

CELLULAR INTERACTIONS IN PULMONARY FIBROGENESIS

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

in the

Faculty of Medicine

University of London

by

ROBERT JAY EMERSON, B.A.

Host Defence Unit
Department of Medicine
Cardiothoracic Institute
Brompton Hospital
London

1980

ABSTRACT

Many pulmonary diseases have fibrosis as their end-stage but the pathogenesis of fibrosis is still obscure.

This thesis describes an animal model which was designed to determine whether mononuclear cell activation plays a role in pulmonary fibrogenesis, paying particular attention to the pulmonary macrophage and collagen content of the lung.

The aim was to introduce too low a number of inhaled asbestos fibres to cause fibrosis by themselves and to study the effect of various additional stimuli which might be expected to activate pulmonary mononuclear cells.

Specific pathogen-free guinea-pigs were exposed to asbestos by inhalation for two eight-hour periods. Some groups of animals were treated with Freund's complete adjuvant by hind footpad injections prior to asbestos exposure, some were infected with a live non-virulent strain of Mycobacterium tuberculosis by subcutaneous injection after asbestos exposure, and some received both these additional stimuli.

The effects of these various treatments were evaluated by light and electron microscopy of lung tissue, light

microscopy of living and fixed mononuclear cells extracted from the lung, biochemical quantitation of lung collagen, measurement of pulmonary macrophage enzyme levels, detection of peripheral blood lymphocyte blast transformation, measurement of lung deoxyribonucleic acid (DNA) levels, measurement of body weight and lung weight, and by gross observations at necropsy.

The principal findings were:

1. Asbestos bodies were demonstrated from three weeks following exposure;
2. Peripheral blood lymphocyte blast transformation and skin testing to PPD provided evidence that these mononuclear cells had become sensitized;
3. Mycobacteria could not be detected in the lung;
4. Varying patterns of a chronic inflammatory response were evident, with the severity and time of development dependent on a particular treatment;
5. Large diffuse increases in reticulin fibres were present in the lungs of groups with activated mononuclear cells, the severity depending on the method used to activate such cells;
6. Increases in lung collagen using Masson's trichrome and Weigert-van Gieson stains were not demonstrable;
7. Extractable lung collagen (hydroxyproline) follows an irregular course, the most significant change being a

- reduction during the early phase of the reactions,
which was dependent on the treatment given;
8. Pulmonary macrophage activation and maturation was demonstrated by light and electron microscopic alterations and by ability to adhere to and spread on glass;
 9. An enzyme marker for activation of pulmonary macrophages (aminopeptidase activity) did increase.

Morphological and biochemical events are discussed in detail in relation to the regulatory nature of the inflammatory process as related to fibrosis and comparisons are made with other in vivo models.

DEDICATION

To Cindy and Tom, for
without their dedication
this thesis could not have
been completed.

One of the most characteristic features of pathological processes is their duality of effect so that they may act sometimes for the benefit of the host, sometimes for his detriment.

W.G. Spector
In Biology of the
Fibroblast 1973

ACKNOWLEDGEMENTS

I wish to give most special thanks for Dr. Peter Cole for his encouragement and extreme kindness.

In addition, I would like to thank:

Professor Bryan Corrin for his helpful advice and support and for allowing me to use the electron microscopy facilities of St. Thomas's Hospital Medical School.

Professor Paul Holt for continued support and providing the facilities for asbestos dust inhalation.

Miss Margaret Rehahn and Professor David Sylwester for advice and teaching of statistical methods.

Mr. Robin J. McAnulty for technical assistance.

The staff of the Audio-Visual Department, Chelsea Collage for printing of photographs.

The staff of the electron microscopy department, St. Thomas's Hospital Medical School for preparation of tissue for electron microscopical examination and for preparation of electron micrographs.

Eric Bateman, Robert J. Emerson and Peter Cole and the publisher of Lung Biology in Health and Disease for the courtesy of allowing me to reproduce Figure 1.

I am also grateful to members of the Host Defence Unit, particularly to Mr. David Roberts for listening to my problems and frustrations and for making me laugh when it seemed impossible.

Finally, I want to give my deepest thanks to my wife who has, without complaint, typed the manuscript and provided me with her continual support throughout this study.

List of Figures

<u>Figure</u>	<u>Page</u>
1. Simplified scheme of collagen biosynthesis	62
2. Electron micrograph of guinea pig lung showing Type I epithelial cell (TPI), Type II epithelial cell (TPII), micro-villie (MV), endothelium (E), interstitium (IN), collagen fibres (CF) and an alveolar mononuclear phagocyte (M).X 7450.	73
3. Schematic representation of the two-stage theory of fibrogenesis.	79
4. Asbestos exposure apparatus.	98
5. Specimen of guinea pig lung six weeks following treatment (Group Asbestos + Freund's complete adjuvant + mycobacteria) showing macroscopic nodules and discoloration. Approx. 3.5 X natural size.	119
6. Guinea pig lung section showing interstitial mononuclear cell infiltration. Haematoxylin and eosin (H) X 200.	171
7. Typical small loss sheet of mononuclear cells with adjacent normal lung. HE.X 100.	171
8. Large sheet of mononuclear cells organized into granulomata, causing obliteration of alveoli. HE. X 100.	173

<u>Figure</u>		<u>Page</u>
9.	Large area of focal granulation tissue infiltrated with mononuclear cells causing complete obliteration of lung architecture. HE. X 100.	173
10.	Sheet of mononuclear cells showing marginated chromatin and nucleoli. Many cells have become confluent causing their plasma membranes to be indistinguishable. HE. X 400.	175
11.	Intra-alveolar and intra-bronchiolar cellular exudate. Note interstitial and peribronchiole mononuclear cell infiltration. HE. X 200.	177
12.	Normal reticulin fibres of guinea pig lung interstitium. Gordon and Sweet's silver impregnation. X 200.	179
13.	Severe mononuclear cell infiltration among which new fine reticulin fibres have developed. Gordon and Sweet's silver impregnation. X 100.	181
14.	Obliteration of lung architecture by massive increases in reticulin fibres which have become thickened and branched. Note argyrophilic material in remaining alveolar spaces (arrow) Gordon and Sweet's silver impregnation X 200.	181
15.	Electron micrograph showing asbestos in a Type I epithelial cell. X 60,000 approx.	183
16.	Minor perivascular lymphocyte cuffing. HE. X 200.	185

<u>Figure</u>	<u>Page</u>
17. Perivascular lymphocytic cuffing similar to Fig. 16 but more intense. HE. X 100.	185
18. Granuloma caused by combination of asbestos inhalation and subcutaneous injection of Freund's complete adjuvant. Oil was contained within the clear spaces (arrows) surrounded by giant cells and has been removed during tissue processing. HE. X 80.	187
19. Higher power view of Fig. 18 showing giant cell (arrow). HE. X 660.	187
20. A section of lung showing asbestos bodies (arrows) among inflammatory cells. Perl's Prussian blue reaction. X 1000.	189
21. Phagosome of alveolar mononuclear phagocyte containing asbestos. X 45,000.	191
22. Typical aggregate of alveolar mononuclear phagocytes seen in lungs of all treated groups. Note numerous pseudopods representative of activated cells and the immature cell (arrow) among the group of mature mononuclear phagocytes. X 4875.	193
23. Electron micrograph of monocyte within the alveolar space. X 21,900.	195
24. Electron micrograph of immature mononuclear phagocyte within alveolar space. X 16,000.	197
25. Electron micrograph of mature mononuclear phagocyte with alveolar space. Note asbestos fibre (arrow). X 11,200.	199

<u>Figure</u>		Page
26.	Photomicrograph of live glass adherent mononuclear phagocytes. Majority of cells are round with numerous cytoplasmic inclusions. Note spreading and elongation of cells (arrows). Saline. X 400.	201
27.		
a.	Cytocentrifuge preparation showing binucleate cell among mononuclear phagocytes with vacuolated cytoplasm. May-Grunwald-Giesma. X 200.	203
b.	Cytocentrifuge preparation showing binucleate cells and mononuclear phagocytes associated with asbestos bodies (arrows). Cytoplasm of these cells is less vacuolated compared to cells seen in Fig. 27a. May-Grunwald-Giesma. X 200.	203
c.	Cytocentrifuge preparation showing mononuclear cell in mitosis. May-Grunwald-Giesma X 1000.	203
28.	Causes of Pulmonary Fibrosis.	239

List of Tables

<u>Table</u>	<u>Page</u>
1. Causes of Interstitial Pneumonia	21
2. Synonyms for Pulmonary Fibrosis	23
3. Causes of Pulmonary Fibrosis 1800-1921	31
4. Animal Models of Pulmonary Fibrosis	41
5. Advantages and Disadvantages of Animal Models of Pulmonary Fibrosis	42
6. Summary of Types, Molecular Structure and Distinguishing Characteristics of Lung Collagen	57
7. Parameters Studied in Thesis	95
8. Pathogens Not Demonstrable in SPF Guinea-Pigs	99
9. Viable <u>M. tuberculosis</u> (H37Ra)	103
10. Histological Stains Used in Thesis	113
11. Summary of Histopathology Following Treatment	121
12. Morphological Criteria Used to Identify Monocytes, Immature Macrophages and Mature Macrophages	137
13. Differential Counts of Harvested A-H Pulmonary Cells	141
14. Mean Total Hydroxyproline Content of Lung (μg)	151
15. Mean Hydroxyproline Content of Lung ($\mu\text{g}/\text{gram}$ wet weight lung)	152
16. Mean Hydroxyproline Content of Lung ($\mu\text{g}/\text{milligram}$ dry weight lung)	154

<u>Table</u>	<u>Page</u>
17. Mean DNA Content of Lung (μg /milligram dry weight lung)	156
18. Leucine Aminopeptidase Activity	157
19. Peripheral Blood Lymphocyte Blast Transformation to PPD Stimulation Index	159
20. Cutaneous Skin Test to PPD Mean Diameter of Induration (mm)	161
21. Mean Body Weight (g)	163
22. Mean Total Lung Wet Weight (g)	164
23. Mean Left Lower Lobe Wet Weight (g)	165
24. Mean Lung Dry Weight (g)	166
25. Examples of Long Time Periods Used for Asbestos Fibre Inhalation	212

CONTENTS

	<u>Page</u>
Abstract	1
Dedication	4
Aknowledgements	6
List of Figures	8
List of Tables	12
 <u>Section I : Introduction</u>	 19
1. Terminology	20
2. History	24
3. Classification	33
4. Existing Models of Fibrosis	36
A. <u>In vitro</u> Models	36
a. Lung Explant Cultures	36
b. Lung Cell Cultures	38
c. Cell-Free Models	39
d. Diffusion Chamber Models	39
B. <u>In vivo</u> Models	40
a. Problems-Advantages-Limitations	40
b. Choice of Animal	45
c. Specific Pathogen Free Status	45
d. Prior Non-Specific Stimulation of Immune System.	46
e. Manipulation of Elements of Immune System	46
5. Interpretation of Models	47
 <u>Section II : Aim of Thesis</u>	 49

<u>Section III : Pulmonary Fibrogenesis:</u>	<u>Page</u>
<u>Structural and Functional Aspects</u>	53
1. Non-Cellular Constituents	55
A. Collagen	55
a. Structure	55
b. Types, Ratios, and Distribution	56
c. Synthesis	56
i. Transcription & Translation	56
ii. Hydroxylation	58
iii. Glycosylation	60
iv. Triple-Helix Formation	61
v. Secretion	61
vi. Tropocollagen & Microfibril Formation	61
d. Degradation	63
B. Elastic Fibres	66
C. Proteoglycans	68
2. Cellular Constituents	68
A. Alveoli and Capillary Lining	69
a. Type I Epithelial Cell	70
b. Type II Epithelial Cell	70
c. Endothelial Cell	71
B. Interstitial Cell	74
a. Fibroblast - Pericyte	74
C. Blood Derived Mononuclear Cells	77
a. Macrophage	77
i. Origin	77
ii. Silica-Macrophage-Fibroblast Interaction	78
iii. Asbestos-Macrophage-Fibroblast Interaction	85
iv. Asbestos-Macrophage Cell Membrane Interaction	86
v. Macrophage Replenishment	89
vi. Complement	90
b. Lymphocytes	90

	<u>Page</u>
<u>Section IV : Materials and Methods</u>	92
1. Experimental Groups and Model Design	93
2. Animals	94
3. Treatments	96
A. Asbestos	96
B. Asbestos Inhalation	96
C. Freund's Complete Adjuvant	101
a. Sensitization of Mononuclear Cells	101
D. <u>Mycobacterium tuberculosis</u> Culture	102
a. <u>M. tuberculosis</u> Inoculation	102
4. Preparation of Pulmonary Mononuclear Cells	102
5. Cytological Examination	105
6. Cell Viability	106
7. Cell Counts	106
8. Morphology of Glass Adherent Cells	106
9. Leucine Aminopeptidase Activity	107
10. Skin Test	108
11. Lymphocyte Blast Transformation	108
12. Hydroxyproline, Deoxyribonucleic Acid (DNA) and Dry Weight Determinations	110
A. Hydroxyproline Determination	110
B. Deoxyribonucleic Acid Determination	111
C. Dry Weight Determination	111
13. Histology	111
14. Electron Microscopy	112
15. Gross Observations	114
16. Index of Pulmonary Response to Insult	114
17. Statistical Methods	115
 <u>Section V : Results</u>	 116
1. Gross Pathology	117

	<u>Page</u>
2. Microscopy	117
A. Histology	117
a. Group Asbestos + Freund's Complete Adjuvant + Mycobacteria (AFM)	120
b. Group Asbestos + Freund's Complete Adjuvant (AF)	125
c. Group Asbestos + Mycobacteria (AM)	128
d. Group Freund's Complete Adjuvant + Mycobacteria (FM)	129
e. Group Asbestos (A)	131
f. Group Freund's Complete Adjuvant (F)	132
g. Group Mycobacteria (M)	134
h. Group Untreated Control (N)	135
B. Electron Microscopy	135
C. Glass Adherent Mononuclear Cells	139
D. Cytology	139
3. Statistical Procedures	140
4. Biochemistry	149
A. Hydroxyproline Determinations	150
a. Total Hydroxyproline	150
b. Hydroxyproline per gram of Lung (wet weight)	150
c. Hydroxyproline per milligram Lung (dry weight)	153
B. Deoxyribonucleic Acid Determination	155
C. Leucine Aminopeptidase Activity	155
5. Sensitization of Lymphocytes	158
6. Weight Relationships	160
7. Summary of Results	167
 <u>Section VI : Discussion of the Results of Treatment</u>	 204
1. Histology	205

	<u>Page</u>
2. Maturation of Pulmonary Mononuclear Phagocytes	215
3. Maintenance of Granulomas and Chronic Inflammation	218
4. Sensitization of Mononuclear Cells	220
5. Activation of Mononuclear Phagocytes	221
6. Histological Estimation of Collagen	224
7. Biochemical Estimation of Collagen	226
8. Weight Relationships	229
 <u>Section VII : Summary and Conclusions</u>	 231
 Appendices	 240
Bibliography	264

Section I : Introduction

Section I : Introduction

Pulmonary fibrosis presents a formidable challenge to contemporary respiratory medicine. It is not a disease unto itself but should be considered the end result of a final common pathway of many biological responses to various insulting agents. It is a source of continuing morbidity and mortality, treatment being largely empirical and palliative in the absence of knowledge of its pathogenetic mechanism(s).

1. Terminology

Pulmonary fibrosis is a general term that encompasses two specific pathological processes that can occur within the peripheral regions of the lung. Interstitial fibrosis is a process whereby changes initially occur within the alveolar wall and intra-alveolar fibrosis is a process whereby changes originate in the lumen of the alveolus. The latter normally occurs following unresolved acute pneumonia when the fibrinous intra-alveolar exudate organizes instead of becoming absorbed. The alveoli become filled with a proliferating mass of fibroblasts which is followed by fibrosis. Interstitial fibrosis is the final common pathway of interstitial pneumonia, caused by a variety of agents (Table 1). Histologically it is represented by a sequence of inflammatory changes. These include

Table 1.Causes of Interstitial Pneumonia

Inorganic dust (i.e. asbestos, silica)

Drugs (e.g. bleomycin, buslphan)

Chemical (e.g. beryllium, paraquat, lipids)

Bacteria (e.g. M. tuberculosis)

Viruses

Radiation

Pulmonary oedema (e.g. uraemic lung)

hyperplasia of Type II pneumonocytes, thickened alveolar septa due to oedema and mononuclear cell infiltration, fibroblast and smooth muscle cell proliferation and fibrosis. As the alveolar walls become thicker they can eventually obliterate the alveolar spaces, thus making it difficult to distinguish between interstitial and intra-alveolar fibrosis.

Pulmonary fibrosis has been discussed under various names for almost two centuries, each name based from gross or histopathological observations of various stages of fibrosis (i.e. cellular - fibrous - honeycomb). For example, "cirrhosis" of the lung was a name that was first accepted in medical literature in 1838 (Clark et al, 1894) and remained in usage well into the second decade of the twentieth century (Powell and Hartley, 1921). This name was chosen following the observation that a severely fibrotic lung had a very nodular appearance resembling a cirrhotic liver. We know today what in fact had been seen was a honeycomb lung. In older literature another name, fibroid phthisis, was frequently used and meant that there was a fibrous consolidation of the lung (Clark et al, 1894).

These names gradually disappeared from the literature, however other names have remained in use for over a century and still other new names have appeared until today we have a variety of synonyms describing what is in the end, fibrosis (Table 2).

Table 2Synonyms For Pulmonary Fibrosis

Usual interstitial pneumonia
Desquamative interstitial pneumonia
Organized interstitial pneumonia
Diffuse interstitial pneumonitis
Chronic interstitial pneumonitis
Interstitial pulmonary fibrosis
Idiopathic pulmonary fibrosis
Idiopathic interstitial pulmonary fibrosis
Chronic interstitial fibrosis of lung
Diffuse interstitial pulmonary fibrosis
Chronic diffuse interstitial fibrosis of lungs
Acute diffuse interstitial fibrosis of lungs
Hamman-Rich syndrome
Muscular cirrhosis
Chronic diffuse fibrosing alveolitis
Cryptogenic fibrosing alveolitis

2. History

Whilst it is probable that Avenbrugger (1761) and Morgagni (1769) were the first to describe the gross pathology of pulmonary fibrosis, it was not until the 19th century that several investigators began to write detailed accounts of its pathogenesis.

In literature of the 19th and first quarter of the twentieth century authors treated the subject of pulmonary fibrosis under a variety of different names, describing different pathological conditions and holding diverse opinions as to its pathogenesis.

Bayle (1815) was the first investigator to describe clinical and pathological features of pulmonary fibrosis which he called phthisis with melanosis. However, he presented no evidence for a cause except when fibrosis was complicated by tuberculosis. He does not mention whether tuberculosis was a primary infection preceeding fibrosis or a secondary infection which developed after fibrosis occurred. In either case, pathologically, the lungs had a thick adherent pleura, black pigmentation and were hard to the extent that they resembled cartilagenous tissue.

Bayle only described six cases of phthisis with melanosis however it is clear that he was in fact discussing pulmonary fibrosis and although he did not discuss the

microscopic changes or theorize as to its pathogenesis, his work laid the foundation for others to build on.

Laennec (1834) considered pulmonary fibrosis to be a secondary complication of tuberculosis since he seldom observed fibrosis except in tuberculosis infections. Hasse (1846), Grisolles (1864) and Stokes (1882) also maintained Laennec's doctrine that fibrosis was a sequela to tuberculosis. These investigators also made brief mention of chronic pneumonia. They described it as a condition in which the lung was gray to black in colour, dense, hard, resisting traction and pressure, sometimes granular and at other times smooth with white bands marking the increases in intralobular tissue. They thought that fibrosis around tubercles was caused by the organisms and not a chronic pneumonia which they considered to be extremely rare.

Andral (1836) however, did not consider chronic pneumonia to be rare, therefore his work established a second school concerning the mechanisms involved in fibrosis. He believed that Bayle's phthisis with melanosis was actually the result of an inflammatory process and that the degree of colouring was related to the chronicity (e.g. red was the early or acute stage of inflammation with gray and black being a direct consequence of the chronic stage). He did not believe that the fibrosis seen around a tubercle was a result of the tubercle itself but was due to the chronic pneumonia. His reasoning was, since tubercles appear in

areas of chronic pneumonia, the pneumonic condition preceded the development of tubercles. Andral also demonstrated that there was considerable variation in the degree of development of chronic inflammatory lesions and these variations could be confined to a small area of lung. He taught that if lung is improperly sectioned for gross and microscopic examination, representative areas depicting all developmental stages of a chronic inflammatory lesion ending in fibrosis may be missed, thus creating an avenue for misinterpretation of the mechanism(s) responsible for fibrosis. He was the first to advance the theory that certain forms of pulmonary fibrosis have no known etiology (i.e. are idiopathic). This is the first discussion of fibrotic lung conditions that are even today given the names idiopathic pulmonary fibrosis (Crystal et al, 1976) or diffuse fibrosing alveolitis (Scadding, 1964; Scadding and Hinson, 1967).

These investigators did not distinguish between inflammatory changes and fibrosis predominantly affecting alveolar septa as opposed to "pneumonia" which is generally used to refer to inflammations of the lung characterized by consolidation by inflammatory exudation filling alveolar spaces. It therefore appears that they considered fibrosis to be a result of intra-alveolar reactions.

Investigations comparing fibrosis with and without mycobacteria present were performed by Addison (1868).

He hypothesized how to distinguish between the two by use of gross and microscopic observations combined with clinical data. He believed that fibrosis whether it was associated with or without mycobacteria had the same origin. This he called "pulmonic inflammation". In his general discussions of fibrosis, he describes three varieties: uniform albuminous, granular and gray indurations, all three of which he considered to be the consequence of acute pneumonia which was either slow to resolve or which became recurrent. Addison also examined the question that has not been answered today. That is why one case of pneumonia may resolve completely while another remains unresolved developing into a fibrous state. His answer to this question may be over simplified in todays terms but in his time, he formulated the following: An individual with unresolved pneumonia remains in such a state because of a general debilitation of the individual, in other words a "diathesis" was involved. Clark et al (1894) have summerized Addisons' opinions as such: "1. that pulmonary indurations were almost always the result of pneumonia or of pulmonic inflammation of some kind, at first free from tubercle; 2. that when found with tubercle, the latter had become engrafted secondarily".

In a work published by Grisolle (1864) the most important feature concerning pulmonary fibrosis was the fact that fibrotic lung lesions may be caused by agents other

than bacteria, especially forms of inorganic irritating dusts.

Between 1867-1894 little is added to the views already known. The journals and textbooks become stereotyped, using descriptions set forth by Bayle, Laennec and Andral. We are left at this time with diverse opinions as to the origin and pathogenetic mechanism(s) of pulmonary fibrosis. Although the end result without question was the same, the disease or conditions described were as varied as the number of authors reporting their individual work.

The first book providing a comprehensive, up-to-date, coherent critique of the clinical and pathological events in pulmonary fibrosis appeared in 1894 (Clark et al, 1894). This volume is appropriately summarized from a paragraph from the authors preface:

"Our first idea was to bring out a book of plates, with short descriptions illustrating the various morbid conditions found in fibroid lungs. Later, as more material came to hand, we decided to enlarge the scope of the work, and to examine more thoroughly the whole subject of fibroid diseases of the lungs. We have, therefore, collected the views to be found in most of the literature upon the subject, and have endeavoured to make the book useful for purposes of reference; based as it is upon an inquiry as searching as possible, and illustrated by a large number of typical instances of the disease than, we venture to think, has ever before been brought together".

A feature of this book is the extensive histopathological description of the entire respiratory system in relation to fibrosis. Credit must be given to these investigators for the accurate histological description of fibrotic lungs, given the quality of tools they had to work with compared to the sophisticated laboratory of today. We can find little fault of what they said concerning the light microscopic appearance of fibrotic lungs.

It was work such as this which laid the cornerstone for present day investigations into the pathogenesis of pulmonary fibrosis.

In Babcocks' text (1907) there is a chapter entitled "Chronic Interstitial Pneumonia - Fibrosis of the Lung - Fibroid Phthisis". The title alone points out the influences of earlier investigators in this area of study and that the questions and controversy pertaining to fibrosis have not been settled. The actual text of this chapter discusses the writings of Bayle and Laennec. It adds nothing to what has previously been mentioned.

In Powells' "Diseases of the Lungs and Pleurae", (1911) a chapter is given the title Chronic Interstitial Pneumonia or Cirrhosis of the Lung - Pneumoconiosis. Again, the title speaks for itself. The views of Laennec and others still persist. There are however, a few points discussed by

Powell that although not new, are brought out more clearly than in earlier works. His description of the morbid changes in pulmonary fibrosis are fairly uniform in all cases and the microscopic changes follow a pattern of cellular infiltration with the eventual disappearance of cells when the fibrotic lesions are fully developed. He also discusses four types of irritating dust that were thought to cause fibrotic lesions: iron, silica, coal and cotton but includes no explanation as to the pathogenetic mechanism leading to fibrosis. His section on pneumoconioses only dealt with the above mentioned dusts possibly because of the types of industry that were emerging at this time and we were just beginning to recognize the vast variety of dusts that could lead to pulmonary fibrotic lesions.

In the sixth edition of Powells' text, his chapter on pulmonary fibrosis has not changed one word with the exception of a case presentation of pneumoconiosis and a somewhat more detailed description of silicosis (Powell and Hartley 1921).

Compared with our present knowledge of the aetiology of pulmonary fibrosis the causes up to this period in history were quite short (Table 3).

During the next nine years, little is added to what was written. This lack of interest in investigations

Table 3Causes of Pulmonary Fibrosis 1800 - 1921

Broncho-pneumonia

Acute croupous pneumonia

Chronic bronchitis

Pleurisy

Inhalation of irritating dust

Collapse of the lung

Syphilis

Bronchiectasis

Trauma

Fibroid diathesis

Alcohol

Tuberculosis

concerning pulmonary fibrosis may be explained if one considers the important areas of medical research taking place between 1894 and the first quarter of the 20th century. The biggest strides at this time were in the areas of surgical antiseptic procedures, bacteriology and the beginning of a new field called immunology. It may have been felt by the individuals working in these areas, that they could not add to the works of Laennec, Andral, and Clark.

A rekindling of research interests in pulmonary fibrosis began to appear around 1930 and has continued at an explosive pace, especially over the past decade. Four apparent reasons for this renewed interest can be cited. First, there has been a continual increase in the number of recognized agents that are capable of causing pulmonary fibrosis. These agents include inorganic dusts, bacteria, radiation, toxic chemicals and fumes, drugs and immunological related insults. Second, the discovery by Hamman and Rich (1935) of a progressive, rapidly fatal, diffuse, interstitial pulmonary fibrosis of unknown aetiology has created an area of intense investigations into the pathogenetic mechanisms responsible for interstitial pulmonary fibrosis (Livingstone et al, 1965; Scadding and Hinson, 1967; Gross, 1962; Stack et al, 1965; Crystal et al, 1976; Fulmer and Crystal, 1976 and Turner-Warwick, 1974). Third, despite the volume of research that has been conducted in

animals, the majority of experiments have attempted to assess the fibrogenicity of agents or to reproduce particular disease entities, (e.g. asbestosis, extrinsic allergic alveolitis). These studies have concentrated on the histopathological changes i.e. the results of, rather than the basic mechanisms of fibrogenesis. Histological studies must be correlated with as many other studies employing as many techniques as possible; for example, histology combined with analysis of collagen synthesis and degradation, analysis of various enzymes and immunological studies. Fourth, despite the fact that the basic mechanisms of fibrosis are not understood and with the increasing recognition of the condition, research conducted with treatment of fibrosis in mind has gained a new impetus.

3. Classification

A list of diagnostic categories has been set forth by Scadding (1974). This classification is also applicable to experimental studies and has the advantage of not being too rigorous and restrictive. In the first category are those diseases that can be defined aetiologically. This is divided into three subcategories. In the first subcategory are those diseases which are caused by inhaled inorganic and organic dust (e.g. asbestos, silica, thermophilic actinomycetes and avian antigens). The second subcategory consists of ingested toxic substances (e.g. paraquat) and

the third subcategory consists of the infectious diseases that may lead to fibrosis. In the second category are those conditions which result from undetermined causes but can be defined histopathologically. This category has been subdivided into two groups. Those which are related to a systemic disease (e.g. sarcoidosis, eosinophilic granuloma) and those found only in association with the respiratory system (e.g. fibrosing alveolitis).

Experimental investigations are performed either to study the effects of a known insult (e.g. asbestos, silica) to the lung or to mimic the processes that have been defined under the histopathologically defined category. Thus the experimentalist should be familiar with such a diagnostic categorization of fibrotic lung diseases.

A second classification scheme based on the histopathology of pulmonary fibrosis has been devised by Liebow (1975). He has classified interstitial pulmonary fibrosis as 1. usual interstitial pneumonia (UIP), 2. desquamative interstitial pneumonia (DIP), 3. bronchiolitis obliterans with usual interstitial pneumonia (BIP), lymphoid interstitial pneumonia (LIP) and giant cell interstitial pneumonia (GIP).

UIP represents the classical histopathologic description of idiopathic pulmonary fibrosis and fibrosis caused by viruses, radiation, drugs, immunological factors (e.g.

hypersensitivity reaction) and toxic chemicals. Initially there is damage to the Type I epithelial cells lining the air spaces and capillary endothelium. Damage to these cells cause leakage of fluids, congestion, oedema and hyaline membrane formation; this is followed by proliferation of Type II epithelial cells, and mononuclear cell infiltration, fibroblast and smooth muscle cell proliferation and fibrosis.

DIP at first appears to be an intra-alveolar reaction, however close examination shows that it is an interstitial reaction consisting of lymphocytic cell infiltration including plasma cells and the occasional eosinophil. The alveolar space becomes filled with cells due to the desquamation of Type II epithelial cells and mononuclear phagocytes.

Since the original description of DIP by Liebow et al (1965) controversy has existed as to whether UIP and DIP are separate disorders or merely represent early and late stages of the same fibrogenic disorder. Carrington et al (1978) drew attention to the differences in mortality, pathology, clinical course and response to corticosteroid treatment between DIP and UIP. They claimed that because of these observed differences each classification represented a separate disorder. However, others believe that DIP is an early stage of a single fibrogenic disorder which merges into the mural pattern of fibrosis seen in UIP (Scadding and Hinson, 1967; Heard, 1976).

The remaining terms in this classification represent rare conditions that do not require further explanation in this thesis.

4. Existing Models of Fibrosis

A. In vitro Models

Because of the enormous complexity created by the multiplicity of cellular and biochemical interactions which occur following insults that develop into fibrosis in in vivo models, less complicated in vitro systems have been established to study these events. In general, in vitro models provide a means to investigate individual relationships between agents, cells and cell milieu. However, it must be remembered that in vitro systems are only models and the events taking place may not reflect what is actually occurring in vivo.

a. Lung Explant Cultures

Explant cultures provide a method to investigate individual anatomical areas of the respiratory system such as trachea, pulmonary vasculature, bronchial tree and the distal respiratory unit. This model has been extensively used to investigate collagen and noncollagen protein synthesis from small quantities (e.g. 100 mg wet weight) of human and

animal lung (Bradley et al, 1974, 1975).

Since the surrounding environment can be controlled it is possible to extract greater than 95% of the collagen synthesized in short term cultures, by incubating the tissue with β -aminopropionitrile, a compound which inhibits the crosslinking of new formed collagen. This enables a more complete analysis of collagen by providing sufficient material to conduct various biochemical investigations. This regulated environment also allows a more precise quantitation of rates of collagen synthesis by controlling such variables as specific activity of isotope within the tissue.

Explant culture can also be used to assess the effects of drugs on collagen synthesis.

Although the above studies have concentrated on normal lung, a more recent investigation by Hext (1979) examined alteration in collagen synthesis following exposure of lung slices to chrysotile asbestos or silica. Results from this work indicate that the major problem is the method of application of particles to the tissue to ensure uniform distribution throughout the slice.

Other disadvantages include: 1. It has not been fully determined what effect removal of lungs, isolation of individual structure and mincing or slicing of tissue has on

the cellular and biochemical events taking place and 2. explants are still very complex structures containing many cell types and therefore it is not possible to delineate specific roles of individual cells (Hance and Crystal, 1976).

b. Lung Cell Cultures

The introduction of techniques to isolate and identify various cell types from lung tissue (Mason, Williams and Greenleaf, 1977; Douglas et al, 1976) provide models that can be used to gain a better understanding of the interaction of agents with individual cells. These cell culture techniques are useful for several reasons. First, they have been useful in understanding factors which influence fibrogenesis by examining the direct interaction of agents with fibroblast (Richards et al, 1971; Richards and Jacoby, 1976; Harington, Miller and MacNab, 1971; Desai, Hext and Richards, 1975); the direct interaction of agents with macrophage with subsequent effects on fibroblast (Allison et al, 1968; Heppleston and Styles 1967; Harington et al, 1973; Burrell and Anderson 1973; Aalto 1976); the initiation of inflammation and tissue injury by insulting agents (Gordon et al, 1976; Snyderman and Mergenhagen, 1976; Houck and Cheng, 1971, Allison and Davies, 1975; Rutherford and Ross, 1976); and antigen-lymphocyte macrophage fibroblast interactions (Wahl and Wahl, 1975; Johnson and Ziff, 1976; Lewis and Burrell, 1976; Davies et al, 1974). A detailed account

of these various types of in vitro interaction has recently been written (Bateman, Emerson and Cole 1980).

Cell culture is also of value in understanding mechanisms of collagen synthesis and degradation by the various lung cells responsible for its control. Although considerable research has been carried out with fibroblast cell lines in this regard, little work has been done with other cell lines (Hance et al, 1976; Langness and Udenfriend, 1974; Houck et al, 1971; Church et al, 1973).

c. Cell Free Models

Cell free models are useful for understanding individual biochemical reactions in collagen synthesis and degradation. It has been used to study prolyl hydroxylase, the enzyme responsible for catalyzing the hydroxylation of proline (Peterkowsky and Udenfriend, 1963) and more recently to obtain information concerning the role of genetic initiation factors, transfer RNA, messenger RNA and ribosomal proteins in controlling collagen synthesis in fibrotic lung disease.

d. Diffusion Chamber Models

The use of diffusion chambers limited by molecular membrane filters which contain a specific cell line (e.g.

macrophage) mixed with a particulate fibrogenic agent (e.g. asbestos) is useful for studying factors released by the cells when they interact with the agent.

This model not only prevents cell-cell contact but by implanting the chambers in the peritoneal cavity of mice, allows for a more natural environment for the reactions to proceed (Bateman, Emerson and Cole, 1979).

B. In Vivo Models

a. Problems - Advantages - Limitations

It is well established that in experimental animals a variety of agents (Table 4) are capable of causing lung damage, short of complete necrosis, which may lead to fibrosis. These agents include toxic chemicals and fumes, drugs, bacteria, inorganic dusts, radiation and immunological insults such as experimentally prepared immune complexes. Despite the volume of research conducted in animals to determine the effects of these agents, surprisingly little information is known of the pathogenetic mechanisms underlying these effects in each case.

There are several advantages in the use of animal models for the analysis of mechanisms by which fibrosis occurs (Table 5). Whole animals are capable of a range of

Table 4Animal Models of Pulmonary Fibrosis

<u>Species</u>	<u>Insult</u>	<u>Reference</u>
Mice	Irradiation	Adamson <u>et al</u> (1970)
Rats	CdCl ₂	Palmer <u>et al</u> (1976)
Rats	Paraquat	Thurlbeck and Thurlbeck (1976)
Rats	Asbestos	Miller and Kagan (1976)
Rats	Silica	Corrin and King (1969)
Hamster	Bleomycin	Snider <u>et al</u> (1978)
Guinea-pig	Asbestos	Holt <u>et al</u> (1966)
Rabbits	Bacteria	Harris <u>et al</u> (1976)
Rabbits	BSA, CMA	Willoughby <u>et al</u> (1976)
Dogs	Irradiation	Pickrell <u>et al</u> (1976)
Monkeys	Asbestos	Wagner (1964)
Baboons	Bleomycin	McCullough <u>et al</u> (1978)

Table 5

Advantages and Disadvantages of Animal Models of Pulmonary Fibrosis

Advantages

Development of methodologies
Similarity of cell types to human
Manipulation
Availability of whole lung
Inbred strains
Large cell quantities
Drug testing
Appropriate controls

Disadvantages

Specie variation to insult
Cell type differences
Complex interactions
Extrapobility

host responses which might be relevant to man. The design of the experiment can be manipulated to allow the mechanism in question to be studied. Of the large number of experiments which have been performed in the past, the majority have attempted to assess the fibrogenicity of agents or to reproduce particular disease entities (e.g. asbestosis, extrinsic allergic alveolitis) and have concentrated on histopathological changes, i.e. the results of, rather than the basic mechanisms, of fibrogenesis. The assessment of mechanisms requires detailed study with as many techniques as possible at frequent intervals both shortly after the insult and over the subsequent evolution of the fibrogenic process. Most studies have not done this; the common problems being study of the event too long after the insult or failure to include sufficient time points.

To obtain full advantage of the freedom permitted by animal models the study should include an assessment of dose-response relationships and temporal course, with observation of such parameters as collagen synthesis and degradation, enzyme analysis, immunological events and changes in the serum complement system. Other factors which modify the fibrogenetic response of the host (e.g. germ and specific pathogen-free status, T-cell depletion, B-cell depletion and macrophage depletion) can also be manipulated.

Availability of the lung as an intact organ allows regional differences in pathology to be studied in their natural setting and even such details as their effect on gas exchange and lung mechanics to be assessed (Snider et al 1978; McCullough et al 1978). This contrasts with the sampling problem attendant to all human studies.

The large cell numbers which are obtained from these animals can be used for a greater variety of tests in vitro than permitted by human samples. By using inbred strains of animals the effect of genetic variability encountered in humans as a result of their "outbred" status, is overcome. This permits conclusions to be drawn from smaller numbers and the pooling of cells and tissues from small animals. An obvious further advantage of the animal model is the facility of screening for the effects of drugs on fibrogenesis. Finally, techniques can be refined in animals for future use in man.

Two important limitations of animal studies are that extrapolation between different species and particularly between animals and man may be invalid; and furthermore that although methods are improving, they are still too insensitive to permit detailed analysis of some of the complex interactions in the lung. However, systematic studies of the type described above provide much useful information.

b. Choice of Animal

Success or failure in answering an experimental question may depend on the type of animal chosen, because of species variability in response to any given agent. An example of this is the variety of animal responses to asbestos exposure. Vorwald et al (1951) found that guinea-pigs developed pulmonary fibrosis and asbestos bodies, rats developed fibrosis but no asbestos bodies, mice formed asbestos bodies but developed no fibrosis, cats developed mild fibrosis but no asbestos bodies and rabbits showed no response. Therefore, logically the most suitable animal to use when attempting to mimic human asbestosis would be the guinea-pig.

c. Specific Pathogen-Free (SPF) Status of Animals

It is well known that the lung response to various infective agents may be considerably altered by the prior colonization of the animals with various specific pathogens (e.g. mycoplasma). Although it is difficult to find well attested evidence of such circumstances unequivocally affecting the outcome of fibrogenetic insults, there is a general consensus that they do. However, the problems encountered in maintaining animals in this state for long term experiments are considerable.

d. Prior Non-Specific Stimulation of the Immune System

Richerson et al (1978) have shown that repeated intravenous injections of heat killed BCG into rabbits, previously sensitized with Freund's complete adjuvant (FCA), resulted in interstitial pulmonary fibrosis; whereas animals not so sensitized failed to develop fibrosis. The reaction produced was an accelerated granulomatous response which could not be produced by other soluble or particulate antigen (i.e. ovalbumin and keyhole limpet haemocyanin). Moore and Myrvik (1974) obtained similar results; both studies demonstrated that the lesions progressed for a period but subsequently resolved. A further example of the protective effect of non-specific host stimulation was the demonstration by Butler (1975) that FCA treated hamsters were protected against the fibrosis induced by paraquat poisoning.

The opposite effect to prior FCA stimulation was observed in experiments by Göthe and Swensson (1970). Intravenous BCG followed by intratracheal instillation of fibrogenic dusts produced lung lesions that were more pronounced than those produced by either treatment alone.

e. Manipulation of Elements of the Immune System

One way of defining the possible role of various elements of the immune system in fibrogenesis is to selectively abrogate such elements in animals and/or to passively re-

store such elements to animals totally deprived of their immune system. Examples of this procedure are the use of T-lymphocyte depleted, B-lymphocyte depleted, macrophage depleted and de complemented mice.

