antibody and peptide together (**CD18 + RGDS**) in fibronectin-coated dishes and the percentage of adherent cells was determined. Cells were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS from *E. coli* 0111:B4 (solid bars). Error bars represent SEM; n = 6 donors. Asterisk indicates p < 0.025 compared with control cells; **, p < 0.025 compared with **CD18** alone and **RGDS** alone.

Figure 30 a
Effect of Anti-CD18 and RGDS on Monocyte Adherence
Patients with Bronchiectasis

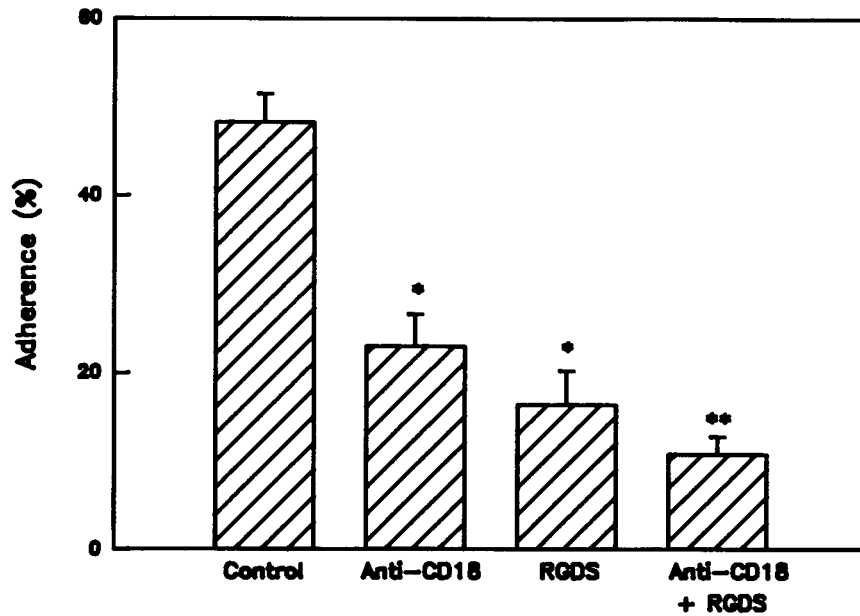


Figure 30 a Monocytes from six patients with bronchiectasis were incubated in fibronectin-coated dishes for 1 h with and without anti-CD18 antibody (4 μ g/ml), RGDS peptide (1 mg/ml) and the antibody and peptide together. Error bars represent SEM. Asterisk indicates $p < 0.025$ compared with control cells; **, $p < 0.025$ compared with anti-CD18 alone and RGDS alone.

Figure 30 b
Specificity of Inhibition by Anti-CD18 and RGDS
Patients with Bronchiectasis

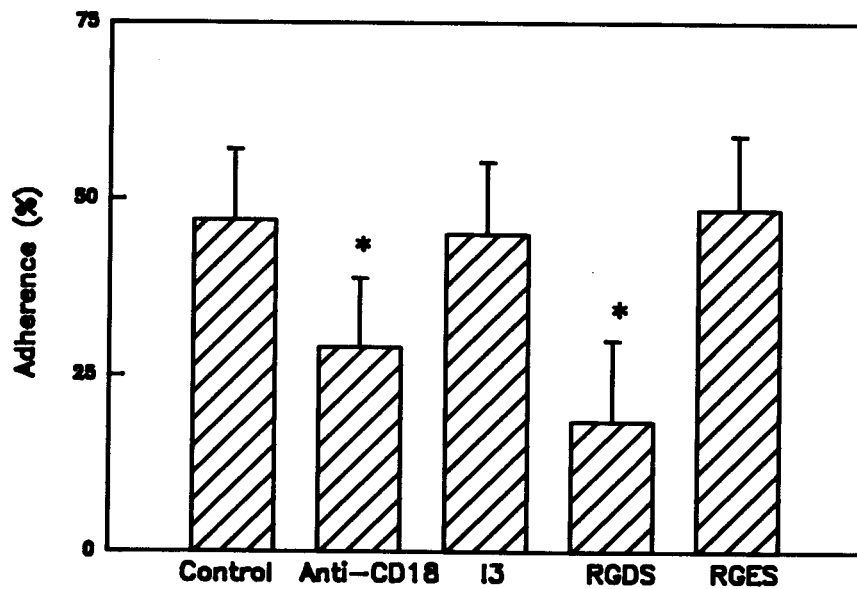


Figure 30 b Monocytes from patients with bronchiectasis were incubated for 1 h in fibronectin-coated dishes with and without anti-CD18 (4 μ g/ml), the control antibody (I3, 40 μ g/ml), RGDS peptide (1 mg/ml) and RGES peptide (1 mg/ml). Error bars represent SEM; $n = 2$ donors.

3.8. Monocyte Phenotype Evaluations

The initial results showed that, on average, only 20 to 25% of monocytes adhered spontaneously to fibronectin and that a further 35 to 40% of the cells could be stimulated by LPS to adhere to fibronectin (figure 2). Therefore, four subpopulations of human monocytes were identified by their differential adherence properties:

- 1) spontaneously adherent monocytes;
- 2) non-spontaneously adherent monocytes;
- 3) LPS-adherent monocytes; and
- 4) non-adherent monocytes.

Further experiments were conducted to test the authenticity of the monocyte subpopulations and to determine whether various functional properties of monocytes are related to their capacity to adhere to fibronectin. Several important antimicrobial functions (phagocytic activity, superoxide anion release and peroxidase content), an immune modulatory function (HLA-DR antigen expression) and the total protein content were compared.

3.8.1. Authenticity of the monocyte subpopulations

To test the authenticity of the monocyte subpopulations, cells were isolated from three healthy subjects and applied to a second identical adherence assay. Non-spontaneously adherent cells were transferred immediately to a second fibronectin-coated culture dish. After incubation for an additional 60 min, 6.9% (\pm SEM 0.6%) of these cells adhered to fibronectin. Spontaneously adherent cells were detached from the plate (by incubating the cells with 5 mmol/L EDTA in a 0.15 mol/L NaCl solution for 45 min at 4°C, followed by gentle scraping with a rubber policeman) then subjected to a second adherence assay. In marked contrast, 85.3% (\pm SEM 1.2%) of these cells adhered to fibronectin. Among the cells that did not adhere in the latter assay, 69.7% (\pm SEM 3.7%) failed to exclude trypan blue dye, suggesting that these cells had been damaged by the detachment process.

The LPS-adherent and non-adherent monocytes were also subjected to a second adherence assay in the presence of 1 µg/ml of LPS from E. coli 0111:B4. Only 2.1% (\pm SEM 15%) of the non-adherent cells adhered to fibronectin in the second assay. In marked contrast, 90.0% (\pm SEM 0.9%) of the LPS-adherent cells adhered

to fibronectin in the second adherence assay. Among cells that did not adhere in the latter assay, 65.3% (\pm SEM 5.2%) failed to exclude trypan blue dye, suggesting that these cells had been damaged by the detachment process. These data confirmed the authenticity of the monocyte subpopulations.

3.8.2. Antimicrobial Functions

3.8.2.1. Phagocytic Activities of the Monocyte Subpopulations

The phagocytic index of the spontaneously adherent monocytes ($197 \pm$ SEM 12.8) was 8-fold greater than that of the non-spontaneously adherent cells (figure 31). In addition, the phagocytic index of the spontaneously adherent cells was 2-fold greater than that of the LPS-adherent cells, which in turn was 7-fold greater than that of the non-adherent cells. These differences were maintained when the results were expressed as the fraction of cells that were positive or as mean number of beads per cell. To control for possible direct effects of LPS on phagocytosis, spontaneously adherent monocytes were also pre-incubated with LPS prior to assessment of their phagocytic activity. Although the phagocytic index of spontaneously adherent monocytes was decreased by pre-incubation with LPS, these cells remained more actively phagocytic than the other groups (figure 31).

3.8.2.2. Superoxide Anion Production

The spontaneously adherent monocytes released $18.3 (\pm$ SEM 4.2) nmol of superoxide anion/ 10^6 cells which was 3-fold greater than that released by the non-spontaneously adherent cells (figure 32). In addition, the spontaneously adherent monocytes released 2.8-fold more superoxide anion than the LPS-adherent cells, which in turn, released an amount of superoxide anion that was similar to that released by the non-adherent cells. To control for possible direct effects of LPS on subsequent superoxide anion release, spontaneously adherent monocytes were also pre-incubated with LPS; this pre-incubation did not alter their superoxide anion release (figure 32).

3.8.2.3. Peroxidase Activity

Spontaneously adherent monocytes contained $182 (\pm$ SEM 4.2) mU/ 10^6 cells of peroxidase activity which was 6-fold greater than that contained in the non-spontaneously adherent cells (figure 33). Furthermore, the

Figure 31
Phagocytic Activity of Monocyte Subpopulations

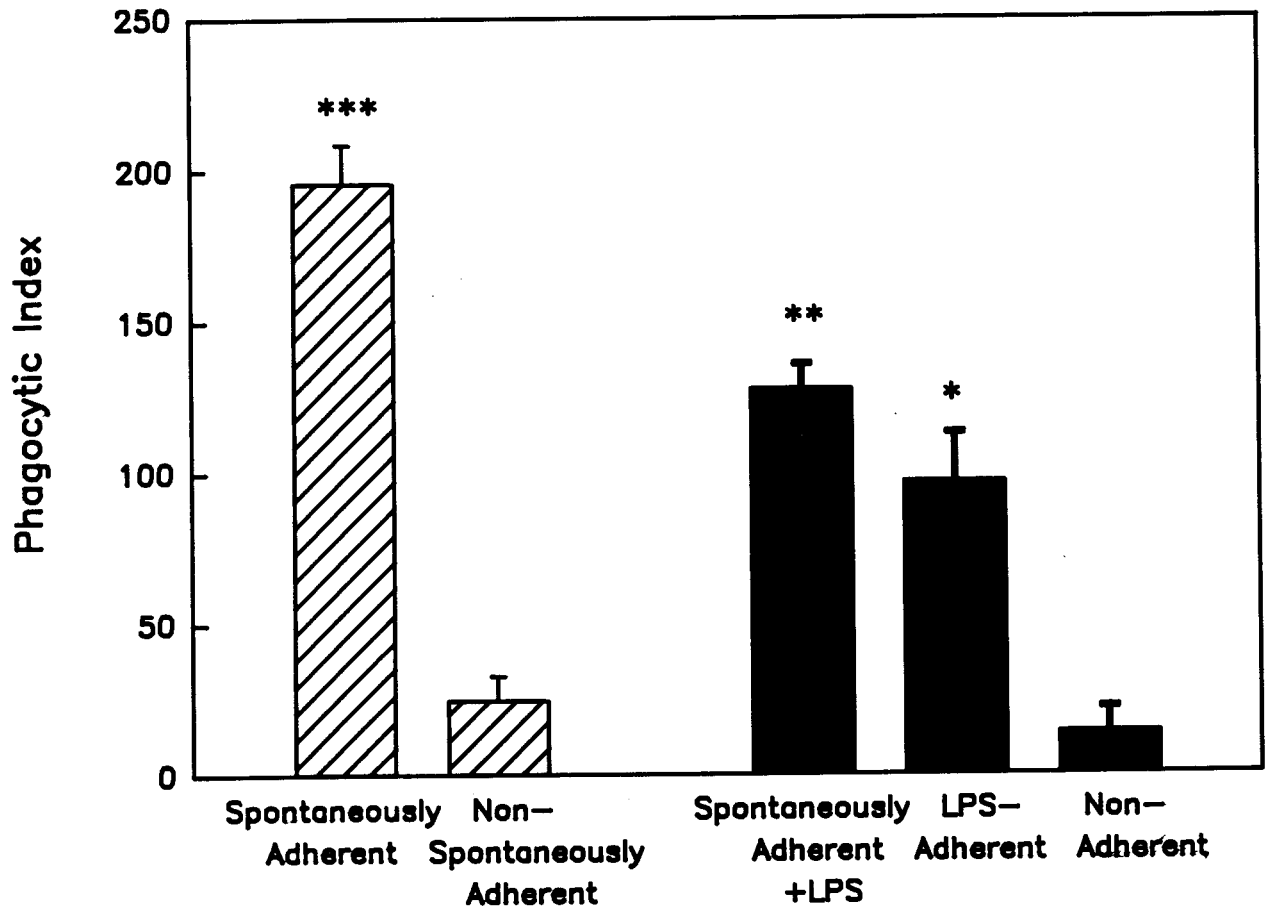


Figure 31 Monocyte subpopulations were incubated with latex beads (3 μ M) for 40 min at 37°C. Phagocytic indices were determined on Wright's stained cytocentrifuge preparations, as described in Methods. Error bars represent SEM; n = 5 donors. Asterisk indicates $p < 0.05$ compared with non-adherent monocytes; **, $p < 0.05$ compared with LPS-adherent cells; ***, $p < 0.05$ compared with non-spontaneously adherent monocytes and LPS-stimulated spontaneously adherent cells.

Figure 32
Superoxide Anion Production by Monocyte Subpopulations

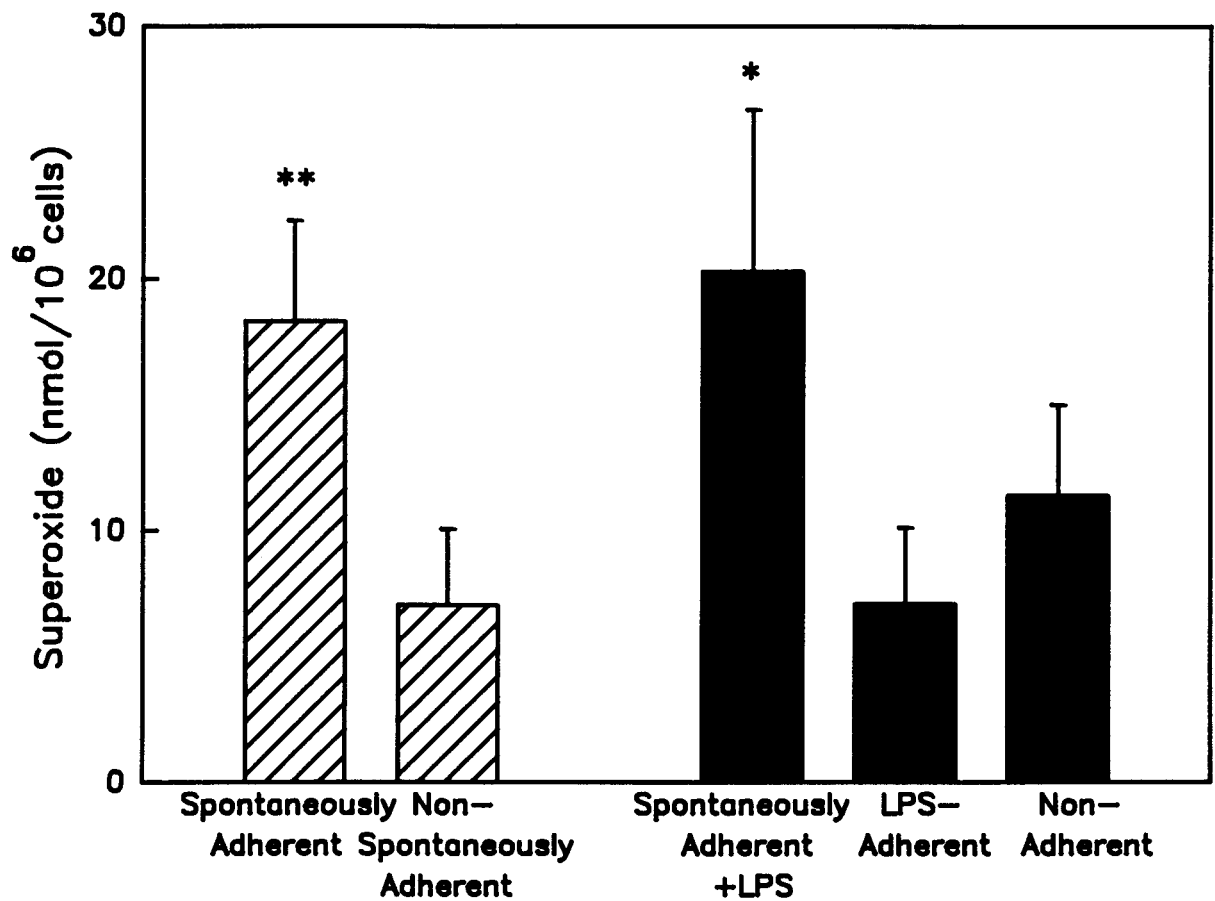


Figure 32 Cells were incubated for 1 h at 37°C and superoxide anion released into the culture media was measured as the superoxide dismutase inhibitable reduction of cytochrome c. Error bars represent SEM; n = 7 donors. Asterisk indicates $p < 0.05$ compared with LPS-adherent and non-adherent monocytes; **, $p < 0.05$ compared with non-spontaneously adherent monocytes.

spontaneously adherent monocytes contained 10-fold more peroxidase activity than the LPS-adherent cells which, in turn, contained 2-fold more peroxidase activity than the non-adherent cells. Pre-incubation of the spontaneously adherent monocytes with LPS did not alter their peroxidase activity (figure 33). Extracts of platelets from two donors were assayed for peroxidase activity since Nycodenz-isolated monocytes were contaminated with platelets. Platelets did not contain peroxidase activity.

To exclude the possibility that the differences in peroxidase content of the monocyte subpopulations could be due to differential release of enzyme into the culture media, all of the culture supernatants were assayed for peroxidase activity. During the spontaneous adherence step, $0.79 (\pm \text{SEM } 0.32) \text{ mU}/10^6$ cells of peroxidase activity were released in 1 h. This was similar to that released by spontaneously adherent cells pre-incubated with LPS ($0.84 \pm \text{SEM } 0.37 \text{ mU}/10^6$ cells/h) and greater than that released during the second adherence assay to separate the LPS-adherent and the non-adherent cells ($0.36 \pm \text{SEM } 0.11 \text{ mU}/10^6$ cells/h). These data indicate that monocyte release of peroxidase during adherence and LPS incubation was minimal; furthermore, the differences among the monocyte subpopulations were not due to varying peroxidase release.

3.8.2.4. HLA-DR Antigen Expression

To compare the immune regulatory function of the monocyte subpopulations, their expression of HLA-DR antigens was assessed since the amount of HLA-DR antigen expressed by mononuclear phagocytes correlates directly with their capacity to present antigen (454,296,507). Monocytes were labelled with **I3**, a murine monoclonal antibody that recognises a non-polymorphic HLA-DR antigen expressed by monocytes (443).

In an initial experiment, the binding of **I3** antibody by pool monocytes was assessed by epifluorescence microscopy. Although greater than **90%** of the pool monocytes were labelled with **I3** antibody, there was marked heterogeneity in the intensity of staining of the cells. HLA-DR antigen expression by pool, spontaneously adherent and non-spontaneously adherent monocytes was then quantified by flow cytometry. Similar proportions of cells from each group were labelled with **I3** antibody (**92%**, **98%** and **98%** for pool, spontaneously adherent and non-spontaneously adherent **cells**, respectively). However, the monocyte subpopulations differed markedly in their intensity of staining for **i3** antibody. The mean fluorescent intensity of the non-spontaneously adherent **cells** was **304 ($\pm \text{SD } 3$)** which was 3-fold greater than that of the spontaneously adherent cells (figure 34). In contrast, only 3% of pool cells were labelled with a control

Figure 33

Peroxidase Content of Monocyte Subpopulations

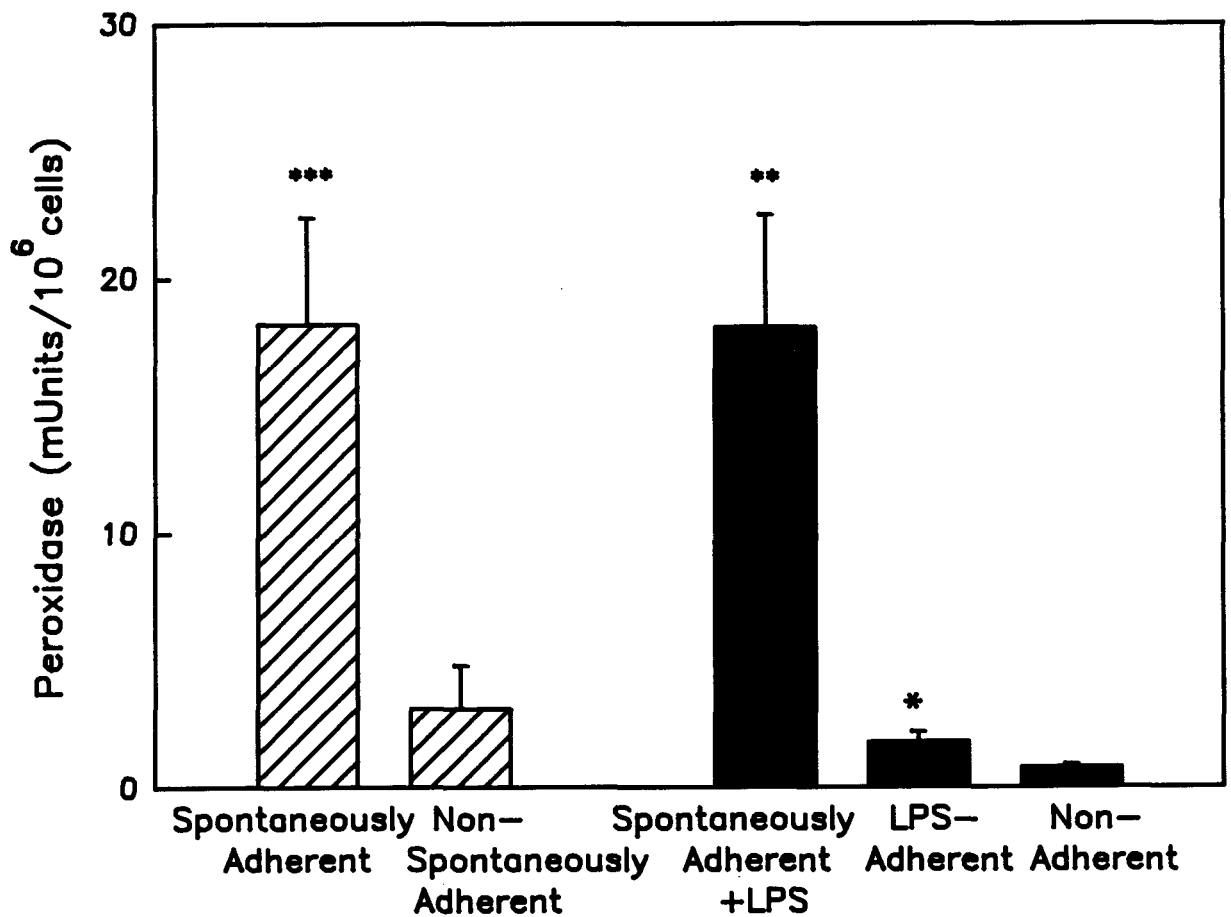


Figure 33 Cells were extracted with PBS containing 0.04% [v/v] Triton X-100, 1 mol/L NaCl, pH 7.4, then assayed for peroxidase activity, as described in Methods. Error bars represent SEM; n = 5 donors. Asterisk indicates p < 0.05 compared with non-adherent cells; *, p < 0.05 compared with LPS-adherent monocytes; **, p < 0.05 compared with non-spontaneously adherent monocytes.

antibody (non-immune murine IgG) and the mean fluorescent intensity of this group of cells was $4 (\pm \text{SD } 2)$. These data excluded the possibility that I3 bound to monocytes by a non-specific mechanism.

The mean fluorescent intensity of the spontaneously adherent and non-spontaneously adherent monocytes was 2-fold and 6-fold greater than that of the pool cells, respectively. It is possible that this was a temperature-related phenomenon since the adherence step to separate the monocyte subpopulations was conducted at 37°C for 1 h, whereas the pool monocytes were held at 4°C for the duration of this assay. To test possibility that incubation of monocytes at 37°C during the adherence assay increased the expression of HLA-DR antigen by the monocyte subpopulations, pool monocytes were incubated at 4°C and at 37°C for 1 h prior to labelling with I3 antibody. Incubation of the pool cells at 37°C for 1 h increased their expression of HLA-DR antigen 2-fold when compared with cells held at 4°C (figure 35). Therefore, in subsequent experiments pool cells were incubated at 37°C for the duration of the adherence assays to separate the monocyte subpopulations.

HLA-DR antigen expression by pool, non-spontaneously adherent, LPS-adherent and non-adherent monocytes was compared. A similar proportion of cells from each monocyte population were labelled with I3 antibody (99.4%, 99.3%, 99.8% and 98.8% of the pool, non-spontaneously adherent, LPS-adherent and non-adherent, respectively). The mean fluorescent intensity of the LPS-adherent and non-adherent cells was similar to that of the non-spontaneously adherent cells (which were incubated at 37°C for the duration of the LPS-adherence assay). In addition, the mean fluorescent intensity of the pool cells (which were incubated at 37°C for the duration of both adherence assays) was similar to that of the non-spontaneously adherent monocytes. There was minimal non-specific binding of the control antibody (non-immune murine IgG) to pool monocytes (figure 36). To control for possible effects of LPS on HLA-DR antigen expression, pool cells were pre-incubated for 1 h with $1 \mu\text{g/ml}$ of LPS prior to labelling with I3 antibody. Exposure of pool cells to LPS did not alter their expression of HLA-DR antigen (figure 36).

3.835. Total Protein Content

The total protein content of the monocyte pool was $36.2 (\pm \text{SEM } 7.5) \mu\text{g}/10^6 \text{ cells}$ (figure 37) which was similar to the that of the non-spontaneously adherent ($30.0 \pm \text{SEM } 1.9 \mu\text{g}/10^6 \text{ cells}$), LPS-adherent ($28.4 \pm \text{SEM } 2.5 \mu\text{g}/10^6 \text{ cells}$) and non-adherent monocytes ($32.9 \pm \text{SEM } 4.5 \mu\text{g}/10^6 \text{ cells}$). However, the total protein content of the spontaneously adherent cells was $46.7 \pm \text{SEM } 6.8 \mu\text{g}/10^6 \text{ cells}$, which was greater than

Figure 34
HLA-DR Antigen Expression by Monocytes

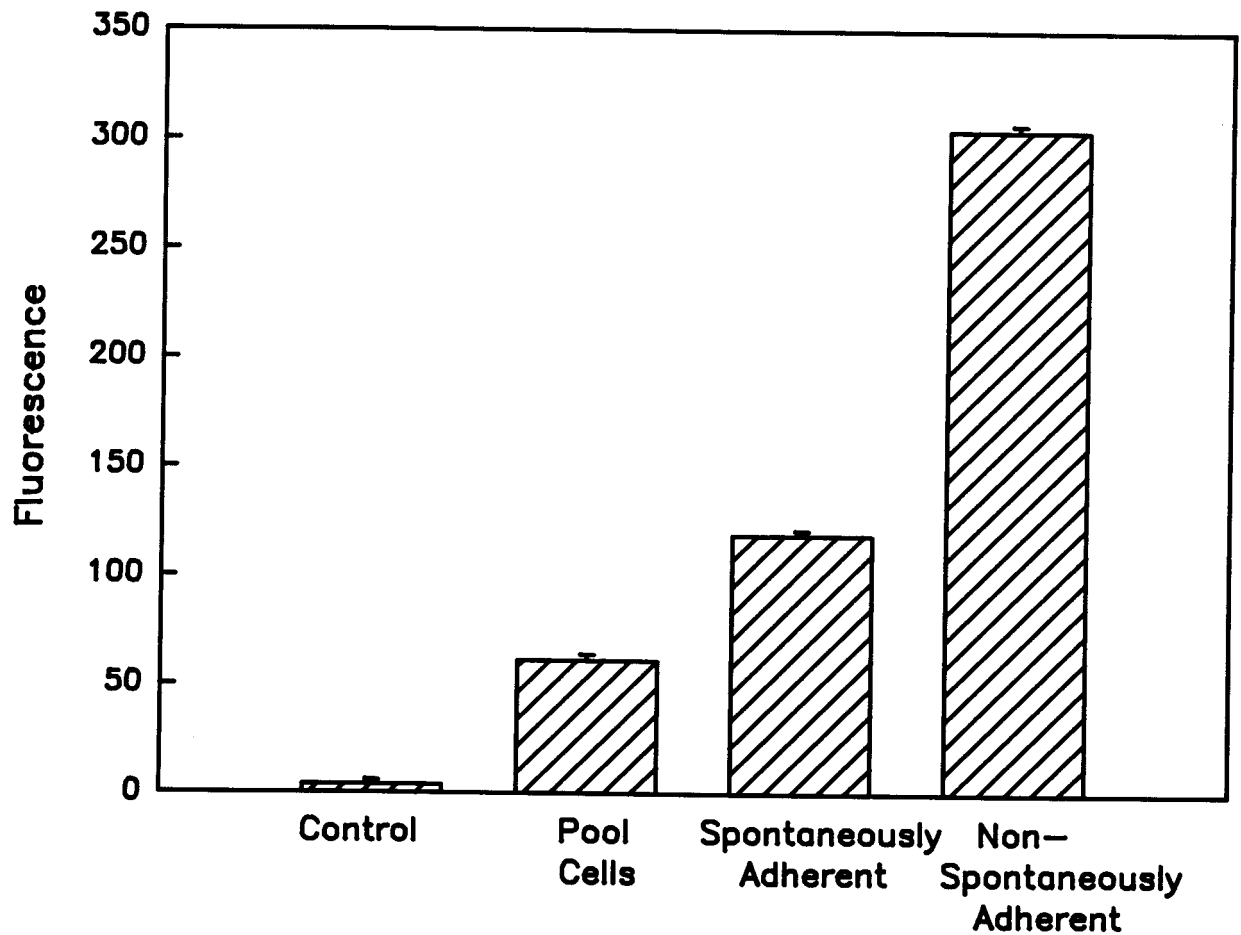


Figure 34 The indirect immunofluorescence technique was used to assess the binding of **I3**, a murine monoclonal antibody that recognises a non-polymorphic **HLA-DR** antigen. Non-immune murine IgG was used instead of I3 as the control antibody (control). Antibody binding was quantified by flow cytometry. Figure shows the results of a typical experiment; 10^5 cells were analysed in each group. Error bars represent SD.