5. Interpretation of Models

Every mechanism observed by in vivo or in vitro experimentation requires evaluation and confirmation by human studies to be certain of its practical significance in human pulmonary fibrosis. Since all non-human studies have this limitation, the methods of studying and analysing the disease process in vivo are being continually improved.

Animal species differ widely in their susceptibility and pattern of response to the same disease producing agent and, even in those species in which the histopathological results most closely mimic man, different mechanisms might be operating to produce this disease. The comparison with human disease, if it is to be useful to research in therapeutics, should preferably be similar at every stage of the disease. Again, a reliable dose-related response in animals does not necessarily imply that the same will be observed in man. The pattern of human disease always involves important modifications resulting from host factors both genetically determined and acquired, such as smoking and co-existent infections or disease. However, it is not essential that

animal models be an exact replica of the human situation. Rather, they should be looked upon as model systems that are being used to develop methods to distinguish abnormal from normal. In this manner they may be helpful in understanding the inability of the human lung to return the fibrotic lung to normal.

Section II : Aim of Thesis

Section II : Aim of Thesis

Sections I and III have reviewed the literature relevant to mechanisms of pulmonary fibrogenesis with special attention to in vitro and in vivo experimental models. Extensive experimental work has been carried out in both model systems but interpretation of mechanisms occurring in vitro must eventually be confirmed by experiments using in vivo models. However, it emerges that:

1. The majority of in vivo models have assessed the fibrogenicity of agents or reproduced particular disease entities, concentrating on histopathological changes rather than mechanisms of fibrogenesis.
2. Where mechanisms have been examined, insufficient parameters have been used to enable understanding of the events that occur in complicated in vivo models.
3. Many studies have not examined events occurring in vivo shortly after insult before obvious pathology occurs; generally, too few time points have been included in many studies.
4. Many of the insulting agents have been administered in high concentration ("unphysiological doses") preventing the assessment of host factors upon which fibrogenesis may partly depend.

The aim of this thesis was to develop an animal model which would overcome as many of these criticisms as possible. Direct insult to the lung was achieved by exposing guinea-pigs to an asbestos dust cloud (5,000 fibres/cc air) for a short period of time (16 hours). Both Freund's complete adjuvant and Mycobacterium tuberculosis (strain H37Ra) were used to modify the host's response to this direct insult and were administered parenterally at a remote site from the lung.

The guinea-pig was chosen as host for two main reasons. First, its response to asbestos is similar to that seen in man (asbestos body formation occurs as well as lung fibrosis). Second, the mononuclear cells of the guinea-pig lung have many characteristics similar to those of man. The alveolar macrophage has surface receptors for the Fc fragment of IgG, surface immunoglobulins, it attaches to and spreads on glass and is a first line lung defense mechanism. It is also capable of secreting enzymes that are found in the human alveolar macrophage. Guinea-pig lung contains a significant number of both B and T lymphocytes in proportions similar to those reported for lymphocytes obtained by bronchopulmonary lavage in man.

Techniques used to assess the effect of the insult to the guinea-pig lung included light and electron microscopy, morphological studies of live glass-adherent pulmonary mononuclear cells, cytological studies of pulmonary mono-

nuclear cells, lung hydroxyproline determinations, enzyme analysis of glass-adherent mononuclear cells and peripheral blood lymphocyte blast transformation studies. A variety of data necessary for interpretation of the data obtained by these techniques was also determined - lung tissue DNA levels, wet lung weight, dry lung weight, body weight and a histological index of inflammation.

These experimental techniques were carried out shortly after administration of the lung insult and at frequent intervals during the evolution of the host's reaction to it.

Section III : Pulmonary Fibrogenesis :
Structural and Functional Aspects

Section III : Pulmonary Fibrogenesis :

Structural and Functional Aspects

At least six cell types within the distal respiratory unit (that area beyond the respiratory bronchioles including the alveolar duct, space and interstitium) are thought to be involved in the pathogenesis of fibrosis. It is understandable therefore that there are a multiplicity of cellular and biochemical events taking place at any one time during fibrogenesis. Fibrosis should be considered to be the end result of a reaction which creates an imbalance in the cellular and biochemical homeostatic mechanisms of the lung. Examination of human biopsy material (Crystal et al, 1976) and study of animals models of pulmonary fibrosis (Tetley et al, 1976; Ryan, 1972; Gross and de Treville, 1967) have clearly shown that the lung response preceeding the development of fibrosis includes chronic inflammatory cellular infiltration, alteration in the parenchymal cell population and derangement of interstitial collagen. It is necessary to define the non-cellular and cellular matrices of the distal respiratory unit which are involved, or thought to be involved, in the pathogenesis of pulmonary fibrosis and to consider the alterations which occur to these constituents as fibrosis develops.

1. NON-CELLULAR CONSTITUENTS

There are three connective tissue elements that provide the structural support of the distal respiratory unit. These are collagen, elastic fibres and the proteoglycans. Of these collagen is the most plentiful, comprising 60% to 70% of total interstitial connective tissue of human lung (Hance and Crystal, 1976). Elastic fibres make up 20% to 30% of the connective tissue, the remaining small percentage consisting of proteoglycans (Pierce and Ebert, 1965). Although elastic fibres and proteoglycans play an important role in maintenance of lung structure and function, their role in pathogenesis of pulmonary fibrosis has not been fully determined.

A. COLLAGEN

a. Structure

The primary structure of collagen, referred to as tropocollagen, is composed of three polypeptide chains called α chains each containing approximately 1,100 amino acids, which are coiled in such a manner as to form a triple helix (Fig. 1).

The major amino acids of the polypeptide chains that constitute the tropocollagen molecule are glycine, proline

and lysine. The glycine moiety occurs at every third position of the peptide, except for a short sequence at the ends of the chains. The two amino acids hydroxyproline and hydroxylysine are important constituents of the collagen molecule and until recently were specific to collagen. It has been demonstrated that elastin (Grant and Prockop, 1972), the C1q component of complement (Porter and Reid, 1978) and the tail structure of acetylcholinesterase (Rosen, Gerry and Richardson, 1977) not only all contain hydroxyproline but have a molecular structure of (x-y-glycine)_n. That is to say when the polypeptide is formed it usually consists of 1,100 amino acids repeating themselves as tripeptide units of (x-y-glycine). The amino acids in the x-y position are most commonly proline-hydroxyproline and lysine-hydroxylysine (Piet, 1976).

b. Types, Ratios and Distribution

Although collagen molecules in the lung are similar in structure, it must be pointed out that collagen is heterogeneous. At present there is strong evidence for the existence of five types of collagen within the lung (Table 6).

c. Synthesis

i. Transcription and Translation

Table 6 Summary of Types, Molecular Structure and Distinguishing Characteristics of Lung Collagen

<u>Type</u>	<u>Distribution in Lung</u>	<u>Molecular Structure</u>	<u>Cell Responsible for Synthesis</u>
I	Bronchi Blood vessels Interstitialium	$[\alpha I(I)]_2 \alpha 2$	Fibroblast Type I epithelial
II	Cartilagenous tissue Trachea Bronchi	$[\alpha I(II)]_3$	Chondrocytes
III	Interstitialium	$[\alpha I(III)]_3$	Fibroblast Type I epithelial
IV	Basement membrane	$[\alpha I(IV)]_3$	Epithelial Endothelial
V	Basement membrane	$\alpha A(\alpha B)_5$	Epithelial Endothelial

The initial event of synthesis is transcription of the genetic code for collagen to messenger RNA (mRNA) within the nucleus of the fibroblast. This mRNA diffuses into the cytoplasm, attaches to ribosomes and translates the message. The newly formed protein (pro α chain) undergoes a number of important post translational modifications: hydroxylation, glycosylation and triple-helix formation. The triple-helical protein formed in this way is procollagen which has peptide extensions at terminal amino and carboxyl groups (Fig. 1). It was originally thought that the function of these extensions was to aid the formation of triple-helical structure but other important functions are now proposed for these extensions including inhibition of formation of premature fibril formation (Grant and Prockop, 1972), a helper role in the assembly of pro α chains and fibrils (Vies et al, 1973) and, following cleavage from the procollagen molecule, their action as a feed-back control of synthesis of new collagen (Lichtenstein et al, 1973).

ii. Hydroxylation

Hydroxylation of proline is essential for the formation of a stable triple-helix. Hydroxylation (Fig. 1) of proline and lysine commences as the amino-terminal end of a pro α chain enters cisternae of the cell's endoplasmic reticulum and continues along the polypeptide. Hydroxylation

of these amino acids requires not only specific enzymes but also presence of ferrous ions, ascorbic acid, α - ketoglutarate and oxygen as co-factors in the reaction (Grant and Prockop, 1972). Other requirements for hydroxylation to take place are:

- a. The substrate to be hydroxylated must be part of a peptide linkage.
- b. The substrate must occupy the correct position in the (x-y-glycine)_n sequence, since a specific hydroxylase will only react with its corresponding substrate when in the correct sequence in the peptide chain. An example of this is the fact that prolyl-4-hydroxylase will only act on proline when it is in the y position of the x-y-glycine sequence.
- c. The pro α chains or procollagen molecule must be in non-helical configuration; and
- d. Long peptides are more easily hydroxylated than short peptides.

The enzymes prolyl-hydroxylase and lysyl-hydroxylase have been the subject of considerable study and many details of their requirements are known (Cardinale and Udenfriend, 1974; Myllyla et al, 1977; Popence and Aronson, 1972).

They exert important intracellular control on rate of collagen synthesis, although it is not known whether they do so together or by independent mechanisms (Prockop et al, 1979). The levels of these enzymes have been observed to change in disease. A three to ten fold increase in prolyl-hydroxylase has been noted in experimental fibrosis and granulomata (Grant and Prockop, 1972).

In anoxic states the incorporation of amino acids into α chains is unaltered but the amount of procollagen hydroxylated is less. Hydroxylation of this procollagen can be completed when the required oxygen tension for hydroxylating enzyme activity is reached (Grant and Prockop, 1972).

Deficiency of vitamin C (ascorbic acid), the cause of scurvy, also results in reduced hydroxylation of α chains and procollagen and ultimately in cessation of procollagen synthesis (Grant and Prockop, 1972).

iii. Glycosylation

The next post-translational step, glycosylation, takes place within the cisternae of the rough endoplasmic reticulum. It involves the addition of galactose and glucose to hydroxyllysine residues and requires activation of specific enzymes (galactosyl transferase and glucosyl transferase), that the pro α chains be in a non helical conformation and presence of a bivalent cation (Prockop et al, 1979).

iv. Triple-Helix Formation

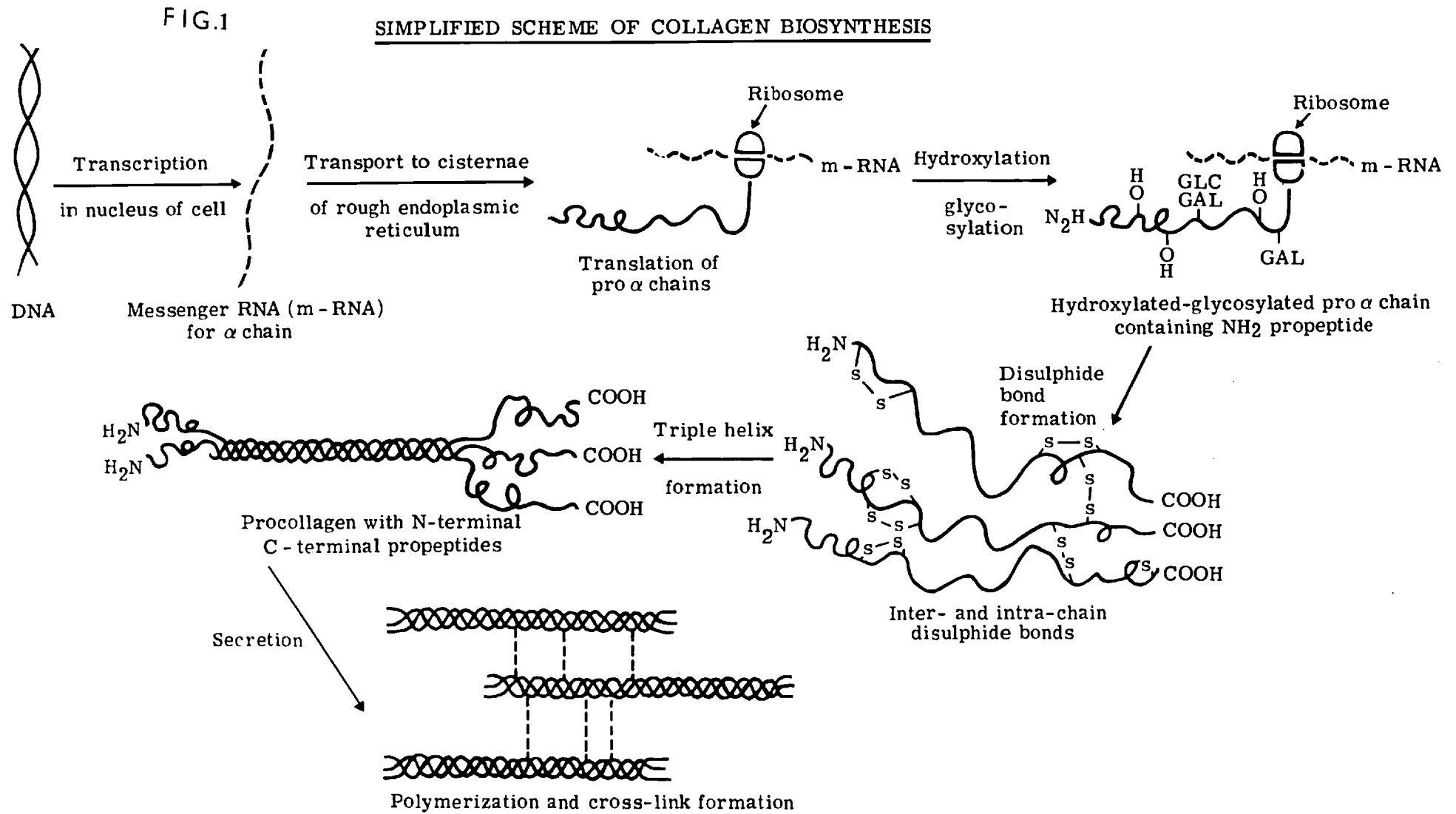
The formation of a triple-helix requires insertion of disulphide bonds within and between the peptide extensions of the pro α chains (Fig. 1), presence of hydroxyproline within the chains and correct association of the latter (Prockop et al, 1976).

v. Secretion

Control of secretion rate depends upon formation of triple-helical procollagen. If the latter is prevented by arrest of hydroxylation or disulphide bond formation synthesis of pro α chains continues for a short time but then slows, while accumulated pro α chains are secreted slowly as a non-helical functionless protein (Prockop et al, 1976).

vi. Tropocollagen and Microfibril Formation

Specific proteases cleave the amino and carboxyl terminal groups of the procollagen molecule in the extracellular space forming tropocollagen. Molecules of tropocollagen have propensity to form microfibrils in the extracellular milieu. Such microfibrils form as a left handed super-helix, comprising five molecules of tropocollagen (Piez, 1976) each overlapping its nearest neighbour by 234 amino acid residues. Microfibrils are microscopically



indistinguishable from mature collagen fibres, but they lack the tensile strength achieved by stable covalent cross-links within and between tropocollagen molecules (Tanzer, 1976).

d. Degradation

It has been shown by Bradley et al (1974) that there is continuous synthesis of collagen in normal adult rabbit lung, yet net concentration of collagen does not change. Therefore, degradation of collagen must occur. Compared with other proteins, collagen has a relatively low rate of turnover, possibly because the triple-helical form is extremely resistant to proteolytic enzyme degradation at physiological pH.

Four neutral proteinases are capable of degrading the triple-helical structure of collagen: bacterial collagenase (Seifter and Harper, 1971), vertebrate collagenase (Harris and Krane, 1974), cathepsin B1 and collagenolytic cathepsin (Etherington, 1977). Vertebrate collagenase cleaves collagen at a glycine-leucine or glycine-isoleucine bond situated one-quarter the length of the molecule from the C-terminal portion (Harris and Krane, 1974). This yields two helical fragments, an N-terminal TC^A fragment and a smaller C-terminal TC^B fragment, which unlike their parent tropocollagen are denatured under physiological conditions.

Catabolism of collagen can be considered to occur in three separate stages. First, the fibroblast is capable of controlling the amount of collagen that leaves the cell. This is achieved by rapid degradation of newly synthesized pro α chains within the fibroblast itself (Bienkowski et al., 1978). Because pro α chains have a non-helical configuration they are a suitable substrate for a variety of lysosomal enzymes which reduce them to small peptides or amino acids. This mechanism is important because the rapid degradation is of sufficient order to be able to influence the total quantity of collagen in an organ (Bienkowski et al., 1978). It is of interest also because intracellular degradation is not a usual mechanism for the regulation of proteins with extracellular functions (Goldberg and St. John, 1976).

Second, the extracellular catabolism of collagen can occur in two ways. Helical tropocollagen molecules are split by collagenases as described above. Insoluble fibrils, composed of multiple crosslinked tropocollagen molecules are split into fibril fragments (TC^A and TC^B linked by inter-molecular crosslinks) by collagenase. This collagenase activity on intact fibres occurs when the enzyme is secreted on the fibre by macrophages, polymorphonuclear leucocytes and even bacteria (Harris and Krane, 1974).

Several factors have an influence on collagen degradation:

1. Conditions which stimulate production and release of collagenase. For example, mechanisms have been described in vitro whereby inflammatory cells (macrophages and lymphocytes) can be induced to release soluble factors which stimulate production and release of collagenase from PMNs and macrophages (Wahl and Wahl, 1975).
2. Conditions which reduce the local concentration of, or compete for binding sites on, serum inhibitors. Serum alpha-2-macroglobulin is the major inhibitor for almost all known proteases acting at neutral pH and blocks nearly all animal collagenases by an almost irreversible binding under physiological conditions (Werb et al., 1974).
3. The susceptibility of collagen to collagenase degradation has been shown to be influenced by anatomical location, age of the tissue, degree of crosslinkage and tissue complexity. The latter refers to the effects of ground-substance components, such as proteoglycans, which may reduce the rate of collagenolysis by their close association with collagen fibres (Harris and Krane, 1974). As a result factors and enzymes unrelated to collagenases (e.g. hyaluronidases) which regulate the rates and types of proteoglycan accumulation also influence collagen degradation. In addition, rates of collagenolysis depend upon collagen

type -- types I, II and III being degraded at markedly different rates by collagenases (Harris and Krane, 1974).

4. Raised tissue temperature as occurs in inflammation also increases the rate of collagenolysis (Harris and Krane, 1974).

Third, the TC^A , TC^B and fibril fragments produced by collagenase activity are phagocytosed by the macrophage and digested in its phagolysosome by the action of collagenolytic cathepsins, such as cathepsin B1, which act at acid pH, and by other proteolytic enzymes (Etherington, 1977).

B. ELASTIC FIBRES

Elastic fibre is the term applied to a mixture of two biochemically distinct connective tissue elements, microfibrils and elastin, which were once thought to have a precursor-product relationship. Several differences between the composition and properties of these constituents have been defined (Ross, 1973). Elastin appears amorphous at the limits of electron microscopic resolution, whereas the microfibril is composed of tubular fibrils 10 to 12 nm in diameter. Elastin stains with anionic stains (e.g. phosphotungstic acid), is rich in non-polar amino acids (alanine, valine, leucine and isoleucine), contains no cysteine or methionine but possesses small amounts of

hydroxyproline and crosslinks of desmosine, isodesmosine and lysinonorleucine. It is derived from tropoelastin which is synthesized in fibroblasts and smooth muscle cells. In contrast, the microfibril stains with cationic stains (e.g. uranyl acetate or lead citrate), is rich in polar amino acids (e.g. aspartic and glutamic acids), contains cysteine and methionine but no hydroxyproline nor crosslinks. The susceptibility of microfibrils to a variety of proteolytic enzymes including trypsin, chymotrypsin and pepsin, contrasts with the resistance of elastin to all except elastase. Hydroxylysine is absent from both components and this distinguishes them from collagen. The ratio of amorphous to microfibrillar components in tissues increases with age and, whereas mature elastin has a very slow turnover rate, newly synthesized elastin can be degraded more rapidly.

Most methods used for quantitation of elastic fibres are based on their relative insolubility. However, uncrosslinked tropoelastin is more soluble and some is lost during extraction. Changes in elastin content of tissues which occur in the course of human disease have not been studied very much, but its concentration, like that of proteoglycans and collagen, has been shown to increase in some animal models of pulmonary fibrosis (Starcher, 1978).

C. PROTEOGLYCANS

Proteoglycans form part of the amorphous matrix (ground substance) composing the interstitium of the lung. Seven types of proteoglycans have been described and all comprise a protein linked covalently to glycosaminoglycans (GAG), a complex polysaccharide. The proteoglycans in the interstitium of the lung are hyaluronic acid, chondroitin-4-sulphate and heparan sulphate. There are no stains which are specific for GAG but they may be demonstrated by the periodic acid-Schiff reaction and toluidine blue for light microscopy, and by ruthenium red for electron microscopy. Fibroblasts, endothelial cells, chondrocytes and possibly epithelial cells synthesize GAG. Proteoglycans may have an important influence on rate of collagen degradation but their other functions in the lung in health and disease are not understood (Silbert, 1973; Buonassisi, 1973).

2. CELLULAR CONSTITUENTS

Forty different types of cells have been identified so far in the respiratory system (Sorokin, 1970), reflecting an extremely complicated network of cellular and biochemical interactions. Consequently it is necessary to describe the specific cell types which make up the particular anatomical area of lung to be investigated in the thesis.

This section reviews cells in the normal distal respiratory unit, emphasising their role in formation and maintenance of the gas exchanging area of lung and outlining their possible function during fibrogenesis.

The cells which constitute the distal respiratory unit can be divided into four identifiable populations: 1. epithelial, 2. endothelial, 3. interstitial and 4. blood derived mononuclear cells. These can be further subdivided as discussed later.

A. ALVEOLI AND CAPILLARY LINING

The function of the interalveolar septum is to bring air and blood into as close contact as possible for the purpose of gas exchange over a large surface area. The air space side of the septum is lined by a continuous sheet of cells denoted as epithelium. The epithelial cells lie on a basement membrane which is separated by the interstitial space from the capillary basement membrane which abutts the endothelial cells lining the capillaries. It was with the use of the electron microscope that Low and Daniels (1952) first demonstrated the existence of the continuous epithelial lining and their results were confirmed a year later by Macklin (1954).

Later Policard (1954) distinguished two types of cells in the epithelial lining which have been termed the Type I

Type II pneumonocyte (Meyrick and Reid, 1968).

a. Type I Epithelial Cell (Pneumonocyte)

Type I cells are simple squamous cells which form a thin layer over most of the alveolar wall, covering 25 times as much surface as Type II cells (Meyrick and Reid, 1970). It has an elongated nucleus with cytoplasm that extends over an area several times the diameter of the nucleus. The cell contains the usual cytoplasmic organelles. The prime function of the Type I cell is to form the blood/gas barrier (Meyrick and Reid, 1970; Kuhn, 1976).

Type I epithelial cells make up approximately 8% of the total cell population of the distal respiratory unit (Weibel et al, 1976), are capable of collagen synthesis (Howard et al, 1976) and following various insults to the lung are significantly reduced in numbers (Adamson and Bowden, 1974). They are replaced by proliferation and differentiation of Type II cells (Okada and Genka, 1964). Consequently a shift of epithelial cell numbers following injury probably affects the relative amount and location of collagen.

b. Type II Epithelial Cell (Pneumonocyte)

The Type II cell is more spherical or cuboidal in shape than the Type I cell. It has a rounded nucleus and

abundant cellular organelles. The most characteristic morphological features of these cells are the projection of micro-villi into the alveolus and the presence of osmiophilic lamellar bodies (Fig.2).

The main functions of the Type II cell are production and secretion of surfactant, a surface-tension lowering substance lining the alveoli, and replacement of Type I cells. Studies have shown that when damage occurs to the Type I cells they are replaced by the massive proliferation of the Type II cell (Okada and Genka, 1964; Kapanai et al, 1969). It was thought at one time that Type II cells might be phagocytic (Corrin, 1969; Esterly and Faulkner, 1970) or become macrophages (Casarett and Milley, 1964) but the evidence for this is scanty.

c. Endothelial Cells

The lung contains at least four histologically distinct types of endothelial cells (Fig.2) as well as the endothelium lining the lymphatic circulation of the lung. It has been shown by Reid (1967) that the alveolar wall contains muscular, partially muscular and non-muscular arteries along with the capillary network. All these vessels are lined by a thin endothelial membrane containing large numbers of vesicles which are thought to be engaged in trans-endothelial transport.

Fig. 2 Electron micrograph of guinea pig lung showing Type I epithelial cell (TPI), Type II epithelial cell (TPII), microvillie (MV), endothelium (E), interstitium ((N), collagen fibres (CF) and an alveolar mononuclear phagocyte (M) X 7450.

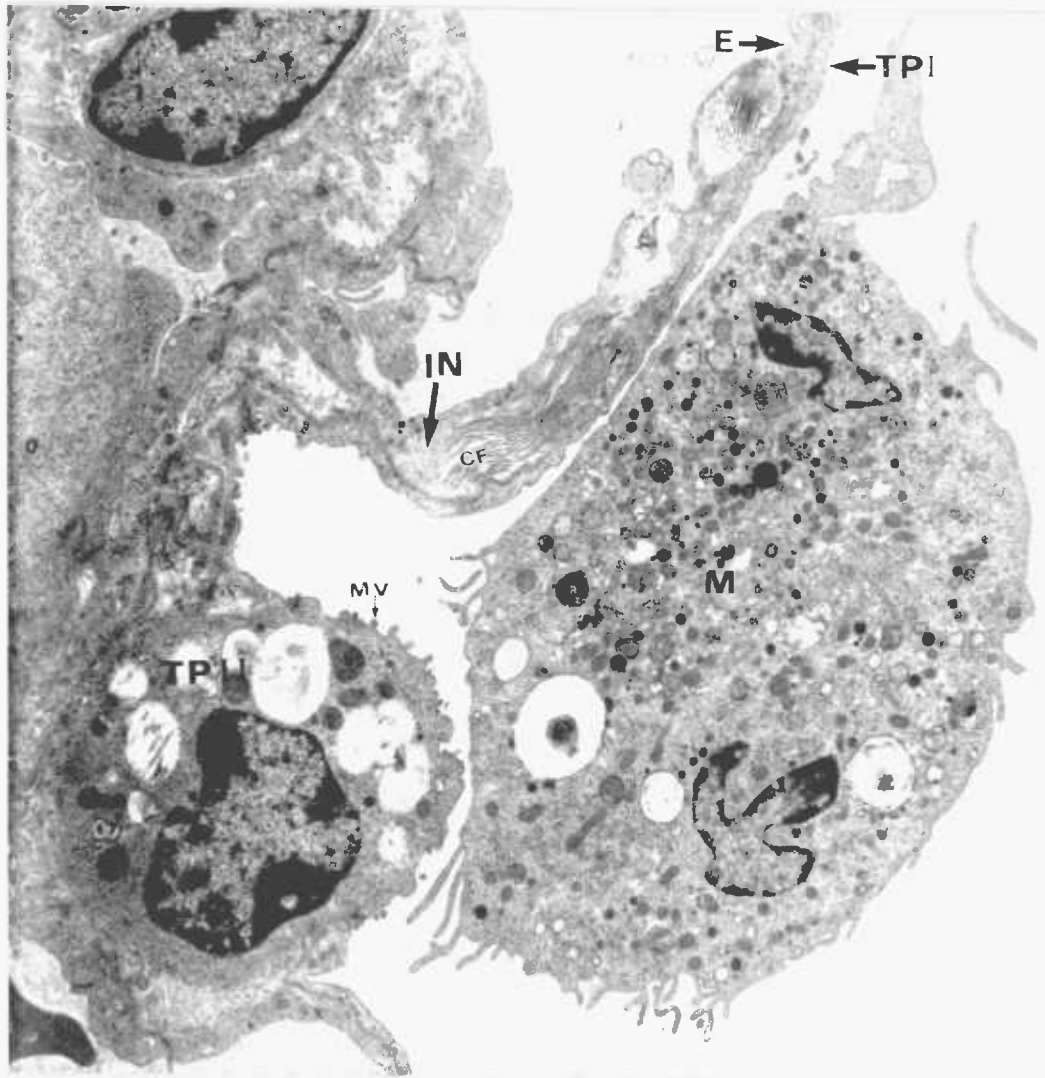


Fig. 2

The main function of the endothelium in conjunction with the Type I epithelial cells is gas exchange. Although their role in fibrogenesis is unknown, it has been established that they are capable of synthesising basement membrane collagen (Howard et al, 1976).

B. INTERSTITIAL CELLS

The interstitial cells are located in the interstitial space within the connective tissue of the alveolar wall. The cells are extravascular and bounded by the basement membrane of the epithelium and endothelium.

It has been calculated from studying rat lungs that the interstitium occupies 47% of the tissue barrier of the lung; of this three-quarters are cells and one-quarter is connective tissue (Weibel et al, 1976).

a. Fibroblast-Pericyte

The prominent fixed cells of the interstitium are the fibroblast and pericyte. Although they can be distinguished from one another by morphological criteria using electron microscopy, they are indistinguishable from each other following their separation from lung tissue. For this reason some investigators prefer to use the term mesenchymal cell, which encompasses both pericyte and

fibroblast. Both cell types have contractile properties (Kapanci et al, 1974) and cytoplasmic extensions that entwine capillaries in some instances. The fibroblast is more often seen in the centre of the interstitium, being associated with the connective tissue fibres (collagen and elastin).

It is well established that the main function of the lung fibroblast is synthesis of Type I and Type III collagen. Recent studies have also demonstrated that the fibroblast is capable of degrading pro-collagen as previously discussed (Bienkowski et al, 1978).

Increasingly, fibroblasts are recognized as having biological activities beside their main function of collagen synthesis. Appreciation of these other properties is necessary to understand fibrogenesis (e.g. accumulation of fibroblasts and the elaboration of collagen, elastin and proteoglycans).

Fibroblasts are motile and migrate in vivo and in vitro under the influence of chemotactic factors (Abercrombie et al, 1971; Harris and Dunn, 1972; Kang, 1978). In vitro assays quantitating fibroblast chemotaxis suggest three different factors to be chemotactic for fibroblasts:

1. A 22,000 molecular weight protein released from antigen or mitogen-stimulated T-lymphocytes, called lymphocyte-

derived chemotactic factor for fibroblasts (LDCFF)
(Postlethwait et al, 1976).

2. Human dermal fibroblasts are strongly attracted by Type I, II, and III collagen, peptides from degraded collagen (including α chains and small peptides split off by bacterial collagenase) and synthetic tripeptides and dipeptides (Postlethwaite et al, 1978). In the course of inflammation, when new collagen is being laid down, these products are generated and might be expected to provide chemotactic stimulus for fibroblasts from adjacent areas.

3. A cleavage fragment of the fifth component of the complement system, generated by activation of the classical and alternative pathways, has been shown to be chemotactic for fibroblasts (Postlethwaite et al, 1977). C5a is the most potent complement-derived peptide for neutrophil and monocyte chemotaxis (Muller-Eberhard, 1975) but the fibroblast chemotactic fragment is larger and thus distinct. Because many events in the inflammatory response to inhaled insults are capable of activating the complement system, some degree of fibroblast chemotaxis is likely to occur.

A second property of the fibroblast is its phagocytic function. Phagocytosis of collagen fragments by human gingival fibroblasts has been observed in vitro (Yajima, 1977). This implicates the fibroblast in the process of

collagen resorption and remodelling which occurs in both normal and pathological states. It also provides a mechanism whereby the fibroblast itself, by producing collagen degradation products, can generate chemotactic fragments.

C. BLOOD DERIVED MONONUCLEAR CELLS

Two cells of bone marrow origin are the macrophage and lymphocyte. In the normal lung they are relatively few in number compared with the increase seen following insult to the lung. Numerous in vitro experiments have confirmed that the macrophage, and possibly also the lymphocyte, are indispensable to fibrogenesis.

a. Macrophage

i. Origin

Although it is fairly well established that the bone marrow is the origin of the pulmonary macrophage (Brain et al., 1977; Van Oud Alblas and Van Furth, 1979) its relationship to the blood monocyte is poorly understood. Also, the mechanism by which the monocyte and immature macrophages tranverse the interstitial space to gain access to the alveolar space is unknown Bowden and Adamson, (1972).

ii. Silica - Macrophage - Fibroblast Interaction

The interaction of silica and macrophages forms the first part of the "two stage" theory of silica-induced fibrogenesis (Fig. 3), suggested by Allison et al (1968), in which silica affects the macrophage in such a way that a factor or factors are released which stimulate collagen biosynthesis by fibroblasts.

Several lines of evidence have been put forward to support this theory but it has proved difficult to isolate the macrophage factor(s) which stimulates fibroblasts. Support for the two-stage theory came from the work of Allison et al (1977) in experiments using Millipore filter diffusion chambers (pore size 0.8 μm) implanted in mouse peritoneum. In theory cells and particles with diameters greater than the diameter of the pores cannot escape from intact chambers but soluble factors can diffuse into and out of the chamber. Their observations included the following: unstimulated mouse peritoneal cells alone and silica alone within the chamber produced only a "slight reaction" which was no different from that elicited by chambers containing saline. By contrast, the combination of small amounts of silica and peritoneal cells produced fibrosis of parietal and visceral peritoneum. Larger amounts ($>50 \mu\text{g}$ m) of silica which resulted in cell death of most of the macrophages in the chamber produced a lesser reaction.

Fig. 3 Schematic Representation of the Two-Stage
Theory of Fibrogenesis

Toxic Particle (e.g. Silica, Asbestos)



1. Particle - Macrophage Interaction



Macrophage



2. Macrophage - Fibroblast Interaction



Fibrosis

Sizing of this grade of quartz was performed by Brown et al (1978) using electron microscopy. Whereas more than 75% of particles was less than 0.8 μm in diameter (the pore size used in Allison's experiments) only 10.7% were less than 0.2 μm .

Lehtonen et al (1973) have shown that Millipore nitrocellulose membranes which, because of their spongy three-dimensional structure, permit cytoplasmic ingrowths and direct cell contact through the membrane. The exclusion of this cell contact is important when confirmation of diffusible macrophage products or factors is sought.

Bateman and colleagues (1979) have obtained different results using a modified diffusion chamber limited by Nuclepore membrane of pore diameter 0.05 μm . The smaller pore size was used to prevent escape from the chamber of the particulate Dörentrup No. 12 quartz used in these experiments and to prevent cell contact by cytoplasmic processes through the membrane filter pores.

The diffusion chamber represents a finite source of macrophages so that any fibrosis observed is the result of particle or fibre interaction with only one generation of macrophages.

They made three observations:

1. Silica alone in the chamber produced no significant fibrosis.
2. Unstimulated mouse peritoneal macrophages alone caused a mild but significant fibrotic response on the outer surface of the diffusion chamber filter.
3. The combination of silica and peritoneal macrophages failed to produce fibrosis over a range of silica from 20 $\mu\text{gm}/10^6$ macrophages to as little as 5 $\mu\text{gm}/10^6$. The presence of lymphocytes did not appear to influence this result. The reason for this result was suggested by very low viable cell counts in the chamber at two weeks compared with those seen in identical experiments performed with asbestos where viable cell counts of up to 25% were observed at this time and fibrosis was produced. This rapid cytotoxic effect of silica, even at this low concentration, is such that insufficient numbers of macrophages survive for long enough to produce fibrogenetic factor(s) in sufficient amounts and/or for adequate duration to produce fibrosis.

Asbestos is less cytotoxic allowing more prolonged stimulation of viable macrophages which in turn can produce or release fibrogenetic factor(s) for a longer period.

Silica appears to require a large continuous recruitment of macrophages for its effect (Heppleston, 1978). In addition, the fibrosis produced in this model by asbestos was maximal at two to four weeks and then resolved completely over the next two months, suggesting that a sustained production of fibrogenetic factor(s) is required for chronic and progressive fibrosis.

Another direction which investigation of fibrogenetic factor(s) from macrophages has taken arose from the work of Heppleston and Styles (1967) who observed that homogenates of rat peritoneal macrophages which had ingested silica were able to increase collagen biosynthesis in chick-tibia fibroblast cultures. Many similar experiments have been performed since (Heppleston, 1978; Harington et al, 1973; and Burrell and Anderson, 1973) but apparently conflicting results may be due to the inherent problems of in vitro systems and the many non-specific factors which can influence collagen biosynthesis in fibroblast cultures (Allison et al, 1977; Harington et al, 1973). In addition, the range of species from which effector and target cells were obtained may explain some differences. Burrell and Anderson (1973), using rabbit alveolar macrophage and human fibroblasts, achieved similar results to Heppleston and Styles (1967) whereas Harington et al (1973) showed an inhibitory effect of suspensions of hamster macrophage extracts on collagen synthesis in hamster fibroblast cultures.

Aalto et al (1976, 1979) have extended this area of research by employing a variety of target fibroblast systems and by fractionating silica-treated macrophages and medium from such macrophage cultures to isolate macrophage fibrogenetic factor(s). The fibroblast systems include:

1. Granulation tissue slices which contain cells in normal relationship to their neighbours but which have problems of restricted access for substances in the medium and a limited cell lifespan.
2. Fibroblasts cultured from granulation slices which are thought to represent cells involved in the repair process with all the synthetic activity which that involves.
3. Preparations of polysomes isolated from granulation tissue which are capable of protein synthesis.

In experiments involving fractionation of the homogenate of silica-stimulated macrophages by differential centrifugation, these workers were able to demonstrate that the active fraction resided in the 20,000 g supernatant. Furthermore, the 700 - 5,000 g sediment of the homogenate from normal macrophages could be induced to produce stimulatory factor by incubating it with silica and this factor could then be separated by repeated centrifugation when it appeared in the 20,000 g supernatant.

Isolated in this way it stimulated collagen synthesis in tissue slices, as measured by ratio of radiolabelled proline to hydroxyproline incorporated into protein, as well as some DNA synthesis. Since the majority of lysosomes are disrupted during the process of freezing and thawing involved in cell fractionation, the macrophage factor was thought not to be liberated hydrolytic enzyme. Another macrophage factor, found in the 100,000 g supernatant of homogenate derived from the cytosol fraction, was of interest because it caused an inhibition of collagen synthesis in tissue slices.

The medium from macrophage cultures has more recently been found to be a "cleaner", more convenient source of the stimulatory macrophage factor, as has also the medium from human peripheral blood monocytes and malignant histiocytes cultured in the presence of silica.

Repeated gel filtration chromatography of this medium yielded a homogeneous protein with a molecular weight of 14,300 which increased the incorporation of tritiated proline and tritiated thymidine into cultured granuloma cells (Aalto, 1979).

The effect of silica on the macrophage is thought by these authors to involve a reduction in macrophage ribonuclease (RNase) activity (Aho and Kulonen, 1977). This

effect is the opposite of its influence on most other hydrolytic enzymes during the process of macrophage stimulation. It is of interest that a similar decrease in acid ribonuclease occurs during phytohaemagglutinin (PHA) stimulation of lymphocytes, when RNA accumulates (Green, 1977). This macrophage RNA could be the fibroblast stimulating factor, which stabilizes polysomes of fibroblasts and increases collagen and non-collagen protein synthesis, including DNA (Aho and Kulonen, 1977).

Further support for these theories was obtained in granulomas produced by sponge containing silica particles and macrophages. Decrease in RNase activity occurred in the developing granulation tissue and also increase in the number of polysomes and in collagen synthesis (Kulonen, 1979). The exact relationships and relative importance of the RNase changes, nucleic acid production and collagen synthesis have yet to be determined.

iii. Asbestos - Macrophage - Fibroblast Interaction

The results of experiments with asbestos in diffusion chambers, which have already been mentioned, confirmed that macrophages are required for fibrosis and provided evidence that asbestos fibrogenicity, like that of silica, operates via a two-stage mechanism. The first is the fibre-macrophage interaction and the second a macrophage-fibroblast

interaction. It is thought that during the first phase prolonged stimulation of macrophages in relation to or containing asbestos fibres results in chronic lysosomal enzyme release. The second stage is thought to involve secretion of macrophage substances, probably lysosomal enzymes and a fibrogenic factor which cause fibroblast to proliferate or increase their collagen synthetic rate or a combination of both. However it has not been established what the direct effect of lysosomal enzymes is on fibroblast nor has a fibrogenic factor been identified (Allison, 1977).

iv. Asbestos - Macrophage Cell Membrane Interaction

The mechanism of interaction of asbestos with cytoplasmic and lysosomal membranes is different from that of silica (Richards & Wasteman, 1974; Allison, 1977) and is thought to be the result of surface magnesium groups on the fibres which interact electrostatically with sialic acid groups on glycoproteins in the membrane. These glycoproteins, which are usually able to migrate within the membrane, are immobilized in the area of contact with the fibre. This aggregation of proteins forms ion-conducting channels which permit the efflux of potassium and influx of sodium ions and water, resulting in osmotic lysis of the cell. This theory is supported by the observation that prior treatment of the cell membranes with neuraminidase (which

removes sialic acid residues) or treatment of the asbestos with agents which preferentially chelate magnesium inhibits haemolysis (Harington et al, 1971). There is still some doubt however whether presence of surface magnesium ions is the correct explanation for the observed effects of asbestos; an alternative is the surface charge (zeta potential) which results from several components of the fibre. Dispersion of fibre in water containing surface-active agents, e.g. acid, (Martinez and Tucker, 1960), and coating the fibres with the lung surfactant component dipalmitoyl lecithin affects this potential. Light and Wei (1979) demonstrated a close correlation between zeta potentials of several asbestos types and their in vitro haemolytic activity. In another area of research on asbestos-cell membrane interactions following asbestos exposure the observation has been made that changes in membrane glycolipids and glycoproteins occur (Newman et al, 1979). Since these effects were not immediate but occurred several hours after exposure to asbestos, they were probably the result of an inhibition of the metabolism of these components. The glycolipid changes, which included a decrease in longer and a concomitant increase in shorter glycolipids, were different for each of the three types of asbestos tested, and correlated with the relative cytotoxicity of the different types. Allison (1971) showed by electron microscopy that fibres of asbestos were phagocytosed by macrophages and remained in phagocytic vesicles and secondary lysosomes for several

hours. Only after one or more days were some fibres seen free within the cytoplasm, presumably as a result of lysosomal membrane lysis. This corresponds to the delayed cytotoxicity of silica but is a slower process with asbestos. A much more rapid cytolysis occurs if protein is absent from the culture medium and this early cytotoxicity is greatest with chrysotile and less with amosite and anthophyllite (Allison, 1977). However, this form of cytolysis is unlikely to have relevance in vivo. Often fibre shape and size allow only incomplete ingestion by macrophages and this allows a more prolonged interaction with plasma membranes of one or more cells (Allison, 1977). Fibres of less than 5 μm are completely ingested, intermediate fibres (5-20 μm) are only occasionally taken up by one macrophage but large fibres (greater than 30 μm) are never totally ingested. Cells may envelop such long fibres at each end but some of the fibre remains in an extracellular position. Experimental evidence regarding fibre length and fibrosis can be summarized as follows: long fibres are probably more effective than short fibres in causing fibrosis. Asbestos also differs from silica in that it induces selective release of lysosomal hydrolases without cell death (Davies et al, 1974). This enzyme release is dose-related in the range of 1 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$, commences within five hours of exposure (Allison, 1977) and increases for more than 24 hours.

v. Macrophage Replenishment

All the fibrogenetic mechanisms discussed have required continuous local replacement of macrophages. Heppleston (1978) has studied the mechanism of macrophage replenishment in silica-treated experimental animals, and has proposed that this process depends on stimulation of the marrow by a lipid component, probably derived from Type II epithelial cells or from silica-bearing macrophages, or both. In support of this hypothesis, lipids of cellular and bacterial origin have been shown to cause increased alveolar macrophage migration (Tainer et al, 1975) and phagocytosis as measured by clearance of an intravenously (i.v.) administered carbon suspension (Conning and Heppleston, 1966). Heppleston (1978) examined the kinetics of the rat mononuclear phagocytic system (MPS) by injecting tritiated thymidine in vivo and examining cell suspensions of marrow from these animals. It was shown that the intravenous administration of the lipid fraction of lungs with silica-induced lipoproteinosis greatly reduced the cell cycle time of the promonocytes, suggesting increased proliferation. In separate experiments in mice comparing cell kinetics of interstitial monocytes from areas of lung containing silica with those of cells from parts which did not, decreased mitotic activity was found in the silica-containing parts. This was considered to be the result of migration of these cells into alveoli at a rate too rapid

to allow cell division to occur. It was concluded that the response to silica is an acceleration of monocyte emigration from blood vessels to alveoli rather than local proliferation, and therefore that the most important source of incoming macrophages is the bone marrow (Heppleston, 1978).

vi. Complement

Chrysotile asbestos fibres in microgram amounts have recently been shown to be capable of activating the alternative pathway of complement in vitro (Saint-Remy, 1979). Since macrophages can synthesize some of the protein components of the complement system, this represents a possible additional mechanism of asbestos-induced fibrogenesis.

b. Lymphocytes

The lymphocyte plays a key role in pathogenesis of granulomata, chronic inflammation and fibrosis associated with delayed hypersensitivity to an antigen. Stimulation of T-lymphocytes results from direct contact with the antigen or from presentation of antigen on macrophage membrane. Secretion of lymphokines initiates a hypersensitivity reaction or granuloma at the site of the antigenic stimulus. Lymphocytes migrate into established areas of chronic inflammation (Smith et al, 1970). T-lymphocytes are present

at the height of the granulomatous reaction, while in the chronic stage an equal number of B and T cells are noted (Boros, 1978).

Antigenic stimulation of lymphocytes has been shown to result in the release of lymphokines which cause fibroblasts to proliferate and produce collagen (Wahl et al, 1978). Cell-free supernatants from non-stimulated lymphocytes do not have this effect. However, Johnson and Ziff (1976) in similar experiments demonstrated that cell-free culture supernatants from mitogen-stimulated lymphocytes contained a lymphotoxin which inhibited proliferation of WI-38 fibroblasts and caused their death. However, the remaining viable cells synthesised increased amounts of collagen. The discrepancy between these experiments may be explained by the difference in fibroblast lines and different culture conditions employed. Wahl et al (1975) observed that treatment of macrophages with lymphokine results in secretion of collagenase-like enzyme. In this way lymphocytes may play a role in the regulation of collagen resorption.

Section IV: Materials and Methods

1. Experimental Groups and Model Design

Guinea pigs were divided into eight groups with 45 animals in each group. The following lettered designations are retained throughout this thesis for the eight groups:

Group E - Animals receiving no treatment.

Group A - Animals exposed to asbestos by inhalation on day 0.

Group F - Animals treated with Freund's complete adjuvant on day 0 (0.1 ml in each of two sites: right and left hind footpad).

Group M - Animals treated with M. tuberculosis (strain H37Ra) on day 0 (0.1 ml subcutaneously of a suspension of 1×10^7 viable organisms per ml in each of two sites: right and left inguinal regions).

Group FM - Animals treated with Freund's complete adjuvant on day 0 and treated with M. tuberculosis (H37Ra) on day 21.

Group AF -- Animals treated with Freund's complete adjuvant on day 0 and exposed to asbestos by inhalation on day 14.

Group AM -- Animals exposed to asbestos by inhalation on day 0 and treated with M. tuberculosis (H37Ra) on day 21.

Group AFM -- Animals treated with Freund's complete adjuvant on day 0 were exposed to asbestos by inhalation on day 14 for two periods of eight hours each and treated with M. tuberculosis (strain H37Ra) on day 21.

Five animals were sacrificed two weeks after a given treatment, thereafter every two weeks for a period of three months and then every three months until one year had elapsed. Two animals were used for light and electron microscopy studies and the remaining three were used for other parameters (Table 7).

2. Animals

Female, specific pathogen free (SPF) Dunkin Hartley guinea pigs, 300-350 grams initial weight were used throughout the study (Redfern Animals Breeders Ltd., Tunbridge Wells, England). All animals not treated with H37Ra were housed

Table 7Parameters Studied in Thesis

Light microscopy of lung tissue

Electron microscopy of pulmonary alveolar macrophages

Cytology of pulmonary mononuclear cells

Morphology of glass adherent cells

Macrophage leucine aminopeptidase activity

Lymphocyte blast transformation to PPD

Hydroxyproline determinations

Miscellaneous

Deoxyribonucleic acid

Skin test to PPD

Lung wet and dry weight

Body weight

in a SPF room for guinea pigs. Animals treated with living H37Ra were housed in the pathogen suite of the Institute. The animals were accredited by Redfern as being category *** (defined as free from all evidence on clinical and post-mortem examination of infectious diseases communicable to man and other animals). Microbiological examination by the breeder revealed no evidence of specific pathogenic organisms (Table 8). Animals were allowed water and standard guinea pig pelleted food ad libitum.

3. Treatments

A. Asbestos

UICC Rhodesian chrysotile A was kindly supplied by Dr. V. Timbrell of the MRC Pneumoconiosis Unit, Llandough Hospital, Penarth, South Glamorgan. The relevant physical and chemical properties of the asbestos sample are listed in Appendix 1.

B. Asbestos Inhalation

The exposure apparatus and facilities for asbestos fibre inhalation were provided by Professor P.F. Holt, Department of Chemistry, University of Reading. The exposure chamber as shown in Figure 4 , accommodated two groups of nine animals each in the front and rear cages. A total

Fig. 4 Asbestos exposure apparatus, H (hopper), AI (Air inlet), HM (Hammer Mill), Rt (Asbestos recirculating tube), AFS (Asbestos freed system), CAFT (Controlled air flow tube)

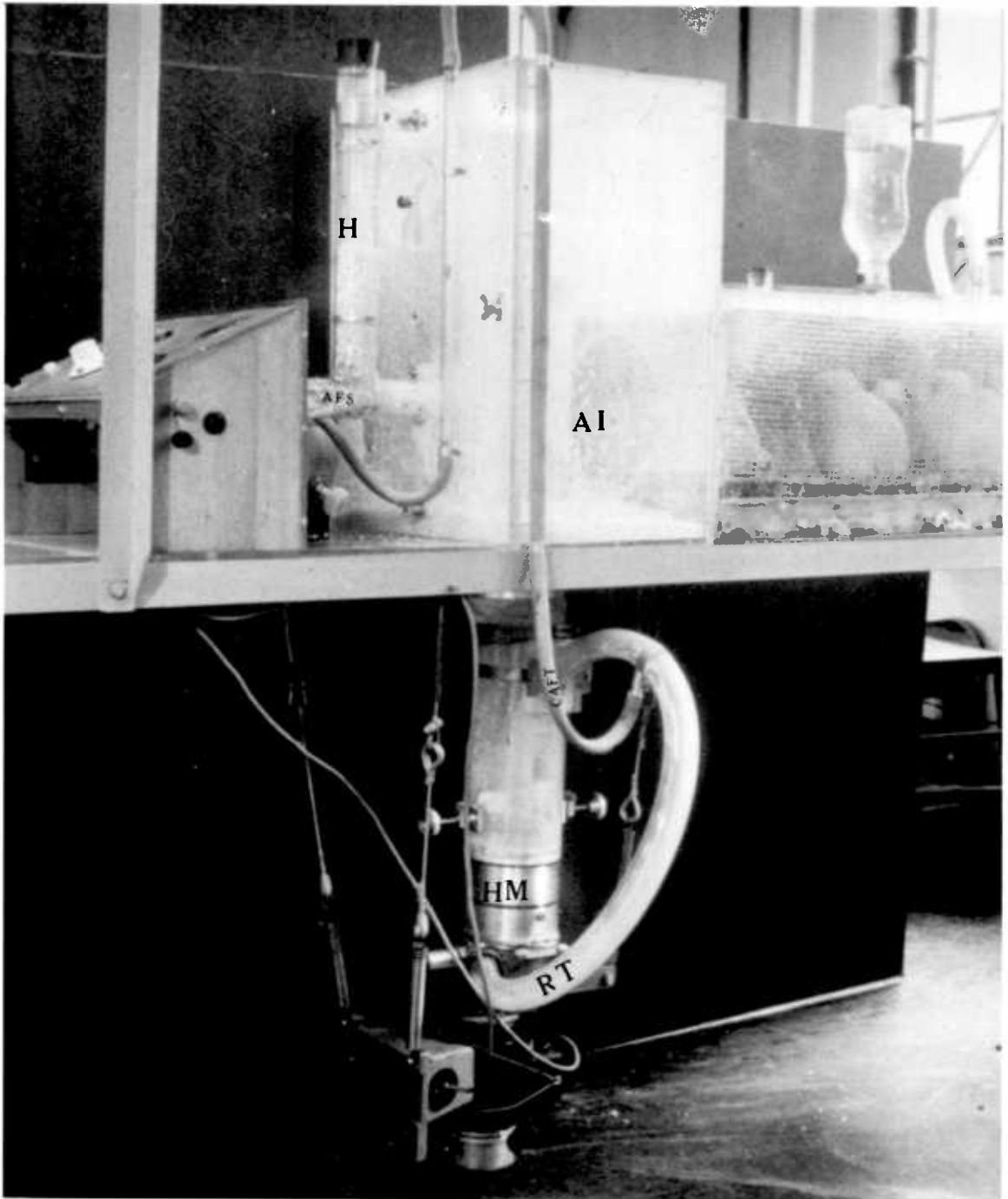


FIG. 4

Table 8.Pathogens Not Demonstrable in SPF Guinea-Pigs

Lymphocytic choriomeningitis virus

All species of Salmonella

Mycobacterium tuberculosis

Yersinia pseudotuberculosis

Leptospira

Listeria monocytogenes

Streptobacillus moniliformis dermatophytes

All Cestods

Pasteurella multocida

Bordetella bronchiseptica

All species of coccidia

All Nematodes except Paraspidodera uncinata

All arthropods

of 36 animals per week were exposed to asbestos by placing two groups of nine animals each in the inhalation chamber for a period of 8 hours on two successive days, with the dust generator operating and dust concentration continually recorded. The animals remained in the inhalation chamber overnight and their own movements generated a further dust cloud during this period. However, no measurement of the asbestos fibre concentration in the atmosphere was taken during this overnight time period. On the third day the dust generator was maintained and on the 4th and 5th day two more groups of nine animals each were exposed for another two eight hour periods. After the exposure period of 48 hours the animals were removed from the exposure chambers and housed in SPF facilities at the University of Reading for one week. This procedure was necessary in order to prevent the asbestos exposed animals from contaminating the colony at the Cardiothoracic Institute on their return. This pattern of exposure was carried out for all asbestos exposed groups.

Since the physical characteristics of the asbestos (length and thickness) are extremely heterogeneous there is no method available to express the dust concentration within the chamber in absolute terms. However the dust concentration was adjusted to give a constant reading of 15 amps on a recording tyndellometer and it has been shown by Holt et al (1964) that this procedure produces a dust concentration

of approximately 5,000 fibres per cc of air.

C. Freund's Complete Adjuvant

Freund's complete adjuvant - H37Ra (Difco 3113 - Detroit, Michigan) contained 1.5 ml of Arlacel A (Mannide monooleate), 8.5 ml Bayol F (paraffin oil) and 10 mg Mycobacterium tuberculosis strain H37Ra (killed and dried). Prior to injection, an adjuvant emulsion was prepared by mixing an equal volume of sterile distilled water with an equal volume of complete adjuvant. Thorough emulsification was effected by drawing the mixture into a glass syringe through a 26 gauge needle and forceably ejecting the mixture a number of times into a sterile beaker until a smooth white emulsion was obtained. The emulsion was considered to be satisfactory for injection when a drop placed on the surface of water did not spread.

a. Sensitization of Mononuclear Cells

Mononuclear cells (lymphocytes and macrophages) were nonspecifically sensitized by injecting 0.10 ml of a 0.50 mg/ml emulsion of Freunds complete adjuvant - H37Ra, into each hind footpad of the respective groups of guinea pigs.

D. Mycobacterial Culture

A strain of M. tuberculosis, H37Ra (TMC 201) which is highly attenuated for the guinea pig was obtained from the Trudeau Mycobacterial Culture Collection, Trudeau Institute, Saranac Lake, New York and maintained by serial passage in Middlebrook 7H-9 broth containing 10% Tween-80 (Difco Laboratories).

a. M. tuberculosis Inoculation

A 7 - 8 day H37Ra (M. tuberculosis) culture maintained by serial passage in Middlebrook 7H-9 broth containing Tween -80 (Difco Laboratories) was washed 3 times in sterile saline, briefly sonicated and diluted to contain approximately 1×10^7 viable organisms per milliliter. A sample of this suspension was used for the determination of viable organisms/ml when plated on 7H-10 media (Table 9). The remainder of the suspension was used for the inoculation. The specified experimental groups given the inoculation were injected subcutaneously in the right and left inguinal regions with 0.1 ml of H37Ra suspension respectively.

4. Preparation of Pulmonary Mononuclear Cells

Guinea pigs were anesthetized by an intraperitoneal injection of sodium pentobarbitone (May and Barker Ltd.,

Table 9.Viable M. tuberculosis (H37Ra)

<u>Group</u>	<u>Viable organisms/ml saline</u>	<u>Viable organisms injected</u>
ATM	1.5×10^7	3.0×10^6
AM	1.5×10^7	3.0×10^6
FM	1.2×10^7	2.4×10^6
M	1.4×10^7	2.8×10^6

Dagenham, England) at a concentration of 50 mg per kilogram of body weight, and exsanguinated by intracardiac puncture into syringes containing 250 i.u. of preservative free heparin (Painer and Byrne Ltd., Greenford, England). The thoracic cavity was then opened and its entire contents removed en bloc and placed in cold phosphate buffered saline (PBS). The lungs were then trimmed free of the heart, trachea and major bronchi and blotted with sterile gauze. The total wet weight of the lungs was measured, the left lower lobe being weighed separately and kept cold until biochemical analysis commenced.

The remaining lobes were finely minced in a small quantity of cold PBS using small pointed scissors. At this stage the lungs of three animals were pooled. Following mincing, the tissue was further dispersed to cell suspension by passing the minced tissue through a fine sterile metal sieve with the aid of a plunger from a 10 ml disposable syringe. The tissue was periodically rinsed with a total of 80 ml of cold PBS during this procedure. This was continued until only the connective tissue elements of the airways remained on top of the sieve. The cell suspension was then pipetted vigorously and passed through four layers of sterile surgical gauze to remove large clumps that were still present. The suspension was then centrifuged at 100 g at 4° for 10 minutes. The supernatant was discarded and the pellet resuspended in 10 ml of fresh PBS. This suspension

was gently layered on a ficoll-triosil gradient (Böyum, 1968) and centrifuged at 500 g at 4° for 15 minutes. Following the ficoll-triosil separation the band of mononuclear cells was carefully collected after first removing the layer above it with a pasture pipet. The band of mononuclear cells was carefully removed, washed twice in TC 199 (Flow laboratories, Scotland) and resuspended in TC 199 containing 10% heat inactivated fetal calf serum, at a concentration of 1.5×10^6 cells/ml. A sample of this cell suspension (1.0 ml) was taken for cytocentrifuge preparations, cell counts and viability. The remaining cells were allowed to adhere to glass cover slips and used for enzyme measurements and morphological evaluation of living mononuclear phagocytes.

5. Cytological Examination

Cytological preparations of pulmonary mononuclear cells were prepared by spinning 100 µl quantities of cell suspension in a Shandon cytocentrifuge (Shandon Southern Products Ltd., Cheshire, England) at room temperature, for 10 minutes at 300 R.P.M. Following centrifugation the air dried smears were fixed in 100% methanol for 15 minutes at room temperature, stained with May-Grunwald Giemsa and mounted. Differential counts were made by counting at least 200 cells per slide using four slides for each group at each time point. Morphological characteristics were also recorded.

6. Cell Viability

Equal volumes of 0.4% trypan blue (Gibco Bio-cult, Glasgow, Scotland) were mixed with equal volumes of cell suspension and incubated at room temperature for a minimum of 5 minutes but no more than 10. At least two hundred cells were counted in a standard haemocytometer. Those cells not taking up trypan blue were scored as viable (Tennant, 1964).

7. Cell Counts

A volume of Turk's diluting fluid (Appendix 2) was added to an equal volume of cell suspension, mixed thoroughly for a few minutes and the cells counted on an improved Neubauer haemocytometer. The number of cells/ml was calculated according to the formula in Appendix 3.

8. Morphology of Glass Adherent Cells

The final cell suspension was remixed and 0.70 ml (1.05×10^6 cells) was carefully placed on each of seven number 3, 22 x 22 mm sterile glass cover slips (Chance Propper Ltd. Warley, England) which had been previously placed in sterile 3.0 cm petri dishes (Sterilin, Ltd., Teddington, England). The cells were incubated in a 5.0% CO₂ humidified atmosphere at 37°. After incubation for

three hours, the original medium was removed and the cover slips vigorously washed with sterile PBS at 37°. Fresh medium (1.0 ml) containing serum was added to each dish and the morphological characteristics were observed before incubating the cells for a further 21 hours. The cells were examined using a Nikon inverted microscope. At 24 hours the medium was removed and the cover slips washed with warm PBS. After washing, 1.0 ml of PBS was added to each dish and the morphological characteristics observed.

9. Macrophage Leucine Aminopeptidase Assay

Glass adherent macrophages were harvested in 1.0 ml of 0.1% Triton X-100 (BDH Chemicals Ltd., Poole, England) by scraping them off the cover slips with a rubber policeman. The samples were distributed into plastic bijoux and stored at -70° until a batch assay was performed in duplicate for each group at each time point.

Enzyme activity was measured using leucine 4-nitroanilide as substrate (Sigma London Chemical CO. Ltd., Dorset, England). Incubation mixtures contained 0.5 ml of cell suspension (enzyme), 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.5 and 0.1 ml of 10 M substrate solution (26.8 mg leucine 4-nitroanilide/10 ml dimethylsulfoxide). After incubation for 30 minutes at 37°, the assay was terminated by addition of 1.0 ml glycine buffer (Appendix 4)

and the optical density read at 405 nM. A blank containing enzyme was prepared for the assay by adding the substrate after addition of the glycine buffer stopping solution. Absolute enzyme activity was calculated by reference to the extinction coefficient of p-nitroaniline (Wachsmuth, 1975).

10. Skin Test

Forty-eight hours before sacrifice, guinea pigs were injected intradermally on the shaved right dorsal flank with 10 µg of PPD in 0.1 ml sterile saline. The shaved left dorsal flank served as a control and was injected with 0.1 ml sterile saline. The skin test was read immediately prior to exsanguination. Cutaneous reactivity was assessed by measuring the mean diameter of induration.

11. Lymphocyte Blast Transformation

A. Preparation of Peripheral Blood Lymphocytes

Lymphocytes used for in vitro (^3H) thymidine assay were obtained from heparinized peripheral blood according to the method of Böyum (1968), following exsanguination of guinea pigs by cardiac puncture. Whole blood was layered over a ficoll-triosil mixture (Appendix 5) in sterile universal tubes and centrifuged at 500 g for 15 minutes at 4°. The band of mononuclear cells was recovered from the

ficoll-triosil plasma interface and washed four times in RPMI-1640 (Flow laboratories, Irvine, Ayrshire, Scotland). This procedure yielded a viability 90% as determined by trypan blue exclusion.

B. (³H) Thymidine Assay

Purified lymphocytes were diluted to a concentration of 1×10^6 cells/ml in RPMI 1640 containing 100 u penicillin per ml, 100 µg of streptomycin per ml and 10% autologous serum. The cells were dispensed in 0.2 ml aliquots in Linbro microfilter plates (Flow Laboratories, Irvine, Scotland). Purified protein derivative (PPD) (Evans Medical Ltd., Liverpool, England) was added to the wells in 0.1 ml aliquots with a final well concentration of 150 µg. Parallel control samples were included in each assay and consisted of incubated cells without PPD.

Plates were incubated at 37° in a humidified atmosphere of 5% CO₂ for six days before adding 1 µCi of (³H) thymidine (Specific activity 2.0 Ci/mmol, Radiochemical Centre, Amersham, England), in a volume of 0.05 ml of RPMI 1640 containing penicillin and streptomycin. Cultures were incubated for a further 24 hours and harvested on glass fibre filters (Reeve Angel, Clifton, New Jersey) after a tap water lysis using a Dynatech miniwash cell harvester (Dynatech

Laboratories Ltd, Billingshurst, Sussex). Filter strips were air-dried for 24 hours and individual filter disks were placed in scintillation vials (Packard, Caversham, Berks., U.K.) with 1.0 ml of scintillation fluid (NE 233, Nuclear Enterprises LTD, Edinburgh, Scotland) and counted for one minute each in a Nuclear Enterprises scintillation counter. Values are expressed as the mean stimulation index of 3 animals for each group at each time point, and were calculated according to the formula in Appendix 6 .

12. Hydroxyproline, Deoxyribonucleic Acid (DNA) and Dry Weight Determinations

The left lower lobe of each guinea pig was minced with scissors, and homogenized in 1.5 ml of 0.5 M acetic acid. The homogenate was made up to 3.0 ml with 0.5 M acetic acid, and 0.5 ml was taken for collagen determination, 1.0 ml for DNA determination, 0.5 ml for dry weight and the remainder stored at -70° .

A. Hydroxyproline Determination

Hydroxyproline was determined by measuring its content on 0.5 ml aliquots of left lower lobe homogenates by the method of Prockop and Udenfriend (1960). The detailed protocol is listed in Appendix 7 .

B. Deoxyribonucleic Acid (DNA) Determination

The extraction of DNA was based on the method of Giles and Myers (1965). Proteins, including DNA was precipitated by adding 14.0 ml of 10% cold trichloroacetic acid (TCA) to 1.0 ml of left lower lobe homogenate in glass screw top tubes. The precipitate was washed once in cold TCA by centrifugation at 100 g for 15 minutes at 4° and resuspended in 5.0 ml of 5% TCA and placed in a 90° water bath for one hour. Following the hot TCA extraction, the suspension was centrifuged at 1,000 for 15 minutes and the supernatant transferred to a clean tube for DNA estimation. DNA was assayed using diphenylamine, according to the method of Giles and Myers (1965).

C. Dry Weight Determinations

Dry weight aliquots (0.5 ml) were added to tared 2.5 ml freeze drying ampoules, lyophilized and reweighed.

13. Histology

Immediately after exsanguination as previously described, the thoracic cavity was opened and the trachea was exposed and cannulated with a stainless steel tube attached to a 20 ml syringe containing 10% buffered formalin (Appendix 8). The lung was slowly inflated with approximately 10-12 ml of

fixative, the trachea ligated and the thoracic contents removed en bloc, and placed in a glass jar containing 10% buffered formalin for at least three days.

Following fixation the lung was first dissected into its respective lobes, right and left upper (apical), right and left lower (diaphragmatic) and the small right and left middle (accessory). The lobes were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. The embedded tissues were sectioned at 5 μ m thickness using a rotary microtome. Staining techniques were performed according to Culling (1974) and are listed in Table 10. A grading system based on visual comparative standards was used to provide a rough assessment of the degree of inflammation from grade 0 (no inflammation) to grade 4+ (severe inflammation).

14. Electron Microscopy

Following inflation of the lung with cold (4°) 5% cacodylate buffered glutaraldehyde pH 7.4 (Appendix 9) it was removed as described under histology and tissue blocks (1 mm³) were cut from the left lower lobe, using a fresh razor blade. The blocks were fixed for one hour in cold fixative and then rinsed and stored in buffered sucrose until final preparation for electron microscopy.

Table 10.Histological Stains Used in Thesis

Haematoxylin and Eosin	(HE)
Perl's prussian blue	(Asbestos bodies)
Ziehl-Neelsen	(Mycobacteria)
Congo red	(Amyloid)
Gordon and Sweet's	
silver impregnation	(Reticulin)
Masson's trichrome	(Collagen)
Weigert-vanGieson's	(Collagen)

The small blocks were post fixed in cold (4°) 1% osmium tetroxide-veronal acetate buffer solution (pH 7.4) containing 1% sucrose, for one hour, dehydrated in graded alcohols and embedded in Epon. The sections were cut on a LKB microtome using glass knives and examined in a Philips 201 transmission electron microscope after staining with lead citrate and uranyl acetate.

The sections of lung not used for electron microscopy were processed for light microscopy as described above.

15. Gross Observations

Macroscopic observation of lung was made as soon as the thoracic cavity was exposed, and following the removal of the lung at which time a sagittal section was made to allow examination of the internal surface. Particular note was made for the presence of lesions, pleural changes, colour changes and size of the lung.

16. Index of Pulmonary Response to Insult

The intensity of the inflammatory response was also assessed by using the following formula (Kawata et al, 1964 and Bhuyan 1974).

$$\text{Indices of Inflammation} = \frac{\text{Lung weight/body weight (experimental)}}{\text{Lung weight/body weight (control)}}$$

17. Statistical Methods

A programme for the two-way analysis of variance with a fractional factorial design was used to analyse biochemical, lymphocyte blast transformation and weight relationships. Details of the analysis are included in Section IV. Data was processed with a Hewlett-Packard programmable calculator (Model 9810A).

Section V : Results

Section V : Results

1. Gross Pathology

White macroscopic nodules visible as pin-point to 3.0 mm subpleural foci were characteristic of the seven treated groups. These lesions were most prominent from four to 12 weeks after treatment. At six weeks Group AFM had a hob-nailed appearance due to the numerous large nodules and showed some gray-black discoloration (Fig. 5). This was the most severe gross pathological change seen for any group at any time. Occasionally lungs from treated groups did not deflate when the thoracic cavity was opened, particularly between eight and 12 weeks and at 26 weeks. At 38 and 52 weeks there were very few visible changes evident in any group.

There were no gross pathologic changes demonstrable in untreated controls.

2. Microscopy

A. Histology

In general, cellular changes as seen by the light microscope examination of lung tissue were not helpful in distinguishing the various experimental groups from each other. However, all treated groups were distinguishable

Fig. 5 Specimen of guinea pig lung six weeks following treatment (Group Asbestos + Freund's complete adjuvant + mycobacteria) showing macroscopic nodules and discoloration. Approx. 3.5 X natural size.



FIG. 5

from the untreated controls during the evolution of the inflammatory process and the treated groups could be distinguished from one another by assessment of the severity of the lesions, especially at two and four weeks following insult (Table 11). Irrespective of treatment, the histological changes differed only quantitatively at a given time. The inflammatory process ranged from widespread focal lesions consisting of interstitial and intra-alveolar mononuclear cell infiltrates which became diffuse, to areas which were consolidated with mononuclear cells causing obliteration of the lung architecture. Also, it was not uncommon to find normal lung adjacent to the lesions. The peak of the inflammatory reaction for any given group was followed by complete resolution. Asbestos bodies were demonstrated after four weeks but mycobacteria were not seen, nor were amyloid deposits nor increases in collagen. Large increases in reticulin were evident, particularly in animals with sensitized mononuclear cells and exposed to asbestos.

a. Group Asbestos + Freund's Complete Adjuvant + Mycobacteria (AFM)

Two Weeks

Variable changes were seen in all sections examined. These ranged from : 1. normal areas, (Fig. 7), 2. areas of interstitial inflammatory reactions made up of mononuclear cells (Fig. 6) and 3. extensive areas of mono-

Table 11.

Summary of Histopathology Following Treatment

Group	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	26 weeks	38 weeks	52 weeks
AFM	+++ E,IM, M, g (1.62)	++++ E,M,g (1.17)	++++ E,M,g,R (1.25)	+++ E,M, g R (1.34)	+++ E,IM,M, g,R (1.17)	+++ E,IM,M, R (1.03)	++ IM,M,R (1.30)	++ IM,M,R (1.58)	t, R (1.38)
AF	++ IM,M, g,R (1.24)	++ M,g,R (1.38)	+++ M,g,R (1.00)	+++ M,g,R (0.97)	+++ M,g,R (1.05)	++ IM,M,g R (0.96)	++ g,R (1.04)	0 (0.94)	0 (1.08)
AM	+ IM (1.17)	+ IM (1.14)	++ IM,M (0.96)	++ M (1.06)	+++ M (1.05)	+++ M (1.10)	++ (1.18)	+	0 (1.73)
FM	+ IM (1.04)	+ IM (1.05)	+++ IM,M,R (1.03)	+++ IM,M,R (1.05)	++ IM,M,R (0.95)	++ IM,M,R (0.99)	++ IM,M,g,R (1.26)	g,R (1.62)	0 (1.41)
A	+ M (1.09)	+ M (1.11)	++ M,g,R (1.38)	++ M,g,R (1.11)	++ M,g,R (1.03)	++ M,g,R (1.03)	+	+	0 (0.92)
F	+ IM,M,g,R (1.23)	++ IM,M,g,R (1.13)	+++ M,g,R (1.26)	++ M,g,R (1.25)	+	+	0 (0.95)	0 (0.93)	0 (1.07)
M	+ IM,M, (1.18)	++ IM,M, (1.04)	+++ IM,M, (1.07)	++ M (0.93)	+++ M,R (1.03)	++ M (0.96)	+++ M,g,R (1.23)	0 (1.58)	0 (1.43)

*1. Severity of lesions graded from 0 (no reaction) to ++++ (severe reaction)

2. E = Eosinophil

3. IM = Immature mononuclear phagocyte

4. M = Mature mononuclear phagocyte

5. g = granulomata

6. R = Reticulin

7. () = Index of pulmonary response to insult

nuclear cells arranged in sheets and packed closely together causing obliteration of the alveolar architecture (Figs. 8,9).

Mononuclear cell infiltration consisted chiefly of mononuclear phagocytes with occasional monocytes, lymphocytes and eosinophils. The mononuclear phagocyte population comprising these lesions was heterogeneous. There were small rounded cells with centrally placed spherical nuclei which had densely stained uniformly distributed chromatin. The cytoplasm was foamy in some instances, pale, basophilic and sparse. The plasma membrane of this cell was usually distinguishable at this stage and this cell type was seldom observed in lesions where the cells had become closely packed together to form large sheets (Fig. 10). The sheets of mononuclear cells consisted of large cells with round-oval eccentric nuclei that had become vesiculated. The chromatin was clumped and marginated toward the nuclear membrane and distinct nucleoli were present. These cells were almost confluent causing their plasma membranes to be indistinguishable.

There was no evidence of giant cell formation at this time.

Many bronchi and bronchioles had become narrowed and contained cellular exudate (Fig. 11).

A few lymphocytic nodules, without germinal centres were also developed at this stage, however there was no lymphocytic perivascular cuffing.

Stains for reticulin demonstrated that the disruptive changes were due to organization of the cellular infiltrate among which were new fine reticulin fibres, otherwise reticulin appeared normal (Figs. 12, 13).

Stains for collagen, mycobacteria, amyloid and asbestos bodies were negative at this time.

Four Weeks

There was little change in the histology present at this time except for an increase in the sheets of mononuclear cells obliterating lung architecture. Reticulin was increased in the interstitium with loose strands projecting into many alveoli.

Perl's stain demonstrated asbestos bodies either lying free in the alveolar lumen, phagocytosed by mononuclear phagocytes or penetrating the interstitial space. Electron microscopy showed that asbestos fibres which had penetrated the interstitial spaces were contained within the Type I epithelial cell (Fig. 15).

Stains for collagen, mycobacteria and amyloid were negative.

Six Weeks

There was no difference from the histology seen at four weeks.

Eight Weeks

The inflammatory response had waned with a less cellular appearance and a reduction in the size of the compact sheets of mononuclear cells.

10 - 52 Weeks

Lesions seen during this period eventually disappeared. They became smaller and the compact sheets of mononuclear cells reverted to the less differentiated interstitial reaction seen at two weeks, the lung eventually returning to an almost normal appearance. At 52 weeks there was minor perivascular lymphocytic cuffing (Fig. 16, 17) and a few small granulomata were present. Although asbestos bodies were still present there was no reaction to them at this time. All other stains were negative.

b. Group Asbestos + Freund's Complete Adjuvant (AF)

Two Weeks

The lungs of these guinea pigs developed mild interstitial oedema and demonstrated discrete peribronchial inflammatory reactions (Fig. 11). There were areas of granulomata organization with increased reticulin in some cases (Figs. 18, 19). Rounded holes lined by mononuclear phagocytes were also present. Reticulin fibres could also be seen branching into alveolar spaces.

Lesions similar to those described for group AFM were also present, but were never as severe or as extensive as in that groups. They consisted of small to moderate sized loose sheets of mononuclear cells, the majority of which were mononuclear phagocytes, amongst which could be found a few lymphocytes. The mononuclear phagocytes forming these sheets showed varying degree of differentiation as previously described with the plasma membranes difficult to distinguish in many instances.

Stains for asbestos bodies, collagen, mycobacteria and amyloid were negative at this time.

Four -- Eight Weeks

Between four to eight weeks there was progressive

increase in severity and extent of the earlier reactions. This was most noticable in peribronchial regions. Two additional features that developed during this time were intra-alveolar cellular exudate and minor pleural thickening.

Asbestos bodies were demonstrated from four weeks onwards.

Stains for mycobacteria and amyloid were negative. Stains for collagen were negative except in one or two cases where granulomata had developed but this was the exception rather than the rule in the majority of granulomata seen.

Ten Weeks

There was slight waning of the inflammatory response with a less cellular appearance but there was little other difference in the lesions from those seen at eight weeks after treatment.

Twelve Weeks

The diffuse cellular reaction was beginning to resolve however the loose sheets of mononuclear phagocytes had coalesced to form well developed granulomata.

In areas where the cellular response was resolving there was extensive proliferation of reticulin fibres which resulted in thickening of alveolar walls and frequent obliteration, or near obliteration, of the alveolar space (Fig.14). The reticulin fibres were fragmented in some instances and condensed in others. Granulomatous areas also showed an extensive reticulin meshwork.

There was no evidence of increased collagen and stains for mycobacteria and amyloid were negative.

26 Weeks

There were numerous well developed granulomata of varying size (Figs. 18,19) with abundant reticulin mesh work. The more generalized inflammatory response was resolving however, increased lymphocytic infiltration was evident. The stains for collagen, mycobacteria and amyloid were negative.

38 Weeks - 52 Weeks

Between these times there was almost total resolution of all the previous lesions and the lung appeared normal except for the occasional mononuclear phagocyte containing material which stained positively with Perl's stain.

c. Group Asbestos + Mycobacteria (AM)

Two to Twelve Weeks

Changes in the lung appeared slowly during the first eight weeks. Little difference except for a slight increase in the initial changes seen at two weeks could be discerned between the appearances at each of the four time points.

Initially, there was minor interstitial oedema with mononuclear cell infiltration and intra-alveolar mononuclear cell exudates (Fig. 11).

The mononuclear cells resembled either monocytes or immature mononuclear phagocytes. The monocytes were small round to oval cells with densely staining nuclei. Nucleoli, if present, were inconspicuous. The cytoplasm was sparse and slightly basophilic in character. The immature mononuclear phagocytes were small round cells with centrally placed nuclei that had become slightly vesiculated. The cytoplasm was eosinophilic and foamy. In some cases the cytoplasm was sparse and in others it was more abundant. There was continual increase in the amount of intra-alveolar cellular exudate and further maturation of mononuclear cells occurred as described in group AFM. Generally the cells were larger with oval nuclei, marginated chromatin, distinct nucleoli and abundant cytoplasm.

Stains for collagen, mycobacteria and amyloid were negative throughout the first 12 weeks. Perl's stain demonstrated asbestos bodies from four weeks onwards. Stains for reticulin demonstrated that the alveolar architecture remained intact, the major reaction being a mononuclear cell infiltration with no granulomata formation.

26 and 38 Weeks

Histology at these times was characterised mainly by lymphoid hyperplasia with lymphocytic cuffing of the vessels, otherwise the lung was normal.

52 Weeks

Lymphocytic cuffing of vessels was still evident but regression of the lesions had taken place without residual fibrosis.

d. Group Freund's Complete Adjuvant + Mycobacteria (FM)

Two and Four Weeks

There was a diffuse interstitial cellular infiltration of immature mononuclear phagocytes and lymphocytes. Scattered foci of bundles of inflammatory cells (predominantly lymphocytes) were present. Reticulin was not increased to any

great extent and stains for collagen, mycobacteria and amyloid were negative. Asbestos bodies were not demonstrable.

Six Weeks

Moderate to severe extensive lesions were present, obliterating the lung architecture. This response was similar to that of group AFM at two and four weeks, although lymphocytes were more prominent in this group. Large lymphocytic nodules were formed in the centre of many of the lesions. There were extensive increases in reticulin, which had become thickened and branched, causing obliteration of the alveolar spaces. Mononuclear phagocytes containing argyrophilic material were also present (Fig. 14). All other stains were negative.

Eight - Twelve Weeks

There was waning of the inflammatory response as judged by reduction in the distribution of the lesions. The reticulin mesh work was still abundant but all other stains were negative.