Figure 35
Effect of Incubation Temperature on HLA-DR Antigen Expression by Monocytes

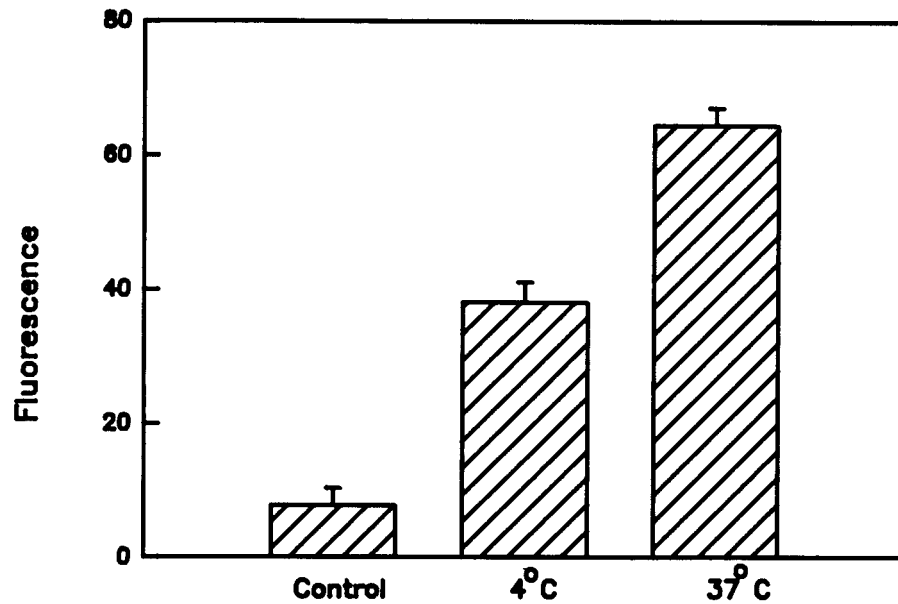


Figure 35 Pool monocytes were incubated at 4°C or at 37°C for 1 h then labelled with I3 antibody. Non-immune mouse IgG was used as the control antibody (control). Figure shows the results of a typical experiment; 10^5 cells were analysed in each group by flow cytometry. Error bars represent SD.

Figure 36
HLA-DR Antigen Expression by Monocyte Subpopulations

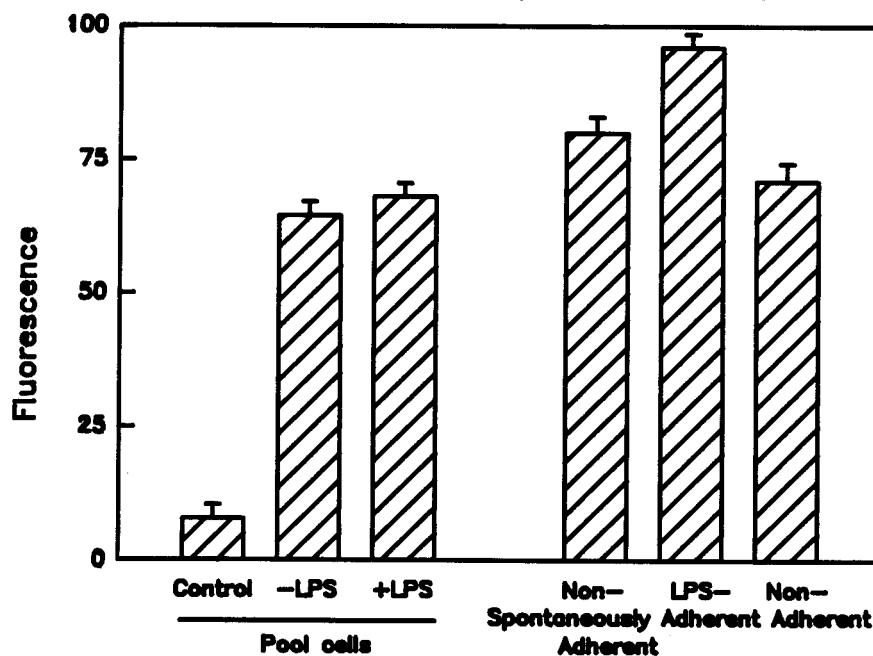


Figure 36 Pool, non-spontaneously adherent, LPS-adherent and non-adherent monocytes were labelled with I3 antibody. Non-immune murine IgG was used as the control antibody (control). To assess the effect of LPS on HLA-DR antigen expression, pool monocytes were pre-incubated with LPS for 1h. Figure shows the results of a typical experiment; 10^5 cells were analysed in each group by flow cytometry. Error bars represent SD.

that of the other subpopulations ($p < 0.05$). Prior to preparation of cell extracts, all of the monocyte populations were greater than 99% viable, as assessed by exclusion of trypan blue dye. Therefore, it is unlikely that the differences in total protein content among the monocyte subpopulations were due to differences in cell viability.

Extracts of the adherent subpopulations for assay were prepared within the fibronectin-coated dishes. To exclude the possibility that the greater total protein content of the spontaneously adherent cells was due to solubilisation of the fibronectin Substrate during preparation of the cells extracts, lysing buffer was added to three fibronectin-coated dishes that had not been incubated with cells, and the total protein content of these extracts was determined. The fibronectin extracts contained **4.4 (\pm SEM 0.1) μg** protein per equivalent of 10^6 spontaneously adherent monocytes which was less than 10% of the mean total protein content of these cells. These data confirm that the greater total protein content of the spontaneously adherent cells, when compared with the other monocyte populations, was not due entirely to solubilisation of the fibronectin Substrate during the preparation of the cell extracts.

Figure 37

Total Protein Content of Monocyte Subpopulations

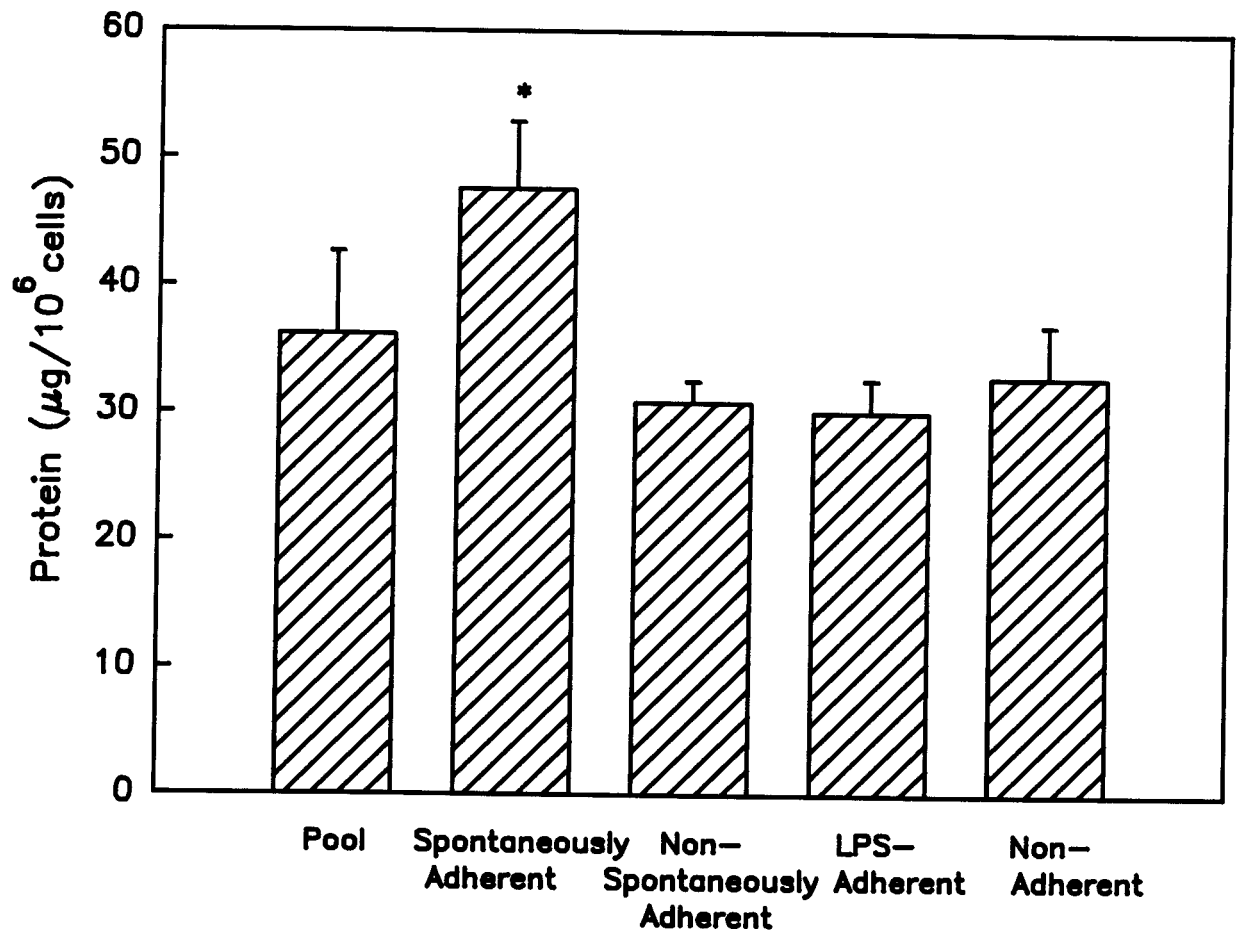


Figure 37 Extracts of the monocyte subpopulations were assayed for total protein content using the Sedmak assay. Error bars represent SEM; n = 5 donors. Asterisk indicates p < 0.05 compared with all other monocyte subpopulations.

3.9. Expression of Human Leukocyte Elastase and Alpha₁-antitrypsin by Monocytes

Tissue destruction in chronic obstructive pulmonary diseases (COPD) is thought to result from an imbalance between lung proteinases and their inhibitors that favors excessive activity of the proteinases. In this respect human leukocyte elastase (HLE) is thought to be the most important proteinase, and α_1 -antitrypsin is its major inhibitor. It is possible that mononuclear phagocytes play an important role in the pathogenesis of COPD since: 1) these cells are present in greatly increased numbers at strategic locations in the lungs of patients with COPD (334); and 2) monocytes contain HLE (81) and synthesise α_1 -antitrypsin (32). It is of particular interest that monocytes have been shown recently to be heterogeneous with respect to their content of HLE. Karg and coworkers (242) demonstrated that only 20% of monocytes stain intensely for HLE. In addition, Campbell *et al.* (81) showed that only 20% of monocytes produce HLE-mediated proteolysis of an extracellular matrix component in the presence of proteinase inhibitors.

The possibility was tested that there is a relationship between the adherence properties of monocytes and their HLE content since: 1) the proportion of monocytes that adhered spontaneously to fibronectin in the current study (20 to 25%) was similar to the proportion of monocytes (20%) that is replete with HLE (81,242); and 2) HLE is stored within the peroxidase positive granules of monocytes (242) and there was a striking direct relationship between the capacity of cells to adhere and their peroxidase content in the current study. The α_1 -antitrypsin content of the monocyte subpopulations was also compared and the effect of several pro-inflammatory mediators on the accumulation of α_1 -antitrypsin by monocytes was investigated.

3.9.1. Expression of HLE by Monocytes

3.9.1.1. Comparison of the HLE Content of Monocyte Subpopulations

The mean HLE content of the spontaneously adherent was $454 \pm \text{SEM } 108 \text{ ng HLE}/10^6 \text{ cells}$ which was 3-fold greater than that of the pool cells, 8 to 9-fold greater than that of the non-spontaneously adherent and LPS-adherent cells, and 22-fold greater than that of the non-adherent cells (figure 38). To control for possible effects of LPS on the HLE content of monocytes, spontaneously adherent cells were also incubated with LPS. Pre-incubation of spontaneously adherent cells with LPS did not affect their HLE content (figure 38).

To exclude the possibility that the differences in HLE content of the monocyte subpopulations were due to differential release of enzyme into the culture media, all of the culture supernatants were assayed for

immunoreactive HLE. During the 1 h adherence step to separate the spontaneously adherent and non-spontaneously adherent monocytes, $1.3 (\pm \text{SEM } 1.1) \text{ ng}/10^6$ cells of immunoreactive HLE were released. When the spontaneously adherent cells were pre-incubated with LPS for 1 h, $21.4 \pm \text{SEM } 4.8 \text{ ng}/10^6$ cells of HLE were released into the culture media and $0.2 (\pm \text{SEM } 0.2) \text{ ng}/10^6$ cells of HLE were released during the second adherence assay to separate the LPS-adherent and the non-adherent cells. These data indicate that differences observed in HLE content among the monocyte subpopulations were not due to varying release of HLE during adherence or in response to exposure to LPS.

To exclude the possibility that the differences among the monocyte subpopulations were due to variable contamination of the subpopulations with platelets, extracts of platelets isolated from three donors were assayed for HLE. Platelets did not contain immunoreactive HLE. To exclude the possibility that the Nycodenz isolation procedure, *per se*, altered the HLE content of monocytes, cells were isolated from a further three donors by both the Nycodenz method and CCE, and extracts of pool, spontaneously adherent and **non-spontaneously** adherent monocytes were assayed for immunoreactive HLE. The HLE content of the monocyte populations isolated by the Nycodenz isolation procedure were similar to those of cells isolated by CCE (figure 39). Moreover, the HLE content of the spontaneously adherent cells ($66.1 \pm \text{SEM } 10.9$ and $632 \pm \text{SEM } 7.7 \text{ ng}/10^6$ cells, for Nycodenz and CCE-isolated cells, respectively) was again substantially greater (7-fold) **than** that of the non-spontaneously adherent **cells** ($9.5 \pm \text{SEM } 5.0$ and $15.9 \pm \text{SEM } 1.6 \text{ ng}/10^6$ cells, for Nycodenz and CCE-isolated cells, respectively). These data indicate that the differences among the monocyte subpopulation were not **a** consequence of **the** Nycodenz isolation procedure.

To compare the relative **amounts** of HLE contained within monocytes and neutrophils, blood was drawn from **three** healthy donors, **monocytes** were isolated by the Nycodenz method, neutrophils were isolated by the Percoll method, and cell extracts were assayed for immunoreactive HLE. The pool monocytes contained $78.1 (\pm \text{SEM } 23.0) \text{ ng HLE}/10^6 \text{ cells}$ which represented 4.6% ($\pm \text{SEM } 0.4\%$) of the amount of HLE contained within neutrophils from the same donor.

Figure 38
HLE Content of Monocyte Subpopulations

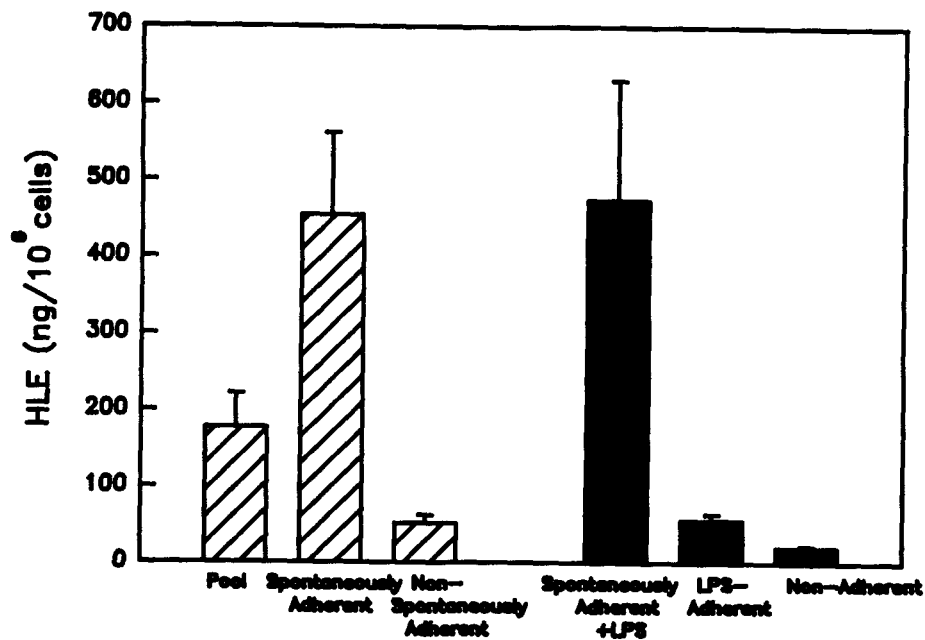


Figure 38 Monocytes were isolated by the Nycodenz method, monocyte subpopulations were separated and cell extracts were assayed for HLE using an indirect binding ELISA. Error bars represent SEM; n = 3 donors.

Figure 39
HLE Content of Monocytes: Comparison of Monocyte Isolation Methods

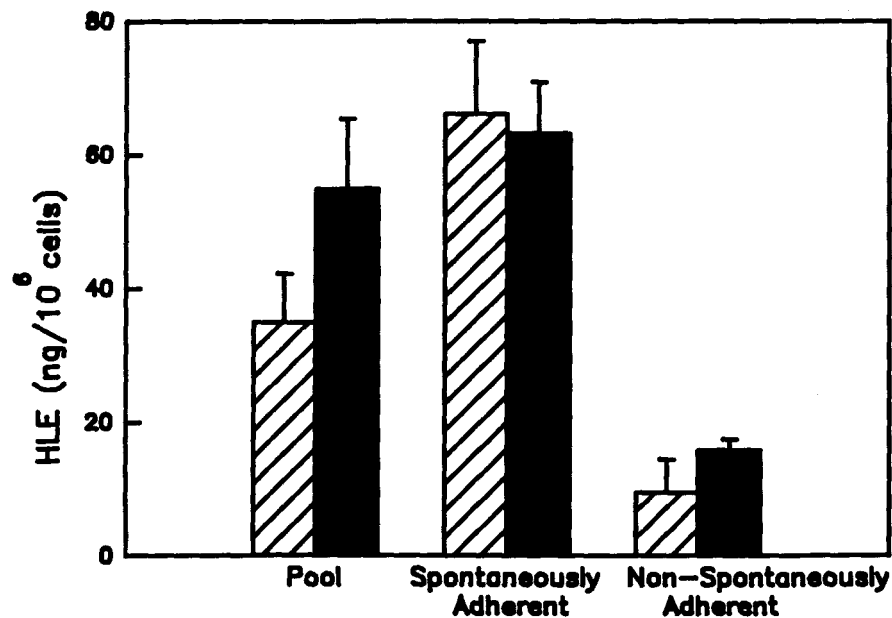


Figure 39 Monocytes were isolated by the Nycodenz method (cross-hatched bars) and by CCE (solid bars), extracts of pool, spontaneously adherent and non-spontaneously adherent cells were assayed for HLE using an indirect binding ELISA. Error bars represent SEM; n = 3 donors.

3.9.1.2. Within-Donor and Between-Donor Variability in the HLE Content of Monocytes

The absolute amounts of **HLE** contained within pool, spontaneously adherent and non-spontaneously adherent cells varied considerably when monocytes were isolated from different donors on different occasions (figures 38 and 39). It is unlikely that these differences were due entirely to intra-assay or inter-assay variability *since*: 1) the within-batch coefficient of variation (C.V.) for the monocyte adherence assay conducted under basal conditions was 3.7%, and the C.V. for spontaneous adherence of monocytes, when cells were isolated from the same donor on different occasions, was 6.7 to 10.3% (table 5 a); and 2) the within- and between-batch C.V.s for the **HLE** indirect binding **ELISA** were 5.6% and **14.2%**, respectively.

To test the possibility that the differences in **HLE** content among the monocyte subpopulations were due to between-donor variability in the **HLE** content of monocytes, the **HLE** content of **pool**, spontaneously adherent and non-spontaneously adherent cells isolated from an additional seven donors were compared. There was considerable variability in the **HLE** content of the monocyte populations isolated from different donors (table 6 a). However, the relative differences in **HLE** content between the monocyte subpopulations were similar to those in figures 38 and 39. The spontaneously adherent cells, on average, contained 7-fold more **HLE** than the non-spontaneously adherent cells ($p < 0.01$).

To investigate whether the variability in **HLE** content of the subpopulation also reflected within-donor variability, the **HLE** content of monocyte populations obtained from the same donor on different occasions were compared. There was considerable within-donor variability in **HLE** content of the monocyte populations (table 6 b). It is possible that these differences are due to day-to-day variation in the **HLE** content of circulating monocytes. This may reflect variations in the maturational stages of circulating monocytes since maturation results in a loss of the expression of **HLE** by monocytes (438). Alternatively, these differences may be due to day-to-day variation in the release of **HLE** during monocyte isolation which may relate to slight differences in the temperature or composition of the solutions used, or in the duration of the monocyte isolation procedure performed on different occasions.

Table 6 a**HLE Content? of Monocyte populations: Between-Donor Variability**

Donor #	Monocyte Population		
	pool	spontaneously adherent	non-spontaneously adherent
1	165 \pm 9	372 \pm 41	39 \pm 3
2	107 \pm 6	324 \pm 31	44 \pm 1
3	259 \pm 25	666 \pm 54	73 \pm 6
4	51 \pm 4	191 \pm 20	27 \pm 3
5	330 \pm 71	262 \pm 30	66 \pm 2
6	124 \pm 20	462 \pm 71	56 \pm 9
7	35 \pm 4	72 \pm 3	17 \pm 1
Mean \pm SEM	153 \pm 24	327 \pm 39*	46 \pm 4

† Results are expressed as ng HLE/10⁶ cells. The samples were assayed in triplicate. Data are mean values \pm SEM. Asterisk indicates p < 0.01 compared with non-spontaneously adherent cells

Table 6 b**HLE Content? of Monocyte Populations: Within-Donor Variability**

Monocyte Population	donor 1		donor 2		donor 3	
	A	B	A	B	A	B
pool	165 \pm 9	309 \pm 30	60 \pm 6	330 \pm 71	107 \pm 6	29 \pm 1
spontaneously adherent	372 \pm 42	438 \pm 39	70 \pm 2	262 \pm 30	324 \pm 30	65 \pm 6
non-spontaneously adherent	39 \pm 3	29 \pm 17	13 \pm 3	66 \pm 2	44 \pm 1	10 \pm 1

† Results are expressed as ng HLE/10⁶ cells. The samples were assayed in triplicate. Data are mean values \pm SEM. A = occasion number 1; B = occasion number 2.

3.9.2. Alpha₁-Antitrypsin Content of Monocytes

3.9.2.1. Comparison of the Alpha₁-Antitrypsin Content of Monocyte Subpopulations

Preliminary studies were undertaken to determine whether platelets contain α_1 -antitrypsin since the Nycodenz-isolated monocytes are always contaminated variably with platelets. Platelets were prepared from four healthy donors by CCE and cell extracts were assayed for α_1 -antitrypsin immunoreactivity using a direct binding ELISA. Platelets contained $0.6 (\pm \text{SEM } 0.3)$ ng of α_1 -antitrypsin/ 10^6 cells. Therefore, to compare the α_1 -antitrypsin content of the monocyte subpopulations, monocytes were isolated by CCE since this method provides monocytes that are not contaminated with platelets.

The mean α_1 -antitrypsin content of the spontaneously adherent cells was $99 (\pm \text{SEM } 11)$ ng/ 10^6 cells which was 2-fold greater than that of the pool cells, 4.5-fold greater than that of the non-spontaneously adherent cells and 7-fold greater than that of the LPS-adherent and non-adherent cells; $n = 3$ (figure 40). To control for possible effects of LPS on the α_1 -antitrypsin content of monocytes, spontaneously adherent cells were also pre-incubated with LPS. Pre-incubation of the spontaneously adherent cells with LPS resulted in a 29% reduction in their content of α_1 -antitrypsin; however, these cells contained more α_1 -antitrypsin compared with the other subpopulation even after exposure to LPS (figure 40). This latter result was due, in part, to the release of α_1 -antitrypsin by spontaneously adherent monocytes since $18 (\pm \text{SEM } 17)$ ng α_1 -antitrypsin/ 10^6 cells were detected in the culture supernatants of spontaneously adherent cells that had been pre-incubated with LPS for 1 h. In contrast, differential release of α_1 -antitrypsin by monocyte subpopulations could not explain the differences in α_1 -antitrypsin content between the spontaneously adherent and other monocyte subpopulations since only $5 (\pm \text{SEM } 3)$ ng α_1 -antitrypsin/ 10^6 cells were released during the spontaneous adherence step and only $5 (\pm \text{SEM } 5)$ ng α_1 -antitrypsin/ 10^6 cells were released during the second adherence assay to separate the LPS-adherent and non-adherent cells.

3.9.2.2. Within- and Between-Donor Variability in the Alpha₁-Antitrypsin Content of Monocytes

Donor variability in the α_1 -antitrypsin content of monocytes was assessed since there was considerable donor variability in their HLE content. To assess between-donor variability, the α_1 -antitrypsin content of pool, spontaneously adherent and non-spontaneously adherent cells isolated from an additional seven donors was determined. Although between-donor variability of α_1 -antitrypsin content of monocytes was observed (table

Figure 40

Alpha₁-Antitrypsin Content of Monocyte subpopulations

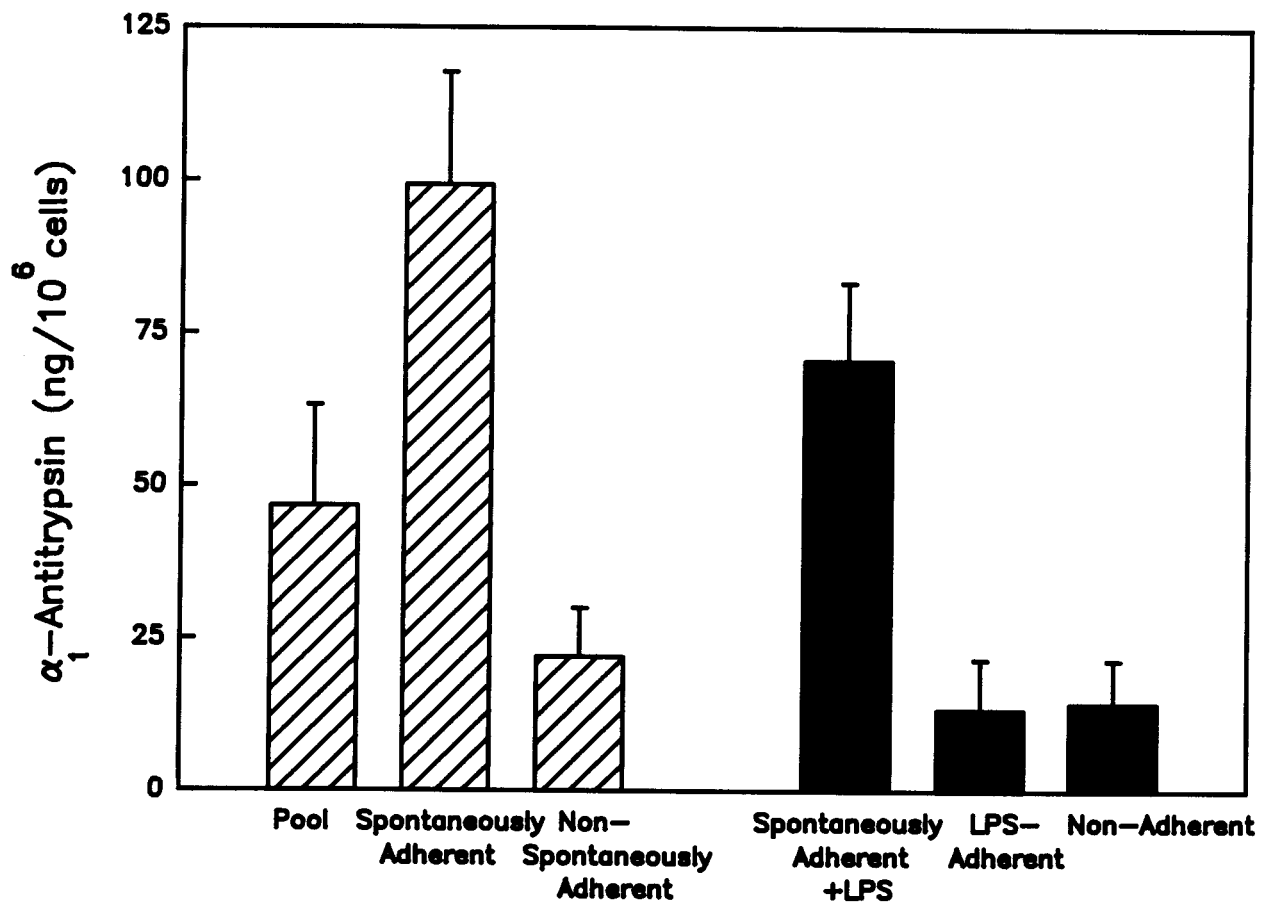


Figure 40 Monocytes were isolated from healthy donors by CCE. The monocyte subpopulations were separated, and cell extracts were assayed for α_1 -antitrypsin using the direct binding ELISA. The results are expressed as ng α_1 -antitrypsin/10⁶ cells. Error bars represent SEM; n = 3 donors.

7 a), it was less marked than that observed for HLE (table 6 a). In addition, the relative differences in the mean α_1 -antitrypsin content of the monocyte populations were the same as those shown in figure 40. The spontaneously adherent cells, on average, contained 2-fold more α_1 -antitrypsin than the pool cells ($p < 0.025$) and 4-fold more than the non-spontaneously adherent cells ($p < 0.01$).

The α_1 -antitrypsin content of monocyte populations obtained from the same donor on different occasions were also compared. Although within-donor variability in the α_1 -antitrypsin content of monocytes was observed (table 7 b), it was again less marked than that observed for HLE (table 6 b).

3.9.3. Comparison of the HLE and α_1 -Antitrypsin Content of the Monocyte Subpopulations

To compare the amounts of HLE and α_1 -antitrypsin contained within the monocyte subpopulations, monocytes were isolated from three healthy donors by CCE, the subpopulations were separated and cell extracts were assayed for both immunoreactive HLE and α_1 -antitrypsin. The results were expressed as pmol/ 10^6 cells and the molar ratios of HLE to α_1 -antitrypsin were calculated.

All of the monocyte populations contained a molar excess of HLE compared with α_1 -antitrypsin; however, the magnitude of the molar excess of HLE varied considerably (table 8 a). The pool monocytes and spontaneously adherent cells contained a substantial molar excess of HLE relative to α_1 -antitrypsin (8-fold and 10-fold, respectively). In contrast, the non-spontaneously adherent cells contained only a 5-fold molar excess of HLE. However, the non-spontaneously adherent cells were heterogeneous in this respect since the LPS-adherent cells contained a molar excess of HLE that was similar to that of the spontaneously adherent cells (11-fold), whereas the non-adherent cells contained only a 3-fold molar excess of HLE relative to α_1 -antitrypsin. This latter result was due to the fact that the LPS-adherent cells contained 3-fold more HLE but similar amounts of α_1 -antitrypsin when compared with the non-adherent monocytes (table 8 a).

The molar ratio of HLE/ α_1 -antitrypsin was also determined for pool, spontaneously adherent and non-spontaneously adherent cells isolated from seven donors. The results confirmed that the spontaneously adherent cells contained a significantly greater excess of HLE compared with non-spontaneously adherent cells ($p < 0.01$; table 8 b).