26 Weeks

There was a further waning of the inflammatory response as demonstrated by the presence of only a few small

scattered granulomata and a less cellular appearance of the interstitial tissue. However, there was development of a diffuse intra-alveolar cellular exudate of moderate proportion. This was never severe enough to cause total obliteration of the air spaces. The reticulin content appeared less than at 12 weeks.

38 - 52 Weeks

The interstitial tissue appeared to be returning to a normal appearance. Most granulomata had resolved and there was only a minor intra-alveolar cellular exudate. Many of the blood vessels were surrounded by lymphocytes which were densely packed together in some cases. All other stains were negative at these times.

e. Group Asbestos (A)

Two - Eight Weeks

As in group AM, the lesions in this group developed slowly but progressively during the first two to eight weeks.

Initially there was a peribronchial and interstitial inflammatory response. The lesions consisted of mononuclear phagocytes among which were found a few lymphocytes and eosinophils. A majority of the mononuclear phagocytes had

round or oval nuclei with margined chromatin and distinct nucleoli (Fig. 10). There was abundant eosinophilic cytoplasm. Aggregates of mononuclear phagocytes were also beginning to form loose sheets of cells as previously described. Stain for reticulin, collagen, mycobacteria and amyloid were negative. Asbestos bodies were demonstrable at four weeks (Fig. 20).

10 - 12 Weeks

The lesions had slightly regressed. Reticulin was moderately increased but all other stains were negative.

26 - 52 Weeks

There was progressive regression of the lesions until the lung appeared normal.

f. Group : Freund's Complete Adjuvant

Initially, guinea pigs in this group developed scattered small patchy areas of interstitial mononuclear cell infiltration. This reaction gradually increased in severity until extensive areas of cellular infiltration caused obliteration of alveolar spaces in some cases. Also, by six weeks bundles of cells containing many lymphocytes were evident, as were numerous eosinophils. In some areas of inflammation rounded holes lined by mononuclear cells were seen (Figs. 18,19).

At two and four weeks the mononuclear phagocytes were more heterogeneous in appearance than at six weeks. Initially there were both immature and mature cells but by six weeks a shift toward predominately mature cells had taken place.

Reticulin progressively increased, particularly where bundles of cells had become organized.

All other stains were negative throughout these time points.

Eight Weeks

The inflammatory reaction was considerably resolved by this time as judged by the less cellular appearance. However, there were still increased numbers of lymphocytes present in the interstitium and reticulin had increased from that seen at six weeks. All other stains were negative.

10 - 52 Weeks

There was complete resolution of the inflammatory response, including the previously noted increase in lymphocytes. The lung appeared to be normal.

g. Group Mycobacteria (M)

Two Weeks

The lung appeared virtually normal at this time except for small scattered foci of intra-alveolar cellular exudate containing mononuclear cells.

Stains for reticulin, collagen, mycobacteria and amyloid were negative.

Four to Six Weeks

Moderate interstitial oedema and cellular infiltration was seen with an increase in intra-alveolar cellular exudate. The cells of these lesions were mainly mononuclear phagocytes which varied in maturity - more immature than mature cells being seen. Numerous fine strands of reticulin were found projecting into alveolar spaces and all other stains were negative.

Eight to Twelve Weeks

There was resolution of the reaction previously described although one animal showed severe cellular infiltration with increased reticulin at ten weeks.

26 Weeks

Perivascular lymphocytic cuffing and organization of granulomata were features at this time. One animal demonstrated severe cellular infiltration causing obliteration of alveoli and there was a general increase in lymphocytes and lymphoid nodules. Numerous areas of normal lung were also demonstrable. Reticulin was increased only in areas of severe cellular infiltration and all other stains remained negative.

38 - 52 Weeks

At both these times the lung appeared virtually normal with only a minor perivascular lymphoid reaction.

h. Group Untreated Control (N)

Control guinea-pigs usually had completely clean lungs throughout the experiment. The occasional lung contained small foci of intra-alveolar cells and scattered small foci of increased interstitial cells. However, this was rare and the alveolar walls were usually thin and devoid of cells that represent an inflammatory process.

B. Electron Microscopy

Ultrastructurally, a number of features were demon-

strated in the alveolar region of treated guinea pigs. In animals exposed to asbestos, fibres were demonstrated in mononuclear phagocytes and occasionally in Type I epithelial cells (Figs. 15,21), whereas mycobacteria could not be demonstrated at any time in animals that were treated with H37Ra or Freund's complete adjuvant. Aggregates of mononuclear phagocytes in the alveolar space were common, (Fig. 22), irrespective of treatment and the presence of increased aggregates correlated with the increased severity of the inflammatory reaction as seen with the light microscope.

Variation in morphology of mononuclear phagocytes was apparent at all times but little difference could be discerned between these cells in the various experimental groups. It was difficult to find mononuclear cells in the untreated control group and when the occasional mononuclear phagocyte was located it was usually seen lying in close proximity to the alveolar wall.

Based on morphological criteria (Table 12), monocytes, immature macrophages and mature macrophages were demonstrated in the alveolar spaces. The majority of these cells appear to be mature macrophages and the occasional mononuclear phagocyte seen in untreated controls was always mature in appearance.

Monocytes were round or oval shaped cells with an

Table 12

Morphological Criteria Used to Identify Monocytes

Immature Macrophages and Mature Macrophages

<u>Cell</u>	<u>Light Microscopic Appearance</u>	<u>Electron Microscopic Appearance</u>
Monocyte	Small round cell, with dark staining nuclei and little cytoplasm	Lobated nuclei, dense heterochromatin; inconspicuous nucleoli, little cytoplasm containing few mitochondria, few short pseudopods.
Immature Macrophage	Nucleus centrally placed, slight vesiculation of chromatin conspicuous nucleoli, foamy cytoplasm, distinct plasma membrane	Nuclear euchromatin, clumping and margination of heterochromatin toward nuclear membrane increase cytoplasmic organelles
Mature Macrophage	Large polygonal cell with eccentric oval nuclei and marginated chromatin, large prominent nucleoli, cells touching causing plasma membranes to be indistinguishable	Large eccentric nuclei, euchromatic chromatin, fine heterochromatin marginated at nuclear membrane, extensive cytoplasm filled with many organelles.

irregular outline (Fig. 23). They had round lobulated centrally placed nuclei with densely clumped heterochromatin and an inconspicuous nucleolus. The cytoplasm was sparse and contained only a few oval or round mitochondria. The cell borders were irregular with numerous small short pseudopods. Small pinosomes were frequently seen in the cytoplasm. These cells were distinguished from the occasional lymphocytes that were present because the latter had less cytoplasm, very round nuclei containing homogeneous heterochromatin and absent pseudopods and pinosomes.

Immature macrophages were larger than monocytes (Fig. 24), round to oval in shape with cell borders that were even except for a few small pseudopods. The nuclei were larger than those seen in monocytes, lobulated, eccentric in some instances, and the heterochromatin was beginning to marginate toward the nuclear membrane. Nucleoli were easily distinguishable in these cells. The cytoplasm was more abundant than that seen in monocytes and contained dense bodies, phagosomes and pinosomes. Except for a few mitochondria other organelles could not be easily distinguished.

Mature macrophages were the largest mononuclear phagocytes seen. These cells had large eccentric round to oval nuclei, which in some instances were elongated and slender

(Fig. 25). Heterochromatin had become completely margined along the nuclear membrane and nucleoli were prominent in many instances. The ratio of cytoplasm to nucleus was greatly increased and the former was filled with a large array of organelles. Numerous mitochondria and lysosomes were present. Phagocytic vacuoles, extensive pseudopod formation and pinocytic vesicles were also a prominent feature of these cells. Endoplasmic reticulum and Golgi apparatus were not a dominant feature.

C. Glass Adherent Mononuclear Cells

A difference in size, tendency to spread and content of cytoplasmic organelles was apparent in the pulmonary mononuclear phagocytes obtained from treated animals as compared with untreated controls (Fig. 26). These differences were most apparent between two and twelve weeks after a given treatment. There was no distinguishable morphological difference between the various treatments and this was true whether the cells were examined three or 24 hours following adherence.

D. Cytology

Throughout this study 88% or more of the cells collected from untreated controls (Group N) were of macrophage like morphology. The remaining population consisted of lymphocytes and an occasional monocyte. There were no binucleate

or multinucleate cells. The mononuclear phagocytes were large cells with abundant cytoplasm that stained a uniform blue-gray. There was little evidence of vacuolation or phagocytosed particles. The nucleus was round to oval with a coarse chromatin that was margined toward the periphery of the nuclear membrane in some cases. Nucleoli were often demonstrable. Lymphocytes were smaller cells with very sparse cytoplasm and a large dark staining nucleus.

In contrast, although the initial number of mononuclear phagocytes and lymphocytes in treated groups were similar to untreated controls, there was an eventual change in numbers of the cell types harvested. This was most pronounced in animals that had received H37Ra and/or FCA, and was seen as decrease in the percentage of mononuclear phagocytes and increase in the percentage of lymphocytes, giant cells and eosinophils. Another feature of the treated groups was the heterogeneous appearance of the mononuclear phagocytes. There were both small and very large cells which exhibited either densely stained or vacuolated cytoplasm. In all groups exposed to asbestos a small percentage of the cells contained asbestos bodies (Table 13) and many of the cells had ruffled plasma membranes and pseudopods (Fig. 27).

3. Statistical Procedures

The most appropriate statistical analysis technique for comparison of several treatments is by methods called

Table 13A. Differential Count of Harvested Pulmonary Cells (%) Group Control (N)

	<u>Macrophages with Asbestos Bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks		88	12				
4 weeks		92	8				
6 weeks		96	4				
8 weeks		88	11			1	
10 weeks		92	6		2		
12 weeks		92	8				
26 weeks		90	10				
38 weeks		87	12			1	
52 weeks		94	6				

Table 13B

Differential Count of Harvested Pulmonary Cells - Group Asbestos (A)

	<u>Macrophages with Asbestos Bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks	4	86	11		1	2	
4 weeks	6	90	7			3	
6 weeks	3	81	10	1		5	3
8 weeks	4	86	8	4		2	
10 weeks	3	91	7	2			
12 weeks	4	84	8		1	6	1
26 weeks	3	82	5	6	1	6	
38 weeks	4	84	16				
52 weeks	1	89	3			8	

Table 13C. Differential Count of Harvested Pulmonary Cells - Group Mycobacteria (M)

	<u>Macrophages with Asbestos bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks		93	6		1		
4 weeks		90	10				
6 weeks		95	2				3
8 weeks		81	14		1	3	1
10 weeks		86	9			5	
12 weeks		91	8		1		
26 weeks		87	12	1			
38 weeks		86	12		2		
52 weeks		87	9			4	

Table 13D. Differential Count of Harvested Pulmonary Cells - Group Freund's Complete Adjuvant (F)

	<u>Macrophages with Asbestos bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks		94	4	2			
4 weeks		91	8		1		
6 weeks		87	11	2			
8 weeks		90	7	3			
10 weeks		93	6	1			
12 weeks		89		11			
26 weeks		84	8	3		5	
38 weeks		90	3		3	4	
52 weeks		66	33		1		

Table 13E. Differential Count of Harvested Pulmonary Cells - Group Freund's Complete Adjuvant +

Mycobacteria (FM)

	<u>Macrophages with Asbestos bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks		91	6	3			
4 weeks		95	4	1			
6 weeks		85	11	2		2	
8 weeks		86	12	2			
10 weeks		84	12	4			
12 weeks		78	16	5		1	
26 weeks		92	4			4	
38 weeks		93	7				
52 weeks		93	7				

Table 13F Differential Count of Harvested Pulmonary Cells - Group Asbestos +

Freunds Complete Adjuvant (AF)

	Macrophages with Asbestos Bodies	Total Macrophages	Lymphocytes	Giant Cells	Monocytes	Eosino- phils	Polymorphonuclear Leukocytes
2 weeks	3	91	7		1	1	
4 weeks	1	91	3	2		3	1
6 weeks	4	92	8				
8 weeks	8	82	11	2		4	1
10 weeks	7	84	10	5			1
12 weeks	4	81	12	4	1	1	1
26 weeks	5	77	9	7	5	2	
38 weeks	3	86	13			1	
52 weeks	5	83	14			3	

Table 13G

Differential Count of Harvested Pulmonary CellsGroup Asbestos + Mycobacteria (AM)

	<u>Macrophages with Asbestos Bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks		84	10	3	1	1	1
4 weeks	1	89	5	4		2	
6 weeks	2	84	8	7	1		
8 weeks	1	91	7		2		
10 weeks	5	99	1				
12 weeks		87	11	2			
26 weeks	3	86	10	4			
38 weeks		82	18				
52 weeks	2	81	18	1			

Table 13H

Differential Count of Harvested Pulmonary CellsGroup Asbestos + Freund's Complete Adjuvant + M. tuberculosis

	<u>Macrophages with Asbestos Bodies</u>	<u>Total Macrophages</u>	<u>Lymphocytes</u>	<u>Giant Cells</u>	<u>Monocytes</u>	<u>Eosino- phils</u>	<u>Polymorphonuclear Leukocytes</u>
2 weeks	3	93	7				
4 weeks	2	85	12	2		1	
6 weeks	3	88	8	3	1		
8 weeks	4	70	24	3	3		
10 weeks	8	86	13	1			
12 weeks	9	89	6	2		3	
26 weeks		90	4	6			
38 weeks	2	89	8	1	2		
52 weeks	2	86	11	3			

analyses of variance. In this study two-way analysis of variance with fractional factorial design was used to examine the biochemical, lymphocyte blast transformation and weight relationships (parameters).

The two-way analysis of variance enabled comparison of 1. one parameter to be made with itself at other time points within the same treatment group; 2. each parameter with the others at each time point within the same treatment group; 3. each parameter with itself in other treatment groups at the same time point. The fractional factorial design was used to show any potentiation of an individual component of treatment upon another within a single treatment group or between two treatment groups. This analysis assumed that the main treatments were asbestos (Group A), M. tuberculosis (Group M) and Freund's complete adjuvant (Group F). Combined treatment was considered to differ in order of interaction according to the number of components present (i.e. Groups AF, AM and FM were second order interactions and Group AFM was a third order interaction). The analysis was made by comparing results at two levels - "treatment present" and "treatment absent".

4. Biochemistry

A. Hydroxyproline Determinations

a. Total Hydroxyproline

Mean values for total hydroxyproline (Table 14) increased significantly with time; the p value between the following times being 0.001: two weeks, four weeks, six to twelve weeks, 26 to 38 weeks and 52 weeks. Two-way analysis of variance showed that means of treatment groups fell into two populations; groups mycobacteria (M), asbestos (A), asbestos + Freund's complete adjuvant (AF) and asbestos + Freund's complete adjuvant + mycobacteria (AFM) were significantly lower in value ($p = 0.05$) than groups Freund's complete adjuvant (F), asbestos + mycobacteria (AM) and untreated controls (N).

Fractional factorial analysis did not indicate any significant potentiation effect of any component of treatment upon another.

b. Hydroxyproline per gram of lung (wet weight)

Hydroxyproline as a concentration per gram of lung (wet weight) followed the pattern described for total hydroxyproline values with respect to time (Table 15). A steady increase with time was observed in all groups. There was no significant difference between the values at two weeks and 52 weeks for groups AF, AM, FM, F, and untreated controls. Group M showed significantly lower

Table 14.

Mean Total Hydroxyproline Content of Lung (ug)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	1497.85	3344.52	2830.21	2866.44	887.84	2852.07	822.57	2590.12
4 weeks	2482.28	2953.99	5528.93	4908.57	1564.85	3815.53	1072.26	4048.13
6 weeks	2432.67	3875.78	3745.08	5720.41	3431.31	3304.81	2329.54	3681.76
8 weeks	4660.17	3589.02	4383.17	5017.28	3739.01	3533.87	2359.93	3703.20
10 weeks	3325.95	4225.50	4097.61	4135.08	4408.81	4210.09	3866.83	4445.34
12 weeks	3257.89	2169.69	4367.79	4215.88	4076.98	4268.69	2852.35	4752.87
26 weeks	6292.59	4260.27	6726.50	6058.89	5156.76	5336.15	5884.78	4790.38
38 weeks	5627.52	5822.41	5764.05	7172.07	6294.57	5305.23	6178.88	6107.17
52 weeks	6734.83	5395.59	8802.46	8084.29	6605.74	6412.10	8462.37	7136.24

Table 15.

Mean Hydroxyproline Content of Lung (µg/gram wet weight)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	448.79	1310.92	1158.31	1375.99	366.25	1365.02	351.85	1380.51
4 weeks	964.64	889.59	2369.89	2024.64	632.09	1725.35	425.17	1838.87
6 weeks	1208.69	1316.29	1427.71	2153.66	1006.23	1378.23	816.68	1596.97
8 weeks	1421.52	1175.45	1705.25	1736.34	1306.02	1123.52	846.69	1535.45
10 weeks	1125.07	1398.71	1639.84	1330.55	1507.88	1536.81	1231.99	1681.45
12 weeks	1110.11	745.62	1303.46	1323.51	1307.87	1502.35	965.35	1836.16
26 weeks	1489.37	1225.87	1980.36	1736.88	1663.36	1682.39	1753.54	1565.44
33 weeks	1143.42	1671.12	1217.56	1364.21	1644.68	1616.78	1425.88	1670.84
52 weeks	1382.71	1508.45	1676.34	1827.78	1783.39	1641.08	1682.58	1929.33

values ($p = 0.001$) at two, four, six, eight and 12 weeks compared to the 26 to 52 weeks values and Group A and AFM had significantly lower values ($p = 0.001$) at two and four weeks compared to their respective 52 week values.

Means of treated groups fell into two populations; groups M, A, AF, and AFM were significantly lower in value ($p = 0.05$) than groups F, AM, FM, and untreated controls.

Fractional factorial analysis showed that group A potentiated the other component or components of the treatment groups AM ($p = 0.05$), AF ($p = 0.01$) and AFM ($p = 0.001$) in their effect.

c. Hydroxyproline per milligram Lung (dry weight)

The use of dry lung weight as a standard against which to express hydroxyproline demonstrated a similar pattern against time as was described for values expressed per lung (wet weight) and values expressed per gram of lung (wet weight) (Table 16). There was a fairly steady increase in hydroxyproline from two weeks onwards. The difference between the values at two and 52 weeks was significant ($p = 0.01$), however it was not possible to find a statistically significant difference between treatment groups. Means of treatment groups fell into two populations; Group M had a significantly lower ($p = 0.05$) amount of hydroxyproline per mg lung (dry weight) than all other groups.

Table 16.

Mean Hydroxyproline Content of Lung (ug/milligram dry weight lung)

<u>TIME</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	5.25	10.90	7.02	8.91	3.79	7.33	3.27	7.70
4 weeks	7.59	6.44	8.23	10.08	5.80	9.75	3.73	10.45
6 weeks	7.52	10.53	8.61	12.40	8.97	8.35	6.76	8.60
8 weeks	10.04	9.23	10.42	9.98	9.09	4.83	5.04	6.66
10 weeks	9.46	10.23	9.79	8.02	11.55	9.44	8.35	7.68
12 weeks	7.80	5.63	6.94	8.22	7.80	10.27	5.72	8.46
26 weeks	9.87	9.09	10.44	10.41	10.38	11.34	10.24	9.65
38 weeks	7.24	10.66	7.69	7.64	9.39	8.23	9.00	9.57
52 weeks	7.68	9.10	9.25	10.79	10.50	9.10	4.28	12.21

B. Deoxyribonucleic Acid (DNA) per milligram Lung (dry weight)

There was a steady decrease in the amount of DNA per milligram lung (dry weight) from two to 52 weeks (Table 17). The first six time points (two, four, six, eight, ten and twelve weeks) formed one population which had significantly higher ($p = 0.001$) amounts of DNA than the second population (26, 38, and 52 weeks). There was no significant difference between values within each of those time populations.

Analysis of the treatment effects showed that the untreated controls and animals receiving only one treatment (i.e. groups A, F, and M formed a population that had significantly lower levels of DNA than group AF and AM ($p = 0.05$) and group FM and AFM ($p = 0.001$). However, groups AM, AF, FM and AFM were not significantly different from each other. The fractional factorial analysis demonstrated significant synergistic effects for the asbestos component of treatment in groups AM ($p = 0.05$) and AF ($p = 0.01$). The p value for the effect of asbestos as compared with no treatment was $p = 0.01$).

C. Leucine Aminopeptidase Activity

The activity of the enzyme, leucine aminopeptidase showed significant differences with regard to both time and treatment.

Table 17.

Mean DNA Content of Lung ($\mu\text{g}/\text{milligram dry weight lung}$)

<u>TIME</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	4.03	3.57	2.83	2.53	2.78	2.60	4.51	3.30
4 weeks	3.81	4.15	1.53	1.59	3.57	2.11	3.04	2.84
6 weeks	3.05	2.80	1.79	2.12	4.00	2.32	3.20	2.09
8 weeks	4.78	4.55	1.81	2.13	3.57	0.91	3.00	1.25
10 weeks	3.67	2.73	3.04	2.37	3.79	1.82	3.07	1.25
12 weeks	3.99	4.91	2.51	2.76	2.32	1.86	2.44	2.19
26 weeks	2.05	2.05	1.34	1.66	2.05	1.45	2.58	1.69
38 weeks	1.92	1.70	1.63	1.85	1.50	1.20	2.25	1.15
52 weeks	2.13	2.12	2.39	2.81	1.60	2.02	1.07	2.13

Table 18. Leucine Aminopeptidase Activity ($\mu\text{mole}/\text{min}/10^9$ cells substrate hydrolyzed)

A. Effect of Time on Activity

<u>Group I</u>		<u>Group II</u>	
<u>Week</u>	<u>Mean Activity</u>	<u>Week</u>	<u>Mean Activity</u>
4	1.208	2	1.113
6	1.257	12	1.148
8	1.199	26	1.139
10	1.198	38	1.138
		52	1.081

$\left. \begin{array}{c} \text{Group I} \\ \text{Mean Activity} \end{array} \right\} P = 0.05 \left\{ \begin{array}{c} \text{Group II} \\ \text{Mean Activity} \end{array} \right.$

B. Effect of Treatment on Activity

<u>Group I</u>		<u>Group II</u>		<u>Group III</u>	
<u>Treatment</u>	<u>Mean Activity</u>	<u>Treatment</u>	<u>Mean Activity</u>	<u>Treatment</u>	<u>Mean Activity</u>
AM	1.282	AF	1.190	A	1.089
AFM	1.245	F	1.156	FM	1.064
M	1.235			Nil	1.052

$\left. \begin{array}{c} \text{Group I} \\ \text{Mean Activity} \end{array} \right\} P = 0.01 \left\{ \begin{array}{c} \text{Group II} \\ \text{Mean Activity} \end{array} \right\} P = 0.001 \left\{ \begin{array}{c} \text{Group III} \\ \text{Mean Activity} \end{array} \right.$

Comparison of enzyme activity of all treatment groups at different time points showed two significantly different populations ($p = 0.05$). A high enzyme activity population existed at four to 10 weeks while a low enzyme activity population was evident at two and 12-52 weeks (Table 18 A).

Mean values for enzyme activity between the treated groups formed three significantly distinct populations. Groups AM, AFM and M formed a population that had the highest enzyme activity and was significant at the 1% level compared with groups AF and F which formed the second population. This second population in turn, had enzyme activities that were significantly higher ($p = 0.001$) than the third population which was made up of groups A, FM and untreated controls (Table 18B).

5. Sensitization of Lymphocytes

The stimulation indices of lymphocytes obtained from groups treated with adjuvant and/or mycobacteria increased with time, usually reaching a peak at 26 weeks and subsequently declining. However, these declining values remained higher than the values at four weeks (Table 19). The means for four, eight, twelve, 38 and 52 weeks were not significantly different from one another; as a group they are lower than the 26 week values ($p = 0.05$).

Table 19Peripheral Blood Lymphocyte Blast Transformation to PPDStimulation Index

<u>Time</u>	<u>Treated Groups</u>						<u>Untreated Groups</u>	
	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>F</u>	<u>M</u>	<u>A</u>	<u>Control</u>
4 weeks	3.41	5.02	11.73	13.06	13.11	1.81	0.93	1.19
8 weeks	6.55	3.14	16.61	88.00	7.27	9.26	1.40	0.95
12 weeks	10.73	16.61	42.38	64.10	11.51	3.94	1.03	1.06
26 weeks	38.84	52.39	63.43	44.85	29.92	7.63	1.84	1.12
38 weeks	1.84	46.93	9.65	5.04	2.84	19.02	1.14	2.73
52 weeks	14.73	2.79	9.67	26.82	10.83	2.80	1.83	2.48

Analysis of treatments showed three distinct groups; AFM, A, M, F, and controls formed one population which had significantly lower stimulation indices than the population AF and AM ($p=0.01$), which in turn was significantly lower than the stimulation index for FM ($p=0.001$).

Cutaneous reactivity (measured between two to twelve weeks) to PPD present 48 hours after skin testing (Table 20) also demonstrate that lymphocytes were sensitised following treatment with FCA and/or H37Ra.

6. Weight Relationships

Two-way analysis of variance demonstrated that there was a general pattern for all weight measurements with respect to time which was independent of the treatment given. The weights between two and twelve weeks did not significantly differ, but there was a significant difference between the weights between two and twelve weeks compared with those between 26 and 52 weeks. The 52 week weights were significantly higher than those between 26 and 38 weeks.

This pattern of a low, middle and high range applied consistently throughout the study to body weight, total lung wet weight, left lower lobe wet weight and lung dry weight. Treatment effects became evident when the total lung wet weight and left lower lobe wet weight were analyzed, however no significant change could be attributed to

Table 20. Cutaneous Skin Test Measurement to PPD Mean Diameter of Induration (mm)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>N (Control)</u>
2 weeks	20.0	15.0	8.0	12.0	0	12.0	5.0	0
4 weeks	20.0	12.0	15.0	12.0	0	18.0	18.0	0
6 weeks	25.0	12.0	17.0	19.0	0	15.0	20.0	0
8 weeks	25.0	12.0	15.0	18.0	0	10.0	22.0	0
10 weeks	27.0	18.0	18.0	22.0	0	15.0	25.0	0
12 weeks	26.0	15.0	24.0	15.0	0	10.0	28.0	0

treatment with respect to lung dry weight or body weight.

A. Body Weight

Analysis of body weights demonstrated a steady progression with time and no difference between treated and untreated controls (Table 21).

B. Total Lung Wet Weight

In untreated controls, lung wet weights increased steadily from two to 52 weeks, with three distinct populations being formed (Table 22). The first population consisted of weights falling between two to twelve weeks (with no significant difference between them). The second population at 26 weeks was significantly increased in weight ($p = 0.05$) compared with the first population, while the third population (38 and 52 weeks) had an even greater increase in weight ($p = 0.001$) compared with the first population. Mean values for treated groups were dependent on the treatment given and progressively increased as the number of components in the treatment increased.

C. Left Lower Lobe Wet Weight

The analysis of left lower lobe wet weight closely paralleled the total lung wet weight. With respect to time the untreated control values increased steadily from two to

Table 21.Mean Body Weight (g)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	394.6	379.8	390.4	376.1	414.8	316.9	366.6	349.2
4 weeks	421.0	449.1	384.5	439.6	413.6	375.5	457.1	408.6
6 weeks	448.5	579.1	529.2	501.1	479.1	369.7	517.9	464.0
8 weeks	516.0	628.2	488.1	563.0	524.4	524.2	600.2	409.2
10 weeks	530.0	594.3	495.2	676.3	557.3	579.1	606.2	518.1
12 weeks	611.3	652.5	658.3	690.0	641.0	504.3	728.1	552.8
26 weeks	769.9	793.3	666.1	640.9	677.2	788.0	643.7	758.2
38 weeks	758.3	906.3	724.8	787.6	914.8	855.6	684.6	888.1
52 weeks	1002.4	939.3	902.2	910.6	1091.7	1050.0	1019.1	1043.3

Table 22.Mean Total Lung Wet Weight (g)

<u>Time</u>	<u>AMF</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	3.43	2.53	2.45	2.10	2.43	2.09	2.32	1.88
4 weeks	2.64	3.33	2.35	2.47	2.45	2.27	2.54	2.19
6 weeks	2.89	2.98	2.62	2.66	3.42	2.40	2.87	2.37
8 weeks	3.34	3.04	2.57	2.94	2.90	3.25	2.79	2.44
10 weeks	2.99	3.02	2.50	3.11	2.92	2.77	3.17	2.65
12 weeks	2.94	2.92	3.39	3.20	3.10	2.86	3.29	2.59
26 weeks	4.24	3.48	3.43	3.43	3.09	3.18	3.36	3.21
38 weeks	4.92	3.49	4.70	5.25	4.16	3.28	4.44	3.65
52 weeks	4.84	3.55	5.43	4.48	3.69	3.93	5.08	3.64

Table 23.

Mean Left Lower Lobe Lung Wet Weight (g)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>AM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 weeks	0.82	0.61	0.55	0.49	0.63	0.54	0.56	0.41
4 weeks	0.62	0.79	0.55	0.59	0.59	0.53	0.59	0.48
6 weeks	0.70	0.72	0.59	0.63	0.76	0.55	0.66	0.56
8 weeks	0.78	0.72	0.63	0.71	0.71	0.76	0.61	0.60
10 weeks	0.66	0.70	0.59	0.75	0.68	0.67	0.74	0.61
12 weeks	0.73	0.70	0.84	0.76	0.71	0.68	0.76	0.59
26 weeks	1.01	0.87	0.80	0.85	0.77	0.80	0.73	0.74
38 weeks	1.27	0.84	1.17	1.28	1.00	0.79	1.08	0.86
52 weeks	1.26	0.87	1.33	1.16	0.98	0.96	1.30	0.92

Table 24.Mean Lung Dry Weight (mg)

<u>Time</u>	<u>AFM</u>	<u>AF</u>	<u>AM</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>Control</u>
2 Weeks	68.48	73.48	90.60	74.60	60.72	99.00	59.78	74.08
4 Weeks	76.72	110.52	155.20	117.60	64.20	92.04	66.80	84.40
6 Weeks	112.44	88.32	101.00	113.80	81.16	90.60	79.24	104.21
8 Weeks	107.81	91.96	102.60	122.80	101.00	172.61	101.00	137.40
10 Weeks	81.45	97.16	98.20	124.00	89.22	112.80	108.80	133.30
12 Weeks	103.96	91.20	155.33	124.80	124.36	96.20	115.52	128.60
26 Weeks	154.00	117.20	150.80	141.20	123.50	118.80	127.80	118.27
38 Weeks	199.00	139.60	186.00	227.60	160.20	155.01	167.80	149.80
52 Weeks	229.78	147.00	246.20	193.80	166.20	171.88	668.00	150.00

52 weeks (Table 23). There was no significant difference between the weights at 38 and 52 weeks, however both these values were significantly greater ($p = 0.001$) than the weights at time points before 38 weeks.

As with total lung wet weights, the mean values for group AFM were significantly higher than all other groups ($p = 0.01$) and the mean weights progressively increased as the number of treatment components increased.

D. Lung Dry Weights

As with both wet weight determinations, the untreated control dry weight values increased steadily from two to 52 weeks (Table 24). The weights between two weeks and 26 weeks were not significantly different from one another but the weights at 38 and 52 weeks were significantly greater than those at earlier time points ($p = 0.05$). The means of all treated groups showed no significant difference and fractional analysis did not demonstrate a treatment component effect.

7. Summary of Results

A. Lymphocyte blast transformation to PPD showed mononuclear cells had become sensitized by treatment with Freund's complete adjuvant and /or M. tuberculosis.

B. The in vitro blast transformation response of peripheral blood lymphocytes to PPD was significantly reduced in animals that inhaled asbestos.

C. Asbestos bodies were demonstrable by light and electron microscopy four weeks after treatment and remained visible until completion of the study.

D. Histologically, the inflammatory process ranged from widespread focal lesions of interstitial and intra-alveolar mononuclear cell infiltration to consolidation with mononuclear cells causing obliteration of the lung architecture. The cells making up these lesions were mainly macrophages and lymphocytes. Granulomata and giant cells were demonstrable but not widespread.

The peak of the inflammatory reaction was followed by complete resolution.

Mycobacteria were not seen, nor were there amyloid deposits nor increases in collagen. Large increases in reticulin were evident, particularly in animals both sensitized to PPD and exposed to asbestos.

E. Mononuclear phagocytes were shown to be activated by their: 1. increased ability to spread on glass, 2. electron microscopic appearance and 3. increased enzyme activity.

F. A heterogeneous population of mononuclear phagocytes was demonstrable in the alveolar spaces of treated animals.

These included monocytes, immature mononuclear phagocytes and mature mononuclear phagocytes. In untreated controls only mature mononuclear phagocytes were found.

G. Biochemically, there was no evidence of significant increase of hydroxyproline above normal levels.

H. Hydroxyproline levels were reduced during the early time points in groups AFM, A and M. however they gradually returned to control levels.

Fig. 6 Guinea pig lung section showing interstitial mononuclear cell infiltration.
Haematoxylin and eosin (HE) X 200.

X Fig. 7 Typical small loss sheet of mononuclear cells with adjacent normal lung. HE.
X 100.

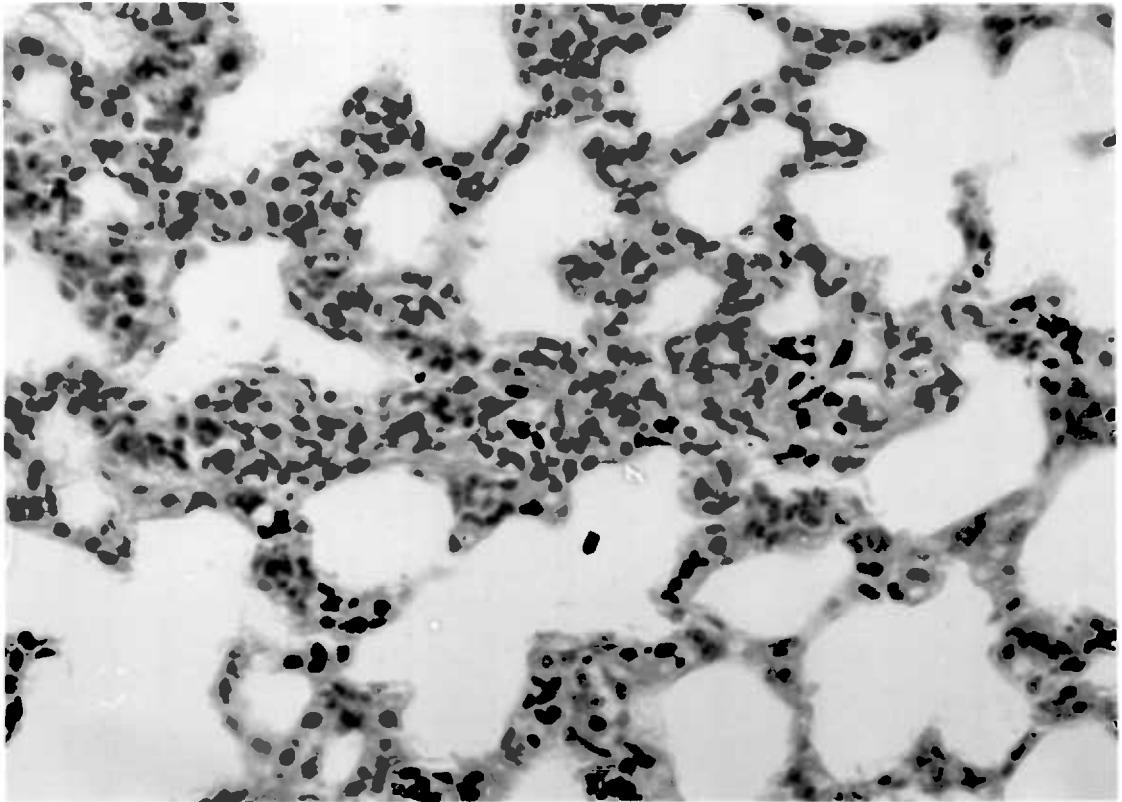


FIG. 6

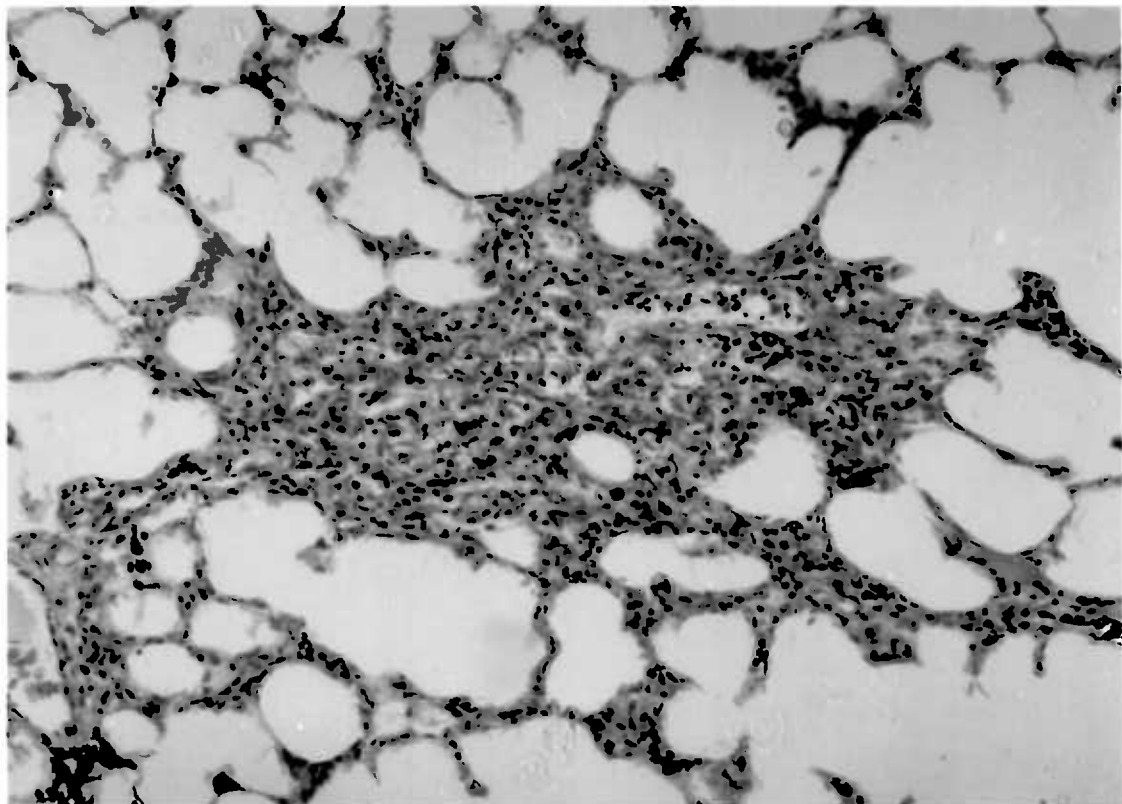


FIG. 7

Fig. 8 Large sheet of mononuclear cells organized into granulomata, causing obliteration of alveoli. HE. X 100.

Fig. 9 Large area of focal granulation tissue infiltrated with mononuclear cells causing complete obliteration of lung architecture. HE. X 100.

NB: Comparison of figures 6-9 show typical development of inflammatory lesions beginning with mild interstitial cellular infiltration and ending with severe lesions obliterating lung architecture. These lesions were found in all treated groups.