Table 7 a

Alpha₁-antitrypsin Content† of Monocyte Populations: Between Donor Variability

Donor	Monocyte Populations		
	pool	spontaneously adherent	non-spontaneously adherent
1	53 ± 3	110 ± 7	22 ± 2
2	28 ± 2	78 ± 7	14 ± 1
3	59 ± 5	110 ± 7	30 ± 6
4	17 ± 2	26 ± 3	7 ± 1
5	19 ± 2	26 ± 0	6 ± 0
6	31 ± 1	26 ± 4	6 ± 0
7	12 ± 10	36 ± 1	8 ± 0
Mean ± SEM	31 ± 7	59 ± 15*	13 ± 3

† Results are expressed as ng α_1 -antitrypsin/10⁶ cells. The samples were assayed in triplicate. Data are mean values ± SD. Asterisk indicates $p < 0.01$ compared with non-spontaneously adherent cells

Table 7 b

Alpha₁-Antitrypsin Content† of Monocyte Populations: Within-Donor Variability

Monocyte Population	Donor 1		Donor 2		Donor 3	
	A	B	A	B	A	B
pool	53 ± 3	82 ± 2	28 ± 1	17 ± 2	28 ± 2	63 ± 2
spontaneously adherent	110 ± 7	73 ± 2	38 ± 4	26 ± 3	78 ± 7	47 ± 5
non-spontaneously adherent	22 ± 2	26 ± 2	18 ± 0	7 ± 1	14 ± 1	21 ± 1

† Results are expressed as ng α_1 -antitrypsin/10⁶ cells. The samples were assayed in triplicate. Data are mean values ± SD. A = occasion no. 1, B = occasion no. 2.

Table 8 a**HLE and Alpha₁-Antitrypsin Content? of Monocyte Populations**

Monocyte Population	HLE (pmol/10 ⁶ cells)	α ₁ -antitrypsin (pmol/10 ⁶ cells)	HLE/α ₁ -antitrypsin ± SEM
pool	7.08 ± 1.81	0.86 ± 0.18	8.14 ± 0.77
spontaneously adherent	18.16 ± 4.34	1.84 ± 0.20	9.79 ± 1.74
non-spontaneously adherent	2.07 ± 0.43	0.41 ± 0.15	5.28 ± 0.84
LPS-adherent	2.12 ± 0.28	0.25 ± 0.15	10.59 ± 3.85
non-adherent	0.83 ± 0.11	0.27 ± 0.13	3.31 ± 0.71

† values are expressed as pmol protein/10⁶ cells. The samples were assayed in triplicate. Data in table are mean ± SEM; n = 3 donors.

Table 8 b**Molar Ratio† of HLE and Alpha₁-Antitrypsin in Monocyte Populations**

Donor	Monocyte Population		
	pool	spontaneously adherent	non-spontaneously adherent
1	6.8	7.3	3.9
2	8.2	9.0	6.8
3	9.4	13.1	5.2
4	4.8	4.0	1.7
5	5.8	15.9	9.8
6	1.3	2.9	1.0
7	8.1	13.0	2.4
Mean ± SEM	6.3 ± 1.0	9.3 ± 1.8*	4.4 ± 1.2

† Data are ratios of HLE (pmol/10⁶ cells)/α₁-antitrypsin (pmol/10⁶ cells) for 7 donors. Asterisk indicates p < 0.01 compared with non-spontaneously adherent monocytes.

3.9.4. Alpha₁-antitrypsin Accumulation by Monocytes

Although de novo synthesis of α_1 -antitrypsin by monocytes has been demonstrated by biosynthetic labelling and immunoprecipitation studies (32), the absolute amount of α_1 -antitrypsin synthesised by monocytes has not been quantified previously. In this study, an indirect binding ELISA was used to quantify immunoreactive α_1 -antitrypsin in monocyte extracts and culture supernatants. However, these data represent the net balance between the synthesis and the catabolism of this protein by the cells. Therefore, the results of this study were expressed as amounts of α_1 -antitrypsin accumulated by monocytes.

3.9.4.1. Experimental Design

Monocytes were isolated by the Nycodenz method for these studies since the CCE method was not available. One potential problem in using Nycodenz-isolated monocytes for these experiments is that the cells are always contaminated with platelets, and platelets contain α_1 -antitrypsin (3.9.2.1). However, it is unlikely that platelets synthesise α_1 -antitrypsin de novo since: 1) platelets contain negligible amounts of DNA and RNA, and 2) any RNA that is present within platelets is in a metabolically inactive form (1). To overcome the problem of platelet contamination, the experiments were designed as follows. The monocyte subpopulations were separated and, after overnight culture, one set of cells (monocytes and their contaminating platelets) from each subpopulation were harvested (time = 0, control cells). Thereafter, cells and supernatants were harvested at intervals, α_1 -antitrypsin concentrations were measured in the samples by direct binding ELISA and the results were expressed as $\text{ng } \alpha_1\text{-antitrypsin}/10^6$ monocytes. The amount of α_1 -antitrypsin contained within the time = 0, control cells (monocytes and their contaminating Platelets) was subtracted from the sum of the results for the cell extracts and culture supernatants for each time interval. This value represented the amount of α_1 -antitrypsin that was accumulated by the monocytes.

3.9.4.2. Effects of Culture and LPS on the Accumulation of Alpha₁-Antitrypsin by Monocytes

The effects of culture and LPS exposure on the accumulation of α_1 -antitrypsin by the four monocyte subpopulations were assessed. To obtain a sufficient number of cells for study, large volumes of blood had to be drawn from the donors and, therefore, the experiment was conducted in two stages. Initially, spontaneously adherent and non-spontaneously adherent cells were isolated from six healthy donors and cultured with and

without LPS. LPS-adherent and non-adherent cells were isolated from an additional five donors at a later date and cultured with and without LPS. To compare the results for monocyte subpopulations isolated from different donors, the amount of α_1 -antitrypsin accumulated by monocytes was expressed as percentage of the amount of α_1 -antitrypsin contained within the time = 0, control cells, as described in Methods.

Spontaneously adherent cells After culture for 24 and 48 h under basal conditions, the spontaneously adherent cells accumulated 522% and 975% more α_1 -antitrypsin, respectively, than the amount contained within the time = 0, control cells (figure 41 a). LPS increased α_1 -antitrypsin accumulation by the spontaneously adherent cells by 1.4-fold after both 24 h and 48 h compared with unstimulated cells.

Non-spontaneously adherent cells After culture for 24 and 48 h under basal conditions, the non-spontaneously adherent cells accumulated 122% and 150% more α_1 -antitrypsin, respectively, than the amount contained within the time = 0, control cells (figure 41 b). In contrast to the spontaneously adherent monocytes, LPS did not alter α_1 -antitrypsin accumulation by non-spontaneously adherent cells.

LPS-adherent cells After culture for 24 and 48 h under basal conditions, the LPS-adherent cells accumulated 135% and 287% more α_1 -antitrypsin, respectively, than the amount contained within the time = 0, control cells (figure 42 a). LPS did not alter α_1 -antitrypsin accumulation by LPS-adherent monocytes.

Non-adherent cells After culture for 24 and 48 h under basal conditions, the non-adherent cells accumulated 25% and 75% more α_1 -antitrypsin, respectively, than the amount contained within the time = 0, control cells (figure 42 b). LPS did not alter α_1 -antitrypsin accumulation by the non-adherent cells.

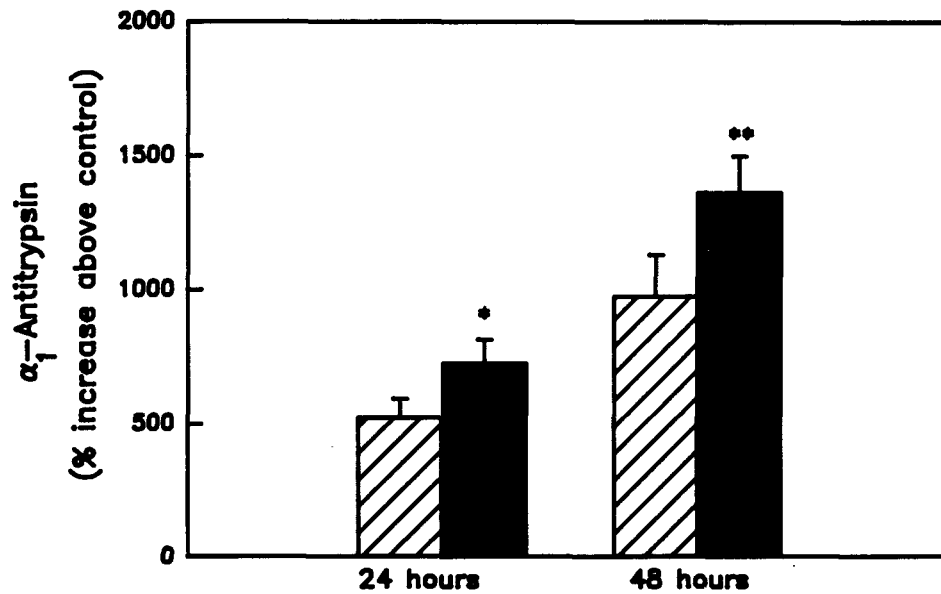
These data indicate that maturation in vitro increases the accumulation of α_1 -antitrypsin by all of the monocyte subpopulations. However, LPS exposure up-regulates the accumulation of α_1 -antitrypsin by only the spontaneously adherent cells.

The data also suggested that the adherent monocyte subpopulations accumulate more α_1 -antitrypsin during culture under basal conditions when compared with the non-adherent subpopulations. However, it is possible that this latter result is an artefact since: 1) the results were expressed as percentage of the amount of α_1 -antitrypsin contained within the time = 0, control cells; and 2) the α_1 -antitrypsin content of the time = 0, control cells for the adherent monocyte subpopulations were substantially lower than those for the non-adherent subpopulations ($65 \pm \text{SEM } 11$ and $387 \pm \text{SEM } 105 \text{ ng of } \alpha_1\text{-antitrypsin}/10^6$ cells for the spontaneously adherent and non-spontaneously adherent cells, respectively [$p < 0.0251$; and $16 \pm \text{SEM } 6$ and $190 \pm \text{SEM}$

Figure 41

Effect of Culture and LPS on Alpha₁-Antitrypsin Accumulation

a. Spontaneously Adherent Monocytes



b. Non-Spontaneously Adherent Monocytes

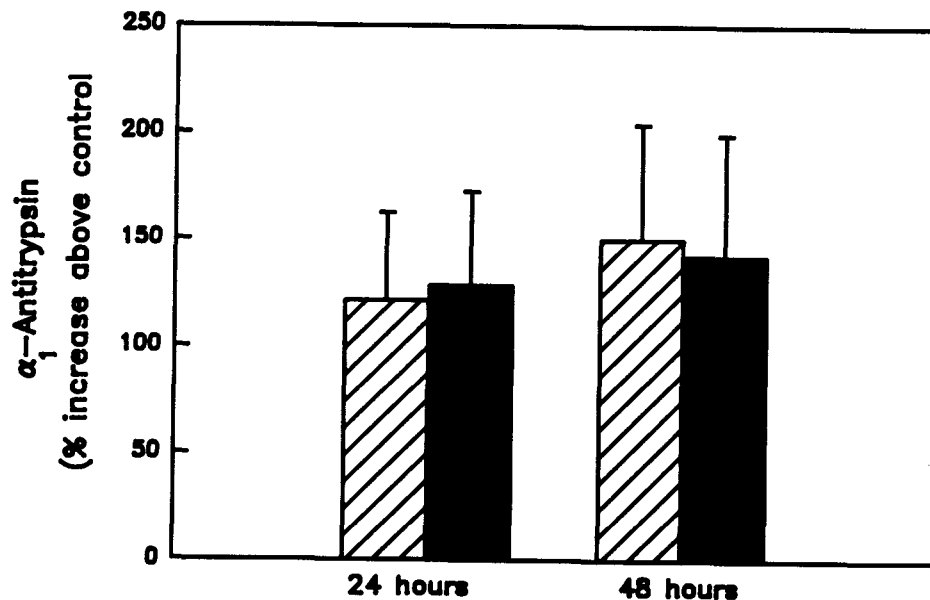
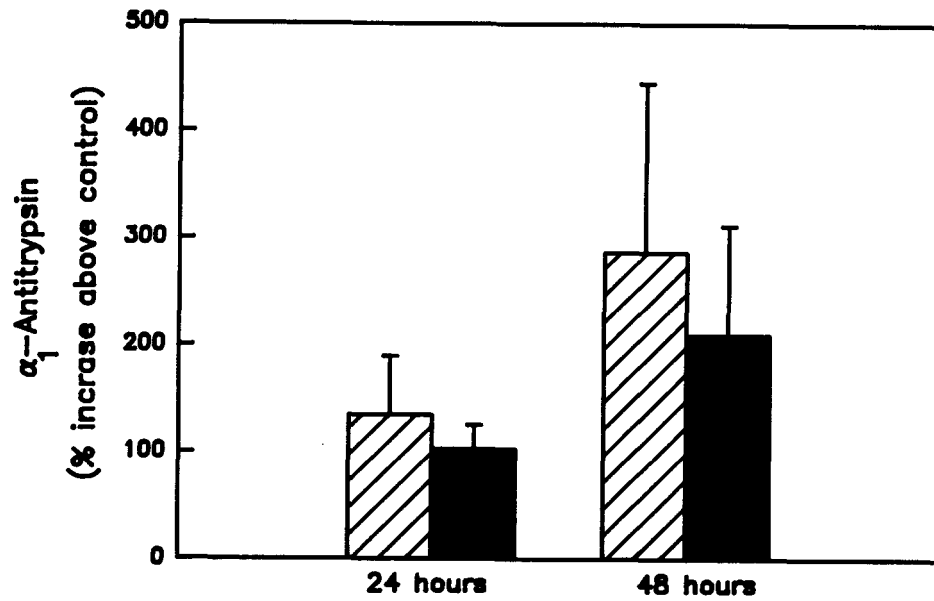


Figure 41 a. Spontaneously adherent and b. non-spontaneously adherent monocytes were cultured for 24 h and 48 h. Cells were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS from *E. coli* 0111:B4 (solid bars). Alpha₁-antitrypsin was measured in the cell extracts and culture supernatants by a direct binding ELISA and the amount of alpha₁-antitrypsin accumulated was expressed as a percentage of the amount contained within the time = 0, control cells, as described in Methods. Error bars represent SEM; n = 6 donors. Asterisk indicates p < 0.05; **, p < 0.025 compared with unstimulated cells.

Effect of Culture and LPS on Alpha₁-Antitrypsin Accumulation

a. LPS-Adherent Monocytes



b. Non-Adherent Monocytes

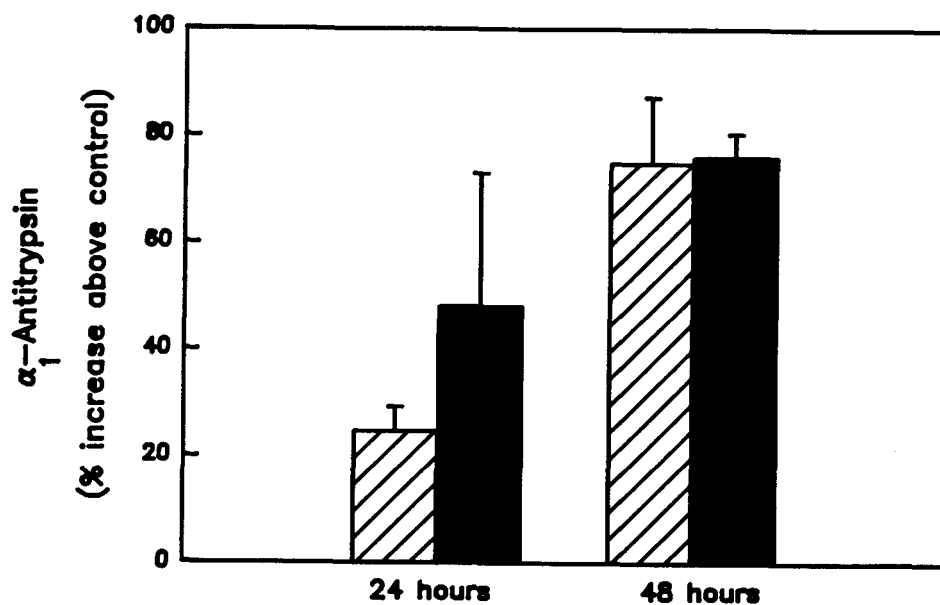


Figure 42 a. LPS-adherent and b. non-adherent monocytes were isolated from five donors and cultured for 24 h and 48 h. Cells were unstimulated (cross-hatched bars) or stimulated with 1 µg/ml of LPS (solid bars). Alpha₁-antitrypsin was measured in the cell extracts and culture supernatants by a direct binding ELISA and the amount of Alpha₁-antitrypsin accumulated was expressed as a percentage of the amount contained within the time = 0, control cells, as described in Methods. Error bars represent SEM.

33 ng of α_1 -antitrypsin/ 10^6 cells for the LPS-adherent and non-adherent cells, respectively ($p < 0.051$). To investigate this possibility, spontaneously adherent and non-spontaneously adherent cells were isolated from the same donors, cultured for 24 h under basal conditions and the absolute amounts of α_1 -antitrypsin accumulated by the cells were measured. The spontaneously adherent and non-spontaneously adherent cells accumulated similar amounts of α_1 -antitrypsin after culture for 24 h ($52 \pm \text{SEM } 7$ and $72 \pm \text{SEM } 8$ ng/ 10^6 cells more than amount contained within the time = 0, control cells, respectively).

Since these preliminary experiments demonstrated that the pattern of α_1 -antitrypsin accumulation by the LPS-adherent and non-adherent monocytes was similar to that of the non-spontaneously adherent cells, subsequent experiments were conducted on only spontaneously adherent and non-spontaneously adherent monocytes.

3.9.4.3. Effect of Time on Alpha₁-Antitrypsin Accumulation by Spontaneously Adherent Monocytes

To assess the effect of time on their accumulation of α_1 -antitrypsin, spontaneously adherent monocytes, cells were incubated with and without LPS ($1 \mu\text{g/ml}$) for 6 h, 12 h and 24 h (figure 43). LPS increased α_1 -antitrypsin accumulation by spontaneously adherent cells within 12 h. Monocytes exposed to LPS accumulated 1.4fold more after 24 h compared with unstimulated cells ($p < 0.05$).

3.9.4.4. Effect of Cytokines on Alpha₁-Antitrypsin Accumulation by Monocyte Subpopulations

To assess the effects of cytokines on α_1 -antitrypsin accumulation, spontaneously adherent and non-spontaneously adherent cells were incubated for 24 h with and without ~~three~~ recombinant cytokines (TNF, IFN, and GM-CSF). Exposure to TNF, IFN and GM-CSF for 24 h stimulated similar increases in α_1 -antitrypsin accumulation by spontaneously adherent monocytes (1.3fold, 1.5fold and 1.6fold, respectively) compared with unstimulated cells (figure 44 a). in contrast, TNF, IFN and GM-CSF had no effect on α_1 -antitrypsin accumulation by non-spontaneously adherent cells (figure 44 b). To determine whether the increase in α_1 -antitrypsin accumulation induced by cytokines was dose-related, spontaneously adherent monocytes were incubated for 24 h with GM-CSF at concentrations ranging from 10 to 75 units/ml. GM-CSF produced a dose-related increase in α_1 -antitrypsin accumulation by spontaneously adherent monocytes with a maximal 1.8 fold increase in the presence of 50 units/ml of GM-CSF (figure 45).

Figure 43

**Effect of Incubation Time on Alpha₁-Antitrypsin Accumulation
Spontaneously Adherent Monocytes**

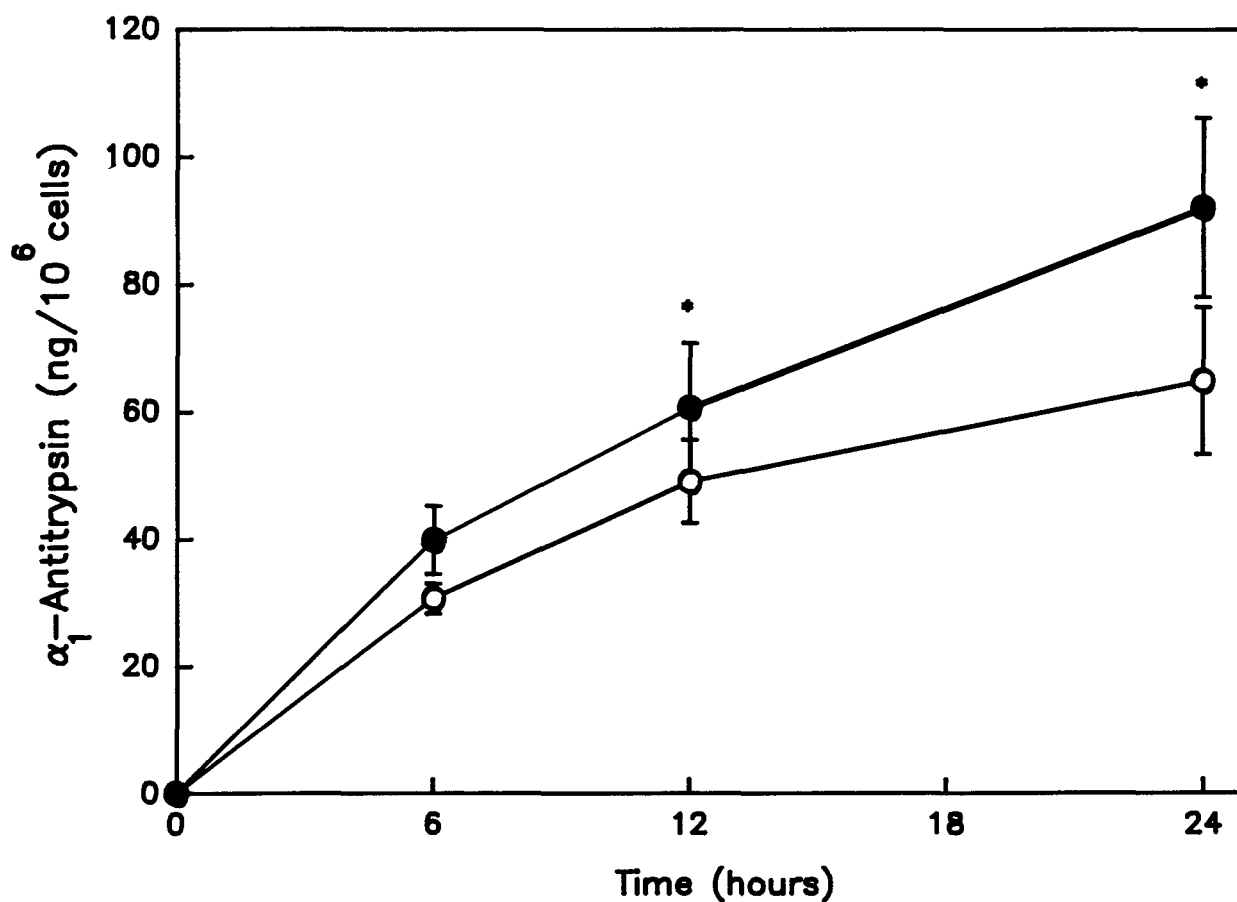


Figure 43 Spontaneously adherent monocytes were cultured for 6 h, 12 h and 24 h. Cells were unstimulated (open circles) or stimulated with 1 μ g/ml of LPS from *E. coli* 0111:B4 (solid circles). Alpha₁-antitrypsin was quantified in cell extracts and culture supernatants using a direct binding ELISA and the amount of Alpha₁-antitrypsin that accumulated (in excess of the amount contained within the time = 0, control cells) was calculated. Results are expressed in ng Alpha₁-antitrypsin/10⁶ cells. Error bars represent SEM; n = 5 donors. Asterisk indicates p < 0.05.

Figure 44
Effect of Cytokines on Alpha₁-Antitrypsin Accumulation by Monocytes

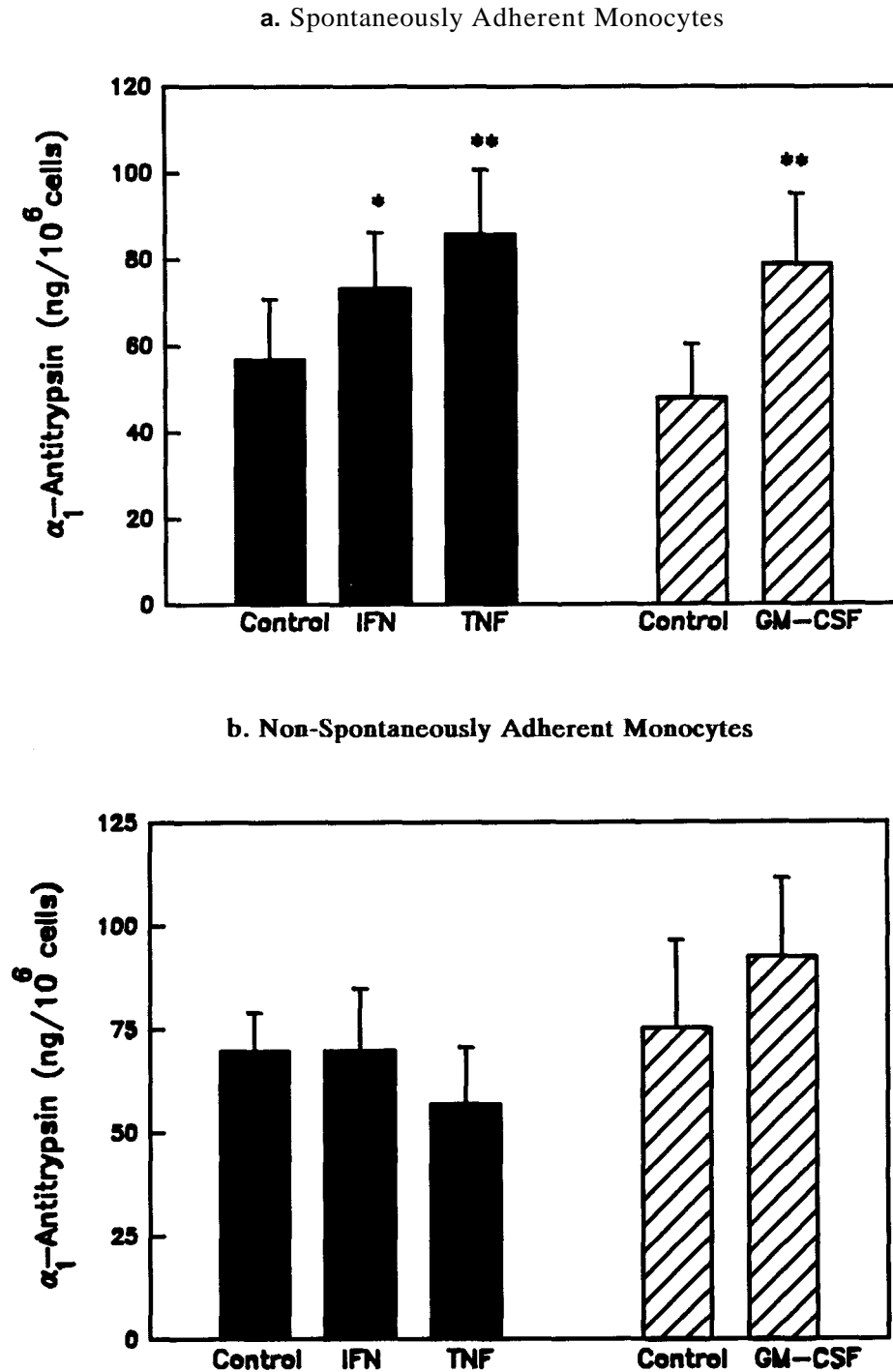


Figure 44 **a.** Spontaneously adherent and **b.** non-spontaneously adherent monocytes were cultured for 24 h. Cells were unstimulated (control) or stimulated with TNF (1000 units/ml), IFN γ (1000 units/ml) and GM-CSF (50 units/ml). Alpha₁-antitrypsin was measured in the cells extracts and culture supernatants using a direct binding ELISA and the amount of α_1 -antitrypsin that accumulated (in excess of the amount contained within the time = 0 control cells) was determined. Error bars represent SEM; n = 6 donors. Asterisk indicates p < 0.05; **, p < 0.025 compared with unstimulated cells.

Figure 45

Effect of GM-CSF on Alpha₁-Antitrypsin Accumulation
Spontaneously Adherent Monocytes

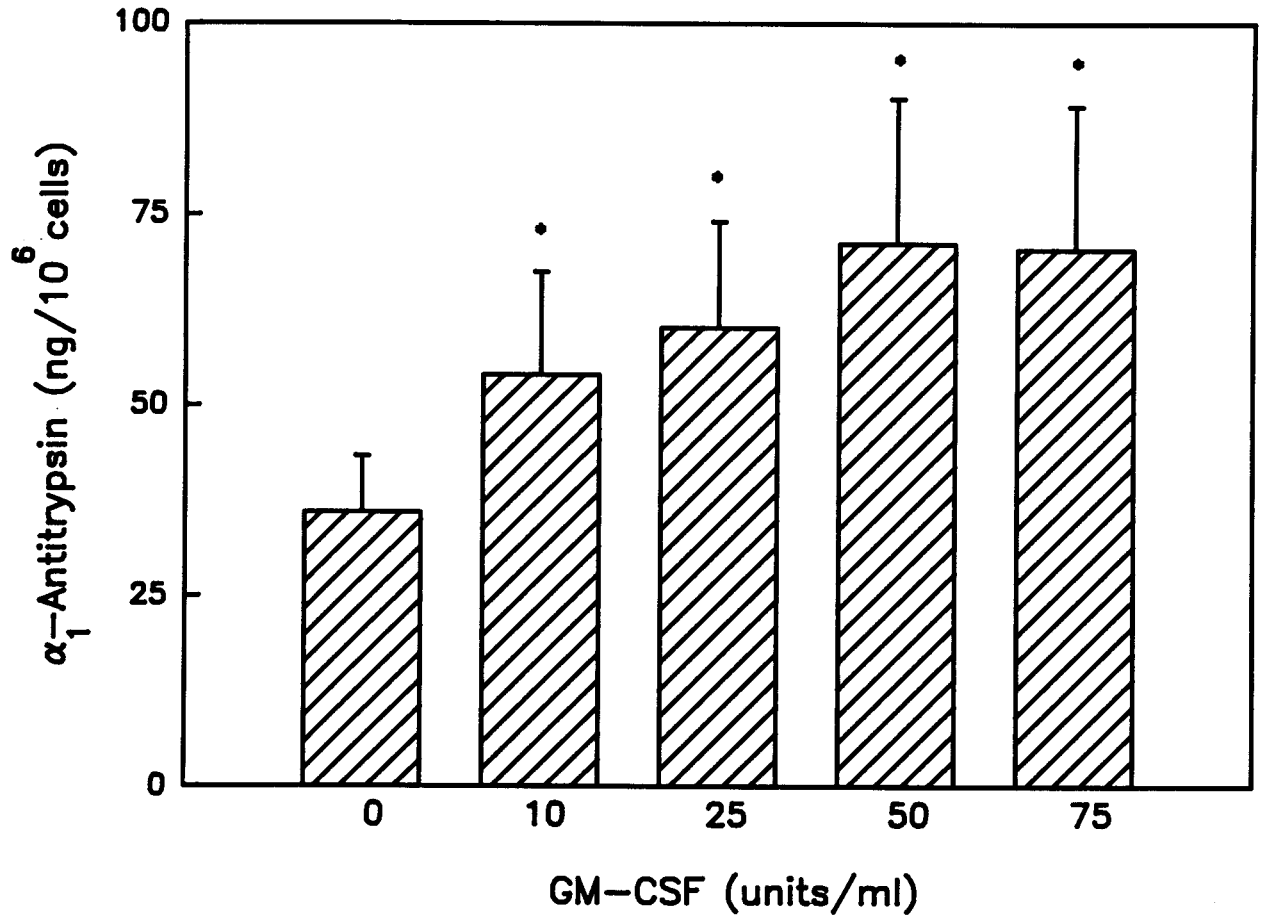


Figure 45 Spontaneously adherent monocytes were cultured for 24 h with concentrations of GM-CSF ranging from 0 to 75 units/ml. Alpha₁-antitrypsin was quantified in cell extracts and culture supernatants using a direct binding ELISA. The amount of Alpha₁-antitrypsin that accumulated (in excess of the amount contained within the time = 0, control cells) was determined. The results were expressed as ng of Alpha₁-antitrypsin accumulation/10⁶ cells. Error bars represent SEM; n = 5 donors. Asterisk indicates p < 0.05 compared with unstimulated cells.

3.10. Expression of the Alpha₁-Antitrypsin Gene by Monocytes

To assess whether LPS and cytokines up-regulated α_1 -antitrypsin accumulation by spontaneously adherent cells by stimulating the transcription of the α_1 -antitrypsin gene, cytoplasmic RNA was extracted from monocytes and subjected to dot-blot analysis using α_1 -antitrypsin and β -actin DNA probes. The results for α_1 -antitrypsin mRNA were expressed as ratios to the value obtained for β -actin to correct for any variation in RNA yields. Preliminary experiments were conducted to: 1) assess the reproducibility and specificity of the dot-blot technique; and 2) confirm that there is a correlation between the amount of RNA applied to the membrane and the autoradiogram signal obtained using both the α_1 -antitrypsin and β -actin DNA probes.

3.10.1. Reproducibility and Specificity of the Dot-Blot Technique

To assess the reproducibility of the dot-blot technique, cytoplasmic RNA extracted from **from** 10^6 cells was dotted onto Gene-screen **TM** membrane **six** times. The blot was probed with the α_1 -antitrypsin DNA probe, subjected to autoradiography and the autoradiogram (figure 46) was scanned with a laser densitometer. The mean autoradiogram signal was **49043** (\pm SD **2588**) arbitrary densitometry units and the within-batch C.V. for the assay was **5.3%**. These **data** confirmed that the dot-blot technique was reproducible.

To confirm the specificity of hybridisation of the α_1 -antitrypsin probe, RNA extracted from the following cell **types** and subjected to dot-blot analysis using the α_1 -antitrypsin and β -actin DNA probes: 1) hepatocytes, monocytes and **U937** cells, cells that express the α_1 -antitrypsin gene (**1,312**); 2) neutrophils which contain α_1 -antitrypsin mRNA at a low copy number per cell (**148**); and 3) lymphocytes as a negative control. To confirm that the DNA probes hybridised to RNA species rather than to any DNA molecules that may have contaminated the sample, an identical aliquot of RNA purified from each cell **type** was incubated with RNAase for 1 h at **37°C** and also subjected to dot-blot analysis using the α_1 -antitrypsin and β -actin DNA probes.

The autoradiogram of the blot hybridised to the β -actin probe demonstrated that all of the RNA samples tested contained β -actin mRNA transcripts (figure 47 a). However, monocytes contained β -actin transcripts at a higher copy number per cell than **U937** cells and lymphocytes which, in **turn**, contained β -actin transcripts at a higher copy number per cell than neutrophils. Incubation of identical samples with RNAase almost completely abrogated the all the autoradiogram signals (figure 47 a), thus confirming that the β -actin probe hybridised to RNA rather than any DNA that may have contaminated the samples.

The autoradiogram of the membrane hybridised to the α_1 -antitrypsin probe demonstrated that: 1) monocytes contained α_1 -antitrypsin mRNA transcripts at a high copy number per cell; and 2) neutrophils and U937 contained relatively low numbers of α_1 -antitrypsin mRNA transcripts per cell when compared with monocytes (figure 47 b). A faint autoradiogram signal was obtained for lymphocytes which may be due to low level expression of the α_1 -antitrypsin gene by lymphocytes or reflect that the lymphocyte preparation contained about 5% contaminating monocytes. RNAase treatment of the samples prior to dot-blot analysis almost completely abrogated all of the autoradiogram signals, which confirmed that the α_1 -antitrypsin probe hybridised to RNA rather than any DNA that may have contaminated the samples (figure 47 b).

3.10.2. Quantification of Alpha₁-Antitrypsin and Beta-Actin mRNA in Monocytes using the Dot-Blot Technique

To confirm that there is a relationship between amount of α_1 -antitrypsin and β -actin mRNA applied to the membrane and the autoradiogram signal, cytoplasmic RNA was extracted from varying numbers of monocytes (10^4 to 4×10^5) and dotted onto two Gene-screen **TM** membranes. The blots were incubated with the α_1 -antitrypsin or β -actin DNA probes, subjected to autoradiography and **scanned** with a laser densitometer. There was a correlation between cell number and the intensity of the autoradiogram signal obtained using both the α_1 -antitrypsin and β -actin DNA probes when cytoplasmic RNA from up to 4×10^6 monocytes was applied to the membrane; $r = 0.985$ and 0.990 , respectively (figures 48 and 49). These data support the validity of using the dot-blot technique to quantify α_1 -antitrypsin and β -actin mRNA concentrations in monocytes.

Figure 46
Reproducibility of the Dot-Blot Technique



Figure 46 Autoradiogram of the dot-blot hybridised to the α_1 -antitrypsin probe. Cytoplasmic RNA from the equivalent of 10^6 human monocytes was applied six times to Gene-screen TM membrane. The membrane was incubated with the α_1 -antitrypsin genomic DNA probe, subjected to autoradiography for 7 days and the autoradiogram was scanned with a laser densitometer.

Figure 47
Specificity of the Dot-Blot Technique

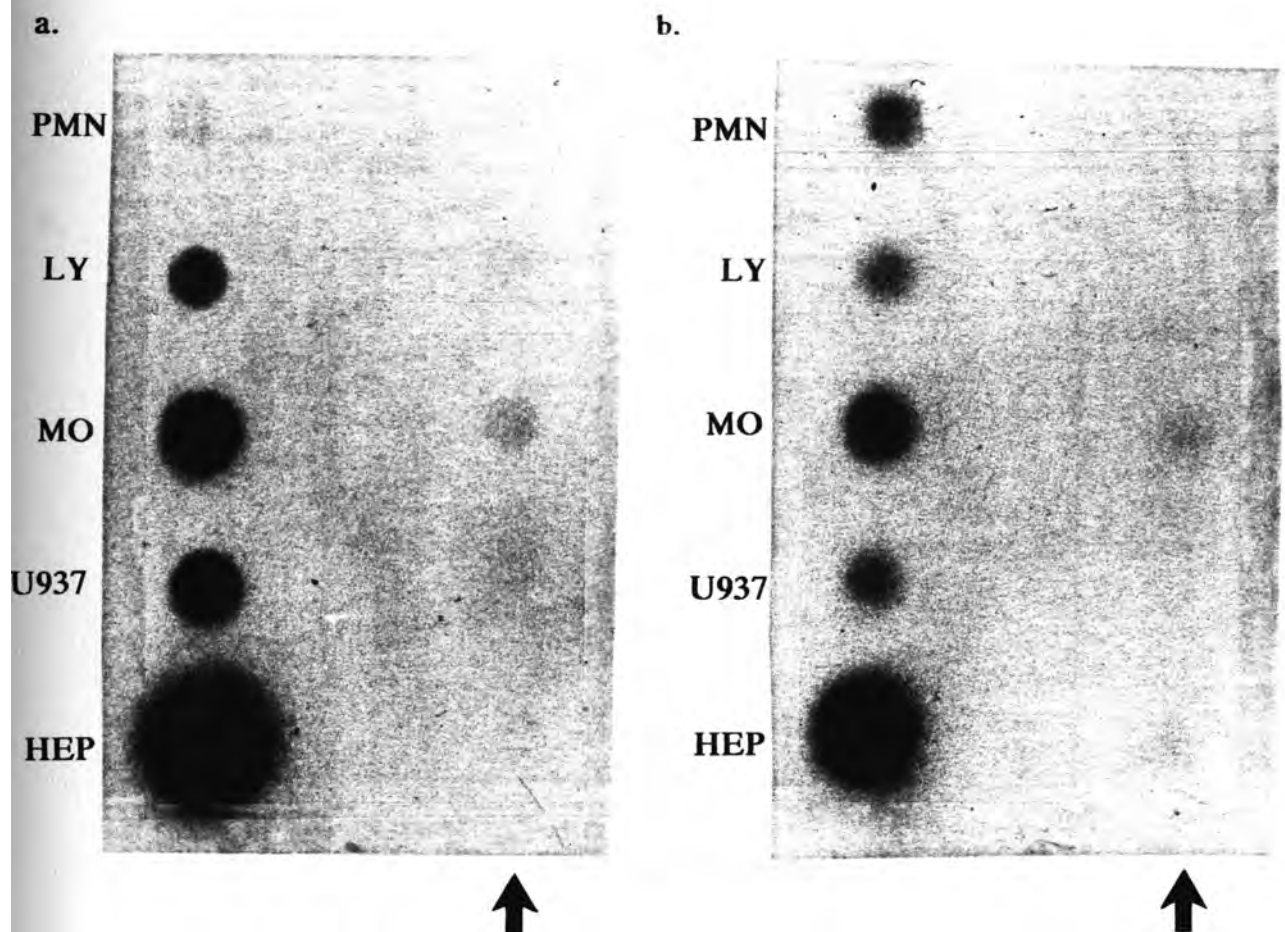
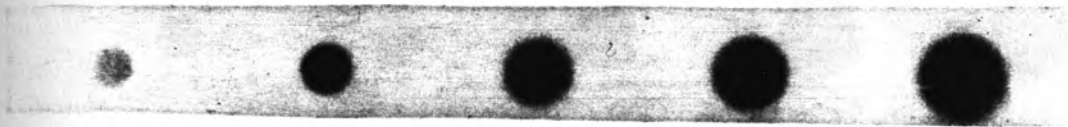


Figure 47 RNA was extracted from human liver (HEP), U937 cells (10^6 cells), 10^6 monocytes (MO), 10^7 lymphocytes (LY) and 10^7 neutrophils (PMN). Duplicate samples were treated with RNAase prior to dot-blot analysis (arrows). The membrane was incubated with the β -actin cDNA probe then stripped and reprobed with the α_1 -antitrypsin genomic DNA probe. **a.** Autoradiogram of the blot hybridised to the β -actin probe. **b.** Autoradiogram of the blot hybridised to the α_1 -antitrypsin probe.

Quantification of Alpha₁-Antitrypsin mRNA in Monocytes

a.



b.

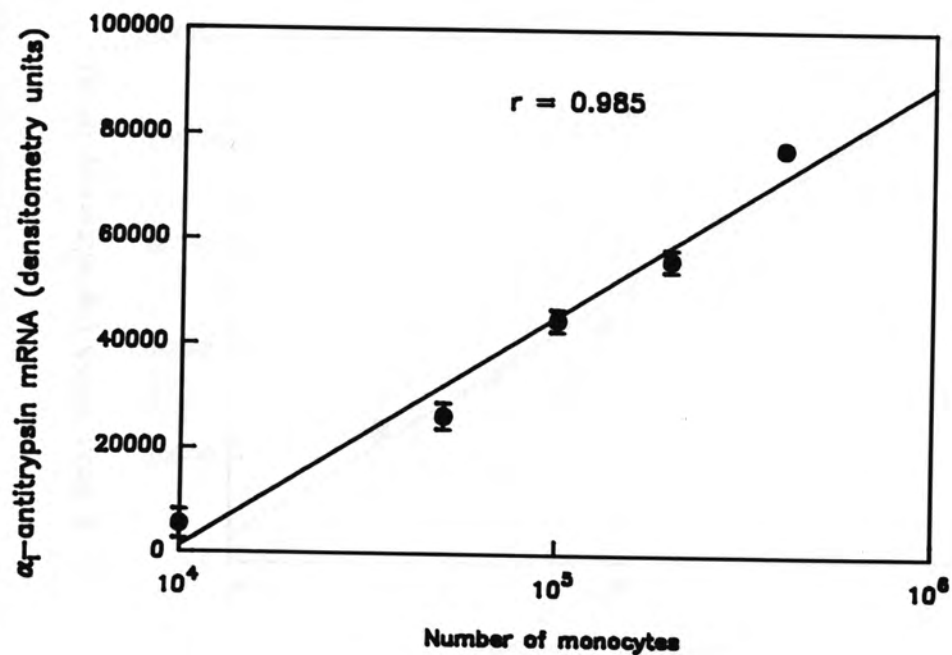


Figure 48 Cytoplasmic RNA was purified from varying numbers of monocytes (10^4 to 4×10^5) and applied to Gene-screen TM membrane. The membrane was hybridised to the α_1 -antitrypsin genomic DNA probe, subjected to autoradiography for 7 days and scanned with a laser densitometer. **a.** Autoradiogram of the dot-blot **b.** Quantification of α_1 -antitrypsin mRNA by densitometry scanning. The signals were scanned in triplicate. Error bars represent SEM, $r = 0.985$.

Figure 49

Quantification of Beta-Actin mRNA in Monocytes

a.



b.

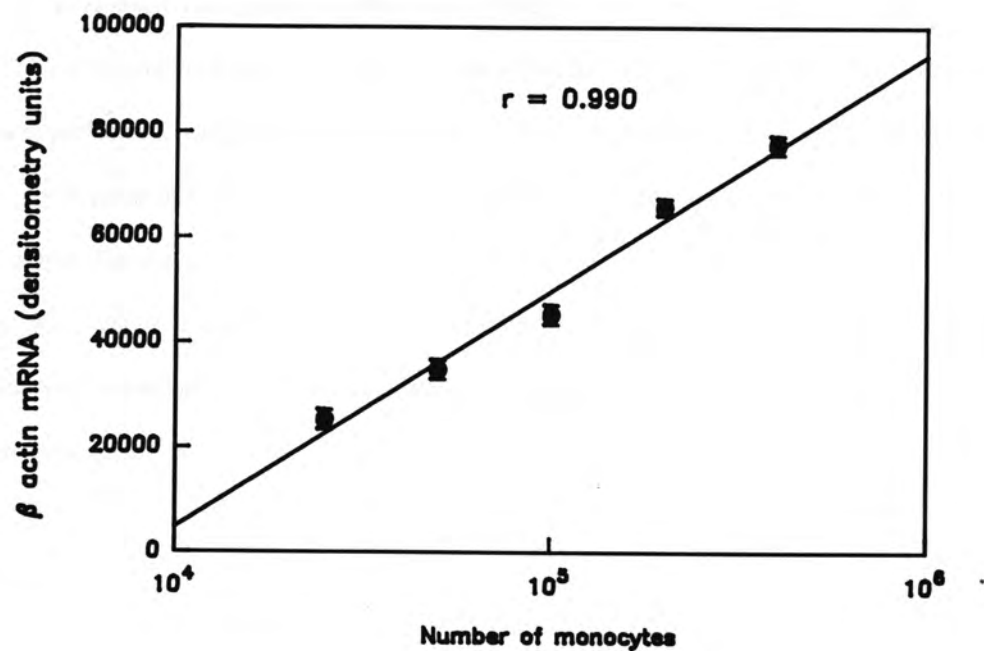


Figure 49 Cytoplasmic RNA was purified from varying numbers of monocytes (2.5×10^4 to 4×10^5) and applied to Gene-screen TM membrane. The membrane was hybridised to the β -actin cDNA probe, subjected to autoradiography for 3 days and scanned with a laser densitometer. **a.** Autoradiogram of the dot-blot **b.** Quantification of β -actin mRNA by densitometry scanning. The signal were scanned in triplicate. Error bars represent SEM; $r = 0.990$

3.10.3. Comparison of Steady State **Alpha₁-Antitrypsin mRNA** Levels in Monocyte Subpopulations

To compare their steady state α_1 -antitrypsin-specific mRNA concentrations, spontaneously adherent and non-spontaneously adherent monocytes were cultured under basal conditions for 48 h. Cytoplasmic RNA was extracted from the cells after culture for 24 h and 48 h, and dot-blot analysis was performed with the α_1 -antitrypsin and β -actin DNA probes. The dot-blots were subjected to autoradiography and the autoradiograms were scanned with a laser densitometer. Spontaneously adherent and non-spontaneously adherent monocytes contained similar steady state concentrations of α_1 -antitrypsin-specific mRNA after culture for 24 h (figure 50). Moreover, culture for up to 48 h under basal conditions did not alter steady state α_1 -antitrypsin-specific mRNA concentrations levels in either the spontaneously adherent or non-spontaneously adherent monocytes.

3.10.4. Effect of LPS and Cytokines on Steady State Concentrations of **Alpha₁-Antitrypsin mRNA** in Monocyte Subpopulations

To investigate whether LPS and cytokines increased monocyte α_1 -antitrypsin by increasing the transcription of the α_1 -antitrypsin gene, spontaneously adherent and non-spontaneously adherent cells were incubated for 24 h with and without LPS, TNF, IFN and GM-CSF. Cytoplasmic RNA was extracted and dot-blot analysis was performed using the α_1 -antitrypsin and β -actin DNA probes. LPS and cytokines did not alter steady state α_1 -antitrypsin mRNA concentrations in either the spontaneously adherent or non-spontaneously adherent monocytes (figures 51 and 52).

These data suggest that LPS and cytokines up-regulate α_1 -antitrypsin accumulation in spontaneously adherent monocytes by translational or post-translational mechanisms rather than by increasing the transcription of the α_1 -antitrypsin gene.

Figure 50
Steady State Alpha₁-Antitrypsin mRNA Concentrations in Monocytes

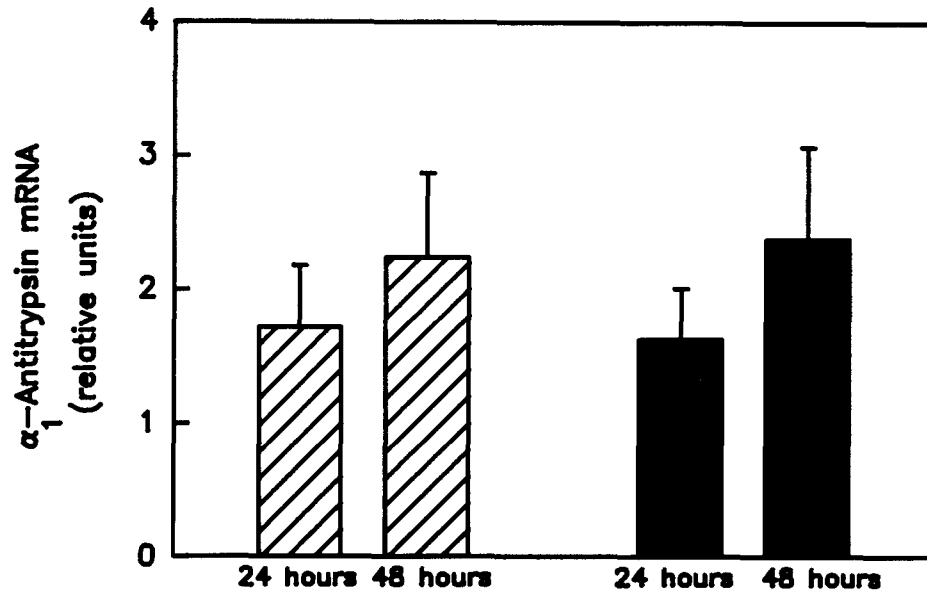


Figure 50 Spontaneously adherent (cross hatched bars) and non-spontaneously adherent monocytes (solid bars) were cultured under basal conditions up to 48 h. Cytoplasmic RNA was extracted and subjected to dot-blot analysis using the α_1 -antitrypsin and β -actin DNA probes. The results for α_1 -antitrypsin mRNA were expressed as ratios to the values for β -actin mRNA to correct for variation in RNA yield. Error bars are SEM; n = 11 donors.

Figure 51
Effect of LPS on Steady-State Alpha₁-Antitrypsin mRNA Levels in Monocytes

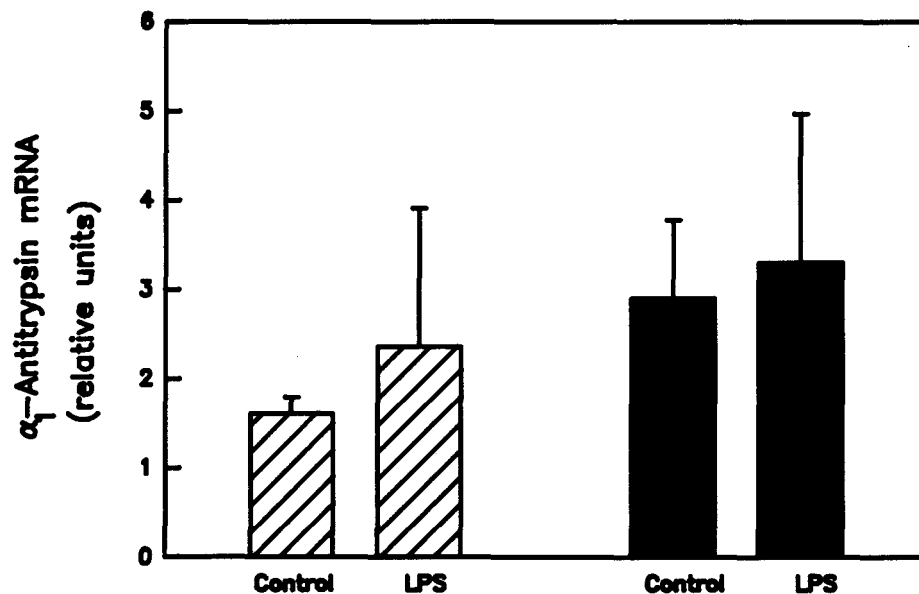
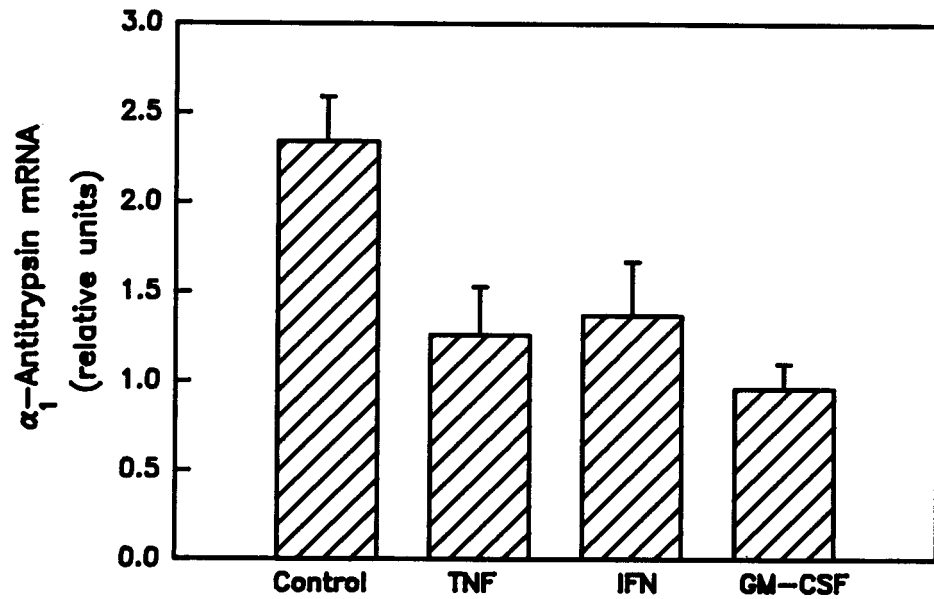


Figure 51 Spontaneously adherent monocytes (cross hatched bars) and non-spontaneously adherent monocytes (solid bars) were cultured for 24 h. Cells were unstimulated (control) or stimulated with 1 μ g/ml of LPS. Cytoplasmic RNA was extracted and subjected to dot-blot analysis using the α_1 -antitrypsin and β -actin DNA probes. The results for α_1 -antitrypsin mRNA were expressed as ratios to the values for β -actin mRNA to correct for variation in RNA yield. Error bars represent SEM; n = 8 donors.

Figure 52
Steady State Levels of Alpha₁-Antitrypsin mRNA in Monocytes
Effect of Cytokines

a. Spontaneously Adherent Monocytes



b. Non-Spontaneously Adherent Monocytes

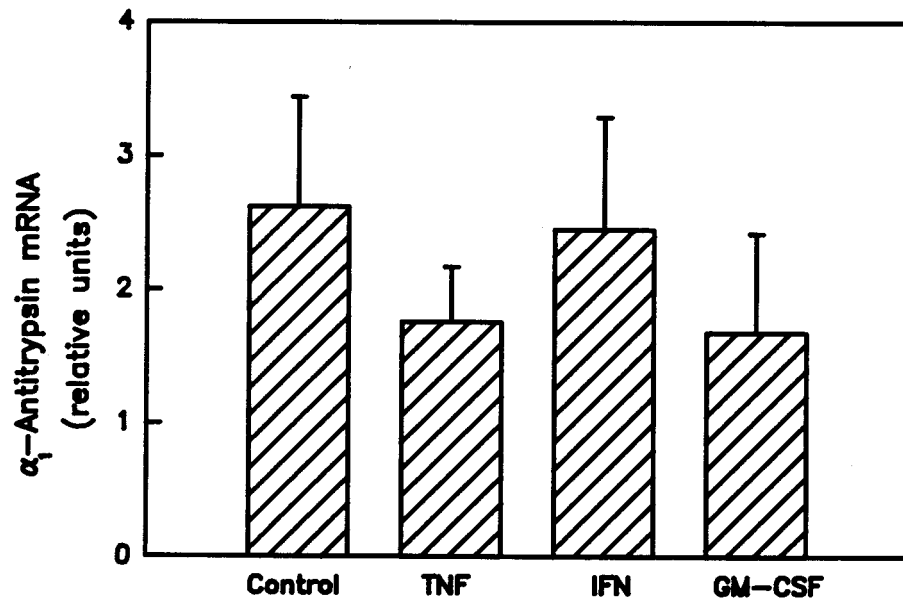


Figure 52 a. Spontaneously adherent and b. non-spontaneously adherent monocytes were cultured for 24 h. Cells were unstimulated (control) or stimulated with TNF (1000 units/ml), IFN (1000 units/ml) or GM-CSF (50 units/ml). Cytoplasmic RNA was extracted and dot-blot analysis was performed using the α_1 -antitrypsin and β -actin probes. The results for α_1 -antitrypsin mRNA were expressed as ratios to the values obtained for β -actin mRNA to correct for variation in RNA yield. Error bars represent SEM; n = 8 donors.

Chapter 4

Discussion

4.1. Overview

In order for monocytes to participate in inflammatory responses, it is necessary for them to be recruited from the vasculature into tissues. This process involves directional migration of monocytes in response to chemoattractant factors released from inflammatory foci, and reversible adherence of monocytes to endothelial cells. The next barriers that monocytes encounter, as they migrate into sites of inflammation, are the extracellular matrix components of the basement membrane and the subendothelial space. It is clear that regulated and reversible adherence of monocytes to extracellular matrix macromolecules is also a prerequisite for the accumulation of these cells at sites of tissue inflammation (172,423). However, the biological and pathological factors that regulate monocyte adherence to extracellular matrix, and the mechanisms involved have not been fully elucidated.

To begin to assess the pathobiological importance of alterations in monocyte adherence to extracellular matrix in inflammatory diseases, the adherence properties of monocytes from patients with an inflammatory lung disease (bronchiectasis) and healthy subjects to a representative matrix component (fibronectin) were compared. In addition, the effects upon monocyte adherence of various signals (LPS and cytokines) that are likely to be released at sites of infection and inflammation were assessed. The potential role of CD11/CD18 integrins in mediating monocyte adherence to fibronectin was investigated. Finally, the possibility was tested that there is a relationship between the adherence properties of monocytes and monocyte functions that are of biological and pathological importance at sites of inflammation.

A minor subpopulation (20 to 25%) of monocytes from healthy subjects adhered spontaneously to fibronectin. The proportion of adherent monocytes from patients with bronchiectasis was 2 to 3-fold greater than that of healthy subjects. Moreover, the adherence properties of monocytes from patients with bronchiectasis were related to the severity of pulmonary inflammation in the patients. LPS and several cytokines up-regulated monocyte adherence to fibronectin. Furthermore, CD11/CD18 integrins were involved in mediating the adherence of monocytes from the patients and healthy subjects to fibronectin. Finally, there was a striking relationship between the adherence properties of monocytes and functions that are of biological and pathological importance at sites of inflammation. Adherent monocytes had an "inflammatory effector" phenotype and non-adherent monocytes had an "immune-modulatory" phenotype. These data begin to provide insight into the mechanisms involved in the accumulation of monocytes at sites of infection and inflammation.

4.2. Monocyte Isolation

To study the adherence properties of monocytes, it was necessary, first, to optimise the monocyte isolation procedure. Although countercurrent centrifugal elutriation (CCE) is the gold standard method for isolating monocytes (293,395), the equipment that is required for this technique is expensive and not universally available. Most investigators, therefore, isolate monocytes by the two step ficoll-Hypaque method which exploits differences in the densities and the adherence properties of monocytes when compared with other blood cells. The first step in the ficoll-Hypaque method involves centrifugation of blood through ficoll-Hypaque which is a continuous density gradient medium. This step separates the mononuclear cells from the other cellular components of blood, since monocytes and lymphocytes have similar densities which are lower than those of granulocytes and erythrocytes (59). An adherence step is then performed to separate monocytes, which are assumed to be adherent cells (4,169), from lymphocytes which are predominantly non-adherent cells (256).

When the ficoll-Hypaque method was used to isolate monocytes for preliminary experiments, the yield of mononuclear cells provided by the ficoll-Hypaque density gradient step was close to the expected yield. However, only about 30% of the potential yield of monocytes were recovered following the adherence step. These data suggested that: 1) not all monocytes have the capacity to adhere in vitro; and 2) the use of an adherence step in the isolation procedure may result in the selection of a subpopulation of monocytes for study.

To test the hypothesis that the use of an adherence step in the monocyte isolation procedure reduces the monocyte yield, the yield of monocytes isolated by the ficoll-Hypaque method was compared with that obtained using Nycodenz osmotic and density gradient centrifugation, an alternative method that does not involve an adherence step (60). Nycodenz is a sterile solution of a tri-iodinated derivative of benzoic acid and is slightly hyperosmotic relative to plasma (mean osmolality 337 ± 5 mOsmol). The Nycodenz procedure exploits the fact that although the densities of monocytes and lymphocytes overlap (and, therefore, it is not possible to establish a satisfactory separation using a continuous density gradient alone), monocytes have a lower mean density than lymphocytes. When blood cells are subjected to hyperosmotic conditions during Nycodenz gradient centrifugation, they expel water, shrink, and their density increases. In this respect, lymphocytes are more sensitive than monocytes and sediment to the bottom of the test-tube along with the erythrocytes and granulocytes, leaving the monocytes at the top of the gradient. The Nycodenz method, therefore, does not require an adherence step to separate monocytes from lymphocytes.