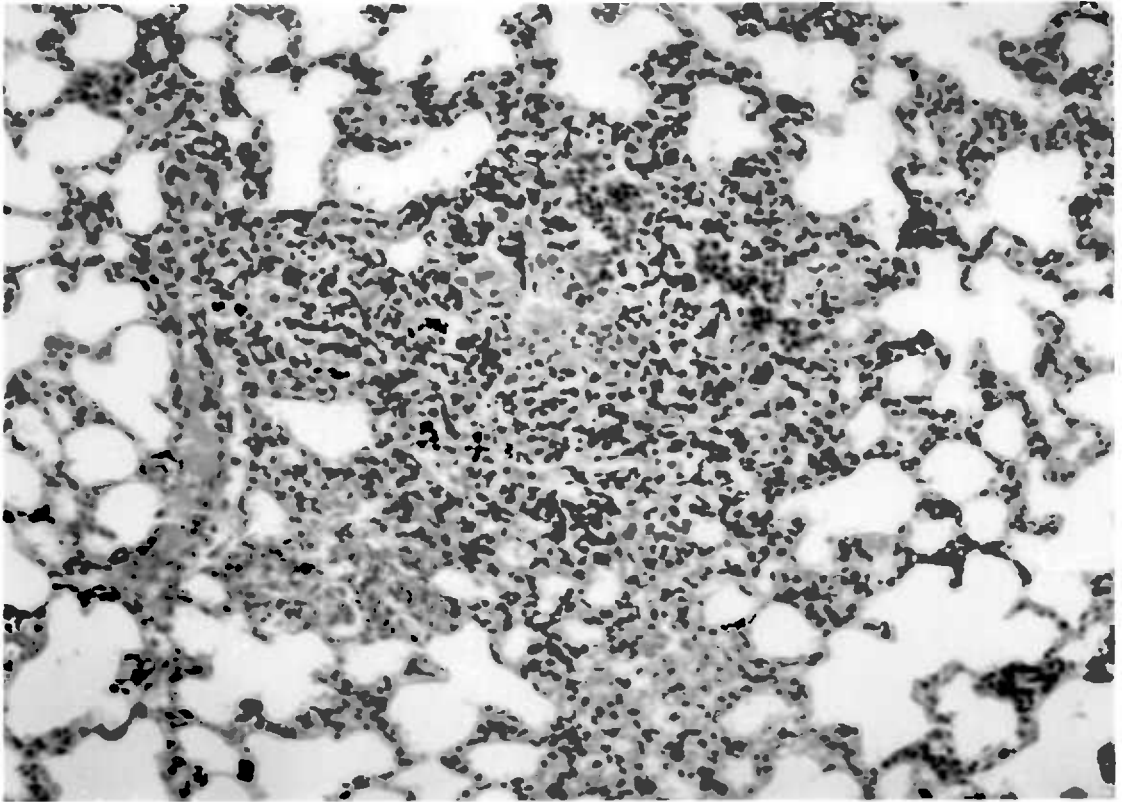


FIG. 8

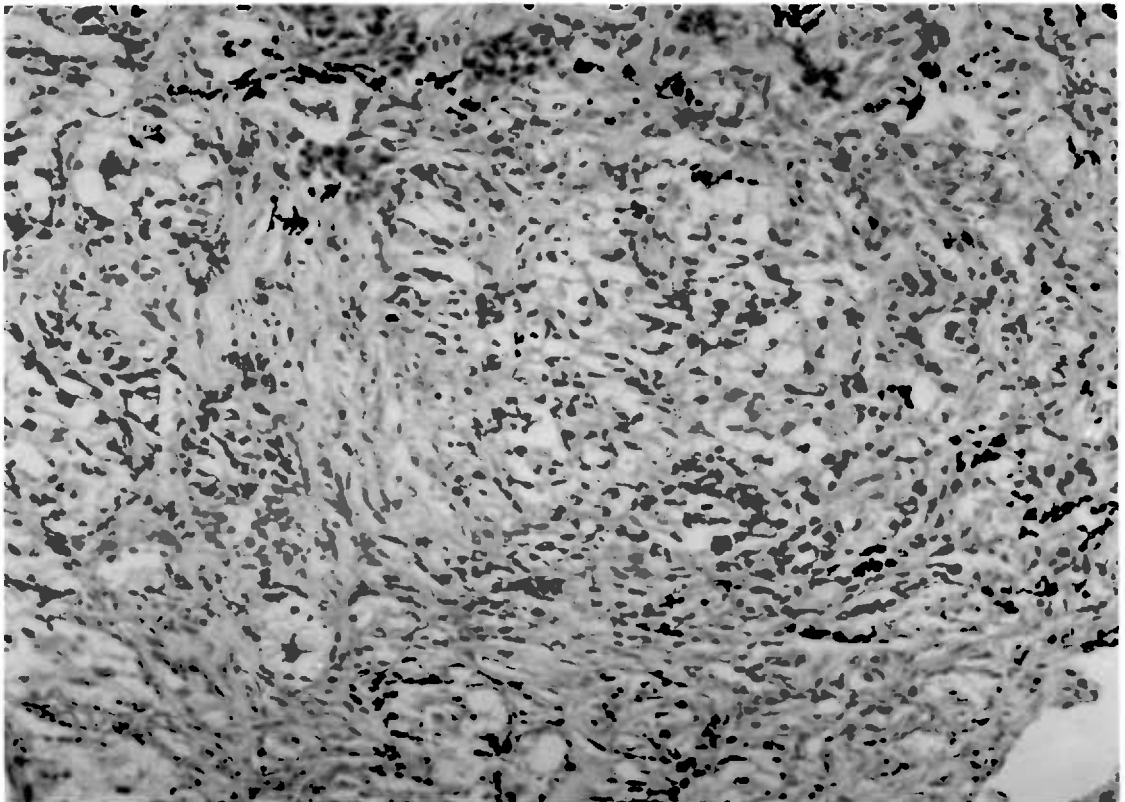


FIG. 9

Fig. 10. Sheet of mononuclear cells showing
marginated chromatin and nucleoli.
Many cells have become confluent
causing their plasma membranes to be
indistinguishable. HE. X 400.

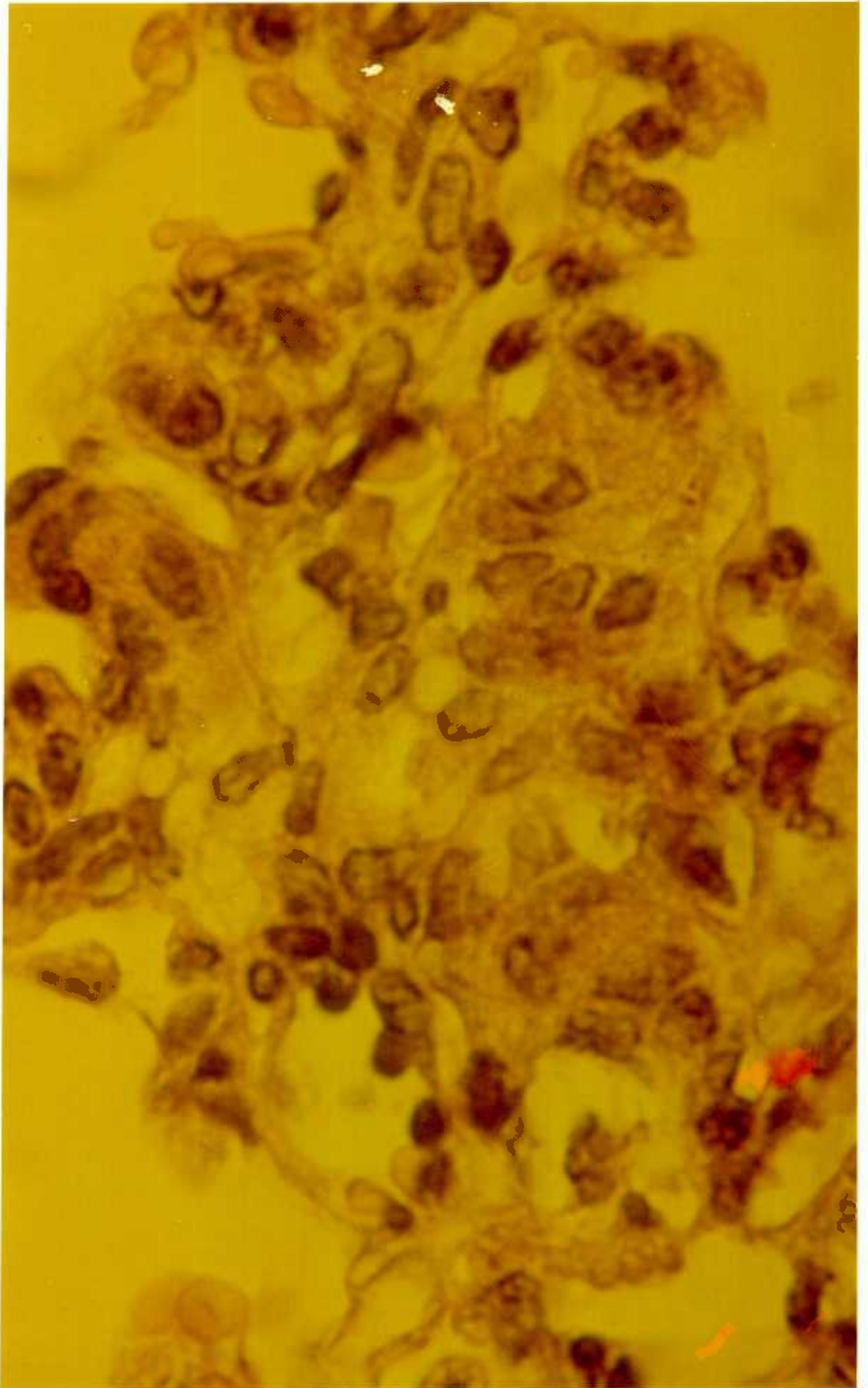


FIG. 10

Fig. 11. Intra-alveolar and intra-bronchiolar cellular exudate. Note interstitial and peribronchiole mononuclear cell infiltration. HE. X 200.

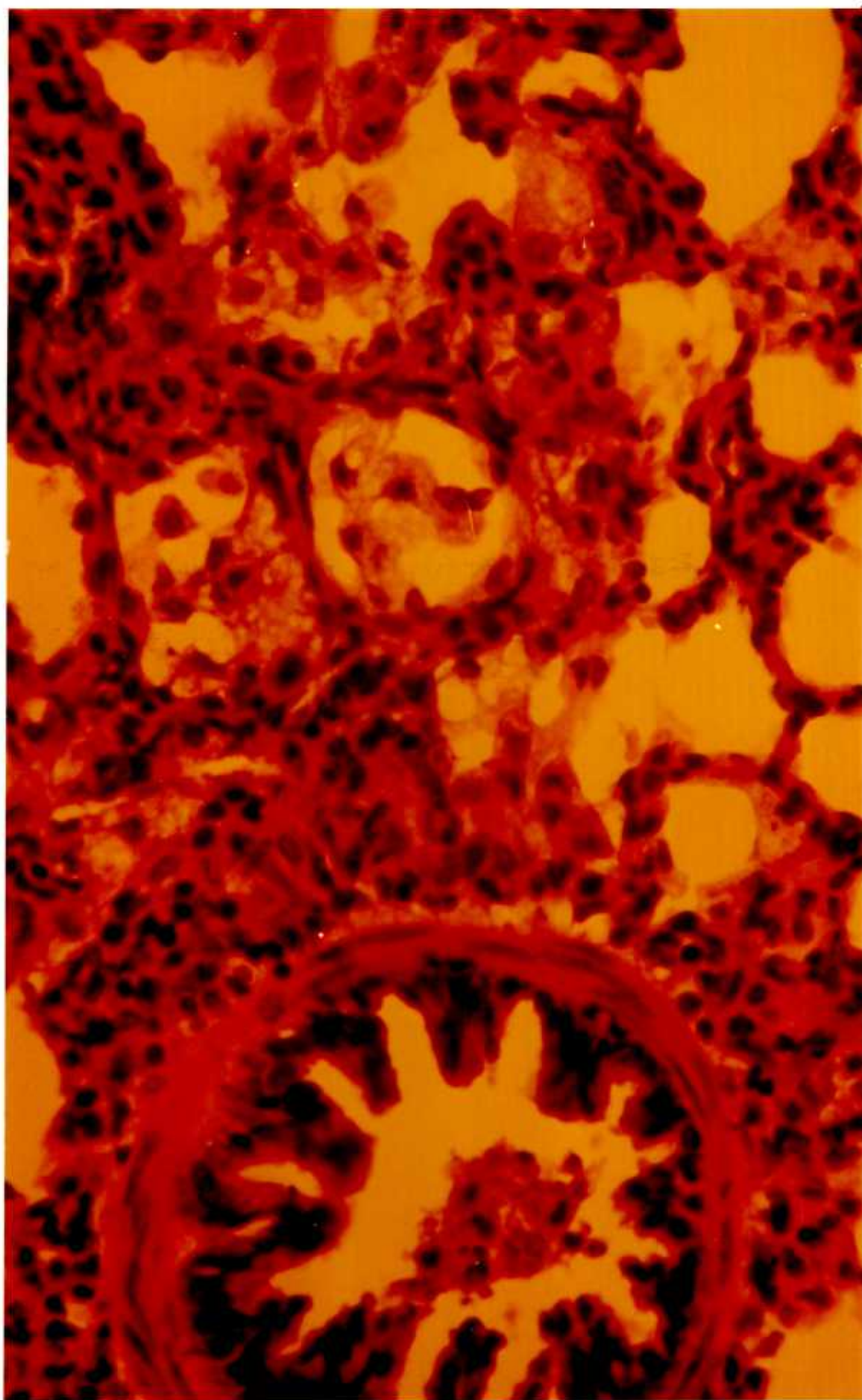


FIG. 11

Fig. 12. Normal reticulin fibres of guinea pig lung interstitium. Gordon and Sweet's silver impregnation. X 200.

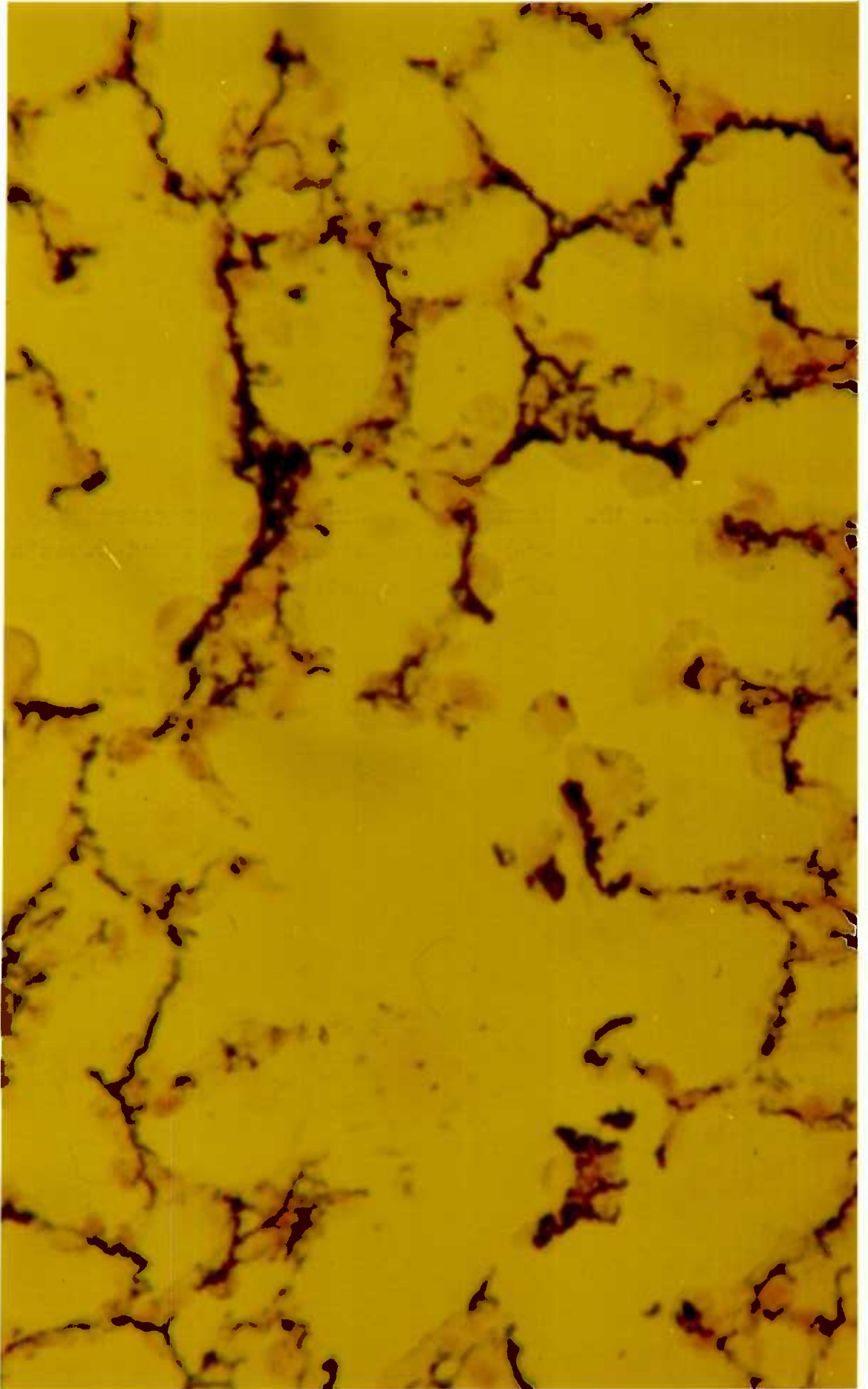


FIG.12

Fig. 13. Severe mononuclear cell infiltration among which new fine reticulin fibres have developed. Gordon and Sweet's silver impregnation. X 100.

Fig. 14. Obliteration of lung architecture by massive increases in reticulin fibres which has become thickened and branched. Note argyrophilic material in remaining alveolar spaces (arrow). Gordon and Sweet's silver impregnation. X 200.

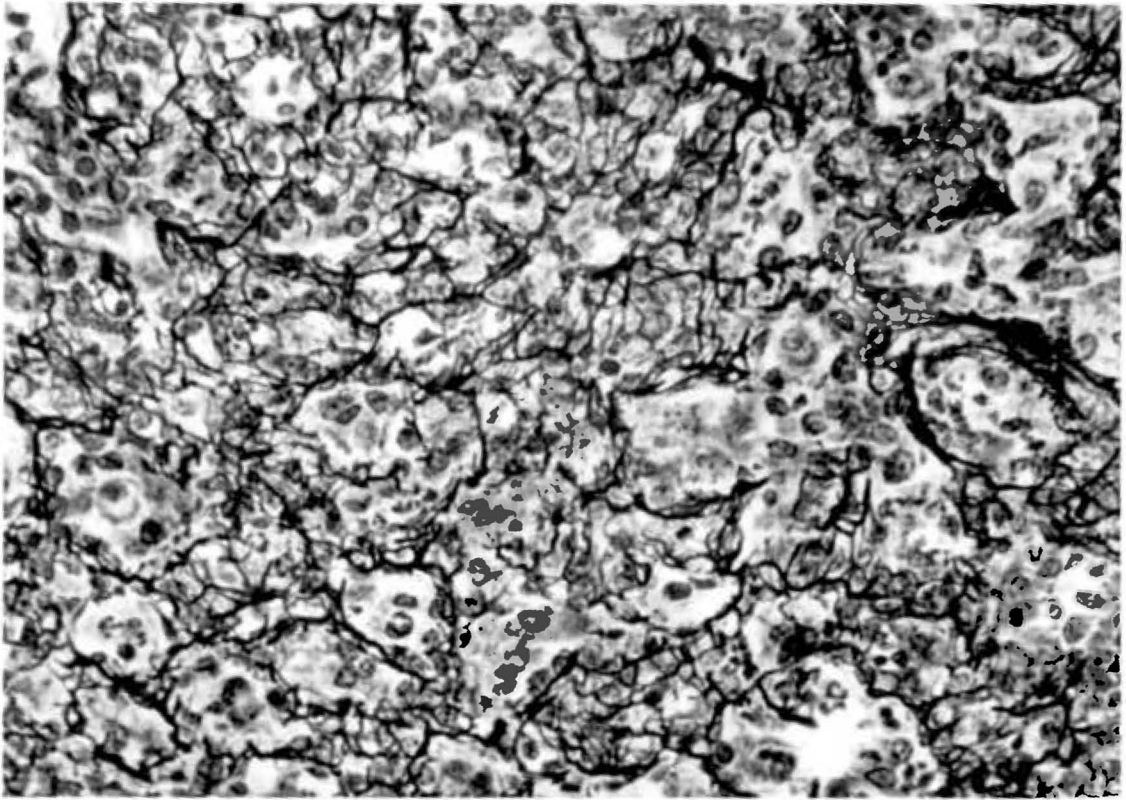


FIG. 13

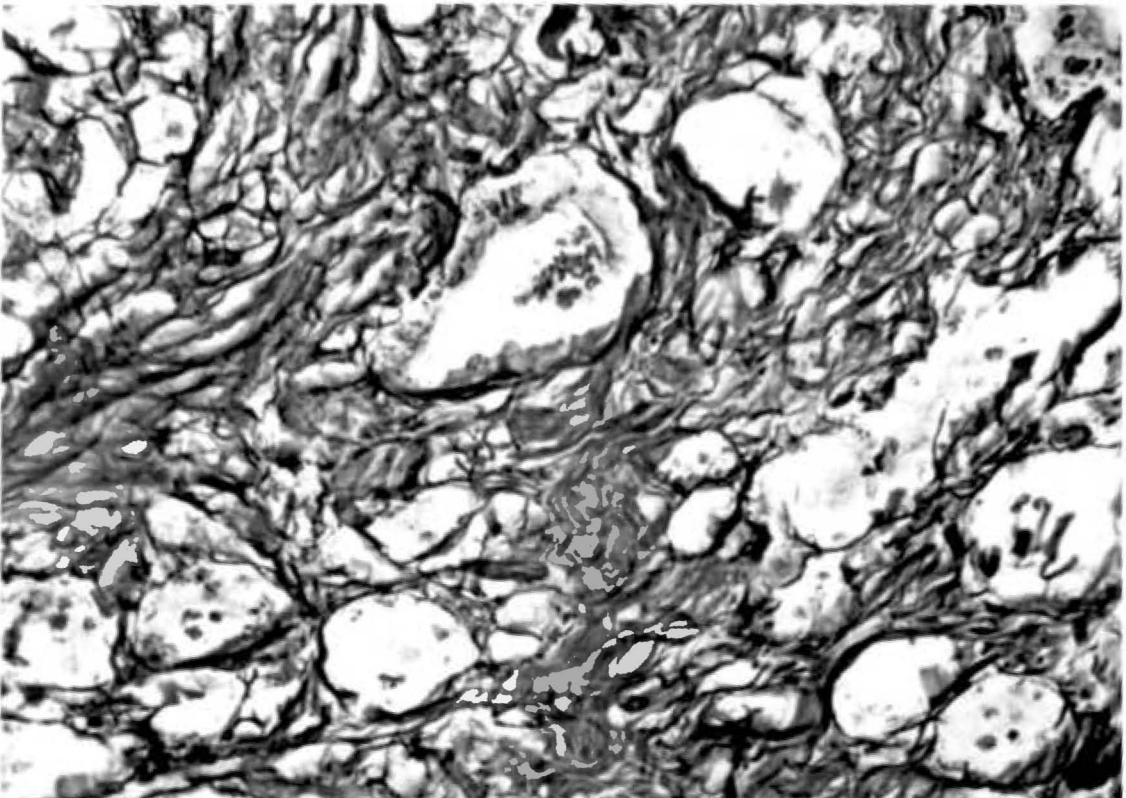


FIG. 14

Fig. 15. Electron micrograph showing asbestos
in a Type I epithelial cell.
X 60,000 approx.

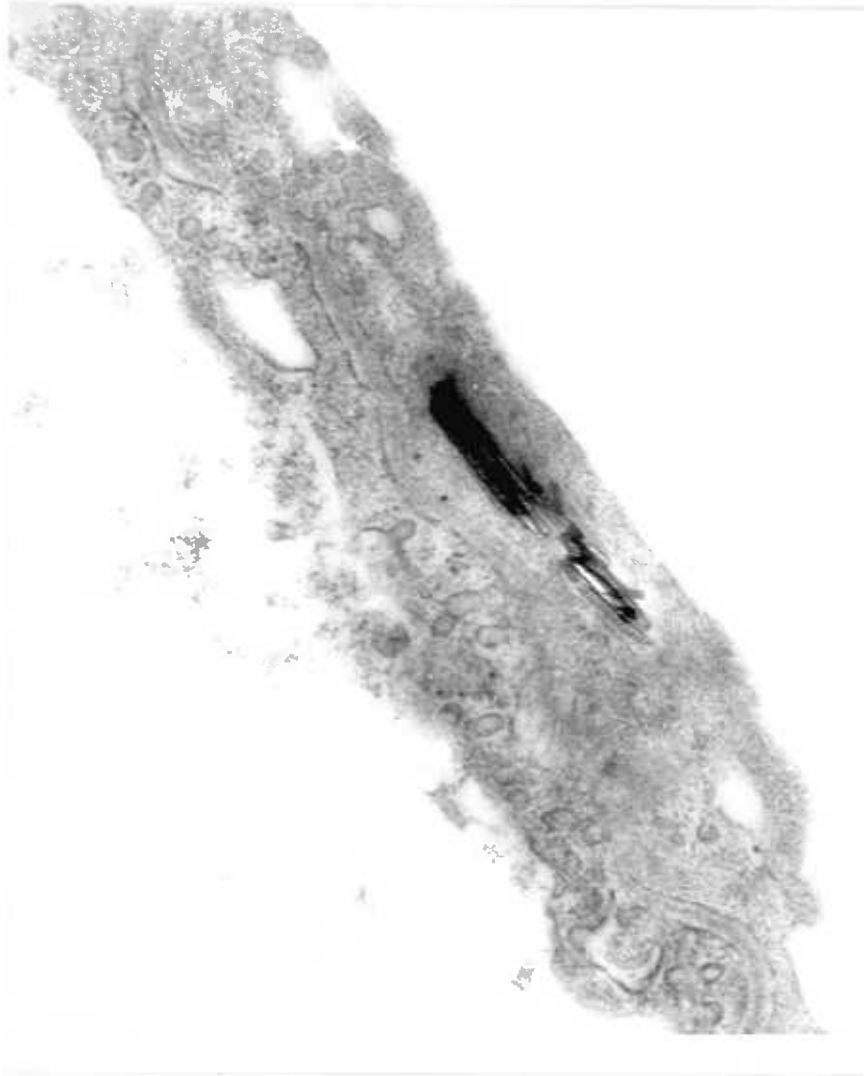


Fig. 15

Fig. 16. Minor perivascular lymphocyte cuffing.
HE. X 200.

Fig. 17. Perivascular lymphocytic cuffing
similar to Fig. 16 but more intense.
HE. X 100.

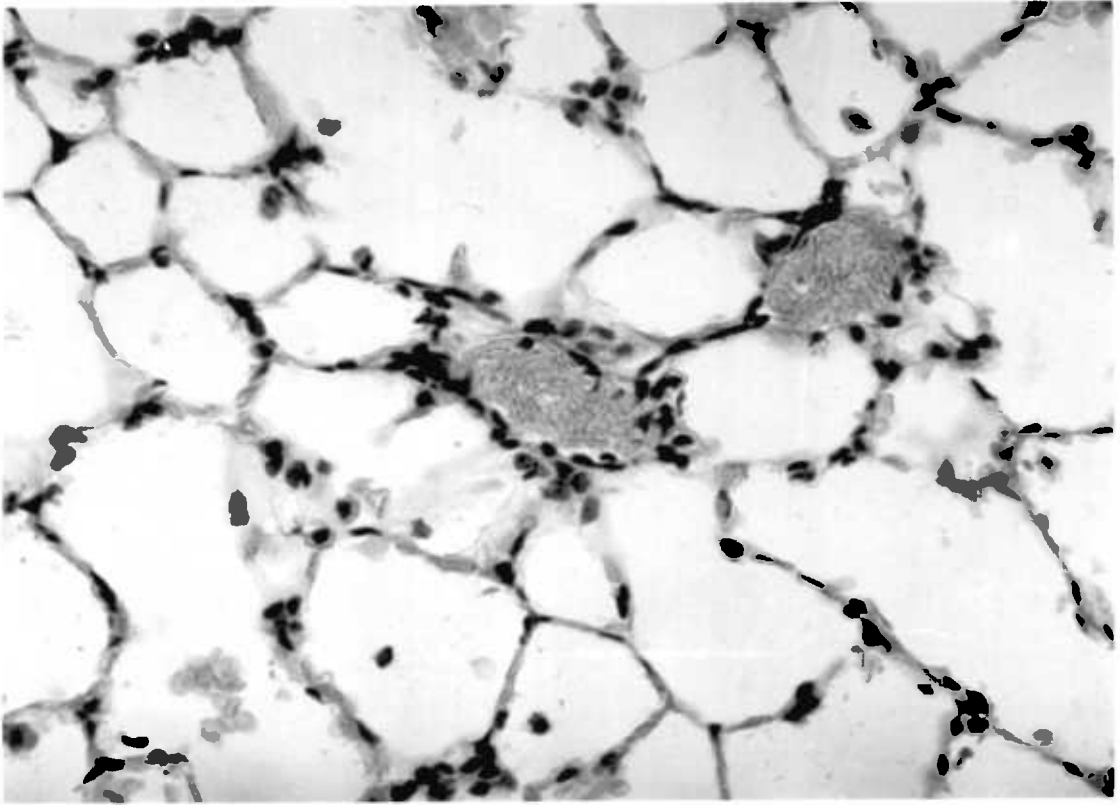


FIG. 16

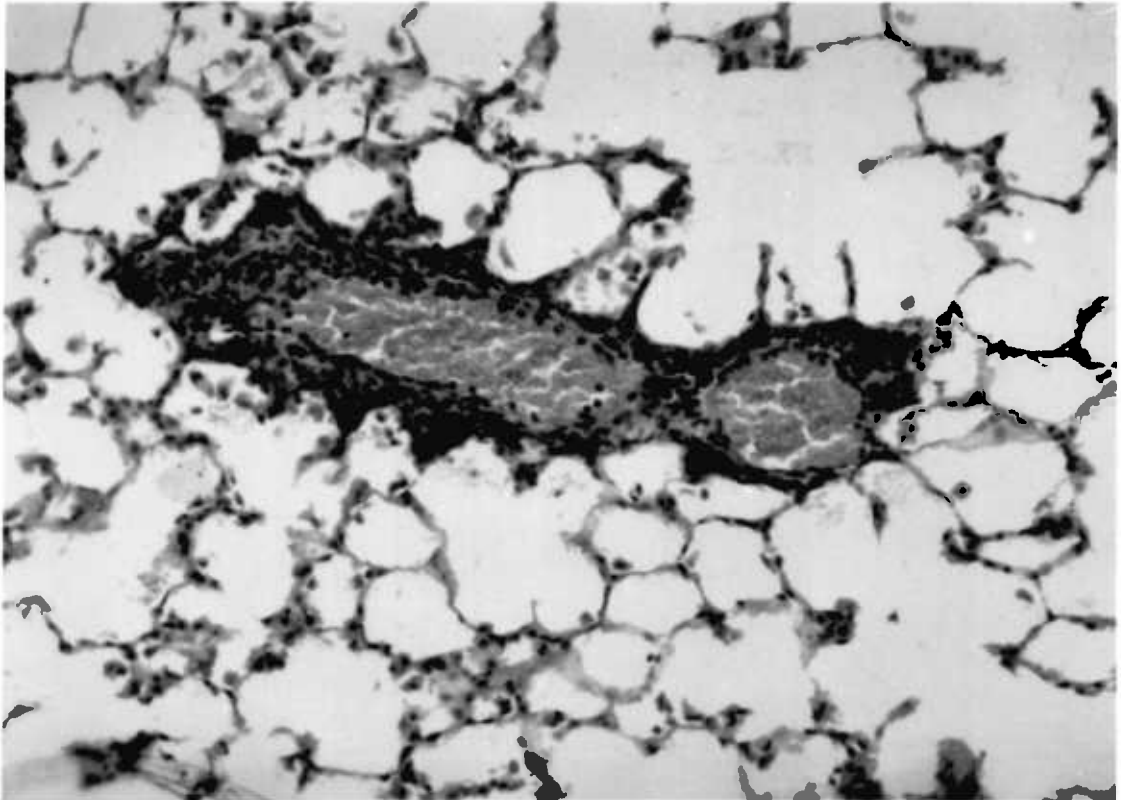


FIG. 17

Fig. 18. Granuloma caused by combination of asbestos inhalation and subcutaneous injection of Freund's complete adjuvant. Oil was contained within the clear spaces (arrows) surrounded by giant cells and has been removed during tissue processing. HE. X 80.

Fig. 19. Higher power view of Fig. 18 showing giant cell (arrow). HE. X 660.

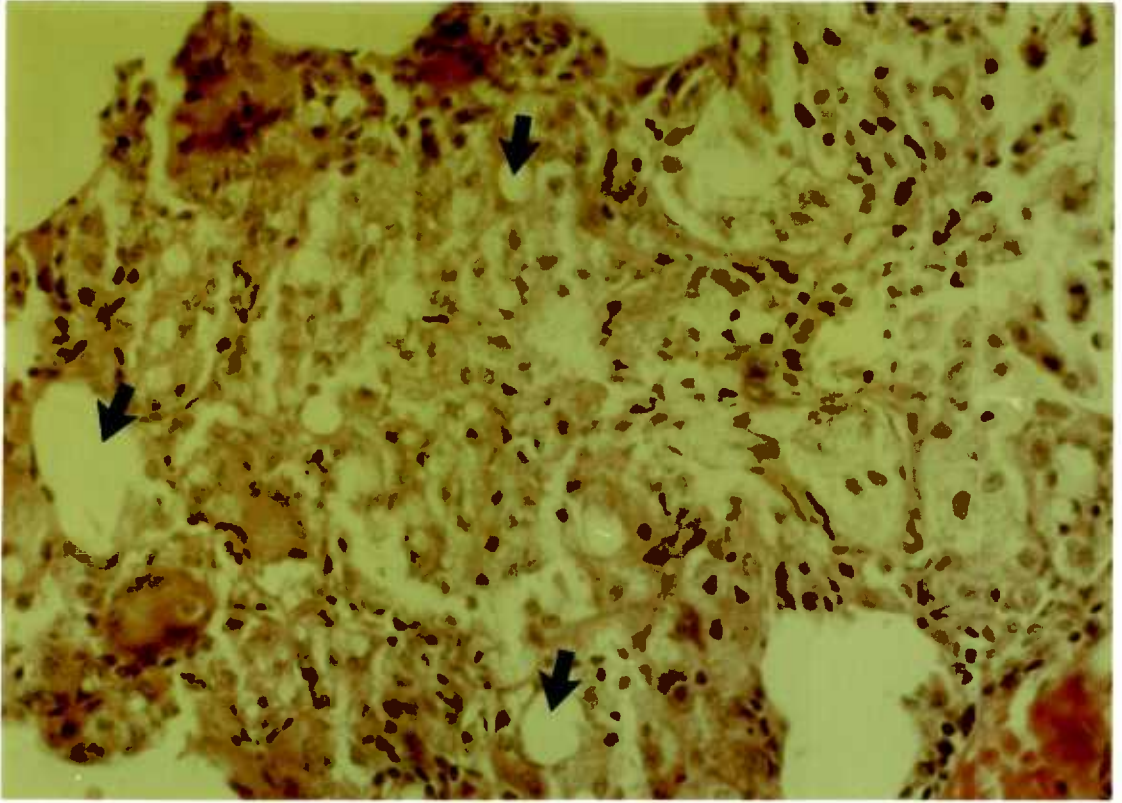


FIG. 18

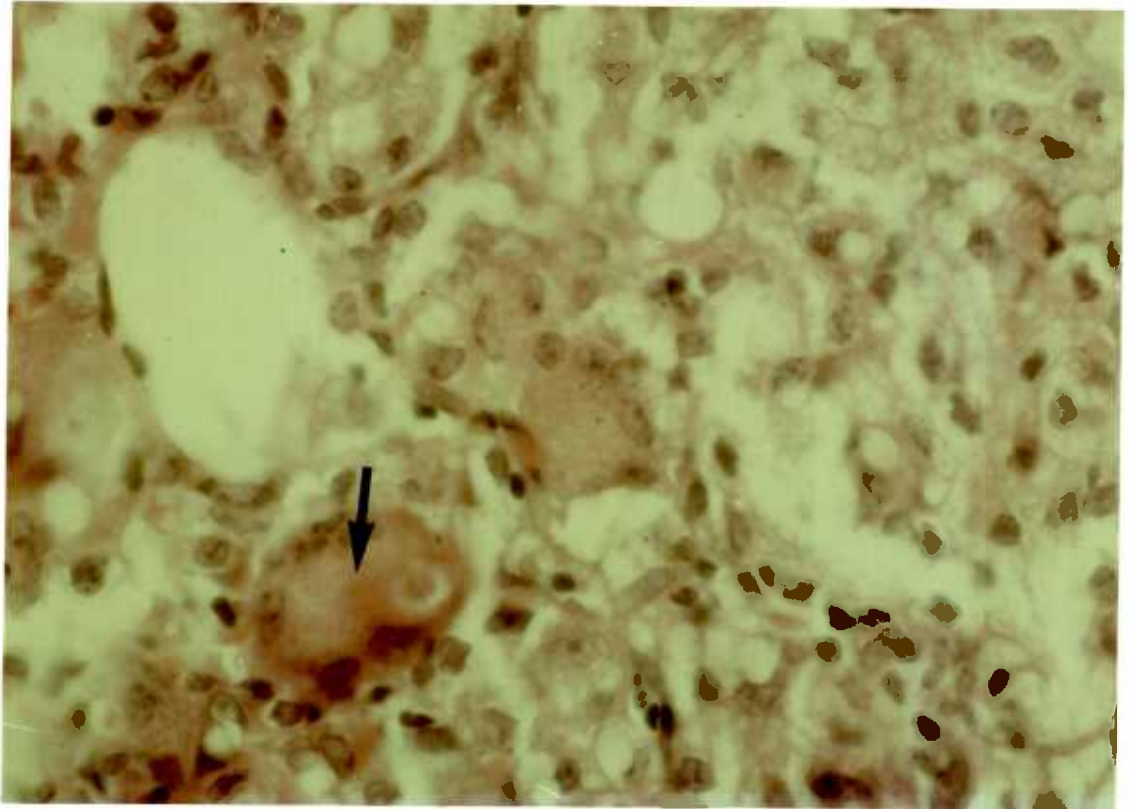


FIG. 19

Fig. 20 A section of lung showing asbestos
bodies (arrows) among inflammatory
cells. Perl's Prussian blue reaction.
X 1000.

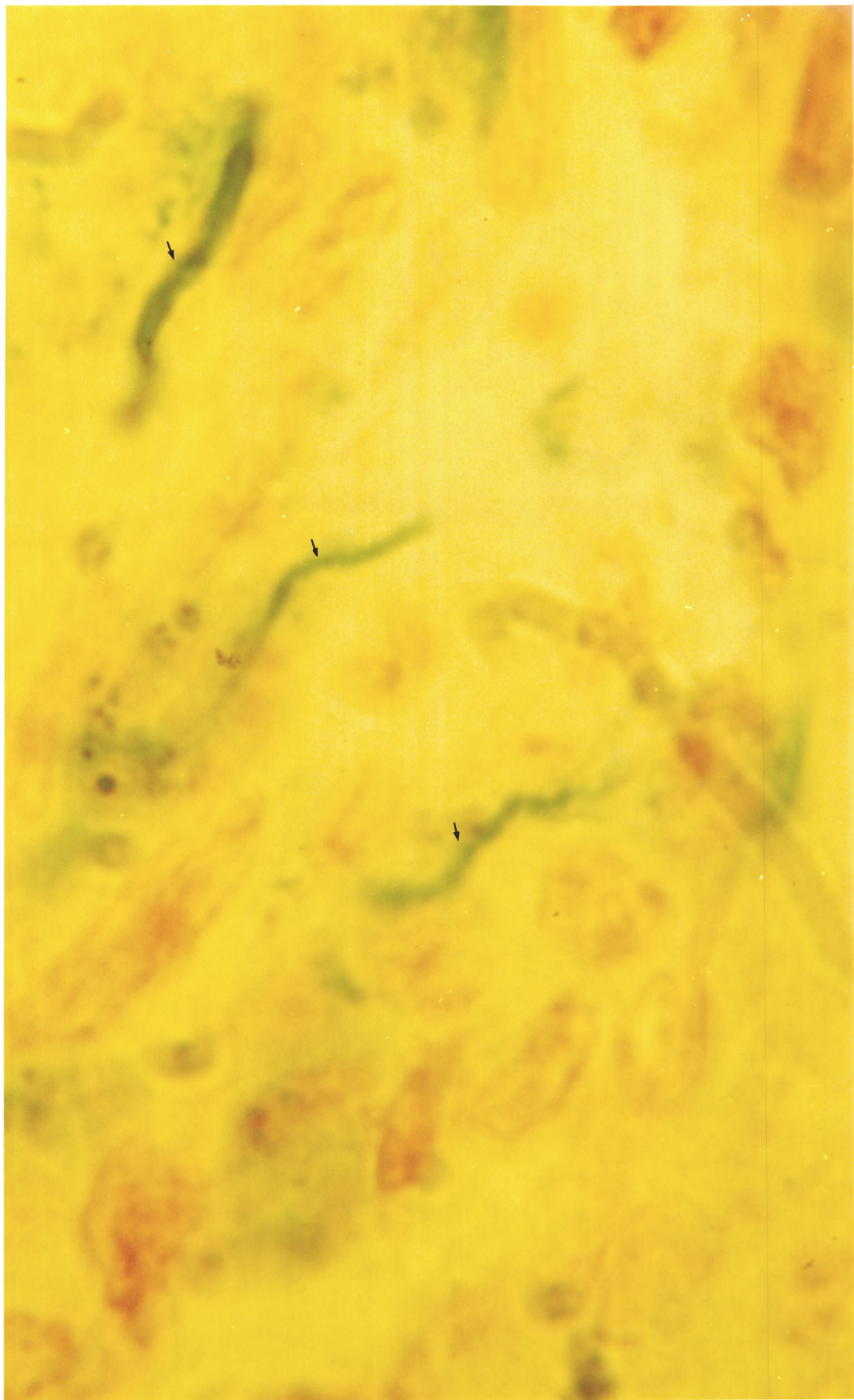


FIG. 20

Fig. 21. Phagosome of alveolar mononuclear
phagocyte containing asbestos
X 45,000.