Monocytes were isolated from **six** healthy subjects by both the ficoll-Hypaque and Nycodenz methods. The average yield of monocytes from the Nycodenz method (**2.4×10^5** cells per millilitre of whole blood) was 2-fold greater than that from the ficoll-Hypaque method (**1.3×10^5** cells per millilitre of whole blood) and represented **69%** of the potential yield of monocytes. These monocyte yields are in excellent agreement with those reported by other investigators for both the Nycodenz (**60,442**) and ficoll-Hypaque (**39**) methods.

The differences in the yield of monocytes isolated by the ficoll-Hypaque and Nycodenz methods could not be attributed to differences in the extent of lymphocyte or neutrophil contamination of the monocyte preparations since both methods provided monocytes that were greater than 90% pure, as assessed by modified Wright's stained cytocentrifuge preparations. In addition, it is unlikely that the low yield of monocytes **using** the ficoll-Hypaque method was due to a reduction in the viability of the cells recovered by this technique since greater than **99%** of the cells that failed to adhere during the adherence step were viable, as assessed by exclusion of trypan blue dye. It is likely that the lower yield of monocytes obtained by the ficoll-Hypaque method, when compared with the Nycodenz technique, is due to the fact that not all monocytes have the capacity to adhere in vitro since: **1)** the yield of mononuclear cells obtained by ficoll-Hypaque gradient centrifugation was approximately 90% of the potential yield of mononuclear cells; and **2)** only **36%** of the potential yield of monocytes was recovered by the adherence step. This hypothesis is supported by the previous findings of Chen and coworkers (**107**) that: **1)** **5** to 25% of non-adherent mononuclear cells are monocytes; and **2)** the yield of monocytes isolated by density gradient centrifugation and an adherence step **can** be increased 3-fold by conducting the adherence step in the presence of LPS. These data indicate that the **use** of an adherence step in the monocyte isolation procedure results in the selection of a minor subpopulation of cells for study.

The Nycodenz method failed to recover about 30% of the potential yield of monocytes. One possible explanation for this loss of monocytes is that these **cells** adhered to the vessels **used** in the isolation procedure. However, this possibility is unlikely since two measures are taken to **minimise** adherence of monocytes during isolation. First, **all** of the solutions used in the Nycodenz method (with the exception of the final washing solution) contain EDTA to chelate divalent cations. The rationale for this is that the adherence properties of monocytes are dependent upon the presence of divalent **cations** (**44**). Second, the entire Nycodenz isolation procedure (with the exception of the initial dextran sedimentation step) is performed at **4°C** which inhibits the

adherence of monocytes to biological substrates (363). An alternative explanation for the failure of the Nycodenz method to recover 30% of monocytes could be related to the fact that monocytes are heterogeneous in their densities (246,357). It is possible that the fraction of monocytes with the highest density sediments to the bottom of the test-tube along with the other cellular components of blood during the Nycodenz gradient step. To test this possibility, the cells that sedimented to the bottom of the test-tube during the Nycodenz isolation procedure were subjected to density gradient centrifugation to separate the mononuclear cells from the erythrocytes and granulocytes. Smears of these mononuclear cells stained for non-specific esterase activity confirmed that monocytes were present in this mononuclear cell layer. These data indicate that at least some of the monocytes that were not recovered by the Nycodenz method were lost as a result of their physical characteristics rather than their adherence properties.

The Nycodenz isolation procedure was used, instead of the ficoll-Hypaque technique, to isolate monocytes for assessment of their adherence properties for three reasons. First, the adherence properties of Nycodenz-isolated monocytes are more likely to be representative of all monocytes than cells isolated by the ficoll-Hypaque method since: 1) the Nycodenz technique recovers about 70% of the potential yield of monocytes, whereas the ficoll-Hypaque recovers only a minor subpopulation (about 36%) of monocytes; and 2) the ficoll-Hypaque method involves an adherence step and, therefore, selects for monocytes that have the capacity to adhere *in vitro*. Second, to assess the adherence properties of ficoll-Hypaque-isolated monocytes, it would be necessary to detach the adherent monocytes by mechanical scraping (62,257) or chemical methods such as exposure to chelating agents (270) or local anaesthetics (371,377). These procedures could induce functional changes in the cells. In contrast, the Nycodenz method does not involve an adherence step and the adherence properties of these cells can be assessed directly. Third, it is likely that monocytes are exposed to LPS during the ficoll-Hypaque isolation procedure and this may influence their adherence properties *in vitro* since: 1) ficoll-Hypaque gradient medium is frequently contaminated with LPS (190); and 2) LPS has been shown to affect the adherence properties of monocytes (141,390,486). In contrast, all of the solutions used in the Nycodenz isolation procedure contained concentrations of LPS that were below the lower limit of detection of the limulus amoebocyte lysate assay (10 pg/ml).

To confirm the purity of Nycodenz-isolated monocytes, smears of monocytes were stained routinely with modified Wright's stain and for non-specific esterase activity. The monocyte preparations were always greater

than 90% pure and contained less than 0.5% neutrophils, as assessed by these methods. In addition, immunohistochemistry was performed on smears of monocytes isolated from five donors, using monoclonal antibodies that recognise monocyte and lymphocyte surface antigens. The results confirmed that the monocyte preparations were greater than 90% pure and that the contaminating nucleated cells were predominantly lymphocytes.

Monocytes isolated by the Nycodenz method are always contaminated with platelets but the extent of platelet contamination varies considerably from donor to donor. Although this platelet contamination can be reduced substantially by centrifuging the cells through autologous plasma, this step reduces the monocyte yield by 25 to 30% and, therefore, was not employed on a routine basis in this study. In all subsequent studies of monocyte function, additional experiments were undertaken to exclude the possibility that contaminating platelets interfered in the assays.

4.3. Monocyte Adherence Assay

A quantitative assay was developed to assess the adherence of monocytes from healthy subjects and patients with bronchiectasis to a representative extracellular matrix component (fibronectin). Monocytes (10^6 cells) were incubated in fibronectin-coated tissue culture dishes for 1 h at 37°C. The non-adherent cells were removed by washing the dishes in a standardised manner. The non-adherent cells were counted using a haemocytometer, and the percentage of adherent cells was calculated from this number and the initial number of cells. Preliminary experiments were conducted to optimise the monocyte adherence assay.

4.3.1. Optimisation of the Monocyte adherence Assay

Fibronectin Substrate

To determine the optimal coating concentration of fibronectin, monocytes were incubated in dishes coated with varying concentrations of fibronectin purified from human serum. A dose-related increase in monocyte adherence to fibronectin was observed with a maximum of 25% of the cells adhering to dishes coated with 20 $\mu\text{g/ml}$ of fibronectin. Specificity of monocyte binding to fibronectin was confirmed by control experiments in which 1) only 2% of monocytes adhered to dishes with human serum albumin; and 2) incubating fibronectin-coated dishes with rabbit anti-human fibronectin **Fab₂** almost completely abrogated the subsequent adherence of monocytes, whereas non-immune rabbit **Fab₂** had no effect. The addition of proteinase inhibitors to the adherence assay did not alter the proportion of adherent monocytes when compared with cells incubated in medium alone. These data indicate that the binding of monocytes to fibronectin is not limited by any degradation of the fibronectin Substrate that may be mediated by monocyte-derived proteinases during the assay.

Other investigators have reported similar optimal coating concentrations of fibronectin for monocyte adherence (390). Moreover, the data on the adherence of monocytes from healthy subjects to fibronectin in **this** study are in close agreement to the previous findings of Brown and coworkers (63) that 25% of monocytes adhere spontaneously to fibronectin-coated plastic. The latter studies were performed using an alternative method to quantify monocyte adherence; monocytes were radiolabelled with ^{111}In and the proportion of adherent cells was determined directly using a gamma counter. The results of Brown *et al.*, therefore, support the validity of quantifying monocyte adherence by the indirect method that was used in the current study.

Alternative Sources of Fibronectin

Several investigators have studied monocyte adherence to plastic coated with autologous serum or plasma (107,139,140). Human plasma and serum contain several proteins to which monocytes adhere including: i) fibronectin (4463,390); 2) fibrinogen (500); 3) albumin (4%); 4) serum amyloid P component (4%); and 5) complement components (498). However, Bevilacqua et al. (44) showed by elegant depletion-repletion studies that when monocytes adhere to plastic coated with plasma or serum, they bind to the fibronectin component. Therefore, the adherence properties of monocyte to the following sources of human fibronectin were compared: 1) fibronectin purified from human serum; 2) complement-depleted autologous serum; and 3) complement-depleted autologous plasma. A greater proportion of monocytes adhered to plastic coated with purified human serum fibronectin (22%) compared with autologous serum (15%) or plasma (14%). These data are in agreement with the findings of Setiadi et al. (412) that a greater number of monocytes adheres to tissue culture plastic that has been coated with purified human fibronectin compared with homologous plasma (412).

It was surprising that a greater proportion of monocytes adhered to dishes coated with purified human serum fibronectin than to dishes coated with autologous serum or plasma, since the concentration of fibronectin in human plasma is 15 to 20-fold greater than the coating concentration of purified fibronectin that was used in this study (209). One possible explanation for these data is that when plastic is coated with plasma or serum, the albumin component competes with fibronectin for binding sites on the plastic surface, and thereby limits monocyte adherence. This hypothesis is supported by two observations: 1) the concentration of albumin is 100 to 200-fold greater than the concentration of fibronectin in serum and plasma (129); and 2) the proportion of monocytes that adhered to human serum albumin was 10 to 12-fold lower than the proportion that adhered to fibronectin in the current study. This possibility could be investigated by comparing the proportion of monocytes that adhere to dishes coated with purified human fibronectin and purified human fibronectin that has been spiked with varying concentrations of human serum albumin. Since a greater proportion of monocyte adhered to purified human serum fibronectin compared with autologous serum or plasma, dishes were coated with the optimal concentration of purified human serum fibronectin (20 µg/ml) in all subsequent experiments.

Culture Medium Additives

Human monocytes are usually cultured in serum-containing media since they are fastidious cells and they are difficult to maintain in a viable state when culture in the absence of serum (8,234). Adherence assays

are most commonly performed in the presence of 10% [v/v] complement-depleted fetal calf serum (**FCS**); however, some investigators ~~use~~ alternative medium additives including: **1)** human serum (**35,44,322**); **2)** human serum albumin (HSA), (**54,261,419**); and **3)** concentrations of autologous, homologous or heterologous serum as high as **20** to **25%** [v/v], (**107,219,390,398**). To assess whether the source, type or concentration of culture medium additive affects monocyte adherence to fibronectin, the adherence properties of cells suspended in medium containing the following supplements were compared: **1)** 10% and **25%** [v/v] **FCS**; **2)** 10% and **25%** [v/v] autologous serum; and **3)** 0.5% [w/v] HSA.

A similar proportion of monocytes adhered to fibronectin in the presence of 10% [v/v] **FCS**, 10% [v/v] autologous serum and 0.5% [w/v] HSA. However, the proportion of adherent monocytes was reduced by **30%** when the concentration of both **FCS** and autologous serum was increased from **10%** [v/v] to **25%** [v/v]. These data indicate that the concentration, rather than the source, of serum influences the adherence properties of monocytes in vitro. These findings are in agreement with the findings of Doherty et al. (**139**) that there was a 70% reduction in the proportion of adherent monocytes when the adherence assay was conducted in the presence of 50% [v/v] plasma when compared ^{with} cells incubated in the presence of with **10%** [v/v] plasma or in the absence of plasma.

It is likely that inhibition of monocyte adherence occurs in the presence of relatively high concentrations of serum (**25%** [v/v] or higher) but in not the presence of 10% [v/v] serum since: **1)** a similar proportion of monocytes adhered to fibronectin in the presence of 10% [v/v] **FCS** and 0.5% [w/v] HSA in the current study; and **2)** a similar proportion of monocytes adhered to serum-coated plastic in the presence 10% [v/v] plasma and in the absence of plasma in Doherty's study (**139**). The reduction in monocyte adherence that was induced by conducting the adherence assay in the presence of 25% [v/v] serum could be the result of plasma protein inhibition by a direct effect such as a stoichiometric competition for adherence sites on the monocyte, or a down-regulation of adherence sites on monocytes. The plasma protein(s) responsible for this inhibitory effect are not known but are unlikely to be: **1)** components of the complement system since **complement was inactivated** (by heating to **56°C** for **30 min**) prior to assay; or **2)** fibrinogen since inhibition of monocyte adherence was observed both in the presence of plasma in Doherty's study (**139**), and in the presence of serum in the current study. It is possible that the fibronectin component of serum-containing medium binds to monocyte fibronectin receptors, and thereby inhibits monocyte adherence to the fibronectin immobilised on the

dish. Serum contains monovalent fibronectin whereas fibronectin adsorbs onto plastic in a multivalent form, and cells bind to immobilised, multivalent fibronectin with substantially greater affinity than to soluble monomeric fibronectin (254). Therefore, one possible explanation for the finding that inhibition of monocyte adherence occurred in the presence of 25% [v/v] serum but not in the presence of 10% [v/v] serum is that there is a sufficient concentration of soluble, monomeric fibronectin in medium containing 25% [v/v] serum (but not in medium supplemented with 10% [v/v] serum) to inhibit monocyte binding to immobilised fibronectin.

Effect of Monocyte Number

To assess the effect of dispensing different numbers of monocytes into fibronectin-coated dishes on monocyte adherence, 5×10^5 to 3×10^6 monocytes were incubated in fibronectin-coated dishes and the proportion of adherent monocytes was determined. When 5×10^5 monocytes were added, 20% of the cells adhered to fibronectin-coated dishes. Visual assessment of the monocytes by phase-contrast microscopy confirmed the integrity of the underlying monocytes which adhered in an evenly distributed and non-aggregated pattern. The addition of up to 3×10^6 monocytes did not alter either the proportion of adherent cells or the appearance of the cells that were adherent to the dishes.

These data suggest that: 1) a minor subset of monocytes is intrinsically more adherent when compared with other monocytes; and 2) about 80% of monocytes remain in suspension during the assay because they do not have the capacity to adhere spontaneously to fibronectin, rather than because there is insufficient fibronectin substrate available to allow all of the cells to adhere. In view of these results, 10^6 to 2×10^6 monocytes were added to fibronectin-coated tissue culture dishes in subsequent experiments.

4.32. Comparison of the Adherence Properties of Monocytes Isolated by Nycodenz and CCE

Two potential problems were identified in assessing the adherence properties of monocytes isolated by the Nycodenz method. First, Nycodenz-isolated monocytes are always contaminated with platelets and one platelet product (platelet derived growth factor) has been shown to enhance monocyte adherence to endothelial cells (309). Therefore, it is possible that either the contaminating platelets or their products may influence the adherence of Nycodenz-isolated monocytes to fibronectin *in vitro*. Second, it is possible that the adherence properties of Nycodenz-isolated monocytes reflect a functional alteration in the cells induced by the Nycodenz isolation procedure, rather than intrinsic differences in their adherence properties. Third, the Nycodenz

procedure fails to recover about 30% of the potential yield of cells, and it is possible that the adherence properties of the cells recovered by the Nycodenz method are not representative of all monocytes. **To** investigate these possibilities, the adherence properties of monocytes isolated from healthy subjects by Nycodenz method were compared with those of monocytes isolated by: 1) the Nycodenz method followed by centrifugation through autologous plasma to remove platelets; and 2) CCE, which is the gold standard method for isolating monocytes. The proportion of monocytes that adhered spontaneously to fibronectin was similar for cells isolated by the three methods (20 to 25%). These results indicate that: 1) the contaminating platelets do not influence the adherence of Nycodenz-isolated monocytes to fibronectin in vitro; and 2) the Nycodenz procedure itself does not alter the subsequent adherence of monocytes to fibronectin in vitro. Moreover, since CCE recovers **a higher proportion** of the potential yield of monocytes (personal communication; Professor E.J. Campbell, University of Utah, Salt Lake City, Utah, U.S.A.), these data indicate that the adherence properties of the monocytes recovered by Nycodenz (about 70% of the potential yield) are representative of **all** monocytes.

4.4. Comparison of the Adherence Properties of Monocytes from Healthy Subjects and Patients with Bronchiectasis

4.4.1. Adherence Properties of Monocytes from Healthy subjects

To investigate the adherence properties of monocytes from healthy subjects, cells were isolated from ~~six~~ healthy donors on ~~six~~ occasions and the proportion of monocytes that adhered spontaneously to fibronectin under optimal assay conditions was determined (10^6 monocytes suspended in medium supplemented with 10% [v/v] FCS were dispensed into dishes coated with 20 $\mu\text{g}/\text{ml}$ of fibronectin). The mean proportion of adherent monocytes from the ~~six~~ donors only varied between 20.0% and 22.8% when the cells were isolated on different occasions. Moreover, there was minimal within-donor variability in the adherence properties of monocytes from these donors. Although between-donor variability in spontaneous monocyte adherence to fibronectin was observed, the proportion of adherent monocytes only varied between 17 to 28%. These data indicate that only a minor subpopulation of monocytes from healthy subjects adheres spontaneously to fibronectin in vitro.

Monocyte adherence to endothelial cells is a prerequisite for their recruitment from the vasculature into tissues. Therefore, to assess whether the capacity of monocytes to adhere to fibronectin in vitro reflects their potential to be recruited into tissues in vivo, monocyte adherence to fibronectin and human umbilical vein endothelial cell (HUVEC) monolayers was compared. Preliminary data, presented in Appendix 3, showed that a similar proportion (25%) of monocytes adhered spontaneously to fibronectin and to HUVEC monolayers. These data support the hypothesis that the capacity of monocytes to adhere to fibronectin in vitro reflects their potential to accumulate in tissues in vivo. Moreover, the data indicate that monocytes from healthy subjects are heterogeneous in their capacity to be recruited into tissues in vivo.

4.4.2. Adherence Properties of Monocytes from Patients with Bronchiectasis

To assess the effect of inflammation on monocyte adherence, the adherence properties of monocytes from healthy donors and patients with an inflammatory lung disease (bronchiectasis) to fibronectin were compared. On average, 20% of monocytes from healthy subjects adhered spontaneously to fibronectin. In comparison, the proportion of adherent monocytes from patients with bronchiectasis was 2 to 3-fold greater than that of healthy subjects. Moreover, the adherence properties of monocytes from bronchiectasis patients were related to the nature of the sputum produced by the patients, which in turn, has been shown to reflect the

severity of pulmonary inflammation(200). A greater proportion of monocytes from patients producing purulent sputum adhered to fibronectin compared with cells from patients producing mucoid or mucopurulent sputum. These data suggest that the increased adherence properties of monocytes may enhance the recruitment of monocytes into the lungs of patients with bronchiectasis.

It is possible that soluble mediators that are released from sites of pulmonary infection and inflammation up-regulated the adherence of the patients' monocytes to fibronectin since bacterial endotoxin has been shown to enhance the accumulation of leukocytes in vivo (140,308). Since the lungs of patients with bronchiectasis are often colonised with gram negative bacteria which are the source of endotoxin, plasma samples from patients with bronchiectasis were assayed for endotoxin activity. The data show that endotoxin activity was detected in all of the plasma samples from patients with bronchiectasis but not in plasma from healthy subjects. These results suggest that endotoxin is released from the lungs of patients with bronchiectasis and plays a role in activating circulating monocytes to adhere to fibronectin in vitro. This hypothesis is strengthened by the additional findings that: 1) plasma endotoxin concentrations increased with increasing clinical evidence of airway inflammation; and 2) there was a striking correlation between the level of endotoxaemia in bronchiectasis and the capacity of the patients' monocytes to adhere to fibronectin in vitro.

4.43. Effect of Pro-Inflammatory Mediators on Monocyte Adherence

To explore the potential role of **LPS** in vivo in increasing monocyte adherence in the patients, monocytes from healthy subjects were incubated with **LPS**. **LPS** exposure in vitro stimulated monocytes from healthy subjects to adhere to fibronectin. **LPS** from *E. coli* 0111:B4 produced a dose-related increase in the adherence of healthy monocytes to fibronectin with a maximum of 56 to 65% of cells adhering in the presence of 1 µg/ml of **LPS**. This was similar to the proportion of monocytes from purulent patient group that adhered spontaneously. These data provide further support for the hypothesis that monocytes from patients with bronchiectasis may have been activated to adhere to fibronectin in vitro by exposure to **LPS** in vivo.

The effects of **LPS** from *E. coli* 0111:B4 and *H. influenzae* on monocyte adherence to fibronectin were compared since: 1) the lungs of patients with bronchiectasis are usually colonised with several bacterial species and it is likely that circulating monocytes of patients with bronchiectasis are exposed to **LPS** from more than one bacterial species in vivo; and 2) **LPS** from different bacterial species have different regulatory effects on

several neutrophil functions in vitro (247). LPS from both bacterial species produced a dose-related increase in monocyte adherence to fibronectin and stimulated a maximum of about 65% of monocytes to adhere at a concentration of **1 µg/ml**. However, at suboptimal concentrations of LPS, monocytes were found to be more responsive to the effects of LPS from *H. influenzae* when compared with LPS from *E. coli*. This latter finding was of particular interest since *H. influenzae* is a common pathogen in bronchiectasis (297) and the biological effects of LPS from this bacterial species are likely to be relevant to the pathophysiology of bronchiectasis. It is likely that monocytes also vary in their responsiveness to LPS released from other gram negative bacterial species that colonise the lungs of patients with bronchiectasis.

The effect of LPS from *E. coli* **0111:B4** on the adherence of monocytes from patients with bronchiectasis was also assessed. LPS stimulated further increases in the adherence of monocytes from patients with bronchiectasis, indicating that monocytes from the patients had been less than maximally activated in vivo. However, even in the presence of optimal concentrations of LPS, a greater proportion of monocytes from the patients adhered to fibronectin compared with LPS-treated cells from healthy subjects. These data indicate that factors in addition to LPS may have activated the patients' monocytes in vivo.

To begin to address the importance of additive effects of in vivo signals, the effect of several recombinant cytokines on monocyte adherence to fibronectin were assessed. GM-CSF, IFN and **TNF** increased the adherence of monocytes from healthy subjects to fibronectin. Moreover, cytokines (**TNF** and **IFN**) and LPS produced additive effects on monocyte adherence. These results suggest that LPS and various cytokines, when released from foci of infection and inflammation in the **lungs** of patients with bronchiectasis, activate circulating monocytes to produce increased adherence of the **cells** to extracellular **matrix** components.

The data show that the adherence of monocytes from patients with bronchiectasis is greater than cells from healthy subjects and is related to the severity of airway inflammation in bronchiectasis. Although it has been shown previously that a greater proportion of monocytes from patients with insulin-dependent diabetes mellitus adhere to fibronectin compared with monocytes from healthy subjects (412), there have been no previous studies of monocyte adherence in inflammatory disease states. It is unlikely that the results in the current study are due to the age difference between the patients and healthy controls (Methods, **section 2.6.**) since Setiadi *et al.* (412) showed that the age of the donor does not affect monocyte adherence to fibronectin. It is likely that the increased adherence of monocytes from patients with bronchiectasis in vitro is the result of

the release of LPS and cytokines from sites of airway infection and inflammation in vivo.

In the current study endotoxin activity was measured in plasma from all of the patients with bronchiectasis and was related to the severity of airway inflammation. Other investigators have ~~used~~ a qualitative limulus assay to demonstrate that endotoxin activity was present in plasma from 50% of patients with bronchiectasis (208). However, endotoxin concentrations in plasma from bronchiectatic patients have not been quantified previously, or related to the severity of airway inflammation.

Two problems have been identified when the limulus assay is used to measure LPS concentrations in plasma. First, plasma samples frequently contain non-specific activators and inhibitors of the limulus enzyme (115). However, in ~~this~~ study, measures were taken to minimise the effects of these factors; dilution and heat inactivation of the plasma samples (115). Second, LPS from different bacterial species differ in their capacity to activate the limulus enzyme (115). Although the lungs of patients with bronchiectasis are usually colonised with several gram negative bacterial species, LPS from *E. coli* 0111:B4 was used as the only reference standard in the assay. Therefore, the results ~~in~~ the current study do not necessarily reflect the absolute quantity of circulating endotoxin in our patients. However, the results demonstrate clearly that endotoxin activity is present in the plasma of bronchiectatic subjects and is related to the severity of airway inflammation.

In the current study, LPS stimulated a 3-fold increase in the proportion of adherent monocytes. These data are in agreement with the studies of Chen and coworkers (107) who showed that LPS exposure results in a 3-fold increase in the yield of monocytes isolated by adherence to serum-coated plastic. Recent data from Roth and coworkers (390) differed somewhat from the results of the current study since they found monocyte adherence to be less responsive to LPS; they reported only a 2-fold increase in adherence following exposure to a 50-fold higher LPS concentration than were maximally effective in the current study. These differences were not due to the use of LPS from different bacterial species since Roth and coworkers also investigated the effects LPS from *E. coli* 0111:B4 on monocyte adherence. It is likely that technical differences ~~in~~ the monocyte separation procedures explain the relatively lower LPS responsiveness reported by Roth and coworkers. In their report: 1) the monocyte separation procedure itself involved an adherence step; and 2) the separation procedure included exposure to ficoll-Hypaque which is contaminated frequently with LPS (190). Thus, it ~~is~~ likely that the monocytes in the study of Roth and coworkers were partially activated during isolation and were consequently less responsive to LPS in the subsequent fibronectin adherence assay.

The different responsiveness of monocytes to **LPS** from *E. coli* and *H. influenzae* is not surprising since Kharazmi *et al.* (247) showed recently that **LPS** purified from multiple strains of a single bacterial species (*P. aeruginosa*) produces different regulatory effects on neutrophil function in vitro. The differences in the biological effects of **LPS** from the different bacterial strains in Kharazmi's study were related to differences in the chemical composition of **LPS**, in particular: 1) in the rhamnose and heptose content of the O-group sugar side chain; and 2) in the alanine content of the core region. It has been postulated that the chemical structure of **LPS** plays a decisive role in the binding of **LPS** by leukocytes (240). **LPS** has been shown to bind to monocytes by specific and non-specific mechanisms (314). Specific interactions result from the binding of **LPS** to specific receptors on the plasma membrane of the monocyte and are believed to be mediated by the O-antigen and core polysaccharide components of the **LPS** molecule. Non-specific binding of **LPS** to the plasma membrane is probably the most common mechanism by which **LPS** enters the monocyte (NO) and probably results from the interaction of the hydrophobic lipid A portion of the **LPS** with the plasma membrane phospholipids, leading to solubilisation of the **LPS** lipids into the membrane (413). It has been postulated that differences in the carbohydrate and lipid composition of **LPS** result in differences in the capacity of **LPS** to bind to monocytes which, in turn, may account for the different biological activities of **LPS** from different species and strains of bacteria (489). It is likely that differences in the chemical composition of **LPS** from *E. coli* and *H. influenzae* account for differences in monocyte responsiveness in the current studies.

The endotoxin activity measured in plasma from the bronchiectatic patients was three orders of magnitude lower than that required to stimulate maximal adherence of healthy monocytes. There are several possible explanations for this discrepancy. First, monocytes passing through the pulmonary circulation in patients with bronchiectasis are likely to be exposed to higher local concentrations of **LPS** than were measured in plasma derived from venous blood. Second, it is likely that circulating monocytes in patients with bronchiectasis are exposed to **LPS** from bacterial species and strains other than those studied here, and it is possible that these endotoxins are more potent at activating monocytes in vivo than **LPS** from *E. coli* and *Haemophilus influenzae*. Third, the concentrations of endotoxin measured in the plasma from patients with bronchiectasis may not reflect the absolute quantity of circulating endotoxin, for the reasons stated previously. Fourth, it is likely that circulating monocytes in patients with bronchiectasis are also activated by cytokines that are released from the inflammatory sites, since several cytokines stimulated monocyte adherence in vitro and had additive effects with

LPS in the current study. Further support for this latter possibility is provided by the findings of Suter et al. (434) who detected several cytokines (TNF, **interleukin-1 α** and **interleukin-1 β**) in plasma from patients with bronchiectasis due to cystic fibrosis. Local release of such cytokines in patients with chronic airway inflammation can be expected to have a variety of other effects, including amplifying the inflammatory response (310), and stimulating degranulation of inflammatory cells (253). In addition, LPS stimulates the release of cytokines such as TNF, GM-CSF from mononuclear phagocytes in vitro (106,441). Thus endotoxin released at sites of airway infection and inflammation may also increase the adherence of circulating monocytes indirectly, by stimulating the release of cytokines from monocytes and macrophages located in the airways and peribronchial space in patients with bronchiectasis.

4.5. Mechanisms Involved in Monocyte Adherence to Fibronectin

The mechanisms involved monocyte adherence to fibronectin were investigated. Monocyte adherence to extracellular matrix components has been thought to be mediated predominantly by the B1 or "very late antigens" (VLA) subfamily of heterodimeric integrin receptors (216,300). The VLA receptors share a common $\beta 1$ chain which associates with different α chains ($\alpha 1$ to $\alpha 6$), (197). For example, VLA 5 ($\alpha 5\beta 1$) is thought to be the most important integrin that mediates monocyte adherence to fibronectin by binding to Arg-Gly-Asp (RGD) sequences in the cell-binding domain of fibronectin (63,195,256,368). However, recent studies have shown that specific anti-VLA 5 antibodies and RGD-containing peptides fail to inhibit monocyte adherence to fibronectin completely, indicating that other integrins may also be involved (256).

The CD11/CD18 or $\beta 2$ subfamily of integrins is involved in mediating the adherence of monocytes to endothelial cells (216). The possibility that CD11/CD18 integrins are involved in mediating monocyte adherence to fibronectin was considered since: 1) LPS, TNF and GM-CSF stimulated monocytes to adhere to fibronectin in the current study; and 2) other investigators have shown that LPS, TNF and GM-CSF increase monocyte cell-surface expression of CD11/CD18 integrins (309,419). Moreover, Bohnsack et al. (55) demonstrated that CD11/CD18 integrins are involved in mediating the adherence of stimulated (but not unstimulated) neutrophils to several extracellular matrix components.

The data in the current study show clearly that CD11/CD18 integrins are involved in mediating monocyte adherence to fibronectin, since an anti-CD18 antibody produced substantial inhibition of both basal and LPS-stimulated adherence of monocytes to fibronectin. In contrast, control antibodies that bind to other monocyte surface antigens had no effect on monocyte adherence. Similarly, non-immune murine IgG also failed to inhibit monocyte adherence. Together, these control experiments exclude the possibilities that the anti-CD18 antibody blocked monocyte adherence non-specifically, for example by steric hindrance or through Fc receptor interactions. Inhibition of monocyte adherence by anti-CD18 was incomplete, indicating that either: 1) the CD18 antibody is not 100% efficient; or 2) other integrins also play a role in monocyte adherence to fibronectin.