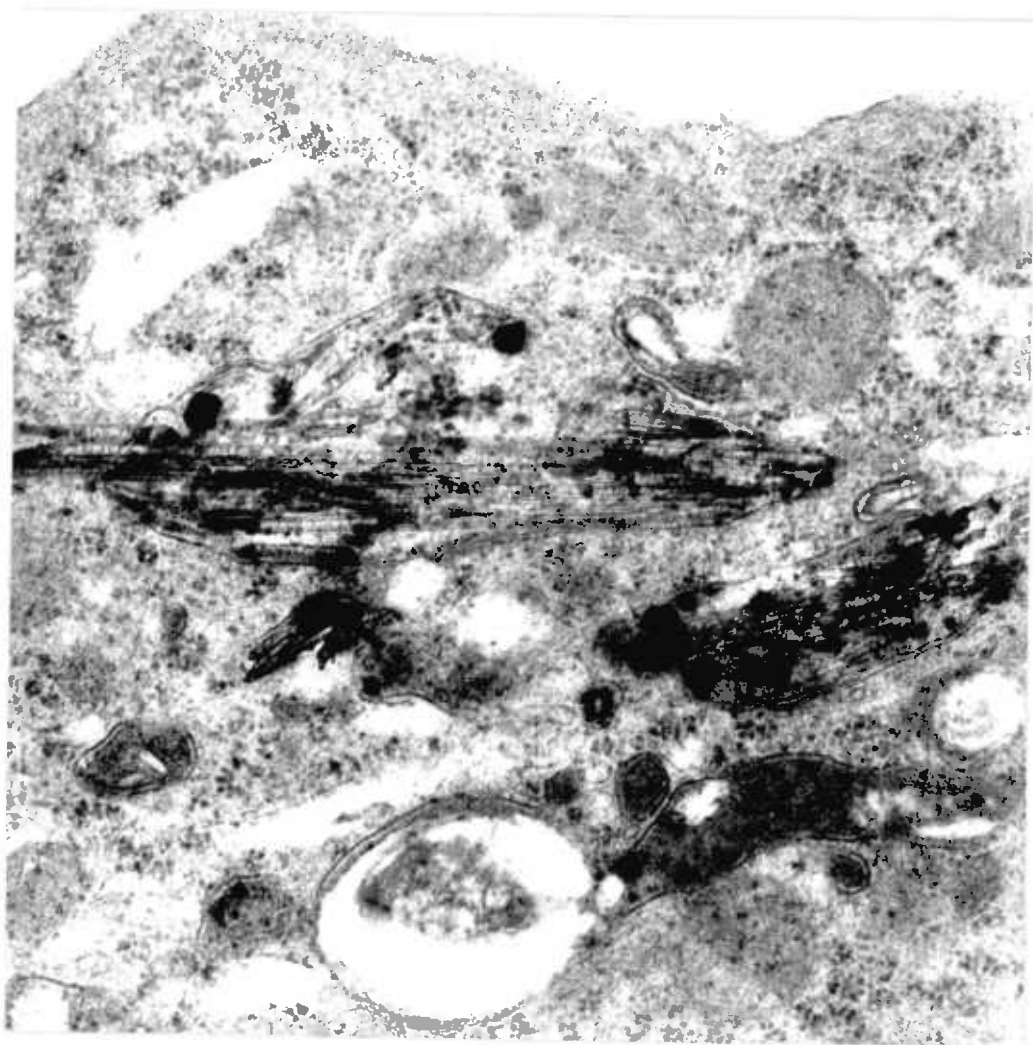


Fig. 21

Fig. 22. Typical aggregate of alveolar mononuclear phagocytes seen in lungs of all treated groups. Note numerous pseudopods, representative of activated cells and the immature cell (arrow) among the group of mature mononuclear phagocytes. X 4875.

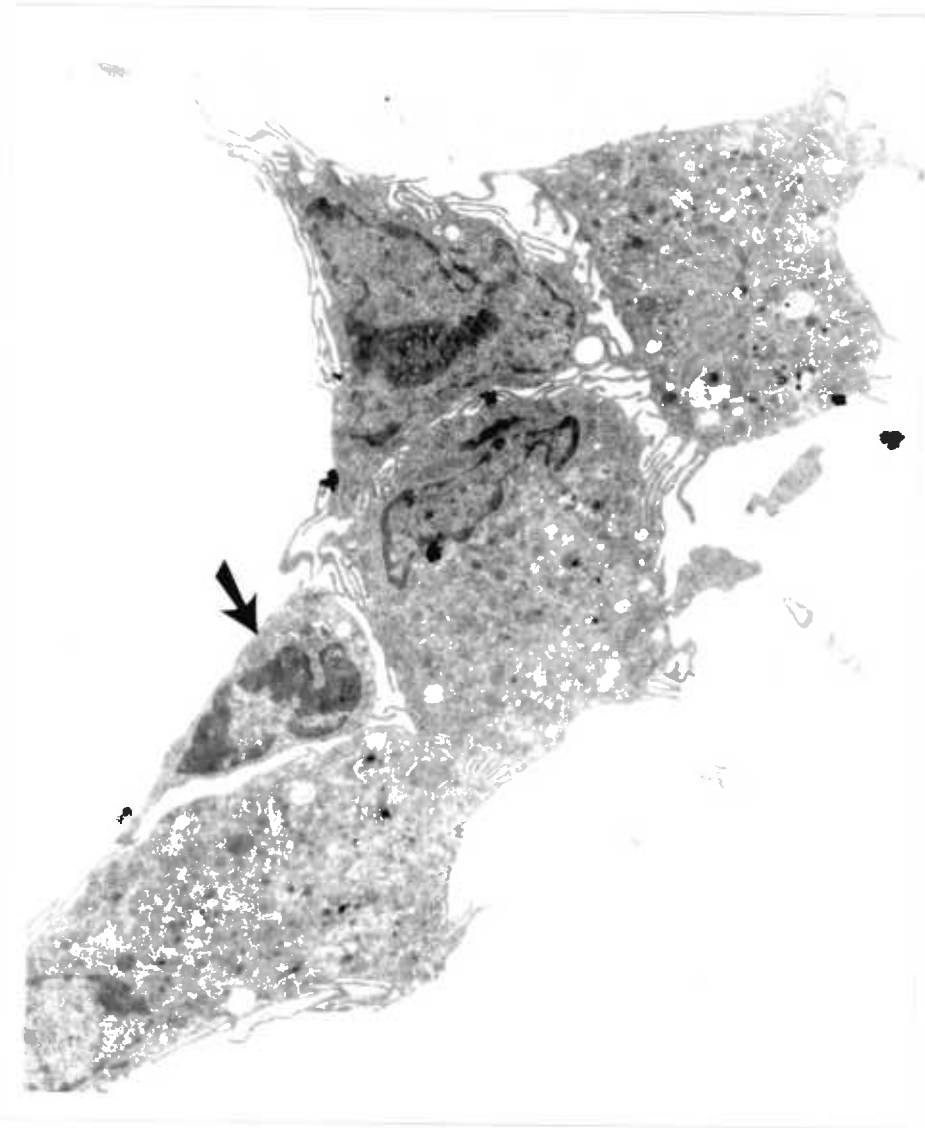


Fig. 22

Fig. 23. Electron micrograph of monocyte with-
in the alveolar space. X 21,900.

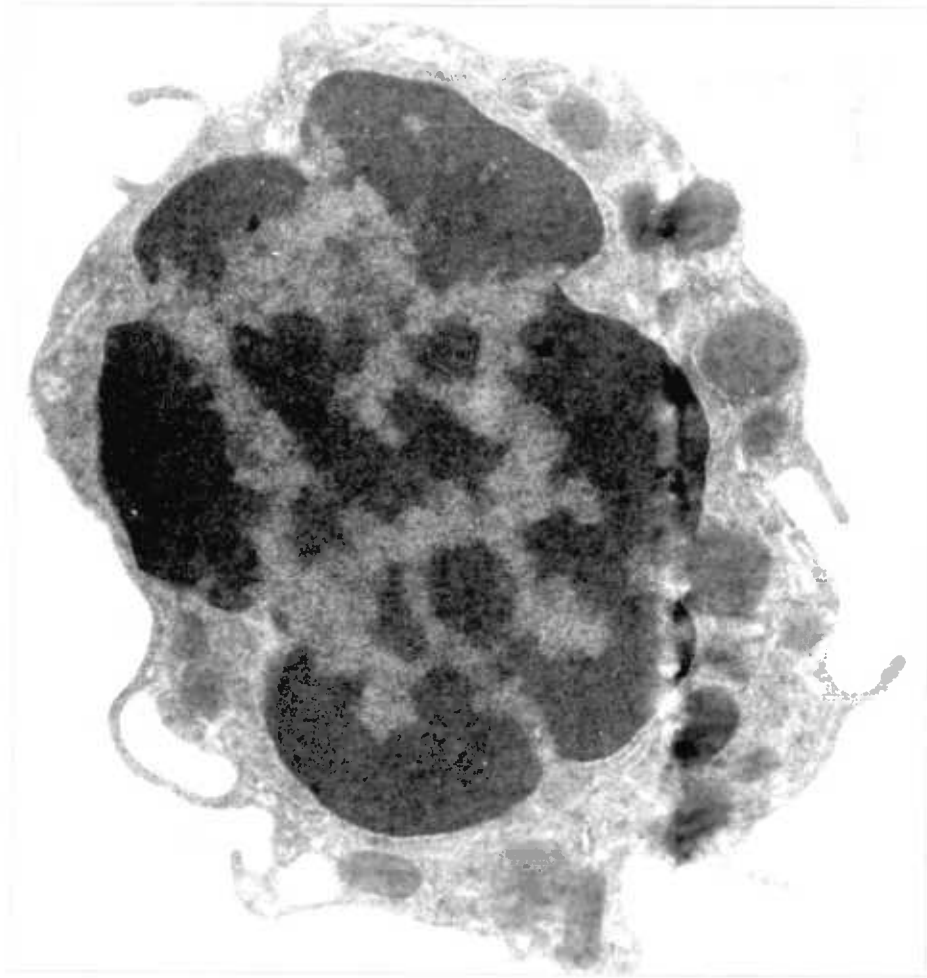


Fig. 23

Fig. 24. Electron micrograph of immature mononuclear phagocyte within alveolar space. X 16,000.

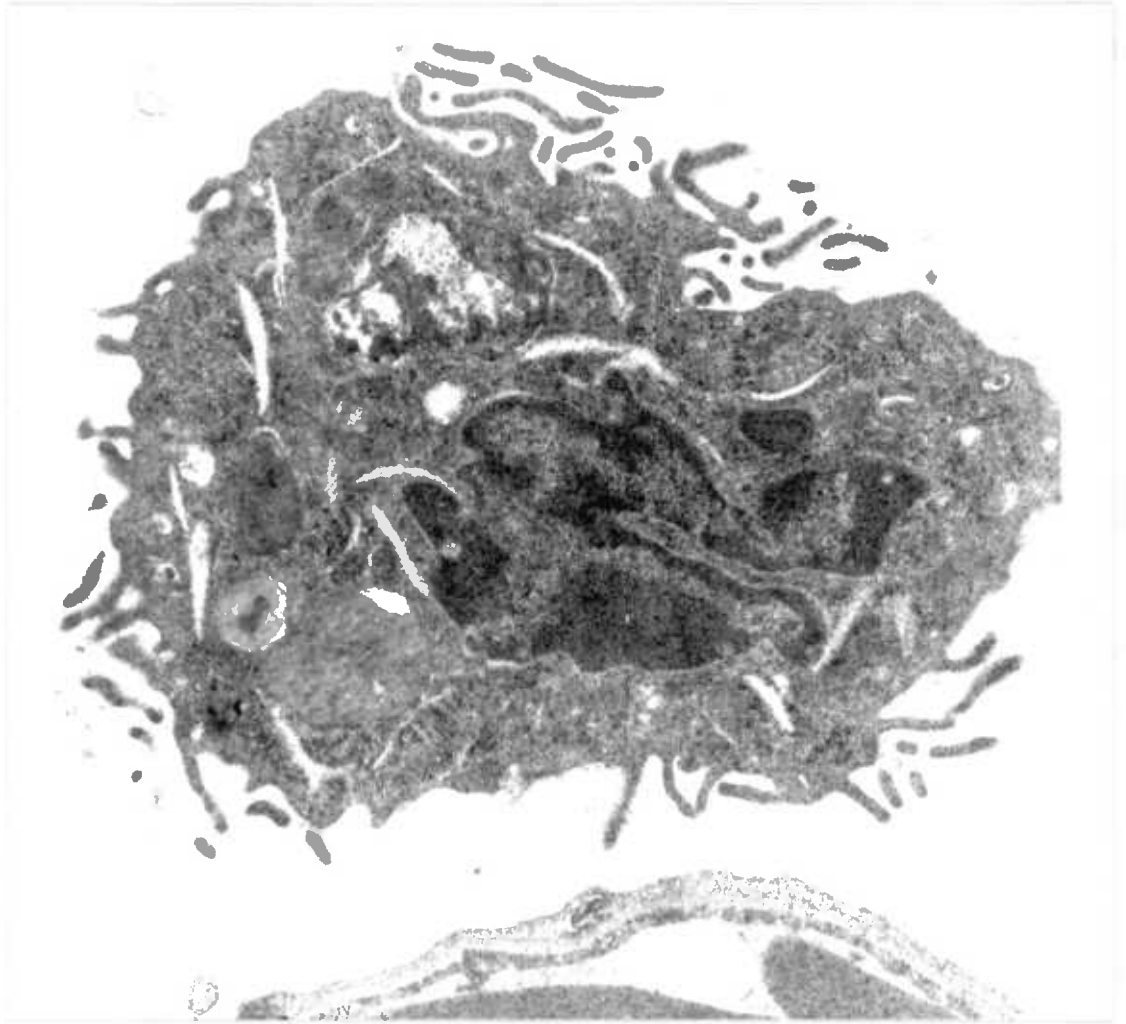


Fig. 24

Fig. 25. Electron micrograph of mature mononuclear phagocyte within alveolar space. Note asbestos fibre (arrow).
X 11,200.



Fig. 25

Fig. 26 Photomicrograph of live glass adherent mononuclear phagocytes. Majority of cells are round with numerous cytoplasmic inclusions. Note spreading and elongation of cells (arrows). Saline. X 400.

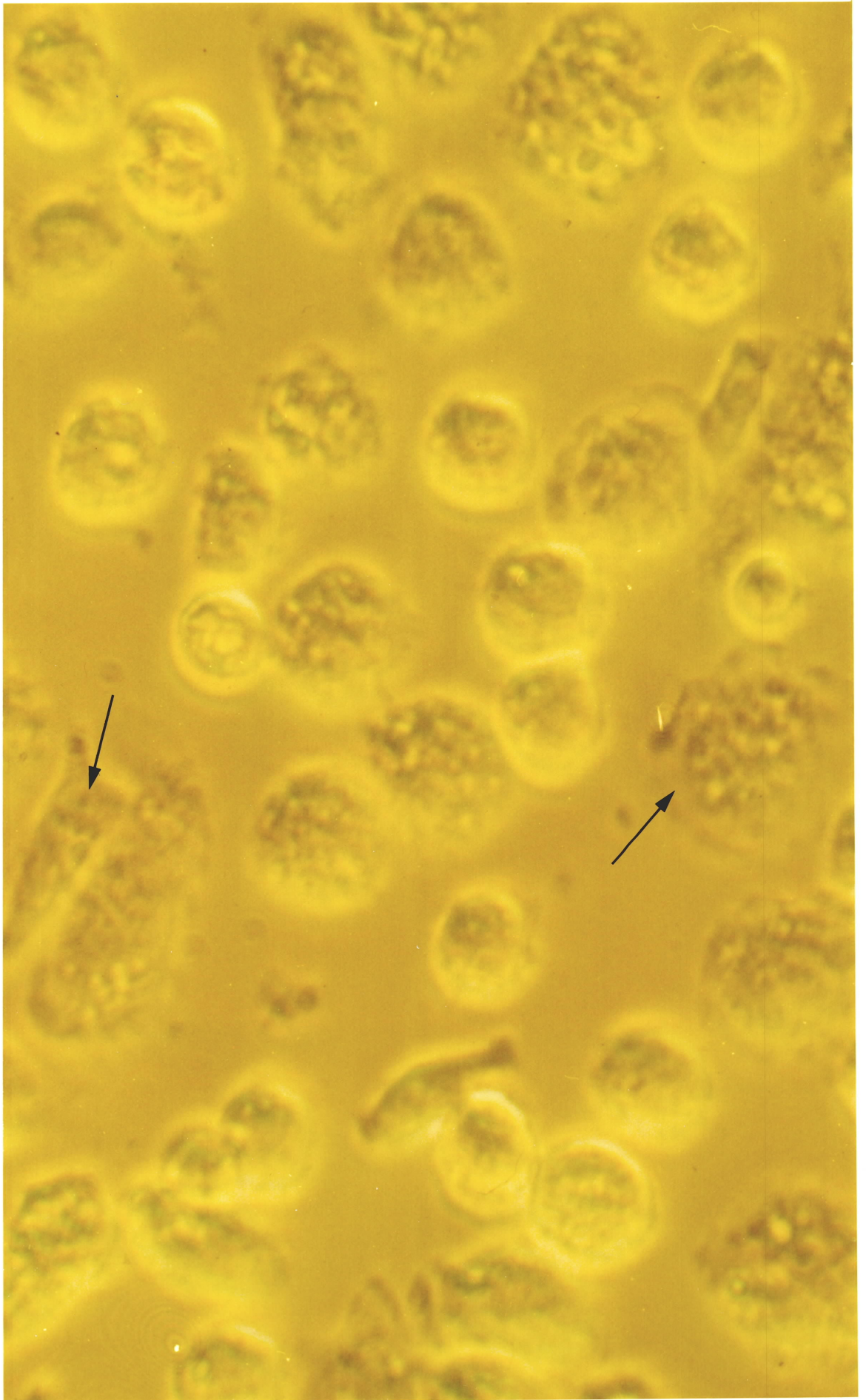
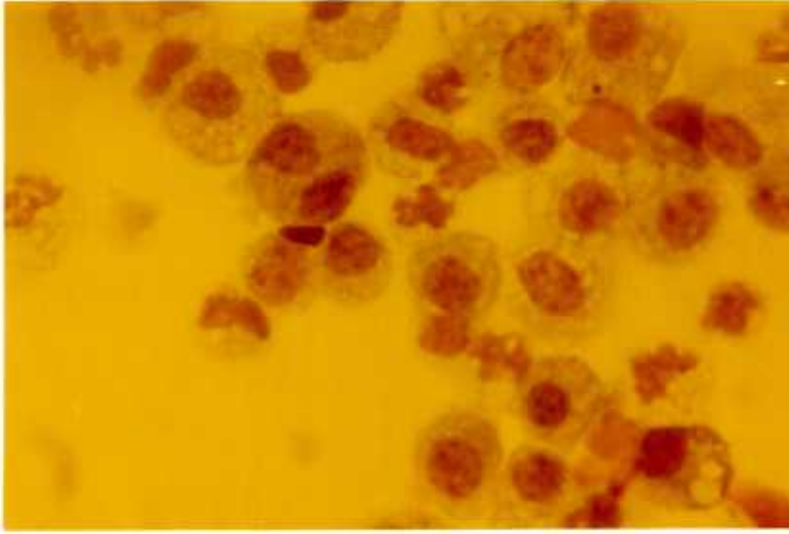


FIG. 26

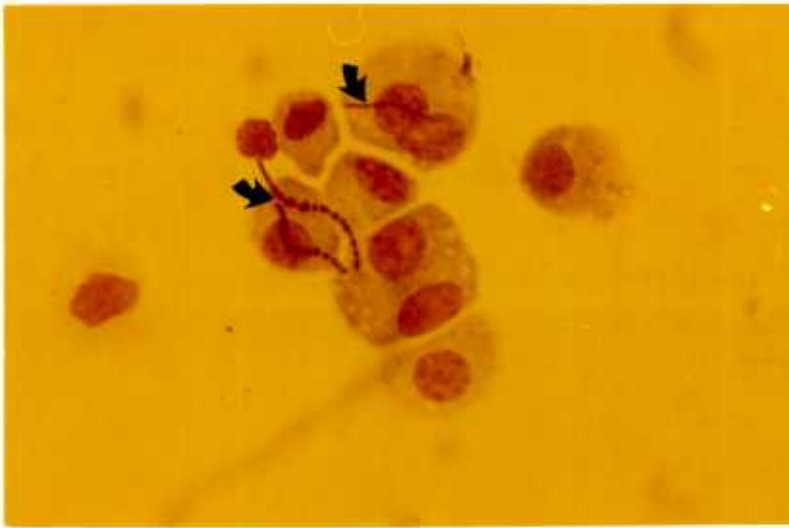
a. Cytocentrifuge preparation showing binucleate cell among mononuclear phagocytes with vacuolated cytoplasm. May-Grunwald-Giesma X 200.

b. Cytocentrifuge preparation showing binucleate cells and mononuclear phagocytes associated with asbestos bodies (arrows). Cytoplasm of these cells is less vacuolated compared to cells seen in Fig. 27a. May-Grunwald-Giesma X 200.

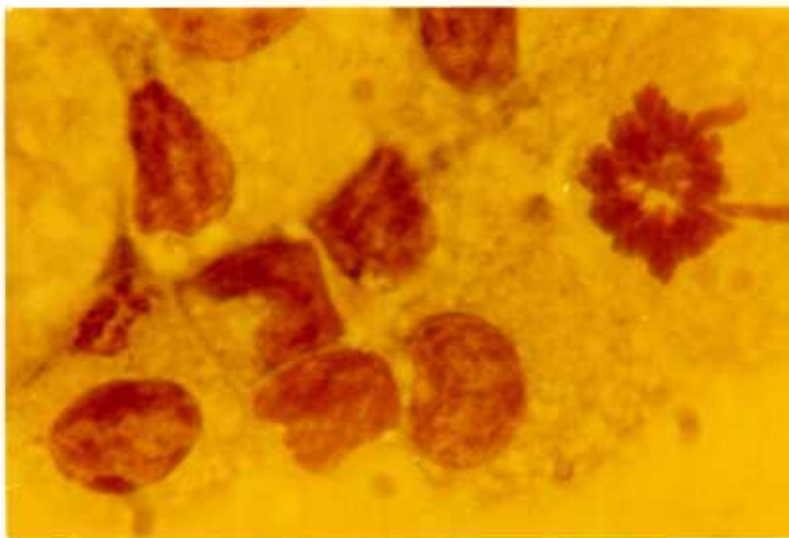
c. Cytocentrifuge preparation showing mononuclear cell in mitosis. May-Grunwald-Giesma X 1000.



A



B



C

FIG: 27

Section VI : Discussion of the Results of Treatment

Section VI : Discussion of the Results of Treatment

1. Histology

Group M : Mycobacterium tuberculosis

These results raise the question - why does live attenuated M. tuberculosis (strain H37Ra) injected subcutaneously at a remote site from the lung cause chronic inflammation in the lung?

First, following subcutaneous injection of mycobacteria the majority of organisms will be trapped by the regional lymph nodes, and an influx of mononuclear phagocytes will destroy most of them. However, it is possible that mononuclear phagocytes containing living bacilli may leave the lymph nodes and enter the circulation via the lymphatics; and they might then enter lung tissue. If such a mononuclear phagocyte died in the lung the mycobacteria would be released and could provoke an inflammatory response.

Second, the fate of relatively small numbers of H37Ra in the lung of guinea pigs must be considered. Alsaadi and Smith (1973) have shown in guinea pigs that infection by H37Ra occurs in three stages in the lung when as few as 60 mycobacteria are retained. A logarithmic growth phase

is evident during the first three weeks following pulmonary challenge. This is followed by two weeks in stationary phase and then a decline in the number of recoverable mycobacteria occurs until virtually no organisms are present at 12 weeks. Development of pathological lesions in the M. tuberculosis (Group M) treated guinea pigs in these experiments followed a similar time course to that of the three growth phases of H37Ra. Although the guinea pigs in Alsaadi and Smith's experiment inhaled H37Ra, a route of administration very different from that used in these experiments, it is possible that following the subcutaneous injection of H37Ra a small number of organisms entered the lung and multiplied in the manner described by them. A single organism may cause some inflammation, but there must be one million bacilli per milliliter of bacterial suspension before microscopic examination reveals more than one organism per ten oil immersion fields (Millard, 1966). Therefore, although in these experiments no mycobacteria could be detected in the lung using Ziehl-Neelsen stain, it does not discount the fact that there may have been sufficient numbers of mycobacteria within the lung to provoke the inflammatory response.

Third, mycobacteria have been shown to be a potent stimulus for generation of a number of soluble factors which alter the function of lymphocytes and mononuclear phagocytes. These factors, also called mediators of delayed hypersensitivity or lymphokines, are elaborated by T-lymphocytes in

draining lymph nodes. The factors provide a means by which the triggering of a very few lymphocytes specifically responsive to a given antigen can be amplified to involve a large number of mononuclear cells. Ward et al, (1971) demonstrated chemotactic factor for monocytes and lymphocytes to be released by sensitized guinea pig lymphocytes following culture of them in the presence of specific antigen. Nelson and Boyden (1963) observed that the intraperitoneal injection of mycobacterial antigen into sensitized guinea pigs, in which peritoneal exudates had been induced, caused macrophages to aggregate and adhere to the mesothelial lining of the peritoneal cavity. The resulting disappearance of mononuclear cells from the peritoneal exudate was analogous to that occurring from the circulation following parenteral administration of antigen to a sensitized animal. Migration inhibition factor (MIF) has been demonstrated to be released from sensitized lymphocytes following their interaction with antigen (David, 1966; Bloom and Bennett, 1966). This factor inhibits the migration of normal macrophages following their entry into an inflammatory lesion.

Once mononuclear phagocytes become stimulated they secrete factors that are capable of perpetuating inflammatory reactions both locally and systemically. These include a group of neutral proteinases, such as plasminogen activator,

collagenase and elastase, products influencing the proliferation of stem cells in the bone marrow and products that regulate the proliferation of lymphocytes in the immune response (Unanue, 1976).

It is likely that the development of inflammatory lesions in the lung, following the subcutaneous injection of live H37Ra at a remote site, involves both a systemically mediated response via soluble factors produced by stimulated lymphocytes and mononuclear phagocytes, and a local tissue response in the lung due to the presence of small numbers of mycobacteria.

Group F : Freund's Complete Adjuvant

When Freund's complete adjuvant (FCA) is given intravenously it elicits an inflammatory reaction in many organs and an extensive proliferation of cells of the reticulo-endothelial system (Laufer, Tal and Behar, 1959; Rupp, Moore and Schoenberg 1960; Steiner, Langer and Schatz, 1960). In these experiments, injection of FCA in the hind footpad produced an inflammatory response in the lung which resembled the lung response following an intravenous injection of FCA. The inflammatory response in the lung following FCA treatment was similar to but more extensive than that seen in the group given Mycobacterium tuberculosis, especially at two and four weeks. A histological feature that distinguished FCA from mycobacteria-treated animals was the

occurrence of occasional sharp-edged rounded holes in the middle of granulomas. These holes were lined by flattened mononuclear phagocytes and occasional giant cells. They represent areas of oil deposits which have been dissolved out in processing the tissue (Spencer, 1958) (Fig. 18).

The studies of FCA by Freund (1956) established that both oil and mycobacteria remain at the injection site for months and that oil droplets and organisms become widely disseminated to sites in the lung and other tissues. He also demonstrated that the cellular reaction to FCA is similar to that caused by living M. tuberculosis. In more recent studies it has also been shown that FCA is a potent stimulant for lymphocytes (B and T cells) and mononuclear phagocytes (Waksman, 1979).

Group FM: Freund's Complete Adjuvant and M. tuberculosis

As might be expected combined treatment with Freund's complete adjuvant and living mycobacteria produced a more extensive cellular reaction which persisted for longer than was seen in the groups of animals given either treatment alone. Again, the explanation for this result occurring in the lung is probably similar to that hypothesised for either treatment alone. However, in the case of combined treatment, FCA adjuvants the cellular response in the lung following the subcutaneous inoculation of mycobacteria.

Group A : Asbestos

Animals which inhale chrysotile asbestos for long periods of time develop lung pathology which has few features to distinguish it from interstitial fibrosis of other aetiology, including cryptogenic fibrosing alveolitis. The only pathological finding which distinguishes late asbestosis from other fibrotic lung diseases is the presence of asbestos fibres and bodies. However, this does not necessarily mean the pathology is asbestos-related, although the likelihood of it being so is increased (Selikoff and Lee, 1978).

Information concerning the early response of the lung to asbestos has come mainly from animal experiments (Wagner, 1963; Holt, 1964; Botham and Holt, 1972). Despite the fact that in man the initial histological reaction of the lung to the presence of asbestos fibres is seldom seen, several animal models have provided a picture which gives an accurate description of the pathological events leading to fibrosis following exposure to asbestos (Vorwald et al, 1951; Wagner, 1963; Holt et al, 1964, 1966; Gross and deTreville, 1967; Botham and Holt, 1972). Initially inflammatory lesions occur following deposition of asbestos fibres in peribronchial regions and in the interstitium and alveolar spaces arising directly from the respiratory bronchioles. The lesions are characterized by oedema, increased numbers of macrophages and lymphocytes and granuloma formation in some instances.

Eventually a network of reticulin is developed which is later replaced by dense collagen causing obliteration of the alveoli. This response is not unique to asbestos but has been described for a variety of lesions causing chronic interstitial pneumonia (Spencer, 1968).

In this study the initial cellular and connective tissue changes in the lung of guinea pigs exposed to chrysotile asbestos for only two eight hour periods were similar to the pathological changes reported by other investigators who exposed animals for periods of weeks to years (Table 25). However, there was resolution of the response without fibrosis.

Based on clinical (Selikoff and Lee, 1978) and experimental (Wagner, 1979) data, a dose-response relationship appears to exist between asbestos exposure and pulmonary fibrosis.

The physical form (i.e. size and shape) of the fibres must be taken into consideration when considering their fibrogenic potential. For example, deposition of asbestos fibres at the level of respiratory bronchioles and alveoli is dependent on the size and shape of the fibres. Also, as discussed earlier, interaction of mononuclear phagocytes with fibres appears to be necessary for fibrogenesis. Fibres which are not completely phagocytosed are able to

Table 25.

Examples of Long Time Periods Used
For Asbestos Fibre Inhalation

Botham & Holt. (1972)	400 hours
Gross & deTreville (1967)	0.25-8 months
Holt <u>et al</u> (1964)	100 hours
Holt <u>et al</u> (1966)	3-40 days
McDermott <u>et al</u> (1977)	80-130 days
Miller <u>et al</u> (1978)	5-7 months
Tetley <u>et al</u> (1976)	15 weeks
Wagner (1963)	1 month-2years

interact with macrophage plasma membranes over a long period and this may be relevant to their biological effect. Allison (1973) has shown that short fibres ($< 5\mu\text{m}$) are readily and completely phagocytosed where as long fibres ($> 30\mu\text{m}$) were never completely ingested. Fibres of intermediate size ($5 - 20\mu\text{m}$) were sometimes completely ingested but not at other times. It is widely believed by most investigators that short fibres ($< 5\mu\text{m}$) are not fibrogenic. However, interstitial pulmonary fibrosis in rats has been produced following the inhalation of asbestos in which 84% of the fibres were $5\mu\text{m}$ or less (Holt et al., 1964).

Asbestos Bodies

Asbestos bodies are asbestos fibres which have become coated with ferroprotein and have been described since the early part of the century. Their appearance has been reported with increasing frequency in both the clinical and experimental situation. Originally they were given the name "asbestosis bodies" but because of the incorrect implication that fibrosis was also present, their name was later changed to asbestos bodies (Selikoff and Lee, 1978).

The conclusions from a number of studies attempting to relate asbestos body content of the lung to fibre content and degree of fibrosis has proved unsuccessful. Less than one fibre in 1,000 that have been phagocytosed by the

macrophage is converted to an asbestos body (Selikoff and Lee, 1978).

In this study, all groups of animals exposed to asbestos showed development of asbestos bodies from 4 weeks as indicated by Perl's stain. Because of the difficulty of accurately counting the bodies, no attempt was made to relate their concentration in lung of exposed guinea pigs to the degree of fibrosis or inflammation. Also, as with the fibres themselves, asbestos bodies are not necessarily accompanied by any fibrotic process. Their presence simply indicates exposure (Elmes et al, 1965; Selikoff et al, 1963).

Group AFM: Asbestos + Freund's Complete Adjuvant +
M. tuberculosis

Injury produced following combined treatment with asbestos, FCA and H37Ra resembled the histological changes seen in all other groups but lesions were more extensive, appeared earlier and were slower to resolve. As discussed above, treatment with FCA is capable of eliciting an inflammatory response in the lung following injection in the hind footpad and, when given 21 days prior to a subcutaneous inoculation of live H37Ra, it potentiates the pulmonary inflammatory reaction to the subcutaneous inoculation of mycobacteria. The pulmonary reaction to these subcutaneously injected antigens resembles the delayed hypersensitivity

reaction seen in the lung following inhalation of purified protein derivative (PPD) in guinea pigs sensitized intramuscularly with FCA (Miyamoto et al, 1971). This together with the fact that skin test and lymphocyte blast transformation to PPD were positive in animals treated with FCA and/or H37Ra, suggests that the reaction seen in the lung following treatment with these agents is immunologically mediated.

In contrast, asbestos is an inorganic fibre that elicits an inflammatory reaction by physical interaction with mononuclear phagocyte cell membranes.

Consequently, the lesions seen in the lung following combined treatment with asbestos, FCA and H37Ra are probably additive.

Maturation of Pulmonary Mononuclear Phagocytes

The origin and maturation of pulmonary alveolar mononuclear phagocytes has been a controversial issue over the past several decades. Current evidence suggests two possible origins for these cells.

One view is that they are derived from blood monocytes and are end cells incapable of cell division under normal circumstances (Godleski and Brain, 1972). A second view

held by Bowden and Adamson (1972, 1976) is that a bone marrow-derived cell population in the interstitium of the lung produces pulmonary alveolar mononuclear phagocytes by local division and maturation. Vijeyaratnam and Corrin (1972) who treated rats with Iprindole, an anti-depressant drug which increases the number of alveolar mononuclear phagocytes demonstrated that interstitial cells represent the immediate precursor of alveolar macrophages. They found that increases of intra-alveolar macrophages is preceded by interstitial cell accumulation which was accompanied by proliferation of these cells. During the early stages of intra-alveolar cell accumulation there was a close ultrastructural resemblance between these cells and the interstitial cells. At later times the intra-alveolar cells were identified as mononuclear phagocytes by their ultrastructural characteristics, the fact that they had phagocytosed thorium particles and high content of oxidative (i.e. nicotinamide-adenine dinucleotide) and hydrolytic (i.e. acid phosphatase) enzymes which are characteristic of normal rat pulmonary mononuclear phagocytes. Although this experiment did not demonstrate the origin of proliferating interstitial cells, their findings are compatible with there being intrapulmonary proliferation of extra pulmonary tissue derived cells.

Recently Van Oud Alblas and Van Furth (1979), by labelling mouse monocytes in vivo with ^3H -thymidine,

demonstrated that influx of blood monocytes is the source of cell renewal for pulmonary mononuclear phagocytes in the normal steady state.

Many of the studies of the origin of the pulmonary alveolar macrophage have been done on animals under normal conditions or in animals where inflammation has been induced directly into the lung. Few studies have been performed in systemically infected animals. Consequently the macrophage population in the lung of systemically infected animals may be composed of cells from origins which depend upon the particular treatment administered. For example, in this study some mononuclear phagocytes responding to the subcutaneous injection of mycobacteria and or Freund's complete adjuvant may be able to reach the lung via the circulatory and lymphatic system. However, there is no direct evidence for this because no labelling experiments were performed.

It has been shown in a mouse model that monocytes are able to migrate from capillaries to the alveolar surface (Murphy, Brody, and Craighead, 1975; Brody and Craighead, 1974). The results reported in this thesis demonstrate that there is a heterogeneous population of pulmonary mononuclear phagocytes within the alveolar spaces. Ultrastructural morphology indicates that there are monocytic

cells, immature macrophages and mature macrophages present. These results indicate that macrophage precursors depend upon monocytes, if the histological and ultrastructural descriptions of the monocyte, immature mononuclear phagocyte and mature mononuclear phagocyte represent sequential alterations of the same cell.

Maintenance of Granulomas and Chronic Inflammation

A histological result of this study in all treated groups was the complete resolution of the inflammatory response including the regression of granulomas. Work from several laboratories has suggested a number of reasons why this may occur. It has been demonstrated that a required property for granuloma development is persistence of the insulting agent (Spector et al., 1968). Also, it has been shown that micro-organisms which are degraded by macrophages in tissue culture evoke only a transient acute inflammatory response in vivo; only those organisms resistant to degradation produce granulomas (Spector et al., 1970). Adams (1975) has shown in studies of mononuclear phagocyte differentiation in vivo following the subcutaneous injection of Mycobacterium tuberculosis, that granulomas only persist as long as the mycobacteria remain within the granuloma itself. In this study FCA and H37Ra, agents which are known to be potent in promoting granuloma development, were not placed directly into the lung and consequently severe or

continued chronic inflammatory response or granuloma formation would not be expected (Spector and Mariano, 1975).

Spector et al., (1968) has shown that persistence of an intracellular irritant is required for the cause or maintenance of chronic inflammation. When the intracellular irritant disappears the reaction resolves. The intracellular persistence of agents capable of causing chronic inflammation is due either to survival of living organisms (i.e. H37Ra), or failure of mononuclear phagocytes to degrade dead organisms or inert material (i.e. asbestos) (Adams, 1976).

Although asbestos persisted in the lung throughout the study, three factors may be responsible for it not eliciting a continuing inflammatory response leading to established fibrosis. First, the dose may not have been sufficient for induction of a rapid massive and selective release of lysosomal enzymes from mononuclear phagocytes (Davies et al., 1975). Lysosomal enzymes such as B-glucuronidase and galactosidase may be responsible for part of the tissue damage seen in chronic inflammation (Davies and Allison 1976). Second, the formation of asbestos bodies (as seen by light microscopy) may be a protective mechanism of the lung against asbestos fibres (Selikoff and Lee, 1978), reducing the already low number of fibres able to interact with mononuclear phagocytes in a detrimental way. Third, asbestos fibres, particularly chrysotile, fragment and components are

leached and appear in cell and tissue fluids (Selikoff and Lee, 1978) again reducing the number of fibres with pathogenetic potential.

In a similar manner, disappearance of FCA and H37Ra would be accompanied by resolution of the inflammatory lesions (Spector et al, 1968).

Sensitization of Mononuclear Cells

It is well established that nonspecific activation of mononuclear cells (lymphocytes and mononuclear phagocytes) can take place following sensitization of the host with Freund's complete adjuvant or living mycobacteria. The response produced by living mycobacteria can be adjuvanted by treatment with FCA. Activation of mononuclear phagocytes is mediated by specifically sensitized lymphocytes at the infective foci in the tissue of the host (Adno, 1973; Truitt and Mackaness, 1971). If antigen or the sensitized lymphocytes become widely disseminated, the mediators of delayed type hypersensitivity (DTH) appear in the blood (Salvin et al, 1973) and phagocytic elements become activated throughout the reticuloendothelial system (Blanden et al, 1969).

In this study, the results of peripheral blood lymphocyte blast transformation to PPD, an in vitro marker for

lymphocyte sensitization, confirmed that the circulating lymphocytes had become sensitized following the injection of FCA or H37Ra alone and that the response was potentiated by combined FCA - H37Ra treatment. However, as measured by blast transformation, systemic DTH response was depressed following the inhalation of asbestos. Kagen and co-workers (1977) have demonstrated a significant depression in mitogen-induced lymphocyte blast transformation in patients suffering from asbestosis. These results have also been confirmed by Haslam et al., (1978). At present it is not understood whether this depression is due to a quantitative alteration in lymphocyte subpopulations or due to a combination of both quantitative and qualitative changes.