Many integrins recognise and bind to RGD sequences within ligands, and some CD11/CD18 recognition events have been shown to be RGD-dependent (498). To assess the role of RGD-dependent integrins, monocytes were incubated with a synthetic RGDS peptide. RGDS, but not a control peptide, produced a dose-dependent inhibition of both basal and LPS-stimulated adherence of monocytes to fibronectin.

However, RGDS also failed to inhibit monocyte adherence to fibronectin completely, suggesting that monocyte adherence to fibronectin has both RGD-dependent and RGD-independent components. Furthermore, since optimal concentrations of RGDS and anti-CD18 together resulted in greater inhibition of basal and **LPS**-stimulated adherence than either alone, CD11/CD18-mediated monocyte adherence to fibronectin is, at least in part, RGD-independent.

The mechanisms involved in the adherence of monocytes from patients with bronchiectasis to fibronectin were also investigated. The data showed that CD11/CD18 integrins are also involved in mediating the adherence of monocytes from the patients to fibronectin since the anti-CD18 antibody, but not a control antibody, produced substantial inhibition of the adherence of monocytes from patients with bronchiectasis to fibronectin. However, anti-CD18 mediated inhibition was again incomplete, indicating that other integrins may be involved. In addition, RGDS (but not a control peptide), also resulted in substantial but incomplete inhibition monocyte adherence to fibronectin. Furthermore, optimal concentrations of anti-CD18 and RGDS together resulted in greater inhibition than either alone. These data indicate that monocytes from patients with bronchiectasis adhere to fibronectin by both RGD-dependent and RGD-independent mechanisms, and that CD11/CD18-mediated adherence of the patients' monocytes to fibronectin is, at least in part, RGD-independent.

The mechanism(s) by which **LPS** and cytokines up-regulate the adherence of monocytes to fibronectin is unknown. However, **LPS** and cytokine-stimulated adherence of normal monocytes to fibronectin was maximal within 30 min and is likely to be independent of new protein synthesis. Monocytes have been shown to have an intracellular store of preformed CD11/CD18 integrins within peroxidase negative granules (309,419). Furthermore **LPS**, GM-CSF and TNF increase the expression of CD11/CD18 integrins rapidly by stimulating the fusion of these granules with the plasmalemma (182,309,419). It is possible that exposure of monocytes to **LPS** and cytokines in vitro or in vivo increases the expression of CD11/CD18 integrins, resulting in increased adherence of monocytes to fibronectin. This concept is supported by the findings of Setiadi et al. (412) that monocytes from diabetic subjects adhere in greater numbers to fibronectin and have increased expression of CD11b/CD18 compared with cells from healthy subjects.

Cytokines and optimal concentration of **LPS** produced additive effects on the adherence of monocytes to fibronectin, indicating that **LPS** and cytokines stimulate monocyte adherence through different mechanisms. In this respect it is of interest that CD11/CD18 integrins possess a binding site for **LPS** that is distinct from the

RGD-binding site (497). It is possible that binding of **LPS** to this site results in increased receptor affinity for fibronectin rather than increased receptor expression.

In summary, these data show that soluble mediators that are released from sites of infection and inflammation have the capacity to alter monocyte adherence to extracellular matrix components, and thereby may regulate the accumulation of monocytes in the lungs of patients with bronchiectasis. The activation of circulating monocytes may promote resolution of tissue infection and inflammation. However, the lungs of patients with bronchiectasis remain colonised with bacteria despite the increased capacity of monocytes to adhere. Since monocytes contain proteolytic enzymes that are capable of degrading connective tissue (81), it is possible that excessive recruitment of monocytes may initiate or potentiate tissue damage. Moreover, once tissue damage has been established, the disease may progress in some patients with bronchiectasis as a result of a continuing "vicious circle". Tissue damage impairs mucociliary clearance leading to mucous retention (279). Bacterial colonisation and phagocyte recruitment into the lung then ensue (124) which result in tissue damage, thereby completing the vicious circle.

4.6. Monocyte Phenotype Evaluations

The initial data showed that monocytes from healthy subjects are heterogeneous in their capacity to adhere to fibronectin. Since movement of cells through tissues depends upon regulated adherence of the cells to extracellular matrix macromolecules (172,423), the data suggest that monocytes are heterogeneous in their ability to accumulate at sites of infection and inflammation. This hypothesis is supported by the preliminary finding that a similar proportion of monocytes adhered to fibronectin and to endothelial cell monolayers in vitro (Appendix 3).

Four subpopulations of monocytes were identified by their differential adherent properties. A minor subpopulation of monocytes (20 to 25%) adheres spontaneously to fibronectin (*spontaneously adherent monocytes*); of the monocytes that do not adhere to fibronectin under basal conditions (*non-spontaneously adherent cells*), a proportion (41 to 56%) can be stimulated to adhere by LPS (*LPS-adherent monocytes*), while the remainder cannot (*non-adherent monocytes*). The authenticity of these subpopulations was confirmed by the **finding** that each retained its original adherence properties when subjected to a second identical adherence assay. To assess the biological relevance of these monocytes subpopulations, further studies were conducted to determine whether there is a relationship between the capacity of monocytes to adhere to fibronectin and phenotypes that are of biological importance at sites of infection and inflammation.

Antimicrobial Function of the Monocyte Subpopulations

The antimicrobial phenotypes of the monocyte subpopulations were compared *since* monocytes have important antimicrobial functions at sites of infection and inflammation (236,267). The antimicrobial phenotypes evaluated were phagocytic activity, and primary and secondary oxidative responses (superoxide anion release and peroxidase activity, respectively). The capacity of monocytes to adhere to fibronectin was related to these antimicrobial functions. The spontaneously adherent monocytes were more active than the non-adherent monocytes with respect to phagocytosis, superoxide release and peroxidase content (14-fold, 2-fold and 20-fold, respectively). Furthermore, the LPS-adherent cells had an intermediate antimicrobial phenotype; the LPS-adherent cells were 4-fold more actively phagocytic, contained 2-fold more peroxidase activity, but released similar amounts of superoxide anion when compared with the non-adherent monocytes.

To control for possible direct effects of LPS on the non-spontaneously adherent cells, spontaneously adherent monocytes were also pre-incubated with LPS prior to assessment of their antimicrobial function. Pre-

incubation with LPS did not alter superoxide release or Peroxidase content of the spontaneously adherent cells. LPS exposure did result in a significant reduction in phagocytic activity; however, the LPS-stimulated spontaneously adherent cells were **still** more actively phagocytic than the non-spontaneously adherent, LPS-adherent and non-adherent monocytes. LPS may be somewhat toxic to freshly-isolated monocytes since it has been reported to decrease the synthesis of intracellular and secreted proteins (77). Although exposure of monocytes to LPS was not associated with a reduction in cell viability, as assessed by exclusion of trypan blue, the reduction in phagocytic activity of the spontaneously adherent cells on exposure to LPS could represent a more subtle toxic effect of LPS. These data indicate that circulating monocytes are heterogeneous with respect to antimicrobial functions, and that there is a relationship between these and the adherence properties of monocytes.

The differences in antimicrobial functions between the monocyte subpopulations could not be explained by differences in cell viability or by neutrophil or lymphocyte contamination of the monocyte preparations (less than 0.5% and 9%, respectively). It is possible that a subpopulation of monocytes could have been activated differentially during the Nycodenz isolation procedure. However, this is unlikely since preliminary results on monocytes isolated by CCE confirm that a minor subpopulation of monocytes adheres spontaneously to fibronectin and that there is a relationship between monocyte adherence and superoxide anion release (Appendix 4). The possibility remains that the subpopulation of monocytes that did not adhere in the presence of LPS were damaged despite their viability, as assessed by exclusion of trypan blue dye.

Although the phagocytic activity of adherent and non-adherent monocytes has not been compared previously, it has been shown that monocytes adherent to immobilised fibronectin have enhanced C3-mediated phagocytic activity compared with cells adherent to human **serum** albumin (4%). It is possible that the interaction of fibronectin receptors with immobilised fibronectin on the basal surface of the monocyte serves to activate or to increase the expression of receptors for phagocytosis on the apical surface, resulting in increased phagocytic activity of the adherent monocyte subpopulations. Whatever the explanation for these differences, the data suggest that LPS released from gram negative bacteria at sites of inflammation, may stimulate the accumulation of a subpopulation of monocytes with an enhanced capacity to ingest microbes, tissue debris and effete cells, and thereby promote the resolution of tissue infection and inflammation.

Although superoxide release by the spontaneously adherent cells in this study was similar to values

reported for superoxide release by monocytes adherent to plastic and collagen (330), superoxide release by non-adherent monocytes has not been measured previously. B-cytochrome, which is involved in superoxide generation, is located within the same intracellular granules as $\beta 2$ integrins (56). It is possible that monocytes with the capacity to adhere to fibronectin spontaneously are enriched for these granules. Alternatively, it is possible that spontaneously adherent monocytes have been activated in vivo, resulting in the translocation of these granules to the plasmalemma. This, in turn, could not only increase their surface expression of fibronectin receptors and promote their adherence to fibronectin, but also facilitate interaction of b-cytochrome with the plasmalemma-associated NADPH oxidase system, and thereby increase superoxide anion release by these cells.

The peroxidase content of adherent and non-adherent monocytes has not been compared previously. However, marked heterogeneity in monocyte staining for peroxidase has been demonstrated; 20% of monocytes stain intensely for peroxidase activity, 60% of monocytes are unstained and the remainder vary greatly in staining intensity (242). The mechanism for the differences between the subpopulations in the current study is not known; however, it is unlikely to be due to differential release of peroxidase activity during the adherence assays, since minimal enzyme activity was detected in the culture supernatants. It is possible that monocytes with the capacity to adhere are enriched for peroxidase containing granules since it has been shown that monocytes are heterogeneous in their content of peroxidase positive granules (9). This possibility could be tested by performing peroxidase histochemistry on cytocentrifuge preparations of the monocyte subpopulations.

Whatever the explanation for the differences in superoxide anion production and peroxidase content among the monocyte subpopulations, the data suggest that LPS released from gram negative bacteria at sites of infection, may stimulate the accumulation of a subpopulation of monocytes with an enhanced capacity to **kill** microbes, and thereby promote resolution of tissue infection and inflammation.

The data demonstrate that there is a striking relationship between the adherence properties of monocytes and several important antimicrobial functions. It is possible that functionally distinct subpopulations of monocytes exist, with adherent cells possessing an "inflammatory effector" phenotype. Alternatively, the subpopulations may represent a continuous spectrum of monocyte function. These functional differences could be related to differences in maturation, differentiation or activation. These possibilities are discussed later (4.8.).

in conclusion, the data show clearly that monocytes are heterogeneous with respect to their adherence properties. In addition, there is a striking relationship between: 1) the capacity of monocytes to adhere to

fibronectin in vitro, which may relate to their potential to be recruited into tissues in vivo; and 2) various antimicrobial functions that are of biological importance at sites of infection and inflammation. Furthermore, LPS released from gram negative bacteria at sites of inflammation may stimulate the recruitment of cells with an "inflammatory effector" phenotype, and thereby promote the resolution of tissue infection and inflammation.

HLA-DR Antigen Expression by the Monocyte Subpopulations

Mononuclear phagocytes are important antigen presenting cells for T lymphocytes and play a critical role in the regulation of immunological responses (454). It has been shown that the amount of HLA-DR antigen expressed by the mononuclear phagocyte correlates directly with their antigen presenting capacity (454,296,507). Therefore, to assess whether the ability of monocytes to adhere to fibronectin is related to their capacity to present antigen to lymphocytes, monocytes were labelled with I3, a murine monoclonal antibody that recognises non-polymorphic HLA-DR antigen (443), using the indirect immunofluorescence technique.

An initial assessment of HLA-DR antigen expression by pool monocytes by phase-contrast and epifluorescence microscopy demonstrated that the majority of monocytes (greater than 90%) expressed HLA-DR antigen. However, there was marked heterogeneity in HLA-DR antigen expression by monocytes. The monocyte subpopulations were subsequently labelled with i3 antibody, and I3 binding was quantified by flow cytometry. There was an indirect relationship between the capacity of monocytes to adhere spontaneously to fibronectin and the expression of the HLA-DR antigen recognised by I3 since the mean fluorescent intensity of the non-spontaneously adherent monocytes was 3-fold greater than that of the spontaneously adherent cells. Moreover, the non-spontaneously adherent and spontaneously adherent monocytes expressed 6-fold and 2-fold more HLA-DR antigen, respectively, than the pool monocytes. However, there were no significant differences in the expression of HLA-DR antigen by non-spontaneously adherent, LPS-adherent and non-adherent monocytes. There was minimal staining of pool cells that were incubated with non-immune murine IgG which excluded the possibility that I3 antibody bound to monocytes by a non-specific mechanism such as Fc receptor interactions. To control for possible direct effects of LPS on HLA-DR antigen expression by non-spontaneously adherent monocytes, pool monocytes were pre-incubated with LPS. Pre-incubation of pool monocytes with LPS did not alter their expression of HLA-DR antigen.

In the initial experiment, the spontaneously adherent and non-spontaneously adherent monocytes

expressed more HLA-DR antigen than the pool monocytes. It is possible that this increased HLA-DR antigen expression by the monocyte subpopulations was temperature-related since: 1) the adherence assay to separate the spontaneously adherent and non-spontaneously adherent cells was performed at 37°C whereas the pool cells were held at 4°C for the duration of this assay; and 2) it has been shown previously that incubation of monocytes at 37°C alters their expression of several surface antigens (146). To test the hypothesis that incubation of monocytes at 37°C induces their expression of HLA-DR antigen, pool monocytes were incubated at 4°C and 37°C for 1 h prior to labelling with I3 antibody. Monocytes incubated at 37°C expressed 2-fold more HLA-DR antigen than cells incubated at 4°C. These data support the hypothesis that the expression of HLA-DR antigen by monocytes is influenced by incubation temperature. This hypothesis is supported further by the subsequent finding that the expression of HLA-DR antigen by pool monocytes was similar to that of the non-spontaneously adherent, LPS-adherent and non-adherent monocytes when the pool cells were incubated at 37°C for the duration of the adherence assays.

These data indicate that: 1) monocytes are heterogeneous in their capacity to present antigen to lymphocytes; 2) there is a striking indirect relationship between the capacity of monocytes to adhere spontaneously to fibronectin *in vitro* and their capacity to present antigen to lymphocytes; and 3) HLA-DR antigen expression by monocytes is influenced by incubation temperature during short-term culture. Other investigators have demonstrated that all monocytes express HLA-DR antigens in widely varying amounts (421); however, HLA-DR antigen expression by adherent and non-adherent monocytes has not been compared previously. The data on the effects of temperature on HLA-DR antigen expression by monocytes in the current study are in agreement with the previous findings of **Smith** et al. (421) that: 1) the expression of HLA-DR antigen by monocytes increases 2 to 3-fold when monocytes are incubated for 12 h at 37°C compared with freshly-isolated cells; and 2) this increase in HLA-DR antigen expression can be inhibited by incubating the cells at 2°C.

Exposure to LPS did not alter HLA-DR antigen expression by monocytes in the current study. **In** contrast, Steeg et al. (427) reported that LPS inhibits HLA-DR antigen expression by macrophages. It is likely that technical factors may explain these differences in LPS responsiveness *since*: 1) Steeg and coworkers studied murine macrophages that had been stimulated with IFN, whereas unstimulated human monocytes were used in the current study; and 2) Steeg and coworkers incubated their cells with LPS for 48 h, whereas monocytes were

incubated with LPS for only 1 h in the current study.

The difference in HLA-DR antigen expression between the spontaneously adherent and non-spontaneously adherent monocytes could not be attributed to differences in cell viability, or by neutrophil or lymphocyte contamination of the monocyte preparations (less than 0.5% and 9%, respectively). In addition, the difference in HLA-DR antigen between spontaneously adherent and non-spontaneously adherent monocytes was not temperature-related since both subpopulations were incubated under identical conditions during isolation and labelling with I3 antibody. In addition, these differences are unlikely to be due to differences in cell size between the subpopulations of monocytes since: 1) there were no significant differences in mean forward scatter for the subpopulations; and 2) Dransfield et al. (146) showed that the mean cell volume of adherent monocytes is similar to that of pool monocytes.

The mechanism(s) for the differences in HLA-DR antigen expression by the spontaneously adherent and non-spontaneously adherent monocytes are not known. However, it is possible that these differences reflect variations in the states of activation of the cells since the pro-inflammatory mediators that stimulated monocytes to adhere to fibronectin in this study (LPS, TNF, GM-CSF and IFN) have also been shown to regulate HLA-DR antigen expression by monocytes (159,202,277,427,469). Therefore, it is possible that the differences in the adherence and immune regulatory properties among the monocytes subpopulations reflect differences in the profile of mediators to which these subpopulations were exposed in vivo. Alternatively, these differences may reflect variations in the maturational states of the cells since there is a progressive reduction in HLA-DR antigen expression by monocytes during maturation in vitro for greater than 24 h (421,463). Finally, it is possible that distinct subpopulations of monocytes exist, with the non-spontaneously adherent cells possessing an "immune modulatory" phenotype. These possibilities are discussed later (4.8)

Whatever the explanation for the differences in HLA-DR antigen expression between spontaneously adherent and non-spontaneously adherent monocytes, it is clear that monocytes are heterogeneous in their capacity to present antigen to lymphocytes. In addition, there is an striking indirect relationship between the ability of monocytes to adhere to fibronectin (which may be related to their capacity to be recruited into inflammatory foci) and their capacity to present antigen to lymphocytes. Moreover, LPS and other mediators that are released from sites of inflammation may stimulate the recruitment of monocytes with an "immune modulatory" phenotype, and thereby promote the resolution of tissue infection and inflammation.

Total Protein Content of the Monocyte Subpopulations

There was also a relationship between the adherence properties of monocytes and their total protein content. The spontaneously adherent cells contained 50% more protein than the non-spontaneously adherent cells which contained similar amounts when compared with pool, LPS-adherent and non-adherent monocytes.

Although the total protein content of adherent and non-adherent monocytes has not been quantified previously, other investigators have shown that monocytes are heterogeneous in their content of protein (10). The higher total protein content of spontaneously adherent monocytes could not be explained by differences in cell viability, since all of the monocytes subpopulations were greater than **99%**, as assessed by exclusion of trypan blue dye. In addition, the results could not be explained by contamination of the monocyte preparations with platelets or other leukocytes since: **1)** the monocytes were isolated by CCE and were not contaminated with platelets; and **2)** the monocytes preparations all contained less than 0.5% neutrophils and less than 9% lymphocytes. Furthermore, the higher total protein content of the spontaneously adherent cells could not be attributed to solubilisation of the fibronectin substrate along with the cells during preparation of the cell extracts since: **1)** minimal amounts of protein were detected in extracts made in identical fibronectin-coated dishes that had not been incubated with monocytes; and **2)** LPS-adherent cells contained similar amounts of protein when compared with the pool, non-spontaneously adherent and non-adherent monocytes.

It is unlikely that the differences in total protein content of the monocyte subpopulations was due to differential rates of synthesis of protein by monocytes during the ~~initial~~ adherence assay, since this would require that the spontaneously adherent **cells** increase their protein content by 50% in 1 h. In addition, it is unlikely that these differences are due **to** the secretion of protein by the non-spontaneously adherent monocytes during the adherence assays because **these** cells contained similar amounts of protein when compared with pool monocytes which were not cultured prior to preparation of cell extracts.

Thus, it is likely that circulating monocytes are heterogeneous in their total protein content and that there is a relationship between the adherence properties of monocytes and their total protein content. Although the mechanism(s) for these differences are unknown, it is possible that they reflect variations in the activation states or maturational stages of these subpopulations since the protein content of monocytes has been shown to increase in response to activation (327) and maturation (322) in vitro. These possibilities are discussed later (4.8).

4.7. Potential Role of Monocytes in the Pathogenesis of Chronic Inflammatory Lung Diseases

Although mononuclear phagocytes play a critical role in the host defence, these cells may also contribute to tissue damage at sites of inflammation because they contain proteolytic enzymes that are capable of degrading a variety of extracellular matrix macromolecules (81,409,483). Proteolytic destruction of connective tissue is the central pathological feature of one chronic inflammatory lung disease (pulmonary emphysema), and is thought to be mediated by enzymes released by inflammatory cells that are recruited into the lungs of these patients (223,333). Human leukocyte elastase (HLE) is widely believed to be the most important enzyme involved in the pathogenesis of emphysema.

Mononuclear phagocytes may be involved in the pathogenesis of emphysema since: **1)** they are present in greatly increased numbers at strategic locations within the lungs of cigarette smokers who are at risk of developing emphysema (334); and **2)** monocytes contain and release HLE (81). However, mononuclear phagocytes synthesise and secrete α_1 -antitrypsin, the major physiological inhibitor of HLE (32,312). Therefore, the balance between the release of HLE and α_1 -antitrypsin by monocytes, as they are recruited into the lung and pass through the interstitial space, may be an important determinant of lung elastin injury and resultant emphysema.

HLE Content of the Monocyte Subpopulations

The possibility that there is a relationship between the adherence properties of monocytes and their HLE content was tested since: **1)** Campbell et al. (81) reported that only **20%** of monocytes from healthy donors are replete with HLE; **2)** a similar proportion of monocytes from healthy subjects adhered spontaneously to fibronectin in the current study; and **3)** these spontaneously adherent monocytes were replete with peroxidase which is located within the same intracellular granules as HLE (242).

The HLE content of the pool monocytes was **4.6%** of that contained within neutrophils from the same donor. Monocytes were heterogeneous in their content of HLE since there was a striking relationship between the capacity of monocytes to adhere to fibronectin and their HLE content. The spontaneously adherent cells, on average, contained 7 to 9-fold more HLE than the non-spontaneously adherent and LPS-adherent monocytes and 22-fold more HLE than the non-adherent cells.

To control for possible direct effects of LPS on the HLE content of non-spontaneously adherent cells,

spontaneously adherent monocytes were also pre-incubated with LPS. Exposure to LPS did not alter the HLE content of spontaneously adherent cells. These data indicate that: 1) freshly-isolated monocytes are heterogeneous in their content of HLE; and 2) there is a direct relationship between the adherence properties of monocytes and their HLE content. Thus, monocytes that may have the capacity to accumulate at sites of infection and inflammation may also possess an enhanced capacity to contribute to HLE-mediated tissue damage.

Although the amount of HLE contained within pool monocytes in the current study is similar to that reported by other investigators (81,408), the HLE content of adherent and non-adherent cells has not been assessed previously. However, these data are in agreement with the findings of Campbell et al. (81) and Kargi and coworkers (242) that only 20% of monocytes from healthy subjects are replete with HLE.

The differences in the HLE content of the monocyte subpopulations did not simply reflect differences in the total protein content of monocytes since: 1) the total protein content of the spontaneously adherent monocytes was only 50% greater than that of the non-spontaneously adherent cells whereas the spontaneously adherent cells contained about 700% more HLE compared with the non-spontaneously adherent cells; and 2) the total protein content of the LPS-adherent and non-adherent cells were similar whereas the LPS-adherent cells contained 3-fold more HLE than the non-adherent cells. The differences in HLE content among the monocyte subpopulations could not be explained by differences in cell viability or by neutrophil contamination (less than 0.5%) of the monocyte preparations. Furthermore, the differences in HLE content of the monocyte subpopulations could not be attributed to differential release of HLE during culture, since minimal amounts of HLE were detected in the culture supernatants. In addition, these differences were not due to platelet contamination of Nycodenz-isolated monocytes since: 1) HLE was not detected in platelet extracts; and 2) monocyte subpopulations prepared from Nycodenz-isolated cells contained similar amounts of HLE when compared with monocyte subpopulations prepared from CCE-isolated cells which were not contaminated with platelets. It is also unlikely that a proportion of monocytes were activated differentially to release HLE during the Nycodenz isolation procedure since similar amounts of HLE were contained within monocytes isolated by the Nycodenz method and by CCE. Finally, the difference in HLE content between the monocyte subpopulations are also unlikely to be due to differential rates of synthesis of HLE by monocytes during isolation or culture since peripheral blood monocytes do not contain mRNA for HLE indicating that endogenous synthesis of this enzyme has been completed prior to release of the cells from the bone marrow (438).

It is possible that the differences in the HLE content of the monocyte subpopulations reflect variations in the number of peroxidase positive granules within the cells since: 1) HLE is located within the peroxidase positive granules of monocytes (242); 2) the relationship between the adherence properties of monocytes and their HLE content was similar to the relationship between the adherence properties of monocytes and their peroxidase content (spontaneously adherent monocytes contained 9-fold more HLE and 10-fold more peroxidase than the LPS-adherent cells, and 22-fold more HLE and 20-fold more peroxidase than non-adherent cells); and 3) monocytes have been shown to be heterogeneous in their content of peroxidase positive granules (9). This possibility could be investigated by performing combined immunogold localisation of HLE and peroxidase histochemistry on cytocentrifuge preparations of the monocyte subpopulations.

The mechanism for the differences in HLE content of the monocyte subpopulations is not known. However, it is possible that these differences are due to variations in the maturational stages of the monocyte subpopulations since maturation of mononuclear phagocytes is associated with the rapid loss of the expression of the HLE gene (438). Thus, spontaneously adherent monocytes could be immature cells, non-adherent monocytes could be the most mature subset of circulating monocytes, and LPS-adherent monocytes could be cells at an intermediate maturational stage. Alternatively, it is possible that these differences reflect variations in the states of activation of monocytes, since activation of monocytes is associated with the release of HLE (81,236). Thus, spontaneously adherent cells could be unstimulated cells, non-adherent could be activated cells and LPS-adherent monocytes may have been partially activated *in vivo* or during isolation *in vitro*. Finally, it is possible that distinct subpopulations of monocytes exist, with spontaneously adherent cells possessing a "inflammatory effector" phenotype. These possibilities are discussed later (4.8).

In summary, the data **suggest** that monocytes that may have the capacity to be recruited into the lung may also have the potential to cause tissue damage at sites of pulmonary inflammation. Moreover, LPS released from sites of pulmonary infection and inflammation may promote the accumulation of cells with an enhanced capacity to degrade matrix macromolecules, and thereby potentiating lung **injury**.

Alpha₁-antitrypsin Content of the Monocyte Subpopulations

The amounts of α_1 -antitrypsin contained within the monocyte subpopulations were compared since monocytes synthesise and secrete α_1 -antitrypsin, the major physiological inhibitor of HLE (32,312), and this may influence their capacity to cause tissue damage. Monocytes were prepared by CCE for these studies since

platelets were found to contain α_1 -antitrypsin and Nycodenz-isolated monocytes are contaminated variably with platelets.

There was a striking relationship between the adherence properties of monocytes and their content of α_1 -antitrypsin. Spontaneously adherent cells contained 4-fold more α_1 -antitrypsin than the non-spontaneously adherent cells, which in turn contained 1.6-fold more α_1 -antitrypsin when compared with LPS-adherent and non-adherent monocytes. To control for the possible effects of LPS on the α_1 -antitrypsin of non-spontaneously adherent cells, spontaneously adherent monocytes were also pre-incubated with LPS. Although LPS exposure resulted in a 30% reduction in their α_1 -antitrypsin content, LPS-stimulated spontaneously adherent monocytes still contained more α_1 -antitrypsin than the other monocyte subpopulations. The latter results was due, at least in part, to the release of α_1 -antitrypsin by the spontaneously adherent cells since immunoreactive α_1 -antitrypsin was detected in the culture supernatants. These data indicate that the monocyte subpopulations are also heterogeneous in their content of α_1 -antitrypsin, and this may influence their capacity to degrade matrix components at sites of infection and inflammation.

The absolute amount of α_1 -antitrypsin contained within monocytes has not been quantified previously. The mechanism for the differences in α_1 -antitrypsin content among the monocyte subpopulations is not known but, as with HLE, did not simply reflect differences in the total protein content of the subpopulations and could not be attributed to differences in cell viability or to neutrophil or lymphocyte contamination of the preparations (less than 0.5% and 9%, respectively). The differences in α_1 -antitrypsin content of the non-spontaneously adherent cells and that of the monocyte subpopulations that were derived by incubating these cells with LPS (i.e. the LPS-adherent and non-adherent cells) were probably due to the release of α_1 -antitrypsin by the non-spontaneously adherent cells during the LPS-adherence assay since immunoreactive α_1 -antitrypsin was detected in these culture supernatants. Moreover, it is likely that LPS stimulated the non-spontaneously adherent cells to release α_1 -antitrypsin during this assay since exposure to LPS induced the spontaneously adherent cells to release α_1 -antitrypsin.

in contrast, the difference in the α_1 -antitrypsin content of the spontaneously adherent and non-spontaneously adherent monocytes is unlikely to be due to differential release of α_1 -antitrypsin alone since only minimal amounts of α_1 -antitrypsin were released into the culture supernatants during this adherence assay. In addition, this difference is unlikely to be due to differential rates of synthesis of α_1 -antitrypsin since this would

require that the spontaneously adherent subset increase their α_1 -antitrypsin content 4-fold during the 1 h adherence assay. It is possible that the difference in the α_1 -antitrypsin content of spontaneously adherent and non-spontaneously adherent monocytes is related to variations in the maturational states of the monocyte subpopulations since maturation of monocytes is associated with an increase in the expression of the α_1 -antitrypsin gene (312). Thus, the spontaneously adherent cells could be a more mature population of cells compared with the non-spontaneously adherent monocytes. However, this would not be consistent with the HLE data which suggested that the spontaneously adherent cells were an immature subpopulation of monocytes. Alternatively, the difference in α_1 -antitrypsin content of these monocyte subpopulations could be related to variations in their states of activation since activation of monocytes in vitro increases their expression of α_1 -antitrypsin (32,352). Thus, it is possible that the spontaneously adherent monocytes are cells that had been activated in vivo or during their isolation in vitro resulting in the ability to adhere spontaneously to fibronectin and an increase in their α_1 -antitrypsin content when compared with other monocytes. These possibilities are discussed later (4.8).

Whatever the explanation for the difference in α_1 -antitrypsin content of the monocyte subpopulations, the data demonstrate that circulating monocytes are also heterogeneous with respect to their content of α_1 -antitrypsin and this is related to their capacity to adhere spontaneously to fibronectin in vitro. Moreover, it is possible that synthesis and release of α_1 -antitrypsin by monocytes that accumulate in the lung during episodes of infection and inflammation, influences their capacity to degrade lung connective tissue.

Comparison of the HLE and

Content of the Monocyte Subpopulations

To assess the potential of the monocyte subpopulations to degrade extracellular matrix components further, the relative amounts of HLE and α_1 -antitrypsin contained within the monocyte subpopulations were compared. Monocytes for these studies were isolated by CCE to overcome the problem of platelet contamination. In addition, HLE and α_1 -antitrypsin immunoassays were performed on the same cell extract samples to overcome the problem of day-to-day variability in the HLE and α_1 -antitrypsin content of monocytes.

AU of the monocyte subpopulations contained a molar excess of HLE relative to α_1 -antitrypsin. However, there were striking differences in the magnitude of the molar ratios of HLE to α_1 -antitrypsin which were related to the adherence properties of the cells. In particular, the mean molar ratio of HLE to α_1 -antitrypsin in spontaneously adherent monocytes was 10 which was 2-fold greater than that in non-spontaneously

adherent cells. However, the latter subpopulation was heterogeneous in this respect since the mean molar ratio of HLE to α_1 -antitrypsin in LPS-adherent monocytes was **11** (which was similar to that of the spontaneously adherent cells) whereas that in non-adherent cells was only 3. The latter result was due the fact that LPS-adherent cells contained 3-fold more HLE but similar amounts of α_1 -antitrypsin when compared with non-adherent cells.

The amounts of HLE and α_1 -antitrypsin contained within monocytes have not been compared previously. However, it is of considerable interest that Campbell et al. (81) demonstrated that: 1) 20% of monocytes express marked but localised HLE-mediated proteolysis of fibronectin in the presence of proteinase inhibitors; and 2) there was a striking indirect relationship between the HLE-mediated proteolytic activity of monocytes and their cell-surface expression of the HLA-DR antigen recognised by **I3** monoclonal antibody. It is likely that the 20 to 25% of monocytes that adhered spontaneously to fibronectin in the current study are identical to the "proteolytic" subpopulation of monocytes identified by Campbell and coworkers since: 1) the spontaneously adherent monocytes were replete with HLE; and 2) there was a striking indirect relationship between the capacity of monocytes to adhere to fibronectin and their cell-surface expression of the HLA-DR antigen recognised by **I3** antibody in this study. This possibility could be investigated further by comparing the proteolytic activities of the monocyte subpopulations against: 1) ~~methoxy-succinyl-alanyl-alanyl-prolyl-valyl-p~~nitroanilide (**MSAPN**), a **chromogenic** Substrate that is specific for HLE (**50%**); and 2) components of the extracellular matrix such as elastin and fibronectin by methods that have been described previously (**29,79,83**).

It is likely that tight adhesion between the cells and the subjacent Substrate is one mechanism that may facilitate extracellular proteolysis of Substrate immediately subjacent to the "proteolytic" monocytes identified by Campbell and coworkers (81). Although not the focus of the present work, several possible mechanisms for ~~this~~ observation have been suggested. First, ~~high~~ concentrations of proteinases may be attained as a result of discharge of granule contents into the subcellular cleft (75). Second, phagocytes that are adherent to substrate may create a closed compartment between the cells and the Substrate. ~~This~~ compartment may exclude α_1 -antitrypsin and other proteinase inhibitors from this subcellular cleft, and thereby protect proteinases released from the cell from inactivation by these proteinase inhibitors (**75,79**). This hypothesis is supported by the previous ~~finding~~ that macrophages that are adherent to a Substrate exclude proteins larger than 50,000 MW from the phagocyte-Substrate interface (**499**). Third, reactive oxygen metabolites that are released by monocytes into

the subcellular cleft may result in oxidative inactivation of α_1 -antitrypsin that has been released by the cell into this compartment. The latter possibility is supported by the previous finding that neutrophils use oxygen metabolites and elastase cooperatively to negate the protective effects of α_1 -antitrypsin to attack extracellular matrix components (476).

In summary, the data indicate that a minor subpopulation of monocytes (20 to 25%), identified by its adherence properties, may be unable or poorly capable of participating in antigen presentation to lymphocytes, but is replete with HLE. The HLE content of these cells may confer unique capabilities upon them that are beneficial to the host such as the ability to penetrate tissue barriers in order to reach sites of infection and inflammation rapidly, or the capacity to participate in tissue remodelling and repair. Alternatively, it is possible that the release of HLE by these cells, as they are recruited into the lung and pass through the interstitial space, may contribute to tissue injury. This latter possibility is supported by the previous findings of Campbell et al. (81) that the minor subpopulation of monocytes that is replete with HLE produces extensive HLE-mediated proteolysis of a matrix macromolecule, even in the presence of proteinase inhibitors. Finally, the data suggest that LPS released during episodes of pulmonary infection and inflammation may promote the accumulation of a subset of monocytes that are replete with HLE, and thereby may potentiate HLE-mediated lung injury.

Accumulation of Alpha₁-Antitrypsin by the Monocyte Subpopulations

Studies were conducted to determine whether the expression of α_1 -antitrypsin can be modulated *in vitro* by maturation or exposure to pro-inflammatory mediators. Monocytes were isolated by the Nycodenz method for these studies since the CCE technique was not available. One potential problem in using the Nycodenz method to isolate monocytes for these studies is that Nycodenz-isolated monocytes are always contaminated with platelets and platelets were found to contain α_1 -antitrypsin in amounts that were consistent with the results from a previous study (320). However, it is unlikely that de novo synthesis of α_1 -antitrypsin by platelets occurs during culture of the cells since: 1) platelets contain negligible amounts of DNA and RNA; and 2) any RNA that is present is in a metabolically inactive form (1). To overcome the problem of platelet contamination, monocytes and their contaminating platelets were harvested at the start of the experiment (time = 0, control cells) and cells and culture supernatants were harvested at intervals thereafter. The amount of α_1 -antitrypsin contained within these samples was quantified using a direct binding ELISA and the results were expressed as ng α_1 -antitrypsin/ 10^6 cells. The value for the time = 0, control cells was subtracted from the sum of the amounts

contained within cell extracts and culture supernatants harvested for each time interval. The results obtained represented the amount of α_1 -antitrypsin accumulated by the monocytes. The results were expressed as amounts of α_1 -antitrypsin accumulated by monocytes since the values obtained reflect the net balance between the rate of synthesis and the rate of catabolism of this protein by the cells.

The effect of cellular maturation on the expression of α_1 -antitrypsin by adherent and non-adherent monocytes was assessed since maturation of monocytes results in the loss of their expression of the HLE gene (77). Culture for 48 h resulted in a progressive increase in the amount of α_1 -antitrypsin accumulated by all of the monocyte subpopulations. After culture for 48 h the spontaneously adherent, LPS-adherent, non-spontaneously adherent and non-adherent cells accumulated 975%, 287%, 150% and 75% more α_1 -antitrypsin, respectively, when compared with the amount of α_1 -antitrypsin contained within the time = 0, control cells. These initial data suggested that the adherent monocyte subpopulations accumulated more α_1 -antitrypsin after 48 h than the non-adherent monocyte subpopulations. However, in a subsequent experiment, the spontaneously adherent monocytes accumulated similar absolute amounts of α_1 -antitrypsin when compared with the non-spontaneously adherent cells. It is likely that the results of the initial experiment are artifacts since: 1) these results were expressed as a percentage of the amount of α_1 -antitrypsin contained within the time = 0, control cells; and 2) the time = 0, control cells for the adherent monocyte subpopulations contained substantially lower amounts of α_1 -antitrypsin than the control cells for the non-adherent monocyte subpopulations. The latter finding probably reflects the fact that platelets contain α_1 -antitrypsin and contaminate the non-adherent fraction of monocytes predominantly.

The data indicate that maturation is associated with an increase in the expression of α_1 -antitrypsin by monocytes. Moreover, it has been shown previously that maturation *in vitro* for 48 h results in the complete loss of the expression of HLE by monocytes (77). Together, these data indicate that maturation of monocytes results in a dramatic change in the balance between their expression of HLE and α_1 -antitrypsin, and thereby alters their capacity to degrade connective tissue during homeostasis and tissue injury. Therefore, it is likely that monocytes only contribute to HLE-mediated lung injury during and shortly after they are recruited into the lung. Further maturation of monocytes within the lung probably results in a loss of the capacity of these cells to produce HLE-mediated lung injury.

To determine whether recruitment of monocytes into inflammatory foci modulates their expression of

α_1 -antitrypsin, monocytes were exposed to several pro-inflammatory mediators that are likely to be released at sites of infection and inflammation (**LPS**, **TNF**, **IFN** and **GM-CSF**). Exposure of spontaneously adherent monocytes to **LPS** and cytokines resulted in **1.4 to 1.8** fold increases in their accumulation of α_1 -antitrypsin compared with unstimulated cells. **LPS** induced a time-dependent increase in α_1 -antitrypsin accumulation by spontaneously adherent monocytes since α_1 -antitrypsin accumulation increased within **12 h** and the effect of **LPS** was still evident after **24 h**. In addition, the increase in α_1 -antitrypsin accumulation by spontaneously adherent monocytes that was induced by **GM-CSF** was concentration-dependent. In contrast, **LPS** and cytokines had no effect on α_1 -antitrypsin accumulation by the non-spontaneously adherent cells. These data indicate that there is a relationship between the adherence properties of monocytes and their capacity to accumulate α_1 -antitrypsin in response to pro-inflammatory mediators.

De novo synthesis and secretion of α_1 -antitrypsin by monocytes has been demonstrated previously using the biosynthetic labelling and immunoprecipitation technique (32); however, the absolute amounts of α_1 -antitrypsin synthesised or accumulated by monocytes has not been quantified previously. Maturation of monocytes was associated with an increase in the accumulation of α_1 -antitrypsin by monocytes in the current study. These data are in agreement with the previous findings of Mornex et al. (312) that maturation of monocytes in vitro is associated with a dramatic increase in their expression of the α_1 -antitrypsin gene.

Although the effect of mediators on α_1 -antitrypsin accumulation by adherent and non-adherent monocytes has not been compared previously, the results in the current study are in agreement with the findings of Barbey-Morel et al. (32) that **LPS** stimulates a time-dependent increase in α_1 -antitrypsin synthesis by adherent monocytes. However, the data of Barbey-Morel and coworkers differed somewhat from the results of the current study since they found that **LPS** induced **8 to 9-fold** increases in the rate of synthesis of α_1 -antitrypsin by adherent monocytes compared with unstimulated cells, which was substantially greater than the 1.4-fold increase in the accumulation of α_1 -antitrypsin by adherent monocytes that was induced by **LPS** in the current study. These differences were not due to differences in the concentration or source of **LPS**, or the duration of exposure to **LPS** since monocytes were incubated with **1 µg/ml** of **LPS** from *E. coli* 0111:B4 for **24 h** in both studies. It is likely that differences in the methods used to quantify α_1 -antitrypsin explain the differences in the results of the two studies. In Barbey-Morel's study, the rate of de novo synthesis of α_1 -antitrypsin by monocytes was measured by the biosynthetic labelling and immunoprecipitation technique. In contrast, the absolute

quantities of α_1 -antitrypsin in cell extracts and culture supernatants were measured by direct binding ELISA in the current study, and these data reflected both the rate of synthesis and the rate of catabolism of α_1 -antitrypsin by the cells. Together, the results of Barbey-Morel's study and the current study suggest that the amount of α_1 -antitrypsin synthesised de novo by monocytes in response to LPS is small relative to the amount of α_1 -antitrypsin accumulated by the cells. Alternatively, LPS may stimulate an increase in the rate of synthesis and a slightly lower increase in the rate of catabolism of α_1 -antitrypsin by spontaneously adherent cells, and thereby result in a small net increase in the amount of α_1 -antitrypsin accumulated by the cells. To investigate the latter possibility, the effect of LPS on both the rate of synthesis and the half-life of α_1 -antitrypsin in spontaneously adherent monocytes could be assessed using the biosynthetic, immunoprecipitation and SDS-PAGE technique (32) and long-term pulse-chase studies (273), respectively.

Perlmutter et al. (352) investigated the effect of cytokines on the expression of α_1 -antitrypsin by adherent monocytes. However, the results of Perlmutter and coworkers were not in agreement with the findings of the current study since these investigators found that **TNF** and IFN had no effect on α_1 -antitrypsin expression by adherent monocytes, whereas these mediators **stimulated 1.4 to 1.8 fold** increases in α_1 -antitrypsin accumulation by adherent cells in the current study. These differences probably also reflect technical differences in the two studies. First, it is possible that different populations of monocytes were studied since monocytes were isolated by adherence of dextran-purified leukocytes to charged tissue culture plastic in Perlmutter's study, whereas Nycodenz-isolated monocytes were allowed to adhere to fibronectin-coated dishes in the current study. This possibility could be investigated by comparing the accumulation of α_1 -antitrypsin by monocytes isolated by both methods. Second, the concentrations of cytokines that were **used** in Perlmutter's study were 10-fold lower than those used in the current study, and this could account for the lack of responsiveness of monocytes to cytokines in Perlmutter's study. This possibility could be investigated by incubating adherent monocytes with and without optimal concentrations of cytokines and measuring their rate of synthesis of α_1 -antitrypsin using the techniques of Perlmutter and coworkers. Third, Perlmutter et al. measured the rate of de novo synthesis of α_1 -antitrypsin by monocytes using the biosynthetic labelling and immunoprecipitation technique, whereas the net amount of α_1 -antitrypsin accumulated by monocytes was measured in the current study. It is possible that cytokines increased the net accumulation of α_1 -antitrypsin by spontaneously adherent monocytes by reducing their rate of catabolism of α_1 -antitrypsin, rather **than** by increasing their rate of synthesis of this protein. This

possibility could be assessed by comparing the effects of cytokines on both the rate of synthesis and the half-life of α_1 -antitrypsin in spontaneously adherent monocytes using the biosynthetic labelling and immunoprecipitation technique (32,352) and long-term pulse-chase studies (273), respectively.

The mechanism for the differences in the capacity of adherent and non-adherent monocytes to accumulate α_1 -antitrypsin in response to pro-inflammatory mediators is not known. However, these differences could not be attributed to differences in cell viability, or to neutrophil or lymphocyte contamination of the monocyte preparations (less than 0.5% and 9%, respectively). Whatever the mechanisms involved, the results suggest that following the accumulation of monocytes at sites of pulmonary infection and inflammation, exposure to cytokines may increase their expression of α_1 -antitrypsin, and thereby may alter their capacity to degrade matrix components and contribute to lung injury.

Expression of the Alpha₁-Antitrypsin Gene by Monocyte Subpopulations

To assess whether maturation *in vitro* and exposure to pro-inflammatory mediators up-regulated α_1 -antitrypsin accumulation in monocytes by a transcriptional mechanism, spontaneously adherent and non-spontaneously adherent monocytes were incubated for 24 h with and without LPS and cytokines, and their steady state α_1 -antitrypsin-specific mRNA concentrations were compared by dot-blot analysis. Hybridisation was performed using a human genomic DNA α_1 -antitrypsin probe and a β -actin cDNA probe. Alpha-antitrypsin mRNA concentrations were expressed as a ratio to the values obtained for β -actin to correct for any variability in RNA yields. The data showed that: 1) freshly-isolated spontaneously adherent and non-spontaneously adherent monocytes contained similar steady state concentrations of α_1 -antitrypsin-specific mRNA; 2) culture for 48 h did not alter the steady state concentrations of α_1 -antitrypsin-specific mRNA in either of these monocyte subpopulations; and 3) exposure to LPS and cytokines did not alter α_1 -antitrypsin-specific mRNA levels in the monocyte subpopulations when compared with unstimulated cells. The data indicate that the increases in monocyte α_1 -antitrypsin accumulation that were induced by short-term culture and exposure to LPS and cytokines are likely to be mediated by either: 1) a translational mechanism such as an increase in the efficiency of translation of α_1 -antitrypsin mRNA transcripts; or 2) a post-translational mechanism such as a decrease in the rate of catabolism of α_1 -antitrypsin protein.

A human genomic α_1 -antitrypsin DNA probe was used to quantify α_1 -antitrypsin-specific mRNA

concentrations in monocytes. It is unlikely that this probe hybridised to α_1 -antitrypsin DNA sequences that may have contaminated the monocyte cytoplasmic RNA samples, since pre-incubation of one of these samples with RNAase prior to dot-blot analysis, almost completely abrogated the autoradiogram signal. In addition, it is unlikely that the genomic α_1 -antitrypsin probe hybridised to ribosomal or transfer RNA molecules rather than mRNA species since other investigators have demonstrated that genomic DNA probes hybridise specifically to mRNA transcripts in blots of total cellular RNA samples (109,393). Therefore, it is likely that the α_1 -antitrypsin genomic probe hybridised to α_1 -antitrypsin-specific mRNA transcripts in the monocyte cytoplasmic RNA samples in the current study. This could be confirmed by: 1) purifying the poly (A)' RNA fraction by oligo (dT) column chromatography prior to dot-blot analysis using the genomic α_1 -antitrypsin probe; 2) repeating these experiment using an α_1 -antitrypsin cDNA probe instead of the genomic probe; or 3) performing Northern blot analysis of monocyte cytoplasmic RNA samples using the α_1 -antitrypsin genomic DNA probe.

Steady state concentrations of α_1 -antitrypsin mRNA in adherent and non-adherent monocytes have not been compared previously. However, other investigators have studied the effects of maturation and LPS on the expression of the α_1 -antitrypsin gene by adherent monocytes. Mornex et al. (312) reported that maturation of adherent monocytes *in vitro* results in a 10-fold increase in steady state concentrations of α_1 -antitrypsin mRNA. These data are not in agreement with the findings in the current study that *in vitro* maturation of adherent monocytes had no effect on monocyte α_1 -antitrypsin mRNA levels and may reflect several technical differences between the two studies. First, Mornex and coworkers cultured their monocytes for 15 days, whereas monocytes were cultured for only 48 h in the current study. This possibility could be investigated by performing longer studies. Second, Mornex and coworkers used an α_1 -antitrypsin cDNA probe to quantify α_1 -antitrypsin-specific mRNA concentrations whereas an α_1 -antitrypsin genomic DNA probe was used in the current study. The potential problems in using a genomic DNA to measure mRNA concentrations have been discussed previously. Third, different methods were used to control for any variation in RNA yield; Mornex and coworkers measured DNA concentrations whereas a β -actin cDNA probe was used in the current study. It is possible that maturation of monocytes is associated with similar increases in α_1 -antitrypsin and β -actin mRNA concentrations which, therefore, would not alter the ratio of α_1 -antitrypsin to β -actin mRNA concentrations. To investigate these possibilities it would be necessary to repeat the experiments using the protocols of Mornex and coworkers to measure steady-state concentrations of α_1 -antitrypsin mRNA in monocytes. In addition, the effect of cellular

maturation on the expression of the β -actin gene by monocytes should be assessed.

Exposure to **LPS**, TNF, IFN and GM-CSF for 24 h did not alter steady-state α_1 -antitrypsin mRNA concentrations in adherent and non-adherent monocytes. These data are in agreement with the previous finding of Barbey-Morel et al. (32) that exposure to **LPS** for 24 h resulted in no change, or only minimal increases in steady state concentrations of α_1 -antitrypsin mRNA in adherent monocytes (32). The effects of TNF, **IFN** and GM-CSF on the expression of the α_1 -antitrypsin gene by monocytes have not been assessed previously.

These data suggest that **LPS** and cytokines up-regulated α_1 -antitrypsin expression in adherent monocytes by translational or post-translational mechanisms rather than by increasing the transcription of the α_1 -antitrypsin gene. This hypothesis could be tested further by assessing the effects of **LPS** and cytokines on the rate of transcription of α_1 -antitrypsin in monocytes using the nuclear run-off technique. However, the possibility remains that **LPS** and cytokines stimulated early, transient increases in α_1 -antitrypsin mRNA levels in adherent monocytes which were missed because the cells were harvested only after culture for 24 h. Although this possibility is unlikely, since other investigators have reported that exposure of monocytes to **LPS** for 12 h did not alter α_1 -antitrypsin mRNA concentrations in monocytes (32), it could be investigated by performing shorter studies. Alternatively, **LPS** and cytokines may have stimulated similar increases in monocyte α_1 -antitrypsin and β -actin mRNA levels which would not change the ratio of α_1 -antitrypsin to β -actin mRNA concentrations in monocytes. This possibility could be investigated by assessing the effect of **LPS** and cytokines on the expression of the β -actin gene by monocytes.

In summary, mononuclear phagocytes may be an important source of α_1 -antitrypsin within the lung since the expression of α_1 -antitrypsin by monocytes is up-regulated by cellular maturation. In addition, signals that arise from inflammatory foci (**LPS** and several cytokines) increase the expression of α_1 -antitrypsin by spontaneously adherent monocytes which may have the potential to be recruited into sites of inflammation. These data suggest that following the recruitment of monocytes into foci of pulmonary infection and inflammation, exposure to pro-inflammatory mediators up-regulates their expression of α_1 -antitrypsin, and thereby may limit proteinase-mediated lung injury.

Conclusions

The data presented above may provide further insight into the mechanisms that may be involved in the pathogenesis of chronic inflammatory lung diseases such as emphysema and bronchiectasis. Preliminary data (presented in Appendix 3) suggest that there is a minor subpopulation of monocytes that may be recruited readily into the lung since: 1) **20 to 25%** of monocytes adhered spontaneously to HUVEC monolayers and fibronectin *in vitro*; and 2) reversible adherence of monocytes to endothelial cells and components of the extracellular matrix is a prerequisite for the accumulation of these cells at sites of inflammation (**172,423**). These spontaneously adherent monocytes contained a substantial molar excess of HLE relative to α_1 -antitrypsin. It is possible that the HLE content of spontaneously adherent monocytes may confer capabilities upon them that are beneficial to the host including: 1) the ability to penetrate tissue barriers in order to migrate into sites of inflammation; and 2) the capacity to participate in tissue remodelling and repair. Alternatively, it is possible that the release of HLE by this subpopulation of monocytes, as they are recruited into the lung and pass through the interstitial space, may contribute to HLE-mediated lung injury. This latter possibility is supported by the previous findings of Campbell *et al.* (**1988**) that a similar proportion of monocytes (**20%**) produces HLE-mediated proteolysis of an extracellular matrix component even in the presence of proteinase inhibitors (**81**). Moreover, reactive oxygen metabolites released by these spontaneously adherent monocytes may contribute to local oxidative inactivation of α_1 -antitrypsin within the lung, and thereby facilitate HLE-mediated tissue injury.

LPS-adherent monocytes also contained a substantial molar excess of HLE relative to α_1 -antitrypsin. These data suggest that LPS released during episodes of pulmonary infection and inflammation, may promote the accumulation of another subset of monocytes that has an enhanced capacity to degrade lung connective tissue, and thereby potentiate lung injury.

It is likely that monocytes only contribute to HLE-mediated lung injury during, or shortly after, the process of recruitment into the lung since maturation of monocytes is associated with a rapid loss of HLE (**77**) and resulted in an increase in their expression of α_1 -antitrypsin in this study. In addition, the capacity of monocytes to contribute to tissue damage may be modulated by mechanisms other than maturation since pro-inflammatory mediators up-regulated the expression of adherent monocytes in this study and HLE (**353,354**) and IL-6 (**352**) have been shown previously to up-regulate the synthesis of α_1 -antitrypsin by adherent monocytes. Thus, exposure of monocytes to pro-inflammatory mediators and HLE, as they are recruited into sites of

pulmonary infection and inflammation, may up-regulate their expression of α_1 -antitrypsin, and thereby limit connective tissue degradation at sites of pulmonary inflammation.

The current study only compared the expression of HLE and α_1 -antitrypsin by monocytes. However, these cells contain other proteolytic enzymes and proteinase inhibitors that may contribute to the protease anti-proteinase balance in the lower respiratory tract. For example, mononuclear phagocytes contain enzymes, other than HLE, that have the capacity to degrade matrix components including: 1) other serine proteinases such as cathepsin G (77,81); 2) metalloproteinases (77); 3) cysteine proteinases such as cathepsin L (294); and 4) plasminogen activator (100,101,103,104,294). Mononuclear phagocytes express proteinase inhibitors, other than α_1 -antitrypsin, that may contribute to the anti-elastase screen including: 1) α_2 -macroglobulin (32); 2) plasminogen-activator inhibitor (462); 3) tissue inhibitor of metalloproteinase (77); and 4) α_1 -antichymotrypsin (39). Moreover, the expression of some of these proteinases and anti-proteinases is regulated by cellular differentiation and activation (32,76,77,81,479). Thus, the capacity of mononuclear phagocytes to contribute to tissue damage depends upon the balance between their expression of all of their proteinases and anti-proteinases which, in turn, depends upon their state of activation and maturational stage. To investigate the capacity of adherent and non-adherent monocytes to contribute to tissue damage further, their expression of other proteinases and anti-proteinases and their capacity to degrade matrix components *in vitro* could be investigated. In addition, the effects of cellular activation and maturation on the capacity of these monocyte subpopulations to degrade matrix components *in vitro* could be assessed.

4.8. Hypotheses for Monocyte Heterogeneity

The data presented in 3.8, 3.9 and 3.10 indicate that human monocytes are heterogeneous in their antimicrobial and immune modulatory functions and also in their expression of HLE and α_1 -antitrypsin. In particular, the capacity of monocytes to adhere to fibronectin is related directly to several antimicrobial functions and their expression of HLE and α_1 -antitrypsin. In addition, there is an indirect relationship between the capacity of monocytes to adhere to fibronectin and one aspect of their immune regulatory function (HLA-DR antigen expression).

There is a substantial literature regarding human monocyte heterogeneity which is summarised in table 4. Although the biological relevance of this heterogeneity is not understood, several investigators have postulated that heterogeneity of monocyte function relates to differences in the degree of maturation, differentiation or activation of monocytes (144,146,449).

A particular strength of the present work is that it demonstrated that there is a relationship between adherence (which probably relates to the ability of cells to reach sites of inflammation) and various phenotypes that are related to important biological activities at sites of infection and inflammation. The mechanism for the functional differences between the monocyte subpopulations is not known. It is possible that these functional differences are artifacts. For example, a subpopulation of monocytes could have been activated differentially during the Nycodenz isolation procedure. However, this is unlikely since preliminary results on monocytes isolated by CCE confirm that a minor subpopulation of monocytes adheres spontaneously to fibronectin and that there is a relationship between monocyte adherence and superoxide anion release (Appendix 4). In addition, the functional differences between the monocyte subpopulations could not be explained by neutrophil or lymphocyte contamination of the monocyte preparations (less than 0.5% and 9%, respectively). However, the possibility remains that the subpopulation of monocytes that did not adhere spontaneously to fibronectin were damaged, despite their viability, as assessed by exclusion of trypan blue dye.

An alternative explanation for the functional differences between the monocyte subpopulations is that the monocyte subpopulations represent a continuous spectrum of monocyte function. These functional differences could be related to variations in their states of activation or maturational stages. For example, it is possible that spontaneously adherent monocytes are a subpopulation of monocytes that have been activated *in vivo* and non-adherent monocytes are unstimulated cells since: 1) activation of monocytes is associated with

increases in phagocytic activity, the release of superoxide anion (236,267), total protein content (327) and the synthesis of α_1 -antitrypsin (32,352); and 2) spontaneously adherent cells are more actively phagocytic, release more superoxide anion and contain more protein and α_1 -antitrypsin than non-adherent monocytes. LPS-adherent cells may represent cells that have been partially activated *in vivo* since they had an intermediate antimicrobial phenotype. However, activation of monocytes is usually associated with an increase in their expression of HLA-DR antigen (236) and a decrease in their HLE content (81), whereas the spontaneously adherent cells express relatively low levels of HLA-DR antigen and contain substantial quantities of HLE. Therefore, it is unlikely that these subpopulations simply reflect differences in the states of activation of circulating monocytes.

Alternatively, the spontaneously adherent cells may represent a more mature subpopulation of cells than non-adherent cells since maturation of monocyte is associated with increases in phagocytic activity (267), total protein content (322), and the expression of α_1 -antitrypsin (312), but a reduction in HLA-DR antigen expression (421). However, it is unlikely that these monocyte subpopulations simply reflect differences in maturational stages of circulating monocytes since maturation of monocytes is also associated with a loss of peroxidase activity and HLE expression (438 458), whereas spontaneously adherent cells contain substantial amounts of peroxidase activity and HLE and the non-adherent monocytes contain only minimal amounts of these enzymes.

Other investigators have proposed that distinct subpopulations of monocytes exist on the basis of differences in the expression of HLA-DQ (177), complement receptors (487) and 5' nucleotidase activity (67). The possibility remains that functionally distinct subpopulations of monocytes exist: adherent monocytes possessing an "inflammatory effector" phenotype and the non-adherent cells an "immune modulatory" phenotype. While the distinction between these phenotypes is not absolute, as in the T and B lymphocyte subset model, it is quite definite. Further studies are required to investigate this possibility, as discussed later (4.10).

4.9. Conclusions and Implications

Initial studies demonstrated that the yield of monocytes isolated by the Nycodenz method was 2-fold greater than that isolated by the conventional ficoll-Hypaque technique followed by adherence of monocytes to fibronectin. Moreover, only a minor subpopulation (20 to 25%) of monocytes isolated from healthy subjects by the Nycodenz method and by CCE adhered spontaneously to fibronectin. Since most investigators isolate monocytes by methods employing an adherence step, these data indicate that many previous studies of monocyte function may have been conducted on a minor, possibly unrepresentative subpopulation of monocytes.

Additional studies demonstrated that the spontaneous adherence of monocytes from patients with a chronic inflammatory lung disease (bronchiectasis) to a representative extracellular matrix component (fibronectin) was 2 to 3-fold higher than that of cells from healthy subjects, and was correlated with the severity of airway inflammation. Since movement of cells through tissues depends upon regulated adherence of the cells to extracellular matrix macromolecules (172,423), the data suggest that circulating monocytes are heterogeneous in their ability to accumulate at sites of inflammation. It is likely that endotoxin and cytokines released from foci of pulmonary infection were responsible for the observed activation of the patients' monocytes since: 1) endotoxin was detected in plasma from all of the patients but none of the controls; and 2) LPS and cytokines stimulated the adherence of monocyte to fibronectin in vitro. These data indicate that signals arising from foci of infection and inflammation can influence the adherence properties of monocytes, and are likely determinants of the accumulation of mononuclear phagocytes in the lungs of patients with chronic inflammatory lung diseases.

The adherence of monocytes from healthy subjects and patients with bronchiectasis to fibronectin was mediated substantially by CD11/CD18 integrins. This is the first report to document the involvement of CD11/CD18 integrins in monocyte adherence to a component of the extracellular matrix. These data provide further insight into the factors that regulate the recruitment of monocytes into inflammatory sites and the mechanisms involved.

There was a striking relationship between the capacity of monocytes to adhere to fibronectin and phenotypes that are of biological importance at sites of infection and inflammation. Spontaneously adherent monocytes had an "inflammatory effector" phenotype, non-adherent cells had an "immune modulatory" phenotype and LPS-adherent cells had an intermediate phenotype. These data indicate that LPS released from the lungs of patients with chronic inflammatory pulmonary diseases, may stimulate the accumulation of a

subpopulation of monocytes with an enhanced capacity to eliminate microbes, and may thereby promote the resolution of pulmonary infection and inflammation.