Activation of Mononuclear Phagocytes

In its resting state, the mononuclear phagocyte is a mobile cell of relative simple structure, but is capable of responding to a variety of stimuli which provoke it into a state of intense physiological activity. This is accompanied by changes in function and morphology which have been collectively referred to as "activation". Mackaness introduced this term in the 1960's to describe the enhanced microbicidal activity of macrophages from animals who had acquired immunity to facultative, intracellular bacterial infection (e.g. *Listeria*, *Brucella*). Mackaness also demonstrated that such an activated macrophage displayed pronounced ruffling of the plasma membrane, increased capacity for adhering to and

spreading on glass, increased phagocytic ability and increased numbers of phagolysosomes and endocytic vesicles. More recently, biochemical criteria have been used as markers for mononuclear phagocyte activation (Karnovsky and Lazdins, 1978). In this thesis pulmonary mononuclear phagocyte activation was assessed by the morphological appearance of glass adherent cells, electron microscopic appearance of mononuclear phagocytes within alveolar spaces and by activity of the plasma membrane-bound enzyme leucine aminopeptidase.

Glass adherent mononuclear phagocytes from all treated groups appeared larger, tended to spread more and contained increased numbers of cytoplasmic organelles. These morphological changes were prominent at the same time that leucine aminopeptidase activity increased at the surface of the same cells. This increase in enzyme activity supports the hypothesis that glass adherent mononuclear phagocytes from treated groups were activated, especially between two and twelve weeks. Using the morphological criterion of glass adherence it was not possible to distinguish whether one of the experimental treatments was more effective than another in activating the cells, but using measurement of leucine aminopeptidase activity this appeared possible. Electron microscopic observations indicated that the cells of controls appear to be in a continuous state of activation. The alveolar surface of the lung is normally sterile (Laurenzi

et al, 1961), even though the lung is continuously exposed to atmospheric and blood-born irritants. Clearance of irritants that reach the alveoli depends almost entirely on resident pulmonary mononuclear phagocytes. The mononuclear phagocytes could therefore be in a state of activation due to their continued phagocytic activity needed to maintain sterility of the lung under normal conditions. All treatments caused an increase in the number of activated mononuclear phagocytes within the alveolar spaces. These were large cells with an eccentric nucleus and margined heterochromatin, a conspicuous nucleolus, increased cytoplasmic organelles and numerous large pseudopods. However, FCA, H37Ra and asbestos, either alone or in combination increased the number of less activated cells of the mononuclear phagocyte system. These cells included the previously described monocyte and immature macrophage.

The most sensitive marker for mononuclear phagocyte activation in this study was activity of the enzyme leucine aminopeptidase for the substrate leucine p-nitroanilide. This enzyme is specific for mononuclear phagocytes and is located on the plasma membrane (Wachsmuth, 1968).

The results show that a minor increase in enzyme activity occurred two weeks after all treatments, with greatest activity occurring between four and ten weeks. It was during this time that the greatest cellular accumulation was seen

in any group. As the inflammatory response waned the enzyme activity also decreased until at 52 weeks values for treated groups were no different than for untreated controls. Treatment groups asbestos-mycobacteria (AM), asbestos-Freund's complete adjuvant-mycobacteria (AFM) and mycobacteria (M) showed the greatest enzyme activity. This was followed by groups asbestos-Freund's complete adjuvant (AF) and Freund's complete adjuvant (F). Group asbestos (A) and Freund's complete adjuvant - mycobacteria (FM) had activities that were not significantly different from those of untreated controls (N).

It can be concluded from these results that pulmonary mononuclear phagocytes were activated. This process may have occurred either because of trafficking of systemically activated mononuclear cells into the lung or by activation of resident pulmonary mononuclear phagocytes. The data also suggests that inhalation of asbestos alone for a short period of time is incapable of causing mononuclear phagocyte activation as judged by enzyme activity.

Histological Estimation of Collagen

In this model, histological evidence of increased collagen was not demonstrable with Masson's trichrome or Weigert-van Gieson stains. However, large diffuse increases of reticulin fibres were present, especially in those groups

with sensitized mononuclear cells and exposed to asbestos.

Histochemical staining of tissue sections is a rapid and simple screening method for studying collagen. It gives only an approximate indication of quantity and cannot distinguish collagen types. Several connective tissue stains have been described and each has its own sensitivity and specificity for connective tissue components. The basis for the tissue affinity of each is an often ill-understood physico-chemical reaction. These methods are fully described in such texts as Pearse (1968). However, a few points are particularly pertinent to the study of fibrosis. The methods in common usage are Weigert-van Gieson's (and other modifications of the resorcinol-fuchsin technique which are useful in distinguishing collagen from elastic fibres and other tissue constituents), Masson's trichrome, Gomori's aldehyde-fuchsin trichrome, and silver impregnation. None of these detects all the collagen present in tissue which can be found on immunofluorescence and electron microscopy, therefore this can result in an under-estimation of collagen, particularly in the early stages in experimental models. The silver impregnation method is the most useful in the later circumstance as it appears to have a particular affinity for newly formed immature collagen. None of these methods stain collagen alone. Thus Masson's-trichrome is useful for detection of early fibrosis because of its sensitivity, but also stains other free proteins such as cell debris and in-

flammatory exudate. Finally, little is known of the changes in staining affinity of collagen which occur in disease.

Reticulin is a histopathological term applying to fibres of connective tissue which are argyrophilic (i.e. stain black with silver impregnation), usually appear delicate and branching, stain only faintly red with van Gieson's stain and are isotropic under polarised light (Pearse, 1968). It is sometimes implied that reticulin is noncollagenous but, while this might be true in respect of its histological and staining characteristics, biochemically and on electron microscopy it appears to be collagen (Huang, 1977). There are extractable components of reticulin which are non-collagenous (Pras and Glynn, 1973) but their exact nature has not been determined. Some fibres adopt the staining characteristics of collagen after a time, possibly because of changes in the chemical characteristics of the fibres. Until this occurs, however, an increase of argyrophilic fibres may be the only light microscopic feature of developing fibrosis.

Biochemical Estimation of Collagen

A unique property of vertebrate collagen is the presence of large amounts of the amino acid hydroxyproline, which until recently was thought to be specific for collagen. It has been established that elastin (Grant and Prockop, 1972), the C1q component of complement (Porter and Reid, 1978) and

the tail structure of acetylcholinesterase (Rosenberry and Richardson, 1977) also contain this amino acid. However, the amounts detectable in these proteins is small compared to that in collagen and does not negate the measurement of hydroxyproline as a simple method of determining lung collagen. A possible criticism of this method is that it does not allow analysis of changes in type or molecular structure of collagen.

Hydroxyproline determinations confirmed the histological observation that there was no apparent increase in lung collagen following any given treatment. Although there was a steady rise in hydroxyproline values with time, this general pattern of increased levels was also demonstrated in the untreated controls and is undoubtedly due to the normal growth of the animal, since a steady rise was demonstrated for body weights and lung weights throughout this study.

The most notable change in hydroxyproline levels was the decrease in amounts in group A (asbestos), M (mycobacteria) and AFM (asbestos-Freund's complete adjuvant-mycobacteria) at two weeks, which was followed by a steady rise back to control values over a period of several weeks. Although synthesis and degradation was not measured in this model, it seems likely that the degradative rate was increased following insult in groups A (asbestos), M (mycobacteria) and AFM (asbestos-Freund's complete adjuvant-mycobacteria).

It is also possible that the synthetic rate could have decreased and the degradation rate remained unchanged.

It may be that asbestos and mycobacteria are capable of activating the mononuclear phagocyte in such a way as to cause them to greatly increase their production and secretion of collagenase. Werb and Cohn (1976) have demonstrated that thioglycolate-stimulated mouse peritoneal macrophages release considerable amounts of collagenase in vitro. The return to untreated control values over a period of several weeks could be explained by the fact that there is traffic to the lung of mononuclear phagocytes that are no longer activated with regard to collagenase production. Consequently, if synthetic rates remained unchanged then the hydroxyproline values could eventually return to normal.

Using measurements of hydroxyproline or collagen concentrations in biopsies, it has been difficult to document that morphologic pulmonary fibrosis involves biochemical fibrosis. Crystal et al (1976) have recently discussed possible explanations for this. There may be no net increase in collagen, but only a change in the organization of the connective tissue or a shift in the ratio of types of collagen molecules. Evidence for a shift in the ratio of Type I to Type III collagen has been found (Seyer et al, 1976). It is also possible that a net increase in any given connective tissue protein may remain undetectable when expressed as a change in protein concentration because diseased

tissue samples may also have a proportional increase in cells and other tissue protein components.

In this study it was not possible to address this problem since at no time was there significant increase in collagen either histologically or biochemically.

Weight Relationships

As with hydroxyproline values, a basic pattern of increasing weight with time was demonstrated for body weight, left lower lobe wet weight, total lung wet weight and lung dry weights. Since untreated controls demonstrated a similar pattern, this increase may be partly due to normal growth. The mean weights in the treatment groups also established that significant increases in left lower lobe and total lung wet weight had taken place. These increases were highest in the AFM group followed by the groups receiving two treatments (i.e. AF, AM, and FM) which in turn were followed by two of the groups receiving only one treatment (i.e. A, F). The data for wet weights correlate with the histological severity of lesions. Also, the percent change in total lung wet weight and left lower lobe wet weight were closely correlated for each treatment group. It could therefore be concluded that it is possible to obtain information of the disease process by examining a defined anatomical

portion of the total lung (e.g. left lower lobe) since the data for this portion is closely correlated with the information obtained from the entire lung. Lung dry weights followed a similar pattern with time as previously described for wet weights, although the changes are not as pronounced and there is no difference between the treated group and untreated controls. Since dry weights are a crude measure of total lung connective tissue components only (in not taking into account cells and fluid) these results are in agreement with the evidence from the hydroxyproline measurements and histological assessment, that in this model there were no increases in collagen above normal values.

Decreases in dry weight of the asbestos (A) and mycobacteria (M) treated groups, at times when hydroxyproline values were decreased for these groups, supports the supposition that these insults have in fact caused a decrease in lung collagen during the first few weeks following insult.

Section VII : Summary and Conclusions

Section VII : Summary and Conclusions

The aim of this thesis was to develop an animal model which would overcome some of the criticisms of other investigations into mechanisms of pulmonary fibrogenesis which has been mentioned in the Introduction. The most serious criticisms of these are: assessment of fibrogenicity of agent rather than mechanisms of fibrogenesis, too few time points and insufficient parameters included in study, insulting agent administered in high concentration preventing assessment of host factors upon which fibrogenesis may partly depend. This aim was partially achieved.

Assessment of host factors was achieved by exposing the lungs of SPF guinea-pigs whose systemic mononuclear cells had been sensitized and whose mononuclear phagocytes had been activated, to asbestos, a known fibrogenic agent which appear to require interaction with cells in order to produce fibrosis, at a dose that by itself did not cause fibrosis.

Asbestos fibres and asbestos body formation were prominent throughout the entire study even though the animals were only exposed for two eight hour periods. Histologically the reaction to asbestos began as a minor peribronchiole mononuclear cell inflammatory reaction at a few scattered individual respiratory bronchioles, but gradually more and more bronchioles became affected with

the reaction spreading to the surrounding alveolar septa. The minor increase in reticulin fibres never developed into collagen fibres and the reaction was short-lived with complete resolution occurring even though asbestos bodies were continually present.

Sensitization of lymphocytes with FCA and/or H37Ra was achieved as demonstrated by lymphocyte blast transformation to PPD. The pulmonary reaction to these agents was a diffuse mononuclear cell infiltration of the interstitium and air space. The severity of the reactions to the various insults was dependent on the treatment given. Although there was no histological evidence of increased collagen, there were diffuse increases in reticulin, particularly in animals with sensitized mononuclear cells which were exposed to asbestos. However, even in the case where severe pulmonary inflammatory changes occurred, complete resolution of the response took place within one year.

Immunological activation of the mononuclear phagocytes was confirmed by criteria based on electron microscopy observation of the alveolar lumen cells, morphology of glass adherent mononuclear cells and measurement of enzyme activity of pulmonary mononuclear phagocytes. It was concluded from these criteria that 1. there was a heterogeneous population of mononuclear phagocytes in the alveolar lumens, a majority of which were mature and appeared

activated, however monocytes and immature mononuclear phagocytes were also present in much smaller numbers;

2. the morphological studies of guinea-pig pulmonary glass adherent mononuclear cells gave inconclusive results as to the state of activation possibly due to the inability of the cells to spread on a glass substrate compared to activated peritoneal macrophages; 3. enzyme activity of the pulmonary mononuclear phagocyte provided the most sensitive method for demonstrating activation of the cells.

The results show that systemic mononuclear cell activation does not enhance establishment of fibrosis in guinea pigs that have inhaled asbestos for a short time. Although in this model the results indicate that mononuclear cell activation may not be an important host factor in fibrogenesis, one must consider that the amount of asbestos capable of interacting with activated cells may be too small to cause fibrosis no matter how much or how the cells were activated. Dose/response studies in a similar model would be needed to answer this question.

Based on observations of human pulmonary fibrosis, Carrington (1968) proposed a set of anatomic criteria that should be met by the ideal experimental model. These included a mixed cellular exudate in the interstitium, protein exudate in air spaces with or without leukocytes, proliferation of epithelial cells, gradual progression to fibrosis and honeycombing, continuing cellular activity

even when partly fibrotic, and widespread focal lesions. Among the criteria that are considered favourable but not essential are the presence of hyaline membranes, smooth muscle cell proliferation, multiple species for the same agent and multiple agents for the same species. Features that the ideal model must not show are abundant polymorphonuclear leukocytes, at least after the first few days, prolonged state of extensive oedema, early respiratory failure and contamination by ordinary bacterial disease.

Although these criteria may meet the ideal animal model, it is not critical that the model be an exact replica of human pulmonary fibrosis. Rather the model should be used as a system to enable investigators to distinguish abnormal from the normal. In this manner a model is helpful in understanding the inability of the human lung to return the fibrotic to normal. Also, animal models have the advantage over human studies of 1. pairing control and fibrotic animals; 2. enabling dose response studies of the insulting agent, 3. studying drug therapy and 4. obtaining the entire lung for comparative morphological, physiological and biochemical studies.

Perhaps it is not surprising that the present model did not meet all the requirements discussed by Carrington.

In man and experimental animals, the progression of interstitial fibrosis from oedema, mononuclear cell infiltration through organization to honeycombing is not always seen after diffuse alveolar damage. Thus it is possible that pulmonary injuries sometimes organize and sometimes resolve. The question of what tips the balance in favour of organization or resolution is yet unanswered.

This study was partially based on the premise that an accumulation of mononuclear cells in the lung is an essential prelude to pulmonary fibrosis. Therefore, keeping the asbestos exposed lung packed with stimulated mononuclear cells, particularly mononuclear phagocytes, should eventually lead to fibrosis. Richerson et al (1978) have taken a similar approach by immunizing rabbits systemically with Freund's complete adjuvant via the foot-pads and followed this with repeated intravenous injections of killed BCG. Their study as with these experiments produced large accumulations of interstitial and intra-alveolar mononuclear phagocytes. However, resolution of lesions occurred unless BCG was given continuously and in either case, fibrosis was scanty. Butler (1975) also thought that the macrophage-laden lung would produce a more intense fibrosis. Following systemic sensitization of mononuclear cells, hamsters were given paraquat intratracheally. Results indicated that the macrophage-laden lungs were protected against paraquat injury, since the

degree of fibrosis was less in animals given BCG plus paraquat compared to paraquat treatment alone.

It appears that unless there is a continuous administration of irritant to the lung resolution of the lesions without fibrosis occurs. It should also be noted that although mononuclear phagocytes may appear to be a prelude to fibrosis their presence alone, even in activated states is insufficient as a stimulus for progressive fibrogenesis. Indeed, the influx of large numbers of mononuclear phagocytes to the lung following insult is usually considered to be a protective mechanism and the presence of concurrent or subsequent fibrosis may be either due to a functional defect of the cell or continued persistence of irritant at concentrations so large that the mononuclear phagocyte is overwhelmed (Fig. 28).

The common problem of complete resolution of the inflammatory and fibrotic response occurring in animal models appears to be overcome only by continual administration of insulting agents throughout a study. This is in direct opposition to what occurs in humans, particularly following exposure to fibrogenic inorganic dusts since once fibrosis has occurred, progression will continue after removal from exposure. It is not known whether this progression is due to continual activity either by the persisting fibrogenic agent or to some unknown self-perpetuating host reaction (i.e. virus). In the case of

inorganic dust, persistence of cytotoxic dust in the lung may in itself be enough to cause continuing fibrosis but in the case of soluble agents such as avian protein, the persistence of antigen is more difficult to accept.

In all cases where exposure to fibrogenic agents occurs (human and animal) there is the question of individual host susceptibility. It has been shown that even among heavily exposed asbestos workers only a proportion developed clinical asbestosis and of those affected the extent of lung involvement varied greatly (Selikoff and Lee, 1978).

Although the results of this thesis are far from satisfactory in answering the questions as to how far fibrosis is occurring as the result of persisting activity of initiating agent (due either to persistent exposure or to persisting activity of retained particles in the lung) and as to how far the perpetuating fibrosis depends on host factors and no longer on the initiating agent, they show enough promise to justify further investigations.

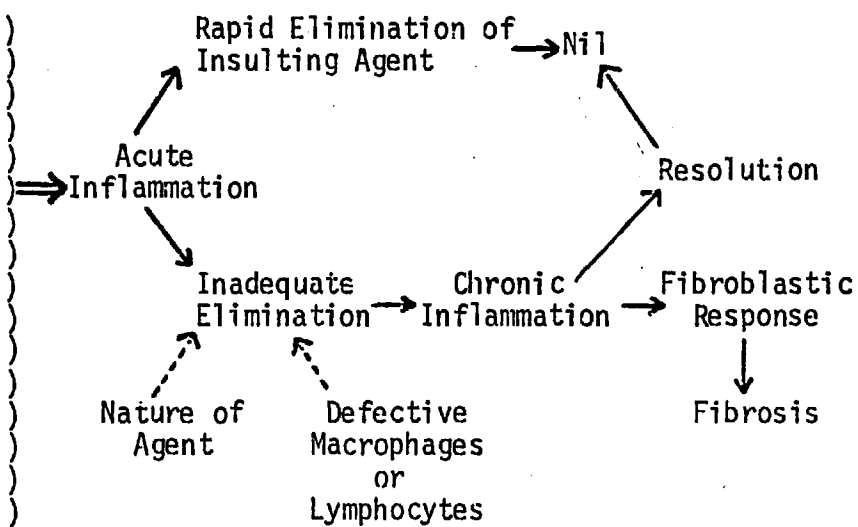
The pulmonary response to insult, preceeding fibrosis is similar to that seen in man and other experimental animal models of fibrogenesis. However, to obtain full benefit from this model dose/response relationships and other methods of macrophage activation should be studied.

Fig. 28

CAUSES OF PULMONARY FIBROSIS

INSULT

1. Immunological
2. Cytotoxic Dust
3. Noxious Inhalants
4. Granuloma Formation
5. Tissue Damaging Organisms



Appendices

Appendix 1.

Rhodesian Chrysotile A Asbestos UICC Standard Reference Sample

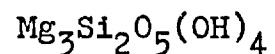
Physical and Chemical Properties

1. Overall % of Length Distribution (Size in microns)

<u>0.2-0.5</u>	<u>0.5-1.0</u>	<u>1.0-2.0</u>	<u>2.0-5.0</u>	<u>5.0-10.0</u>	<u>10.0-25.0</u>	<u>25.0-50.0</u>	<u>50.0-100.0</u>	<u>100.0-200.</u>
20.70	34.90	23.10	15.20	2.83	2.49	0.62	0.16	0.00

2. Chemical Formula and Composition (Excluding Trace Elements)

a. Molecular formula



b. Composition

SiO_2	41.8 - 42.0%	Fe_2O_3	0.2 - 1.3%	K_2O	0. - 0.1
MgO	41.8 - 42.8%	FeO	0.1 - 1.6%	H_2O	13.6 - 14.0
Al_2O_3	0.1 - 0.5%	CaO	0 - 0.1%		

Source: Pneumoconiosis Research Unit, Cardiff and Johannesburg

Appendix 2Turk's Diluting Fluid

Glacial acetic acid 3.0 ml

Gentian violet 1.0 ml

Distilled water 96.0 ml

Appendix 3Formula for Calculation of Cell Concentration per mlUsing a Neubauer Haemocytometer

$$\frac{\text{No. of Cells Counted}}{\text{No. of large corner squares used to count cells (e.g. 2 or 4)}} \times 10^4 \times \text{dilution factor} = \text{Cells/ml}$$

Appendix 4Leucine Aminopeptidase Assay

Reference: Wachsmuth, E., Expt. Cell Res. 96:409,1975

Substrate: leucine 4-nitroanilide

Reagents: 10 mM leucine 4-nitroanilide in DMSO MW 268
(26.8 mg/10 ml) Buffer: 0.1 M phosphate pH 7.5

Stopping Solution: 250 ml each of:

0.8 M glycine	)	
0.8 M NaOH	)	adjust to pH 10.4
0.8 M NaCl	)	make to 1 liter

Harvest cells in 0.1: Triton X-100 in saline

Assay: 0.5 ml cell suspension
0.5 ml buffer
0.1 ml substrate

Incubate at 37°C for 30 minutes. Stop with 1 ml of glycine buffer.

*Blank is assay solutions without substrate - add substrate after adding glycine.

Read OD at 405 nM. Absolute activity calculated by reference to extinction coefficient of p-nitroaniline (see reference).

Appendix 5Ficoll-Triosil Preparation

Ficoll 9% solution in sterile distilled water

Triosil 34% solution (9.6 ml sterile distilled
 water + 30 ml of 50% Triosil)

Solutions are kept separate at 4° until use. Just prior to use mix 10 parts of 34% Triosil with 24 parts 9% Ficoll. Layer 12 ml blood onto 9 ml Ficoll-Triosil mixture.

Appendix 6Formula for Calculation of Lymphocyte Blast TransformationStimulation Index

$$\text{Stimulation Index} = \frac{\text{CPM of PPD stimulated cells}}{\text{CPM of nonstimulated cells}}$$

Appendix 7STANDARD HYDROXYPROLINE PROTOCOL

1. Begin with 0.5 ml samples in 13 x 100 hydrolysis tubes. Keep frozen at -20°C until ready to begin assay.
2. Thaw samples; add 0.5 ml 12 N HCL to each sample and cap tightly (teflon-lined caps); vortex each sample.
3. Hydrolyze samples in autoclave (120°C) for 16 hours.
4. Add small amount (75-100 mg) carbon decolorizing neutral "Norit" (Fisher Scientific) to each sample; mix and transfer quantitatively through a no. 2 Whatman 4.25 cm filter paper under vacuum to large (25 x 200 mm) culture tubes (with teflon-lined caps). (Use 9" pasteur pipette).
5. Wash hydrolysis tubes with 1.0 ml deionized water and transfer to culture tube as in step no. 4.
6. Repeat step no. 5 two times.
7. Make up chloramine-T solution: 5.634 grams chloramine-T per 100 ml ethylene glycol; Need 2 ml per sample.
8. Adjust pH of all samples to 8.3 - 8.9 using phenolphthalein indicator.
 - a. Add 1-2 drops phenolphthalein solution.
 - b. Add 10-20 drops 10 N KOH until sample turns pink.
 - c. Add 6 N HCL drop-by-drop until sample becomes clear.
 - d. Alternate between 1 N KOH, 0.5 N HCL, 0.3 N KOH, 0.1 N HCL and 0.05 N KOH dropwise until sample is

- very pink in colour.
- e. Vortex tubes and recheck colour.
9. Equalize volumes in culture tubes "by eye" with deionized water.
10. Add 1.0 ml 10% alanine.
11. Add 2.0 ml borate buffer.
12. Vortex tubes.
13. TIMED STEP.
- a. Total reaction is 20 minutes.
 - b. Samples are to be treated in groups of 4 (four).
 - c. Over 60" interval to each of 4 samples add 2.0 ml chloramine-T solution. Vortex each tube.
 - d. At end of 60" repeat process with next set of 4 samples, etc.
 - e. At end of 20 minutes (from time chloramine-T was added to first sample) add 6.0 ml 3.6 M sodium thiosulfate to each of 4 samples over 60" interval; vortex each tube.
 - f. At end of 60" repeat process with next set of 4 samples; etc.
14. Saturate samples with KCL (about 12 grams). (3 heaping spatulas).
15. Add 10 ml toluene to each sample.
16. Cap tightly (teflon-lined caps) and shake on shaker for 5 minutes.
17. Aspirate toluene down to interface, being careful not to aspirate aqueous phase.

18. Cap tightly and place in boiling water bath for 30 minutes.
19. Cool on ice to room temperature.
20. Add 12.0 ml toluene to each sample, resaturate with KCl, if necessary, cap tightly and shake for 5 minutes on shaker.
21. Remove 5.0 ml from each vial and transfer to large (16 x 150) test tube. Add 2.0 ml Erlich's reagent to each test tube and vortex. Let sit for 30 minutes then read absorbance at 560 nm using sample marked "blank" as zero reference.

Appendix 810% Neutral Buffered Formalin Fixative

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	14.8 g
Na_2HPO_4	24.0 g
Formalin	370 ml
Distilled water	2330 ml

Dissolve Na_2HPO_4 in H_2O , add $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and dissolve with aid of gentle heating. Add formalin after phosphate buffer solution has been cooled to room temperature.

Appendix 9Buffered Glutaraldehyde Fixative (pH 7.4)Materials

Sodium cacodylate	2.14g/100 ml distilled water
25% commercial glutaraldehyde	
Solution I Sodium cacodylate	50.0 ml
0.1N HCl	2.7 ml
Distilled water	47.3 ml

Fixative prepared just prior to use

25% commercial glutaraldehyde	5.0 ml
Solution I	20.0 ml

Buffered sucrose solution for storage of specimens

Sucrose	4.5 g	
Sorensen's phosphate buffer (pH 7.4)		100 ml

Appendix 10

Letter Code for Individual Data appendices 11-18

A = Body Weight
B = Total Lung Weight (wet) (gram)
C = Left Lower Lobe Wet Weight (gram)
D = Lung Dry Weight (milligram)
E = Total Hydroxyproline (ug)
F = Hydroxyproline (ug/g lung wet weight)
G = Hydroxyproline (ug/mg lung dry weight)
H = Deoxyribonucleic Acid (ug/mg lung dry weight)

Appendix 11.

Individual DATA - Group N

	A	B	C	D	E	F	G	H
2 weeks	315.6 346.8 385.3 347.2	1.73 1.80 2.10 1.88	0.39 0.39 0.76 0.41	68.52 74.52 79.20 74.08	2352.94 2420.09 2353.04 2590.12	1353.38 1353.38 1374.76 1380.51	7.87 7.24 7.98 7.70	3.24 3.07 3.57 3.30
4 weeks	387.0 400.0 439.0 408.6	1.95 2.29 2.33 2.19	0.45 0.45 0.53 0.48	72.00 93.24 87.96 84.40	3255.22 4290.54 4558.62 4048.13	1669.34 1873.60 1973.46 1835.87	10.43 9.04 11.89 10.45	2.98 2.94 2.59 2.84
6 weeks	444.1 460.0 488.0 464.0	2.42 2.48 2.20 2.37	0.55 0.52 0.60 0.56	100.10 115.20 97.33 104.21	3575.00 4011.07 3158.40 3681.76	1606.24 1617.69 1572.00 1596.97	8.80 7.30 9.69 8.60	2.22 2.16 1.90 2.09
8 weeks	531.2 470.8 468.6 490.2	2.73 2.12 2.47 2.44	0.69 0.53 0.59 0.60	144.60 128.40 139.20 137.40	3238.40 3553.90 4314.40 3793.20	1181.90 1677.74 1746.72 1535.45	5.64 6.93 7.40 6.66	1.39 1.24 1.12 1.25
10 weeks	510.2 499.0 545.2 518.1	2.80 2.55 2.60 2.65	0.62 0.60 0.60 0.61	122.44 129.96 147.49 133.30	4336.18 4547.42 4450.42 4445.34	1549.35 1783.30 1741.70 1681.45	7.85 8.23 8.96 7.68	1.91 1.43 1.37 2.57
12 weeks	526.9 565.2 566.2 552.8	2.61 2.55 2.61 2.59	0.59 0.60 0.59 0.59	115.80 136.20 133.80 128.60	4222.36 5037.28 4978.98 4752.87	1612.76 1995.40 1955.32 1836.16	8.24 8.70 8.45 8.46	2.92 1.95 1.70 2.19
26 weeks	771.6 680.0 822.9 758.2	3.02 2.97 3.66 3.21	0.70 0.69 0.83 0.74	112.80 121.20 120.80 118.27	4578.82 4772.04 4650.28 4720.58	1638.68 1623.14 1434.80 1565.44	10.17 9.24 9.54 9.65	1.86 1.44 1.76 1.69
38 weeks	759.1 970.8 934.0 888.1	3.50 3.83 3.63 3.65	0.80 0.90 0.87 0.86	132.00 162.00 155.40 142.80	5576.22 6337.88 6467.42 6102.17	1576.06 1654.80 1781.66 1620.84	9.55 9.19 9.98 9.57	1.02 1.17 1.27 1.15
52 weeks	1110.0 1057.2 962.8 1043.3	3.59 3.45 4.03 3.44	0.97 0.83 0.95 0.92	149.40 145.80 154.40 150.40	5898.88 7101.34 8408.52 7136.24	1643.14 2058.36 2086.48 1927.33	10.67 13.16 12.80 12.21	2.48 1.27 2.64 2.13

Appendix 12.

Individual Data - Group A

	A	B	C	D	E	F	G	H
2 weeks	374.1 447.2 423.0 414.8	2.31 2.48 2.50 2.43	0.58 0.63 0.68 0.63	60.15 58.72 63.30 60.72	924.57 859.74 874.20 887.84	402.41 346.67 349.68 366.25	3.88 3.72 3.76 3.79	2.78 3.07 2.18 2.78
4 weeks	386.5 426.9 422.4 413.6	2.24 2.53 2.59 2.45	0.55 0.59 0.62 0.59	57.96 60.36 74.28 64.20	1118.70 1772.19 1803.65 1584.85	499.12 700.47 696.39 632.09	4.74 6.85 5.81 5.80	3.26 4.20 3.24 3.57
6 weeks	543.6 454.9 438.9 479.1	3.00 4.09 3.18 3.42	0.74 0.85 0.70 0.76	70.56 98.64 89.28 81.16	2891.67 4072.50 3329.75 3431.31	963.89 995.72 1047.09 1002.23	10.11 8.58 8.21 8.97	5.00 3.55 3.44 4.00
8 weeks	536.3 469.4 567.4 524.4	2.84 2.62 3.23 2.90	0.70 0.63 0.79 0.71	86.28 106.80 109.92 101.00	3485.79 4119.14 3608.10 3737.01	1228.80 1572.19 1117.06 1306.02	9.47 9.27 8.03 9.09	5.61 3.95 3.16 3.57
10 weeks	576.6 537.7 555.7 567.3	3.09 2.72 2.96 2.92	0.74 0.62 0.69 0.68	97.44 79.56 90.66 89.22	4059.22 3777.93 5407.27 4408.81	1307.19 1389.68 1826.18 1507.88	9.93 10.83 13.90 11.55	3.81 3.98 3.57 3.79
12 weeks	670.3 661.8 596.8 641.0	3.10 3.43 2.77 3.10	0.74 0.78 0.61 0.71	166.80 104.64 101.64 124.36	4268.55 4719.17 3212.81 4076.98	1377.08 1375.85 1170.67 1307.87	6.11 10.25 7.03 7.08	1.63 2.93 2.39 2.32
26 weeks	740.9 588.1 702.5 677.2	3.14 2.95 3.18 3.09	0.77 0.77 0.76 0.77	120.60 129.90 120.00 123.50	6104.16 4110.09 5256.03 5756.76	1944.00 1393.25 1652.84 1663.36	12.41 8.26 10.47 10.38	2.83 1.63 1.67 2.05
38 weeks	932.0 965.8 846.6 914.8	3.80 6.05 2.62 4.16	0.95 1.41 0.63 1.00	157.80 184.20 144.60 160.20	4663.66 7962.00 6263.04 6294.57	1227.28 1314.38 2392.38 1644.68	7.68 10.06 10.42 9.39	1.36 1.78 1.35 1.50
52 weeks	1125.0 1050.2 1100.0 1091.7	3.70 3.92 3.45 3.69	0.98 1.00 0.95 0.98	172.80 183.00 142.80 166.20	6858.91 7470.42 5487.88 6605.74	1853.76 1905.72 1590.69 1783.39	10.51 10.41 10.58 10.50	1.20 1.83 1.78 1.60

Appendix 13.

	Individual Data - Group F							
	A	B	C	D	E	F	G	H
2 Weeks	321.9	2.10	0.52	97.68	2878.10	1320.52	7.29	3.20
	311.9	2.14	0.61	101.28	2567.58	1144.80	7.24	2.43
	<u>312.1</u>	<u>2.04</u>	<u>0.48</u>	<u>98.04</u>	<u>310.52</u>	<u>1524.76</u>	<u>7.47</u>	<u>2.17</u>
	316.9	2.09	0.54	99.00	2852.07	1365.02	7.33	2.60
4 Weeks	369.1	2.59	0.58	94.44	3280.51	1266.62	7.78	1.92
	354.1	1.99	0.45	94.68	4576.48	2291.74	10.93	2.10
	<u>403.4</u>	<u>2.23</u>	<u>0.57</u>	<u>87.00</u>	<u>3589.58</u>	<u>1609.68</u>	<u>10.55</u>	<u>2.31</u>
	378.5	2.27	0.53	92.04	3815.53	1725.35	9.75	2.11
6 weeks	417.0	2.55	0.61	84.60	3411.22	1349.50	9.73	2.55
	373.2	2.25	0.53	93.00	3163.14	1408.06	8.02	2.32
	<u>315.0</u>	<u>2.40</u>	<u>0.50</u>	<u>94.20</u>	<u>3305.08</u>	<u>1377.12</u>	<u>7.31</u>	<u>2.08</u>
	369.7	2.40	0.55	90.60	3304.81	1378.13	8.35	2.32
8 Weeks	539.6	3.25	0.74	160.20	4077.06	1254.48	5.79	1.04
	504.3	2.63	0.63	184.80	3530.72	1342.48	4.58	0.97
	<u>528.8</u>	<u>3.87</u>	<u>0.92</u>	<u>172.80</u>	<u>2973.84</u>	<u>773.60</u>	<u>4.12</u>	<u>0.71</u>
	524.2	3.25	0.76	172.61	3533.87	1123.52	4.83	0.91
10 Weeks	598.3	2.68	0.66	88.80	4809.36	1794.54	13.33	2.41
	572.6	2.89	0.71	124.80	3761.98	1302.76	7.41	1.61
	<u>569.3</u>	<u>2.74</u>	<u>0.64</u>	<u>124.80</u>	<u>4053.84</u>	<u>1513.12</u>	<u>7.59</u>	<u>1.43</u>
	579.1	2.77	0.67	112.80	4210.09	1536.81	9.44	1.82
12 Weeks	510.8	3.23	0.80	105.60	4305.78	1333.06	10.10	1.85
	472.2	2.56	0.62	91.80	3763.72	1470.20	9.14	1.92
	<u>530.0</u>	<u>2.78</u>	<u>0.62</u>	<u>91.20</u>	<u>4736.56</u>	<u>1703.80</u>	<u>11.58</u>	<u>1.80</u>
	504.3	2.86	0.68	96.20	4268.69	1502.35	10.27	1.86
26 Weeks	794.0	3.23	0.83	130.20	5332.08	1650.80	10.52	1.57
	765.1	3.03	0.73	106.80	5800.14	1944.24	13.08	1.59
	<u>805.0</u>	<u>3.29</u>	<u>0.84</u>	<u>119.40</u>	<u>4876.24</u>	<u>1482.14</u>	<u>10.42</u>	<u>1.70</u>
	788.0	3.18	0.80	118.80	5336.15	1682.33	11.34	1.45
38 Weeks	882.5	3.18	0.73	156.00	5597.50	1760.22	8.32	1.43
	926.3	3.65	0.91	168.60	5711.76	1565.14	8.45	1.01
	<u>758.0</u>	<u>3.02</u>	<u>0.73</u>	<u>140.40</u>	<u>4605.44</u>	<u>1524.48</u>	<u>7.93</u>	<u>0.86</u>
	855.6	3.28	0.79	153.01	5305.23	1616.78	8.23	1.20
52 Weeks	1000.0	3.85	0.97	172.80	6012.48	1862.98	8.22	1.95
	1100.0	4.53	1.11	187.44	7237.68	1890.70	9.42	2.11
	<u>1050.0</u>	<u>3.38</u>	<u>0.80</u>	<u>153.40</u>	<u>5781.14</u>	<u>1709.86</u>	<u>9.11</u>	<u>2.00</u>
	1050.0	3.93	0.96	171.88	6412.10	1641.08	9.10	2.02

Appendix 14.

Individual DATA - Group M

	A	B	C	D	E	F	G	H
2 Weeks	351.5 401.8 <u>346.5</u> 366.6	2.37 2.48 <u>2.40</u> 2.32	0.59 0.56 <u>0.52</u> 0.56	60.32 56.54 <u>62.48</u> 59.78	756.79 862.36 <u>812.55</u> 811.57	319.32 346.42 <u>384.31</u> 351.85	3.12 3.44 <u>3.24</u> 3.22	5.39 4.90 <u>3.24</u> 4.51
4 Weeks	499.2 471.5 <u>400.6</u> 457.1	2.69 2.62 <u>2.32</u> 2.54	0.63 0.59 <u>0.54</u> 0.59	69.96 69.00 <u>61.44</u> 66.80	950.46 1107.87 <u>1158.45</u> 1072.26	353.33 422.85 <u>499.33</u> 425.17	3.18 3.62 <u>4.39</u> 3.73	3.31 3.08 <u>2.73</u> 3.04
6 Weeks	558.6 498.7 <u>496.3</u> 517.9	3.27 2.51 <u>2.82</u> 2.87	0.67 0.64 <u>0.68</u> 0.66	69.00 77.64 <u>91.08</u> 79.24	2133.61 1733.78 <u>3121.23</u> 2527.54	652.48 690.75 <u>1106.82</u> 816.68	6.34 5.69 <u>8.26</u> 6.76	3.72 3.10 <u>2.78</u> 3.20
8 Weeks	582.9 545.8 <u>672.0</u> 600.2	2.88 2.66 <u>2.84</u> 2.79	0.64 0.62 <u>0.56</u> 0.61	95.40 100.56 <u>107.04</u> 101.00	1944.00 2379.58 <u>2756.22</u> 2359.93	675.00 894.58 <u>970.50</u> 846.69	4.53 5.52 <u>5.07</u> 5.04	2.97 3.10 <u>2.92</u> 3.00
10 Weeks	609.6 584.2 <u>624.9</u> 606.2	3.32 3.31 <u>2.89</u> 3.17	0.72 0.74 <u>0.75</u> 0.74	106.56 98.40 <u>121.44</u> 108.80	3721.16 3718.31 <u>4021.03</u> 3886.83	1120.83 1183.76 <u>1391.36</u> 1231.77	7.57 8.90 <u>8.59</u> 8.35	2.96 3.30 <u>2.96</u> 3.07
12 Weeks	681.3 774.1 <u>729.0</u> 728.1	3.23 3.31 <u>3.34</u> 3.29	0.74 0.78 <u>0.72</u> 0.76	110.76 120.84 <u>114.96</u> 115.52	2552.93 2761.69 <u>3042.44</u> 2852.35	790.58 894.77 <u>910.41</u> 865.35	5.28 5.78 <u>6.10</u> 5.72	2.54 2.51 <u>2.26</u> 2.44
26 Weeks	628.2 681.2 <u>621.6</u> 643.7	3.77 3.33 <u>2.92</u> 3.36	0.73 0.76 <u>0.71</u> 0.73	105.60 148.20 <u>129.60</u> 127.80	6605.04 5815.25 <u>5234.06</u> 5884.78	1752.00 1746.32 <u>1762.31</u> 1723.54	12.11 8.96 <u>9.66</u> 10.24	2.84 2.33 <u>2.56</u> 2.58
38 Weeks	740.3 726.8 <u>586.6</u> 684.6	5.20 4.55 <u>3.52</u> 4.44	1.32 1.10 <u>0.83</u> 1.08	180.60 179.40 <u>143.40</u> 167.80	6051.40 6353.44 <u>6131.30</u> 6178.88	1163.73 1376.47 <u>1717.45</u> 1425.88	8.51 8.56 <u>9.94</u> 4.00	2.37 2.32 <u>2.05</u> 2.25
52 Weeks	959.6 972.8 <u>1125.0</u> 1019.1	5.32 4.46 <u>5.46</u> 5.08	1.41 1.12 <u>1.38</u> 1.30	948.00 408.00 <u>648.00</u> 668.00	6925.75 8881.47 <u>9579.68</u> 8462.37	1301.87 1991.36 <u>1754.52</u> 1682.88	3.64 5.47 <u>3.74</u> 4.28	0.68 1.66 <u>0.88</u> 1.07

Appendix 15.