Patients with bronchiectasis remain colonised with bacteria, and progressive lung damage occurs in some patients with bronchiectasis and emphysema despite the increased recruitment of mononuclear phagocytes into the lungs of these patients. It is possible that excessive recruitment of mononuclear phagocytes into the lungs of patients with chronic inflammatory lung diseases may initiate or perpetuate HLE-mediated tissue, since monocytes that may have the capacity to be recruited into the lung are replete with HLE. Moreover, these cells produce large amounts of reactive oxygen metabolites which may contribute to local inactivation of α_1 -antitrypsin, and thereby may lower the anti-elastase screen in the lower respiratory tract.

It is likely that monocytes only contribute directly to HLE-mediated lung injury during or shortly after their recruitment into the lung since: **1)** maturation of monocytes is associated with a rapid loss of the expression of HLE (77); and **2)** maturation of monocytes was associated with a progressive increase in their expression of α_1 -antitrypsin in the current study. Moreover, several pro-inflammatory mediators also increased the expression of α_1 -antitrypsin by adherent monocytes. Therefore, it is likely that exposure of monocytes to pro-inflammatory mediators, following their accumulation at sites of pulmonary infection and inflammation, also limits their capacity to produce HLE-mediated lung injury directly. However, mononuclear phagocytes may perpetuate lung injury subsequently by two mechanisms. First, these cells may contribute indirectly to HLE-mediated lung injury since LPS released from inflammatory foci stimulates monocytes to release TNF (106) which is chemotactic for neutrophils (310), and also stimulates the release of proteolytic enzymes from neutrophil granules (253). Second, mononuclear phagocytes contain proteolytic enzymes, other than HLE, that are capable of degrading connective tissue components in vitro (76,77,81,125,479). In addition, the expression of some of these proteinases is up-regulated by cellular maturation and activation (77,125,479).

Finally, if monocyte recruitment is an important determinant of the outcome of chronic inflammatory lung diseases, factors that alter monocyte adherence to biologically relevant substrates may have a role in the management of these diseases.

4.10. Further Studies

These studies have provided insight into: 1) the factors that regulate monocyte adherence to fibronectin; 2) the mechanisms involved; and 3) the relationship between the adherence properties of monocytes and other monocyte functions that are of biological and pathological importance at sites of infection and inflammation. However, several questions remain to be answered. The following account outlines studies that could be performed to address these uncertainties.

Adherence Properties of Monocytes

1. Preliminary data, presented in Appendix 3, suggest that the capacity of monocytes to adhere to fibronectin reflects their potential to be recruited into tissues. To test this hypothesis, monocytes could be isolated from additional donors, and their adherence to fibronectin and HUVEC monolayers under basal conditions and in response to pro-inflammatory mediators could be compared. In addition, the chemotactic responsiveness of adherent and non-adherent monocytes could be compared since the recruitment of monocytes from the vasculature into inflammatory foci involves monocyte chemotaxis in addition to the adherence properties of these cells. The chemotactic responsiveness of adherent and non-adherent monocytes to chemoattractants such as f-MLP and C5a could be compared using standard techniques (110).
2. The data suggested that pro-inflammatory mediators released from the lungs of patients with bronchiectasis increase the adherence properties of their monocytes and this may enhance the recruitment of these cells into the lungs of these patients. To test this hypothesis, the adherence properties and chemotactic responsiveness of monocytes isolated from patients with other inflammatory lung diseases such as emphysema, acute bacterial pneumonia, cystic fibrosis and pulmonary fibrosis could be compared with those of cells isolated from age-matched healthy subjects and patients with a non-inflammatory lung disease such as carcinoma of the bronchus.
3. What are the relative roles of CD18 and VLA-5 integrins in mediating monocyte adherence to fibronectin? This question could be addressed by comparing the effect of the anti-CD18 and an anti-VLA-5 antibodies on basal and LPS-stimulated adherence of monocytes from healthy subjects and patients with bronchiectasis. In addition, the expression of CD11/CD18 and VLA-5 integrins by monocytes from healthy subjects and patients with bronchiectasis could be assessed. Cells could be labelled with the anti-CD18 and anti-VLA-5 antibodies by the indirect immunofluorescence technique and receptor expression quantified by flow cytometry.

Monocyte Subpopulations

1. It is possible that the monocyte subpopulations identified in this study represent differences in the states of activation of the cells. To test this hypothesis, adherent and non-adherent monocyte subpopulations could be separated and assayed for their specific activities of two enzymes that are markers of mononuclear phagocyte activation: 5' nucleotidase and acid phosphatase (67,357). In addition, the monocyte subpopulations could be activated in vitro to determine whether cellular activation induces non-adherent monocytes to develop the characteristics of adherent cells or vice versa. Cells could be exposed to stimuli such as LPS, TNF and IFN, and their antimicrobial function and expression of HLA-DR antigen could be quantified, as described in Methods.
2. It is possible that the monocyte subpopulations represent differences in the maturational stages of monocytes. To test this hypothesis, the expression of surface antigens that are correlates of mononuclear phagocyte maturation by the monocyte subpopulations could be compared. Cells could be labelled with the following monoclonal antibodies by the indirect immunofluorescence technique: 1) KB23, which recognises p150/95 (203); 2) UCHM1 which reacts with a monocyte marker; and 3) RFD7 which reacts with a macrophage marker (204,227). The expression of these surface antigens by adherent and non-adherent monocytes could be quantified by flow cytometry. In addition, the monocyte subpopulations could be assayed for their specific activities of two enzymes that are markers of mononuclear phagocyte maturation: macrophage tissue transglutaminase (317) and cathepsin B (66). The monocyte subpopulations could also be matured in vitro to determine whether non-adherent monocytes develop some of the characteristics of adherent monocytes when induced to mature or vice versa. The monocyte subpopulations could be cultured for one to three weeks with and without PMA, and their antimicrobial function and expression of HLA-DR antigen could be quantified at intervals, as described in Methods.
3. It is possible that functionally distinct subpopulations of monocytes exist. If the studies outlined above failed to demonstrate that there are differences in the states of activation or maturational stages of the monocyte subpopulations, this would support the hypothesis that functionally distinct subsets of monocytes exist. In addition, human mononuclear phagocyte bone marrow precursor cells could be studied to determine whether they have distinct pro-inflammatory and immune-modulatory phenotypes. Mononuclear phagocyte precursor cells could be isolated, as described previously (233,315) and stained for: 1) HLE and peroxidase by combined immunogold localisation and enzyme histochemistry (242); and 2) HLA-DR antigen by the indirect

immunofluorescence technique, as described in Methods.

Monocyte Proteinases and Anti-Proteinases

1. The data presented in this thesis suggested that a minor subpopulation of monocytes may have the capacity to contribute to tissue injury during or shortly after their recruitment into the lung. To investigate this possibility, the following experiments could be performed.

a. The proteolytic activities of the monocyte subpopulations isolated from healthy subjects could be compared. Extracts of the monocyte subpopulations could be prepared and their elastase activity could be measured against MSAPN, a chromogenic substrate that is specific for HLE (50,96) and ³H-elastin (29,83). In addition, the proteolytic activity of viable cells could be assessed in the presence of proteinase inhibitors using a solid phase fibronectin assay (79).

b. The effect of maturation in vitro and pro-inflammatory mediators on the expression of α_1 -antitrypsin by monocyte subpopulations could be assessed further. Monocytes could be isolated by CCE instead of the Nycodenz method to circumvent the problem of platelet contamination. To gain further insight into the mechanisms involved in the increased expression of α_1 -antitrypsin induced by maturation and pro-inflammatory mediators, cells could be cultured for up to 15 days with and without pro-inflammatory mediators and the following measured: 1) the accumulation of α_1 -antitrypsin by direct binding ELISA; 2) the rate of de novo synthesis of α_1 -antitrypsin by the biosynthetic labelling and immunoprecipitation technique; 3) the half-life of α_1 -antitrypsin in monocytes by long-term pulse-chase studies; 4) steady-state α_1 -antitrypsin-specific mRNA concentrations by dot-blot and Northern blot analyses using an α_1 -antitrypsin cDNA probe and DNA concentrations to correct for any variations in RNA yield; and 5) the rate of α_1 -antitrypsin gene transcription by nuclear run-off assays.

c. To assess the capacity of adherent and non-adherent monocytes to contribute to lung injury further, their expression of other proteinases and anti-proteinases could be compared since: 1) mononuclear phagocytes express proteinases, other than HLE, that may degrade matrix macromolecules (76,77,184,479); and 2) these cells contain proteinase inhibitors, other than α_1 -antitrypsin, that may contribute to the anti-proteinase screen in the lower respiratory tract (32,39,77,462). In addition, the effects of cellular activation and maturation on the capacity of these cells to degrade matrix components in vitro could be investigated since the expression of several of these proteins is modulated by activation and maturation of monocytes (32,39,77,125,479).

d. To assess the capacity of monocytes from patients with chronic inflammatory lung diseases to contribute to lung **injury**, adherent and non-adherent monocytes could be isolated from healthy subjects and patients with bronchiectasis and emphysema and the following could be compared: 1) their HLE and α_1 -**antitrypsin** content; 2) the effect of maturation and pro-inflammatory mediators on α_1 -**antitrypsin** expression; 3) their expression of other proteinases and anti-proteinases that contribute to the **proteinase-antiproteinase** balance in the lower respiratory tract; and **4)** their capacity to degrade a variety of matrix macromolecules in vitro.

Appendix 1

Materials and Suppliers

Material	Supplier
acetic acid (giacai)	Sigma Chemical Company, Poole, Dorset, U.K.
actinomycin D	Flow Laboratories, Irvine, Scotland, U.K.
agarose	Sigma Chemical Company, Poole, Dorset, U.K.
alkaline phosphatase - anti-alkaline phosphatase (APAAP) complex	Dakopatts, High Wycomb, U.K.
alpha₁-antitrypsin (human)	B.D.S. Biologicals Ltd., Birmingham, U.K.
ampiciltin	Beecham Research Laboratories, Brentford, Middlesex, U.K.
bacto-tryptone	Difco Laboratories Limited, East Mosely, Surrey. U.K.
BamH 1 restriction enzyme	Sigma Chemical Company, Poole, Dorset, U.K.
boric acid	Sigma Chemical Company. Poole, Dorset, U.K.
bovine serum albumin	Sigma Chemical Company, Poole, Dorset, U.K.
bromophenol blue	Sigma Chemical Company, Poole, Dorset, U.K.
N-butanol	Sigma Chemical Company, Poole, Dorset, U.K.
caesium chloride	Sigma Chemical Company, Poole, Dorset, U.K.
calcium chloride (CaCl₂)	Sigma Chemical Company. Poole. Dorset, U.K.
CD3, CD18, CD22 and p150/95 monoclonal antibodies	Dakopatts, High Wycombe U.K.
chloroform	Sigma Chemical Company, Poole, Dorset, U.K.
Coomassie brilliant blue G250 dye	Eastman Kodac Co , Rochester, New York , U.S.A.
cytochrome c	Sigma Chemical Company, Poole, Dorset. U.K.
dextran T 500	Pharmacia, Uppsala, Sweden.
Odianisidine (3,3'-dimethoxy benzidine)	Sigma Chemical Company, Poole, Dorset, U.K.
N,N-dimethyl formamide	Sigma Chemical Company, Poole, Dorset, U.K.
Eppendorf tubes	LIP (Equipment and Suppliers) Ltd, Shipley, Yorkshire , U.K.
ether	Sigma Chemical Company, Poole, Dorset, U.K.
ethidium bromide	Sigma Chemical Company, Poole, Dorset, U.K.
ethylene diamine tetracetic acid	Sigma Chemical Company, Poole, Dorset, U.K.
fast red substrate	Sigma Chemical Company, Poole, Dorset, U.K.
fetal calf serum	Flow Laboratories, Irvine, Scotland, U.K.
ficoll	Sigma Chemical Company, Poole, Dorset, U.K.
ficoll-Hypaque	Pharmacia, Uppsala, Sweden

fibronectin (human)	Sigma Chemical Company, Poole, Dorset, U.K.
rabbit anti-human fibronectin Fab₂	Organon Teknika-Cappel Corporation, Durham, NC, U.S.A.
formaldehyde	Sigma Chemical Company, Poole, Dorset, U.K.
formamide	Sigma Chemical Company, Poole, Dorset, U.K.
Gene-screen TM membrane	NEN Research, Boston, MA, U.S.A.
gentamicin	Flow Laboratories, Irvine, Scotland, U.K.
glacial acetic acid	Sigma Chemical Company, Poole, Dorset, U.K.
glucose	Sigma Chemical Company, Poole, Dorset, U.K.
glutamine	Flow Laboratories, Irvine, Scotland, U.K.
glycagel	Dakopatts, High Wycomb, U.K.
glycerol	Sigma Chemical Company, Poole, Dorset, U.K.
goat anti-mouse-FITC	Coulter Immunology, Hialeah, Florida , U.S.A.
goat IgG	Organon Teknika-Cappeli, West Chester, Pennsylvania, U.S.A.
granulocyte-macrophage colony stimulating factor	Genzyme, Boston, MA, U.S.A.
guanidinium isothiocyanate	Sigma Chemical Company, Poole, Dorset, U.K.
haemaccel	Hoechst U.K. Limited, Hounslow, Middlesex, U.K.
Hind III lambda DNA digest (DNA molecular weight markers)	Sigma Chemical Company, Poole, Dorset, U.K.
horse radish peroxidase type IV (HRP)	Sigma Chemical Company, Poole, Dorset, U.K.
human leukocyte elastase (HLE) purified from sputum	Dr. E.J. Campbell Salt Lake City, Utah, U.S.A.
human serum albumin (HSA)	American Red Cross, Washington D.C. U.S.A.
hydrogen peroxide (H₂O₂)	Fisher Scientific, Salt Lake City, Utah, U.S.A.
hydroxyquinolone	Sigma Chemical Company, Poole, Dorset, U.K.
Hypave	Winthrop Laboratories-Breon Laboratories, New York, U.S.A.
I3 monoclonal antibody and I3 conjugated to FITC	Coulter Immunology, Hialeah, Florida, U.S.A.
isoamyl alcohol	Sigma Chemical Company, Poole, Dorset, U.K.
interferon gamma	Genzyme, Boston , MA, U.S.A.
interleukin-1 β	Genzyme, Boston , MA, U.S.A.
latex beads (3 μ M diameter)	Sigma Chemical Company, Poole, Dorset, U.K.
levamisole	Sigma Chemical Company, Poole, Dorset, U.K.
limulus amoebocyte lysate kit (Coatest)	KabiVitrum, Stockholm, Sweden
lipopolysaccharide from E. Coli 0111 B4	Sigma Chemical Company, Poole, Dorset, U.K.
lipopolysaccharide from H. Influenzae	Dr. R. Wilson. National Heart and Lung Institute, London U.K.
lysozyme	Sigma Chemical Company, Poole, Dorset, U.K.
α_2-macroglobulin	B.D.S. Biologicals Ltd., Birmingham, U.K..

magnesium chloride (MgCl₂)	Sigma Chemical Company, Poole, Dorset, U.K.
β mercaptoethanol	Sigma Chemical Company, Poole, Dorset, U.K.
microtitre plates (96 well)	NUNC, LIP Ltd, Shipley, Yorkshire, U.K.
2-N-morpholinoethane sulphonic acid (MES)	Sigma Chemical Company, Poole, Dorset, U.K.
modified Wright's stain (Diff Quik)	Travenol Laboratories, Compton, U.K.
murine ascites (non-immune)	Liver Research Laboratories, Queen Elizabeth Hospital, Birmingham, U.K.
murine IgG (non-immune)	Coulter Immunology, Hialeah, Florida, U.S.A.
multi-test slides	Flow Laboratories, Irvine, Scotland, U.K.
MY7 monoclonal antibody	Coulter Immunology, Hialeah, Florida, U.S.A.
naphthol As-Mx phosphate	Sigma Chemical Company, Poole, Dorset, U.K.
Nonidet P40	Sigma Chemical Company, Poole, Dorset, U.K.
nonspecific esterase kit	Sigma Chemical Company, Poole, Dorset, U.K.
Nycodenz	Nycomed, Birmingham , U.K.
α³²P-dCTP	Amersham International PLC, Aylesbury, Bucks, U.K.
pancreatic RNase	Sigma Chemical Company, Poole, Dorset, U.K.
paraformaldehyde	Sigma Chemical Company, Poole, Dorset, U.K.
perchloric acid	J.T. Baker, Phillipsburg, New Jersey, U.S.A.
Percoll	Pharmacia, Uppsala, Sweden
polyoxyethylenesorbitan monolaurate (Tween-20)	Sigma Chemical Company, Poole, Dorset, U.K.
polypropylene RIA vials	Sarstedt, Princeton, NJ, U.S.A.
polypropylene tubes	Becton Dickinson Labware, Lincoln Park, NJ, U.S.A.
polyvinylpyrrolidone	Sigma Chemical Company, Poole, Dorset, U.K.
potassium chloride (KCl)	Sigma Chemical Company, Poole, Dorset, U.K.
potassium dihydrogen phosphate (KH₂PO₄)	Sigma Chemical Company, Poole, Dorset, U.K.
potassium hydroxide (KOH)	Sigma Chemical Company, Poole, Dorset, U.K.
phenol	Sigma Chemical Company, Poole, Dorset, U.K.
proteinase K	Sigma Chemical Company, Poole, Dorset, U.K.
rabbit anti-mouse immunoglobulin	Dakopatts, High Wycomb, U.K.
rabbit anti-sheep IgG conjugated to horse radish peroxidase	Organon Teknika–Cappel Corporation, Durham, N.C., U.S.A.
rabbit Fab₂ (non-immune)	Organon Teknika–Cappel Corporation, Durham, N.C., U.S.A.
random primer DNA labelling kit	Boehringer Mannheim U.K., Lewes, East Sussex , U.K.
RGDS and RGES peptides	Sigma Chemical Company, Poole, Dorset, U.K.
ribonucleoside vanadyl complexes	Sigma Chemical Company, Poole, Dorset, U.K.

RNAase	Sigma Chemical Company, Poole, Dorset, U.K.
RPMI 1640 medium	Flow Laboratories, Irvine, Scotland
salmon sperm DNA	Sigma Chemical Company, Poole, Dorset, U.K.
sephadex G50	Pharmacia. Uppsala, Sweden
sheep anti-human α_1 - antitrypsin (highly purified IgG fraction)	B.D.S. Biologicals Ltd., Birmingham, U.K.
sheep anti-human HLE pyclonal antiserum	B.D.S. Biologicals Ltd. , Birmingham, U.K.
sodium acetate	Sigma Chemical Company, Poole, Dorset, U.K.
sodium azide	Sigma Chemical Company, Poole, Dorset, U.K.
sodium borohydride	Sigma Chemical Company, Poole, Dorset, U.K.
sodium carbonate (Na₂CO₃)	Sigma Chemical Company, Poole, Dorset, U.K.
sodium chloride (NaCl)	Sigma Chemical company, Poole, Dorset, U.K.
sodium chloride solution (sterile, 0.15 mol/l)	Phoenix Pharmaceuticals, Gloucester, U.K.
sodium citrate	Sigma Chemical Company, Poole, Dorset, U.K.
sodium hydroxide	Sigma Chemical Company, Poole, Dorset, U.K.
sodium dihydrogen phosphate (Na H₂PO₄)	Sigma Chemical Company, Poole, Dorset, U.K.
sodium dodecyl sulphate (SDS)	Sigma Chemical Company, Poole. Dorset, U.K.
sodium hydrogen carbonate (Na HCO₃)	Sigma Chemical Company, Poole, Dorset, U.K.
sodium lauryl sarkosinate	Sigma Chemical Company, Poole, Dorset, U.K.
sodium periodate	Sigma Chemical Company, Poole, Dorset, U.K.
sodium pyrophosphate	Sigma Chemical Company, Poole, Dorset, U.K.
sucrose	Sigma Chemical Company, Poole, Dorset, U.K.
sulphuric acid	Sigma Chemical Company, Poole, Dorset , U.K.
superoxide dismutase	Sigma Chemical Company, Poole, Dorset, U.K.
thiomersal	Sigma Chemical Company, Poole, Dorset, U.K.
tissue culture dishes (35 x 10 mm)	Becton Dickenson, Lincoln Park, New Jersey, U.S.A.
tris (hydroxymethyl-aminomethane)	Sigma Chemical Company, Poole, Dorset, U.K.
trichloroacetic acid (TCA)	Sigma Chemical company, Poole, Dorset, U.K.
Triton X-100	Sigma Chemical Company, Poole, Dorset, U.K.
trypan blue	Sigma Chemical Company, Poole, Dorset, U.K.
tumour necrosis factor-α (TNF)	Genzyme, Boston , MA, U.S.A.
water (sterile distilled)	Phoenix Pharmaceuticals, Gloucester, U.K.
yeast extract	Difco Laboratories Ltd, East Mosely, Surrey, U.K.

Appendix 2

Demographic and Spirometrie Data for Patients with Bronchiectasis

Sputum Characteristics											
Mucoid				Mucopurulent				Purulent			
sex	age	FEV ₁ *	FEV1 FVC (%)	sex	age	FEV ₁ *	FEV1 FVC (%)	sex	age	FEV ₁ *	FEV1 FVC (%)
F	50	92.4	79.2	F	43	26.8	35.1	F	36	45.5	46.8
M	55	19.6	32.8	F	52	126.3	78.7	F	39	111.4	80.1
M	63	38.4	30.8	M	55	60.5	60.3	F	41	25.8	39.3
M	63	95.5	72.4	F	57	47.5	53.6	F	44	106.8	75.4
M	63	55.2	41.7	M	59	59.0	46	F	46	94.9	74.3
M	64	46.4	44.7	M	60	81.4	69.7	F	54	19.9	31.5
M	64	122.8	77.8	M	61	103.4	71.1	F	56	47.1	51.1
M	67	91.9	69.2	M	65	79.4	50.5	F	58	36.4	36.4
F	68	96.9	55.0	M	65	62.1	47.9	F	66	72.4	78.8
M	70	62.7	51.8	F	68	31.5	34.7	F	67	61.2	44.6
M	72	48.8	61.2	F	69	36.6	37.8	F	68	31.5	34.7
M	73	21.3	18.0	F	71	89.8	75.4				
M	75	92.4	74.6	F	74	42.5	41.0				
Mean	65.2	68.0	54.6		61.5	64.2	55.0		52.3	59.4	53.9
S.D.	7.0	32.6	19.9		8.5	30.1	15.1		11.7	32.7	19.3

* % predicted
 FEV₁ : forced expiratory volume in 1 second
 FVC : forced vital capacity

Appendix 3

Comparison of Monocyte Adherence to Fibronectin and to Endothelial cells

Methods

Adherence Substrates

Tissue culture dishes were coated with the optimal concentration of human fibronectin, as described in Methods. Human umbilical vein endothelial cells (HUVEC) were grown to confluence in 24 well tissue culture dishes, as described previously (309). The HUVEC monolayers were kindly supplied by Dr. A. Sturrock, Division of Respiratory, Critical Care and Occupational Pulmonary Medicine, University of Utah, Salt Lake City, Utah, U.S.A. The monolayers were cultured overnight in RPMI medium supplemented with 10% [v/v] FCS and gentamicin (20 $\mu\text{g/ml}$) and the culture supernatants were discarded immediately prior to the adherence assay.

Monocyte Isolation and Adherence Assays

Monocytes were isolated from two healthy donors by the Nycodenz method, as described in Methods. The monocyte preparations were greater than 92% pure, as assessed by modified Wright's staining, and greater than 99% viable, as assessed by exclusion of trypan blue dye. Monocytes were suspended at a concentration of 10^6 cells/ml in supplemented RPMI medium and 1 ml aliquots of the cell suspension were dispensed into two wells containing HUVEC monolayers and two fibronectin-coated tissue culture dishes. The cells were incubated for 1 h at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. Non-adherent cells were removed by vigorous washing with sterile, ice-cold 0.15 mol/L NaCl solution, centrifuged (600 g for 5 min), re-suspended in medium and counted using a haemocytometer. The proportion of adherent cells was calculated from the initial number of cells added to the assay and the number of non-adherent cells.

Results and Discussion

The results showed that similar proportions of monocytes adhered to fibronectin-coated dishes and to HUVEC monolayers (table 9). To exclude the possibility that HUVEC had been detached along with non-adherent monocytes during the washing procedure, cytocentrifuge preparations of the non-adherent cells obtained from the HUVEC-containing wells were prepared. The cytocentrifuge preparations were stained with

modified Wright's stain and examined under oil immersion. Less than 0.5% of the non-adherent cells were HUVEC. These data indicate that a similar proportion of monocytes from healthy subjects adheres spontaneously to fibronectin and to HUVEC monolayers in vitro.

Since monocyte adherence to endothelial cells is a prerequisite for their recruitment from the vasculature in vivo, these preliminary data suggest that the capacity of monocytes to adhere to fibronectin in vitro is related to their capacity to be recruited into tissues in vivo.

Table 9

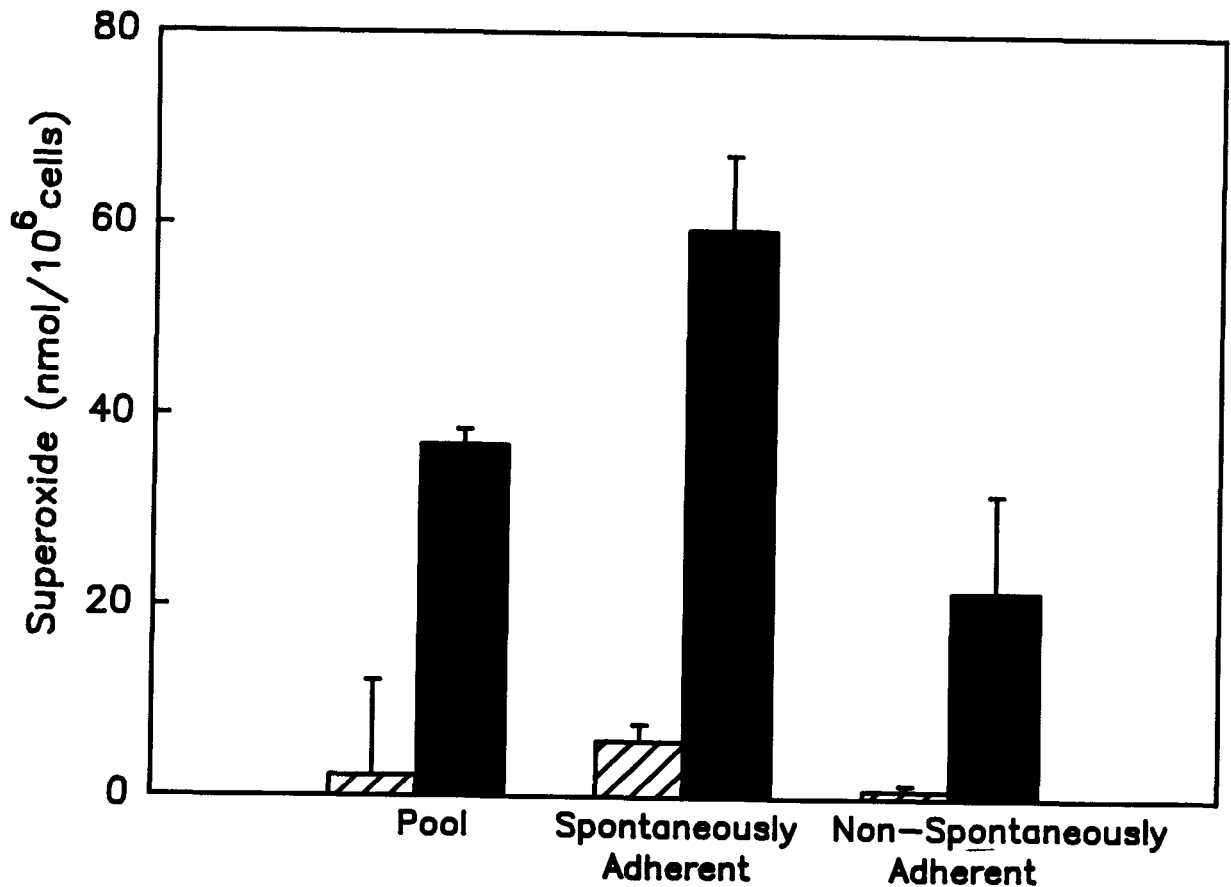
Comparison of Monocyte Adherence to Fibronectin and HUVEC Monolayers

Donor #	Percentage of Monocytes Adhering to:	
	Fibronectin	HUVEC monolayers
1	27%	25%
2	25%	25%
Mean \pm SD	26 \pm 1%	25 \pm 0%

Monocytes were isolated from two healthy donors and the proportion of cells that adhered spontaneously to fibronectin and HUVEC monolayers was compared.

Appendix 4

Adherence Properties and Superoxide Anion Release by CCE-isolated Monocytes



Appendix 4 Figure shows the relationship between monocyte adherence to fibronectin and superoxide production by monocytes. Monocytes were isolated by CCE and incubated in fibronectin-coated dishes for 1 h at 37°C to separate the spontaneously adherent and non-spontaneously adherent cells, as described in Methods. Superoxide anion production by pool monocytes and the monocyte subpopulations was measured in triplicate, as the superoxide dismutase-inhibitable reduction of cytochrome *c*, as described in Methods. Cells were unstimulated (cross-hatched bars) or stimulated with phorbol myristate acetate (PMA) at a final concentration of 50 ng/ml (solid bars). Note the relationship between monocyte adherence to fibronectin and superoxide anion production. The spontaneously adherent cells produced more superoxide than the non-spontaneously adherent cells both under basal conditions and in response to stimulation with PMA. Data are mean values; error bars represent SEM; *n* = 3 donors.

Appendix 5

Publications

Abstracts

1. Owen C.A., S.C. Afford, D. Burnett and R.A. Stockley (1988). Monocyte adherence in bronchiectasis and the effect of bacterial lipopolysaccharide. Clin. Sci., 75 (suppl 19): 45.
2. Afford S.C., C.A. Owen, R.A. Stockley and D. Burnett (1988). Synthesis of α_1 -antiproteinase inhibitor by the promonocytic U937 cell line. Thorax, 43: 820-821.
3. Owen C.A., S.C. Afford, L.W. Poulter, D. Burnett and R.A. Stockley (1989). Expression of surface markers and superoxide production by monocyte subpopulations. Am. Rev. Respir. Dis., 139 (No 4): 159
4. Owen C.A., S.C. Afford, D. Burnett and R.A. Stockley (1989). Differential α_1 -antitrypsin synthesis by monocyte subpopulations. Am. Rev. Respir. Dis., 139 (No 4): 201.
5. Owen C.A., S.C. Afford, S.L. Hill, D. Burnett and R.A. Stockley (1989). Monocyte adherence in bronchiectasis. Am. Rev. Respir. Dis., 139 (No 4): 127
6. Owen C.A., S.C. Afford, D. Burnett and R.A. Stockley (1989). Functional heterogeneity of circulating monocytes. Clin. Sci. 77 (suppl 21): 16
7. Owen C.A., S.C. Afford, D. Burnett and R.A. Stockley (1989). α_1 -antitrypsin synthesis by monocyte subpopulations. Thorax 44 (No 10):874
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