Individual Data - Group FM

	A	B	C	D	E	F	G	H
2 Weeks	410.3 376.6 341.6 <u>376.1</u>	2.16 2.25 1.89 <u>2.10</u>	0.50 0.53 0.43 <u>0.49</u>	72.00 79.80 72.00 <u>74.60</u>	272.10 2802.92 3004.30 <u>2866.44</u>	1241.64 1245.74 1589.58 <u>1375.99</u>	8.98 8.27 9.49 <u>8.91</u>	2.56 2.36 2.67 <u>2.53</u>
4 Weeks	476.3 495.3 347.3 <u>438.6</u>	2.62 2.75 2.03 <u>2.47</u>	0.60 0.67 0.51 <u>0.59</u>	116.00 120.60 121.20 <u>117.60</u>	5418.68 4989.48 4817.56 <u>4904.59</u>	2068.20 1632.54 2373.18 <u>2024.04</u>	11.18 9.07 9.49 <u>10.08</u>	1.41 1.63 1.74 <u>1.59</u>
6 Weeks	517.3 456.8 529.3 <u>501.1</u>	2.55 2.69 2.75 <u>2.66</u>	0.60 0.63 0.66 <u>0.63</u>	82.80 138.00 120.60 <u>113.80</u>	5622.76 5938.50 5599.48 <u>5722.41</u>	2205.20 2219.62 2036.36 <u>2153.66</u>	15.98 10.08 11.14 <u>12.40</u>	2.61 1.75 1.97 <u>2.12</u>
8 Weeks	487.2 628.7 573.1 <u>563.0</u>	2.45 3.32 3.05 <u>2.94</u>	0.58 0.80 0.74 <u>0.71</u>	104.40 126.00 138.00 <u>122.80</u>	5180.14 5353.50 4508.20 <u>5017.28</u>	2118.42 1612.50 1478.10 <u>1736.34</u>	11.77 10.44 7.93 <u>9.98</u>	2.28 1.95 2.16 <u>2.13</u>
10 Weeks	630.0 660.3 738.5 <u>676.3</u>	3.17 3.02 3.14 <u>3.11</u>	0.75 0.73 0.76 <u>0.75</u>	118.80 120.00 133.20 <u>124.00</u>	3948.56 4175.04 4251.64 <u>4135.08</u>	1245.60 1322.46 1363.58 <u>1330.55</u>	7.86 8.41 7.78 <u>8.02</u>	2.60 2.44 2.07 <u>2.37</u>
12 Weeks	642.9 668.5 758.7 <u>690.0</u>	3.38 2.90 3.34 <u>3.20</u>	0.77 0.69 0.82 <u>0.76</u>	123.00 133.20 118.20 <u>124.80</u>	4425.04 4371.36 3831.09 <u>4215.88</u>	1309.14 1514.26 1141.02 <u>1323.51</u>	8.20 8.49 7.47 <u>8.22</u>	2.54 2.13 3.62 <u>2.76</u>
26 Weeks	658.7 584.4 678.6 <u>640.9</u>	3.03 3.39 3.82 <u>3.43</u>	0.75 0.73 1.06 <u>0.85</u>	114.60 122.40 186.60 <u>141.20</u>	3865.32 6539.44 7762.92 <u>6058.89</u>	1275.68 1429.04 2005.42 <u>1736.58</u>	8.34 11.51 11.39 <u>10.41</u>	1.63 1.57 1.77 <u>1.66</u>
38 Weeks	858.8 753.3 750.7 <u>787.6</u>	5.77 5.57 4.42 <u>5.25</u>	1.38 1.36 1.09 <u>1.28</u>	228.00 239.40 215.40 <u>227.60</u>	7837.62 7715.20 5963.58 <u>7172.07</u>	1358.34 1325.12 1344.18 <u>1364.21</u>	8.22 7.77 6.83 <u>7.64</u>	1.84 2.02 1.58 <u>1.85</u>
52 Weeks	853.3 891.2 987.3 <u>910.6</u>	4.98 4.24 4.21 <u>4.48</u>	1.34 1.04 1.09 <u>1.16</u>	209.40 181.20 190.80 <u>193.80</u>	7153.35 8755.76 8343.72 <u>8084.99</u>	1436.42 2065.04 1981.88 <u>1827.78</u>	9.19 11.85 11.32 <u>10.78</u>	3.33 2.65 2.45 <u>2.81</u>

Appendix 16.

Individual DATA - Group AF

	A	B	C	D	E	F	G	H
2 Weeks	364.9 367.3 407.1 379.8	2.27 2.62 2.70 2.53	0.58 0.62 0.63 0.61	63.24 71.28 85.92 73.48	2520.16 3479.78 4035.04 3344.54	1110.20 1323.14 1454.46 1310.92	10.18 11.55 10.96 10.90	4.15 3.57 3.00 3.57
4 Weeks	507.5 459.3 380.4 449.1	3.55 3.39 3.04 3.33	0.86 0.88 0.64 0.79	111.48 132.84 87.24 110.52	3212.32 2784.28 2865.88 2453.49	904.88 821.32 912.56 888.59	6.98 5.44 6.91 6.44	4.59 3.28 4.59 4.15
6 Weeks	625.0 600.0 512.2 579.1	3.21 3.09 2.64 2.98	0.84 0.73 0.59 0.72	87.24 84.24 93.48 88.32	3453.44 4045.30 4124.60 3875.78	1075.84 1309.16 1563.86 1316.29	10.36 11.35 9.82 10.53	3.04 2.66 2.70 2.80
8 Weeks	611.1 606.8 666.5 628.2	2.86 2.79 3.47 3.04	0.66 0.63 0.88 0.72	83.04 80.64 112.20 91.96	2891.18 3435.52 4444.36 3589.02	1040.90 1235.80 1279.64 1175.45	8.04 9.62 10.04 9.23	4.64 4.56 4.46 4.55
10 Weeks	533.5 643.4 606.0 594.3	2.80 3.10 3.16 3.02	0.66 0.72 0.73 0.70	80.52 108.00 102.96 97.16	3874.20 4399.40 4402.90 4225.50	1383.44 1419.16 1393.32 1399.71	11.34 9.46 9.88 10.23	2.83 2.65 2.70 2.73
12 Weeks	665.6 572.8 719.1 652.5	2.71 2.72 3.33 2.92	0.66 0.63 0.82 0.70	88.80 89.88 94.92 91.20	1974.56 2157.34 2352.18 2169.69	736.00 793.14 707.86 745.62	5.47 5.56 5.87 5.63	4.45 5.40 4.57 4.91
26 Weeks	840.0 832.0 702.1 743.3	3.61 3.41 3.41 3.48	0.86 0.94 0.82 0.87	98.40 136.20 123.00 119.20	4335.04 3912.64 4533.12 4260.27	1200.84 1147.40 1329.36 1225.82	10.49 7.92 9.86 9.09	2.60 1.54 2.02 2.05
38 Weeks	826.9 870.9 1021.0 906.3	3.52 3.30 3.66 3.49	0.89 0.80 0.83 0.84	132.00 140.40 146.40 139.60	5632.14 6028.44 5966.44 5822.41	1600.04 1926.56 1586.46 1671.12	10.79 10.41 8.99 10.06	1.48 1.57 2.05 1.70
52 Weeks	964.7 949.6 903.5 939.3	3.60 3.50 3.62 3.55	0.85 0.86 0.92 0.87	127.80 145.20 168.00 142.00	5882.94 4732.36 5566.48 5585.59	1635.54 1322.10 1532.20 1588.45	10.88 8.01 8.42 9.10	2.18 2.21 1.58 2.21

Appendix 17.

Individual DATA - Group AM

	A	B	C	D	E	F	G	H
2 weeks	374.2 406.1 <u>391.0</u> 390.4	2.37 2.57 <u>2.40</u> 2.45	0.50 0.59 <u>0.56</u> 0.55	89.40 96.60 <u>85.50</u> 90.60	2964.00 2846.68 <u>2679.94</u> 2830.21	1250.64 1107.66 <u>1116.64</u> 1158.31	6.99 6.77 <u>7.29</u> 7.02	2.62 2.96 <u>2.90</u> 2.83
4 weeks	404.2 400.9 <u>348.5</u> 384.5	2.44 2.30 <u>2.31</u> 2.35	0.56 0.55 <u>0.53</u> 0.55	117.60 192.00 <u>156.00</u> 155.20	3579.48 6267.68 <u>6739.62</u> 5528.93	1467.00 2725.08 <u>2917.53</u> 2369.89	6.99 7.80 <u>9.91</u> 8.23	2.04 1.09 <u>1.45</u> 1.53
6 weeks	474.2 517.7 <u>595.6</u> 522.2	2.62 2.61 <u>2.64</u> 2.62	0.58 0.60 <u>0.60</u> 0.59	83.40 113.40 <u>106.20</u> 101.00	3609.10 3883.68 <u>3742.46</u> 3745.08	1377.52 1488.00 <u>1417.60</u> 1427.71	9.95 7.87 <u>8.01</u> 8.61	1.95 1.77 <u>1.65</u> 1.79
8 weeks	513.3 518.3 <u>432.8</u> 488.1	2.67 2.63 <u>2.41</u> 2.57	0.68 0.60 <u>0.61</u> 0.63	91.80 106.80 <u>109.20</u> 162.60	3856.12 5319.76 <u>3973.42</u> 4383.17	1444.44 2011.80 <u>1648.72</u> 1705.25	10.70 11.36 <u>9.21</u> 10.42	2.04 1.71 <u>1.67</u> 1.81
10 weeks	542.2 451.1 <u>492.2</u> 495.2	2.64 2.41 <u>2.46</u> 2.50	0.65 0.59 <u>0.52</u> 0.59	91.80 106.80 <u>96.0</u> 99.20	4007.26 3972.84 <u>4312.72</u> 4097.4	1517.90 1648.48 <u>1753.14</u> 1637.84	10.75 9.11 <u>9.50</u> 9.79	3.16 2.97 <u>3.00</u> 3.04
12 weeks	669.1 651.8 <u>654.0</u> 658.3	3.50 3.84 <u>2.83</u> 3.39	0.85 1.03 <u>0.65</u> 0.84	147.00 169.20 <u>150.00</u> 153.33	4018.30 4774.36 <u>4260.01</u> 4367.29	1156.80 1248.58 <u>1505.34</u> 1303.46	6.69 7.60 <u>6.52</u> 6.94	2.53 2.59 <u>2.42</u> 2.51
26 weeks	772.2 617.5 <u>608.7</u> 666.1	3.76 3.35 <u>3.20</u> 3.43	0.84 0.80 <u>0.76</u> 0.80	123.0 174.6 <u>155.4</u> 153.80	5671.66 7224.45 <u>7236.40</u> 6726.50	1508.46 2155.66 <u>2277.00</u> 1930.36	10.30 9.88 <u>11.14</u> 10.44	1.52 1.12 <u>1.37</u> 1.34
38 weeks	707.6 635.5 <u>831.3</u> 724.8	4.33 4.45 <u>5.31</u> 4.70	1.02 1.14 <u>1.36</u> 1.17	160.80 172.20 <u>225.00</u> 186.00	4903.12 5142.78 <u>7246.24</u> 5764.05	1132.36 1153.64 <u>1364.64</u> 1217.56	7.18 7.65 <u>8.25</u> 7.69	1.69 1.86 <u>1.33</u> 1.63
52 weeks	917.7 833.1 <u>955.9</u> 902.2	4.65 6.73 <u>4.50</u> 5.43	1.16 1.73 <u>1.11</u> 1.33	175.00 328.20 <u>236.20</u> 246.20	8176.10 8108.18 <u>19123.10</u> 8802.46	1758.30 1204.78 <u>2065.94</u> 1676.34	11.64 6.35 <u>9.75</u> 8.25	2.70 2.05 <u>2.42</u> 2.39

Appendix 18.

Individual DATA - Group AFM

	A	B	C	D	E	F	G	H
2 Weeks	409.0 340.1 384.7 394.6	3.12 2.88 4.29 3.43	0.77 0.66 1.04 0.82	73.56 66.72 65.16 69.48	1614.28 1332.92 1496.36 1497.85	5124.0 480.18 348.80 448.79	5.42 4.75 5.57 5.25	4.36 3.97 3.75 4.03
4 Weeks	426.7 418.3 418.1 421.0	2.96 2.51 2.44 2.64	0.72 0.57 0.58 0.62	74.28 72.84 83.04 76.72	1751.84 2793.74 2901.26 2482.28	591.84 1113.04 1189.04 964.64	5.74 8.71 8.31 7.59	4.16 3.50 3.76 3.81
6 weeks	460.6 398.4 486.5 448.5	3.08 3.31 2.27 2.89	0.71 0.80 0.59 0.70	94.92 128.68 113.52 112.44	3485.70 3662.18 3150.62 3432.67	1131.72 1106.40 1387.94 1208.69	8.47 6.87 7.21 7.52	3.78 2.50 2.86 3.05
8 Weeks	525.0 475.0 508.0 576.00	3.72 3.41 2.89 3.34	0.86 0.80 0.68 0.78	99.84 111.96 111.36 107.81	3332.46 5983.06 4664.98 4660.17	895.82 1754.56 1614.18 1421.52	7.72 12.54 9.86 10.04	5.35 4.86 4.14 4.78
10 weeks	572.3 495.9 521.8 530.0	3.25 2.97 2.74 2.99	0.70 0.64 0.63 0.66	79.08 92.04 73.22 81.45	2974.02 3398.00 3605.84 3325.95	915.08 1144.12 1316.00 1125.07	8.10 8.97 11.32 8.46	3.73 3.28 3.99 3.67
12 weeks	666.6 558.4 609.0 611.3	2.98 2.87 2.97 2.94	0.73 0.71 0.76 0.73	94.63 114.00 103.22 103.56	2807.40 3640.48 3325.80 3257.89	942.08 1268.46 1118.80 1110.11	7.26 7.90 8.25 7.80	3.93 3.72 4.32 3.99
26 Weeks	841.2 719.6 749.0 769.9	4.27 4.64 3.80 4.24	1.03 1.12 0.88 1.01	153.60 186.60 121.80 154.00	5919.92 6889.84 6067.82 6292.59	1386.40 1484.90 1588.32 1489.37	9.30 8.77 11.54 9.87	1.94 1.86 2.34 2.05
38 Weeks	763.4 752.2 754.7 758.3	4.87 4.97 4.92 4.92	1.16 1.38 1.26 1.27	175.80 203.40 212.80 199.00	4842.44 5380.52 6658.40 5627.52	994.34 1082.60 1353.34 1143.42	6.56 7.34 7.83 7.24	2.02 1.99 1.76 1.92
52 Weeks	905.7 896.1 1203.4 5002.4	4.49 4.51 5.52 4.84	1.13 1.15 1.49 1.26	168.54 247.20 273.60 227.78	5533.86 6367.30 8313.54 6734.83	1220.26 1711.82 1506.04 1382.71	8.27 6.57 8.20 7.68	2.54 1.72 2.13 2.13

Appendix 19.

Individual Data - Lymphocyte Blast Transformation
Stimulation Index

	<u>AFM</u>	<u>AM</u>	<u>AF</u>	<u>FM</u>	<u>A</u>	<u>F</u>	<u>M</u>	<u>N</u>
4 weeks	3.44 3.46 <u>3.32</u> 3.41	17.86 8.98 <u>9.36</u> 11.73	6.45 6.60 <u>2.00</u> 5.02	3.70 3.16 <u>32.32</u> 13.46	0.93 1.06 <u>0.80</u> 0.93	3.56 10.05 <u>25.71</u> 13.11	2.88 1.27 <u>1.24</u> 1.81	0.59 1.45 <u>1.54</u> 1.19
8 weeks	6.86 1.58 <u>11.20</u> 6.55	6.67 2.02 <u>41.13</u> 16.61	2.51 3.90 <u>3.02</u> 3.14	107.98 66.19 <u>89.83</u> 88.00	0.79 1.76 <u>1.64</u> 1.40	3.83 5.46 <u>12.53</u> 7.27	1.05 22.68 <u>4.04</u> 9.26	0.77 0.83 <u>1.23</u> 0.95
12 weeks	8.70 17.94 <u>5.54</u> 10.73	72.45 34.46 <u>20.22</u> 42.38	20.85 5.53 <u>23.44</u> 16.61	28.17 51.19 <u>113.85</u> 64.10	1.30 0.80 <u>0.88</u> 1.03	12.09 3.81 <u>18.62</u> 11.51	4.72 3.56 <u>3.58</u> 3.94	0.92 1.23 <u>1.03</u> 1.06
26 weeks	12.84 19.36 <u>84.22</u> 38.84	23.74 46.42 <u>120.14</u> 63.43	37.58 12.66 <u>106.94</u> 52.39	315 8204 <u>44.37</u> 44.85	2.79 1.60 <u>1.13</u> 1.84	25.46 50.79 <u>13.52</u> 29.42	2.21 8.38 <u>11.28</u> 7.63	0.78 1.39 <u>1.18</u> 1.12
38 weeks	1.20 2.72 <u>1.57</u> 1.84	23.87 3.70 <u>1.39</u> 9.65	38.71 31.16 <u>20.92</u> 46.93	10.00 1.11 <u>3.91</u> 5.04	1.33 1.12 <u>0.98</u> 1.14	0.40 5.25 <u>2.57</u> 2.84	35.32 0.48 <u>21.28</u> 12.03	2.84 2.46 <u>2.80</u> 2.73
52 weeks	30.98 0.98 <u>12.23</u> 14.73	10.07 0.86 <u>18.07</u> 9.67	5.46 1.39 <u>1.53</u> 2.79	77.53 1.91 <u>1.02</u> 26.82	3.83 1.27 <u>0.60</u> 1.83	2.00 24.29 <u>3.86</u> 10.03	0.65 1.71 <u>6.05</u> 2.80	3.07 2.45 <u>1.93</u> 2.48

Appendix 20.

Leucine Aminopeptidase Activity - Individual Data

	AFM		AF		AM		FM		A		F		M		N	
	O.D.	Activity	O.D.	Activity	O.D.	Activity	O.D.	Activity	O.D.	Activity	O.D.	Activity	O.D.	Activity	O.D.	Activity
2 weeks	0.140	1.02	0.155	1.13	0.152	1.11	0.155	1.13	0.140	1.02	0.170	1.24	0.160	1.16	0.135	0.98
	0.135	0.98	0.140	1.02	0.165	1.20	0.170	1.24	0.165	1.20	0.160	1.16	0.155	1.13	0.150	1.09
		1.00		1.08		1.16		1.19		1.11		1.20		1.15		1.04
4 weeks	0.205	1.49	0.270	1.96	0.180	1.31	0.145	1.06	0.150	1.09	0.135	0.98	0.135	0.98	0.128	0.98
	0.210	1.53	0.190	1.38	0.155	1.13	0.160	1.16	0.150	1.09	0.150	1.09	0.150	1.09	0.145	1.06
		1.51		1.67		1.22		1.11		1.09		1.04		1.04		1.07
6 weeks	0.180	1.31	0.173	1.26	0.161	1.17	0.150	1.09	0.188	1.37	0.205	1.49	0.180	1.31	0.150	1.09
	0.210	1.53	0.185	1.35	0.200	1.46	0.150	1.09	0.145	1.06	0.150	1.09	0.185	1.35	0.150	1.09
		1.42		1.31		1.32		1.09		1.22		1.29		1.33		1.09
8 weeks	0.172	1.25	0.190	1.38	0.160	1.16	0.130	0.95	0.190	1.09	0.150	1.09	0.190	1.38	0.168	1.27
	0.190	1.38	0.170	1.24	0.170	1.24	0.165	1.20	0.180	1.24	0.165	1.20	0.160	1.16	0.145	1.06
		1.32		1.31		1.20		1.08		1.13		1.15		1.27		1.14
10 weeks	0.175	1.27	0.160	1.16	0.240	1.75	0.140	1.02	0.150	1.09	0.150	1.09	0.185	1.35	0.140	1.07
	0.175	1.27	0.150	1.09	0.200	1.46	0.130	0.95	0.160	1.16	0.155	1.13	0.170	1.24	0.155	1.13
		1.27		1.13		1.61		0.99		1.13		1.11		1.30		1.08
12 weeks	0.175	1.27	0.130	0.95	0.235	1.71	0.140	1.02	0.140	1.02	0.165	1.20	0.150	1.09	0.130	0.95
	0.165	1.20	0.145	1.06	0.190	1.38	0.150	1.09	0.145	1.06	0.130	0.95	0.185	1.35	0.145	1.06
		1.24		1.01		1.55		1.06		1.04		1.08		1.22		1.01
26 weeks	0.180	1.31	0.140	1.07	0.170	1.24	0.150	1.09	0.140	1.02	0.165	1.20	0.150	1.09	0.155	1.13
	0.170	1.24	0.135	0.98	0.165	1.20	0.130	0.95	0.145	1.06	0.130	0.95	0.185	1.35	0.130	0.95
		1.29		1.00		1.27		1.02		1.04		1.08		1.22		1.24
38 weeks	0.140	1.07	0.160	1.16	0.180	1.31	0.150	1.09	0.140	1.02	0.160	1.16	0.175	1.27	0.150	1.09
	0.165	1.20	0.155	1.13	0.150	1.09	0.140	1.02	0.149	1.08	0.165	1.20	0.185	1.35	0.140	1.07
		1.11		1.15		1.20		1.06		1.05		1.18		1.31		1.06
52 weeks	0.130	0.95	0.145	1.06	0.150	1.09	0.145	1.06	0.150	1.09	0.150	1.09	0.150	1.09	0.135	0.98
	0.170	1.24	0.150	1.08	0.145	1.06	0.130	0.95	0.135	0.98	0.160	1.16	0.180	1.31	0.150	1.09
		1.10		1.08		1.08		1.01		1.04		1.13		1.20		1.04

Appendix 21FORMULA FOR CALCULATION OF LEUCINE AMINOPEPTIDASE ACTIVITY

$$C = \frac{\text{optical density}}{\text{extinction coefficient}^*} \times 10^3 \times 2.1$$
$$= \text{umoles/30 min/10}^6 \text{ cells}$$

* Extinction coefficient = 9620

BIBLIOGRAPHY

BIBLIOGRAPHY

- Aalto, M. and Kulonen, E. (1979). Fractionation of connective tissue-activating factors from the culture medium of silica-treated macrophages. *Acta Pathol. Microbiol. Scand. Sect. C*, 87, 241-250.
- Aalto, M., Potila, M., and Kulonen, E. (1976). The effect of silica-treated macrophages on the synthesis of collagen and other proteins in vitro. *Exp. Cell Res.*, 97, 193-202.
- Abercrombie, M., Heaysman, J.E.M. and Pegrum, S.M. (1971). The locomotion of fibroblasts in culture, IV, electron microscopy of the leading lamella. *Exp. Cell Res.*, 67, 359-367.
- Adams, D.O. (1975). The structure of mononuclear phagocytes II. The effects of Mycobacterium tuberculosis. *Am. J. Pathol.*, 80, 101-113.
- Adams, D.O. (1976). The granulomatous inflammatory response. *Am. J. Pathol.*, 84, 164-191.
- Adamson, I.Y.R. and Bowden, D.H. (1974). The type 2 cell as progenitor of alveolar epithelial regeneration: a cytodynamic study in mice after exposure to oxygen. *Lab. Invest.*, 30, 35-42.
- Adamson, I.Y.R., Bowden, D.H. and Wyatt, J.P. (1970). A pathway to pulmonary fibrosis: a ultrastructural study of mouse and rat following radiation to the whole body and hemithorax. *Am. J. Pathol.*, 58, 481-487.
- Addison, T. (1868). A collection of the published writings. edited by Dr. Wilks and Dr. Daldy, London. The New Sydenham Society. 39-64.

- Aho, S. and Kulonen, E. (1977). Effect of silica-liberated macrophage factor on protein synthesis in cell-free systems. *Exp. Cell. Res.*, 104, 31-38.
- Allison, A.C. (1971). Lysosomes and the toxicity of particulate pollutants. *Arch. Inter. Med.*, 128, 131-139.
- Allison, A.C. (1973). Experimental methods - cell and tissue culture: effects of asbestos particles on macrophages, mesothelial cells and fibroblasts. In *Biological Effects of Asbestos*, Proc. working conf. Int. Agency for Research on Cancer, Lyon, France, October, 1972. Edited by P. Bogovski, J.C. Gilson, V. Timbrell and J.C. Wagner. Lyon, IARC Scientific Publications No. 3, 1973, 89-93.
- Allison, A.C. (1977). Mechanisms of macrophage damage in relation to pathogenesis of some lung diseases. In Respiratory Defense Mechanism, Part II, edited by J.D. Brain, D.F. Proctor and L. Reid, New York, Marcel Dekker, 1075-1102.
- Allison, A.C., Clark, I.A. and Davies, P. (1977). Cellular interactions in fibrogenesis. *Ann. Rheum. Dis.* 36, (suppl.) 8-13.
- Allison, A.C. and Davies, P. (1975). Increased biochemical and biological activities of mononuclear phagocytes exposed to various stimuli, with special reference to secretion of lysosomal enzymes. In Mononuclear Phagocytes in Immunity, Infection and Pathology. Edited by R. van Furth, Oxford, Blackwell, 487.
- Allison, A.C., Harington, J.S. and Birbeck, M. (1968). An examination of the cytotoxic effects of silica on macrophages. *J. Exp. Med.*, 124, 141-154.

- Alsadi, A. and Smith, D.W. (1973). The fate of virulent and attenuated mycobacteria in guinea pigs infected by the respiratory route. *Am. Rev. Respir. Dis.*, 107, 1041-1046.
- Ando, M. (1973). Macrophage activation in tuberculin reactions of rabbits with primary BCG infection and reinfection. *J. Reticuloendoth. Soc.*, 14, 132-145.
- Andral, G. (1836). *The clinique medicale*. Translated by D. Spillan. London, Henry Reushaw Publisher, 413-423, 497-502.
- Avenbrugger, L. (1761). *Inventum novum ex percussione thoracis humani ut signo abstrusos interni pectoris morbos detegendi*. Translated by J. Forbes, (1824), London, 34-37.
- Babcock, R.H. (1907). *Diseases of the lung*. 1st ed., London, Sidney Appleton Publisher, 362-370.
- Bateman, E.D., Emerson, R.J. and Cole, P. (1979). The use of diffusion chambers to examine the biological effects of mineral dusts. In Proceedings of the International Workshop on the in vitro Effects of Mineral Dusts, held at Cardiff, September 4-7, 1979.
- Bateman, E., Emerson, R.J. and Cole, P. (1980). Mechanisms of fibrogenesis, In Occupational Lung Diseases: Research Approaches and Methods, Edited by H. Weill and M. Turner-Warwick, New York, Marcel Dekker, in press.
- Bayle, G.L. (1815). *Researches on pulmonary phthisis*. Translated by W. Barrow, Liverpool, G.F. Harris's Widow and Brothers Printers. 233-260.

- Bhuyan, U.N. and Ramalingaswami, V. (1974). Systemic macrophage mobilization and granulomatous response to BCG in the protein deficient rabbit. *Am. J. Pathol.*, 76, 313-322.
- Bienkowski, R.S., Baum, B.J., Crystal, R.G. (1978). Fibroblasts degrade newly synthesised collagen within the cell before secretion. *Nature* 276, 413-416.
- Blanden, R.V., Lefford, M.J. and Mackaness, G.B. (1969). The host response to Calmette Guerin bacillus infection in mice. *J. Exp. Med.*, 129, 1079-1087.
- Bloom, B.R. and Bennett, B. (1966). Mechanism of reaction in vitro associated with delayed-type hypersensitivity. *Science*, 153, 80-82.
- Boros, D.L. (1978). Granulomatous inflammations. *Prog. Allergy*, 24, 183-267.
- Botham, S.K. and Holt, P.F. (1971). Asbestos-body formation in the lungs of rats and guinea-pigs after inhalation of anthophyllite. *J. Pathol.*, 107, 245-252.
- Botham, S.K. and Holt, P.F. (1972). The effects of inhaled crocidolites from transvaal and north-west cape mines on the lungs of rats and guinea-pigs. *Brit. J. Exp. Pathol.*, 53, 612-620
- Bowden, D.H. and Adamson, I.Y.R. (1972). The pulmonary interstitial cell as immediate precursor of alveolar macrophage. *Am. J. Pathol.*, 68, 521-528.
- Bowden, D.H. and Adamson, I.Y.R. (1976). The alveolar macrophage delivery system: kinetic studies in cultured explants of murine lung. *Am. J. Pathol.* 83, 123-134.

- Böyum, A. (1968). Separation of lymphocytes and erythrocytes by centrifugation. *Scand. J. Clin. Lab. Invest.* 20, Suppl. 97., 1-5.
- Bradley, K.H., McConnell, S.D. and Crystal, R.G. (1974). Lung collagen composition and synthesis - characterization and changes with age. *J. Biol. Chem.*, 249: 2674-2683.
- Bradley, K., McConnell-Breul, S. and Crystal, R.G. (1975). Collagen in the human lung - quantitation of rates of synthesis and partial characterization of composition. *J. Clin. Invest.*, 55, 543-550.
- Brain, J.D., Sorokin, S.P. and Godleski, J.J. (1977). Quantitation, origin and fate of pulmonary macrophages, In Respiratory Defense Mechanisms (Part II). Edited by J.D. Brain, D.F. Proctor and L. Reid, New York, Marcel Dekker, 840-892.
- Brody, A.R. and Craighead, J.E. (1974). Pathogenesis of pulmonary cytomegalo-virus infection in immunosuppressed mice. *J. Infect. Dis.*, 129, 677-689.
- Brown, R.C., Chamberlain, M., Griffiths, D.M. and Timbrell, V. (1978). The effects of fibre size on the in vitro biological activity of the three types of amphibole asbestos. *Int. J. Cancer*, 22, 721-727.
- Buonassisi, V. (1973). Sulfated mucopolysaccharide synthesis and secretion in endothelial cell cultures. *Exp. Cell Res.*, 76, 363-368.
- Burrell, R., and Anderson, M. (1973). The induction of fibrogenesis by silica-treated alveolar macrophages. *Environ. Res.*, 6, 389-394.

- Butler, G. (1975). Pulmonary interstitial fibrosis from paraquat in the hamster. *Arch. Pathol.*, 99, 563-507.
- Cardinale, G.J. and Udenfriend, S. (1974). Prolyl hydroxylase. *Adv. Enzymol.*, 41, 245-300.
- Carrington, C. (1968). Organizing interstitial pneumonia, definition of the lesion and attempts to devise and experimental model. *Yale J. Biol. Med.*, 40, 352-363.
- Carrington, C.B., Gaensler, E.A., Coutu, R.E., Fitzgerald, M.X. and Gupta, R.G. (1978). Natural history and treated course of usual desquamative interstitial pneumonia. *N. Engl. J. Med.*, 298, 801-809.
- Casarett, L.J. and Milley, P.S. (1964). Alveolar reactivity following inhalation of particles. *Health Phys.* 10, 1003-1011.
- Church, R.L., Tanzer, M.L. and Pfeiffer, S.E. (1973). Collagen production by a clonal line of Schwann cells. *Proc. Natl. Acad. Sci.*, 70, 1943-1946.
- Clark, A., Hadley, W.I. and Chaplin, A. (1894). Fibroid diseases of the lung including fibroid phthisis. London, Charles Griffin and Co. Ltd.
- Conning, D.M. and Heppleston, A.G. (1966). Reticuloendothelial activity and local particle disposal. *Brit. J. Exp. Pathol.*, 47, 388-400.
- Corrin, B. and King, E. (1969). Experimental endogenous lipid pneumonia and silicosis. *J. Pathol.*, 97, 325-330.
- Corrin, B. (1969). Phagocytic potential of pulmonary alveolar epithelium with particular reference to surfactant metabolism. *Thorax*, 25, 110-115.

- Crystal, R.G., Fulmer, J.D., Roberts, W.C., Moss, M.L., Line, B.R., Reynolds, H.Y. (1976). Idiopathic Pulmonary Fibrosis, clinical, histologic, radiographic, physiologic, scintigraphic, cytologic and biochemical aspects. *Ann. Intern. Med.*, 85, 760-787.
- David, J.R. (1966). Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc. Natl. Acad. Sci. U.S.A.*, 56, 72-81.
- Davies, P. and Allison, A.C. (1976). Secretion of macrophage enzymes in relation to the pathogenesis of chronic inflammation. In Immunobiology of the Macrophage. Edited by David Nelson, London, Academic Press, 427-462.
- Davies, P., Allison, A.C., Ackerman, J., Butterfield, A. and Williams, S. (1974). Asbestos induces selective release of lysosomal enzymes from mononuclear phagocytes. *Nature*, 251, 423-425.
- Davies, P., Page, R.C. and Allison, A.C. (1974). Changes in cellular enzyme levels and extracellular release of lysosomal acid hydrolases in macrophages exposed to group A streptococcal cell wall substance. *J. Exp. Med.*, 139, 1262-1282.
- Desai, R., Hext, P.M., and Richards, R.J. (1975). The prevention of asbestos-induced haemolysis. *Life Science*, 16, 1931-1938.
- Douglas, W.H.J., Del Vecchio, P., Teel, R.W., Jones, R.M. and Farrell, P.M. (1976). Culture of Type II alveolar cells, In Lung Cells in Disease. Edited by A. Bouhuys, Oxford, North-Holland Publishing Co. 53-68.

- Elmes, P.C. McCaughey, W.T.E. and Wade, O.L. (1965).
Diffuse mesothelioma of the pleura and asbestos. *Brit. Med. J.*, 1, 350-353.
- Esterly, J.R. and Faulkner, C.S. (1970). The granular pneumonocyte. Absence of phagocytic activity. *Am. Rev. Respir. Dis.*, 101, 869-876.
- Etherington, D.J. (1977). Collagen degradation. *Ann. Rheum. Dis.*, 36, suppl. 2, 14-17.
- Freund, J. (1956). The mode of action of immunologic adjuvants. *Adv. Tuberc. Res.*, 7, 130-148.
- Fulmer, J.D. and Crystal, R.G. (1976). The biochemical basis of pulmonary function. In The Biochemical Basis of Pulmonary Function. Edited by R.G. Crystal, New York, Marcel Dekker, 419-466.
- Giles, K.W. and Myers, A. (1965). An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature*, 206, 93.
- Godleski, J.J. and Brain, J.D. (1972). The origin of alveolar macrophages in mouse radiation chimeras. *J. Exp. Med.*, 136, 630-643.
- Goldberg, A.L. and St. John, A.C. (1976). Intracellular protein degradation in mammalian and bacterial cells, part 2. *Ann. Rev. Biochem.*, 45, 747-803.
- Gordon, S., Bray, M.A. and Morley, J. (1976). Control of lymphokine secretion by prostaglandins. *Nature*, 262, 401-402.

- Gotho, G.J. and Swenson, A. (1970). Effects of BCG on lymphatic lung clearance of dusts with different fibrogenicity. *Arch. Environ. Health*, 20, 579-585.
- Grant, M.E. and Brockop, D.J. (1972). The biosynthesis of collagen. *N. Engl. J. Med.*, 286, 194-199, 242-249, 291-300.
- Green, R.C. (1977). Changes in acid ribonuclease and other acid hydrolases during lymphocyte stimulation. *Exp. Cell. Res.*, 110, 215-223.
- Grisolle, A. (1864). *Traite de la pneumonie*. 2nd ed. Paris, J.B. Bailliere Publisher, 115.
- Gross, P. (1962). The concept of the Hamman-Rich syndrome. A critique. *Am. Rev. Respir. Dis.*, 85, 828-832.
- Gross, P. and de Treville, T.P. (1967). Experimental asbestosis - studies on the progressiveness of the pulmonary fibrosis caused by chrysotile dust. *Arch. Environ. Health*, 15, 638-649.
- Gross, P., McNervey, J.M., Westrick, M.L. and Babyak, M.A. (1962). Resolution of chronic interstitial pneumonitis. *Arch. Pathol.*, 74, 93-99.
- Hamman, I. and Rich, A.R. (1935). Fulminating diffuse interstitial fibrosis of the lungs. *Trans. Am. Clin. Assoc.*, 51, 154-163.
- Hance, A.J., Bradley, K. and Crystal, R.G. (1976). Lung collagen: synthesis of Type I and Type III collagen by rabbit and human lung cells in culture. *J. Clin. Invest.*, 57, 102-111.

- Hance, A.J. and Crystal, R.G. (1976). Collagen, In The Biochemical Basis of Pulmonary Function. Edited by R.G. Crystal, New York, Marcel Dekker, 215-271.
- Harrington, J.S., Miller, K. and Mac Nab, G. (1971). Haemolysis by asbestos. *Environ. Res.*, 4, 95-117.
- Harrington, J.S., Ritchie, M., King, P.C. and Miller, K. (1973). The in vitro effects of silica-treated hamster macrophages on collagen production by hamster fibroblasts. *J. Pathol.*, 109, 21-37.
- Harris, J.O., Bice, D. and Salvaggio, J.E. (1976). Experimental granulomatous pneumonitis: bronchopulmonary response to Micropolysporia faeni in the rabbit. *Chest*, 69, suppl. 2, 287-288.
- Harris, A. and Dunn, G. (1972). Centripetal transport of attached particles on both surfaces of moving fibroblasts. *Exp. Cell, Res.*, 73, 519-523.
- Harris, E.D. and Krane, S.M. (1974). Collagenases. *N. Engl. J. Med.*, 291, 557-563, 605-609, 652-664.
- Haslam, P.L., Lukoszek, A., Merchant, J.A. and Turner-Warwick, M. (1978). Lymphocyte responses to phytohaemagglutinin in patients with asbestosis and asbestosis and pleural mesothelioma. *Clin. Exp. Immunol.*, 31, 178-188.
- Hasse, C.E. (1846). An anatomical description of the diseases of the organs of circulation and respiration. Translated by W.E. Swaine. London. The Sydenham Society.
- Heard, B.E. (1976). Pathology of interstitial lung diseases with particular reference to terminology, classification and trephine lung biopsy. *Chest*, 69, suppl. 2, 252-253.

- Heppleston, A.G. (1978). Cellular reactions with silica, In Biochemistry of Silicon and Related Problems. Edited by G. Bendz and I. Linguist, Plenum Press, 357-379.
- Heppleston, A.G. and Styles, J.A. (1967). Activity of a macrophage factor in collagen formation by silica. *Nature*, 214, 521-522.
- Hext, P.M. (1979). The effect of mineral dusts on lung slices in vitro. In Proceedings of the International Workshop on the in vitro Effects of Mineral Dusts, held in Cardiff, September 4-7 1979 in press.
- Holt, P.F., Mills, J. and Young, D.K. (1964). The early effects of chrysotile asbestos dust on the rat lung. *J. Pathol. Bact.*, 87, 15-23.
- Holt, P.F., Mills, J. and Young, D.K. (1966). Experimental asbestosis in the guinea-pig. *J. Pathol. Bact.*, 92, 185-195.
- Houck, J.C. and Chang, C. (1971). The chemotactic properties of the products of collagenolysis. *Proc. Soc. Exp. Biol. Med.*, 138, 69-75.
- Houck, J.C., Sharma, V.K. and Hayflick, L. (1971). Functional failures of cultured human diploid fibroblasts after continued population doublings. *Proc. Soc. Exp. Biol. Med.*, 137, 331-333.
- Howard, B.V., Macerak, E.J., Gunson, D. and Kefalides, N.A. (1976). Characterization of collagen synthesized by endothelial cells in culture. *Proc. Natl. Acad. Sci.*, 73, 2361-2364.

- Huang, T.W. (1977). Chemical and histochemical studies of human alveolar collagen fibers. *Am. J. Pathol.* 86, 81-98.
- Johnson, R.L. and Ziff, M. (1976). Lymphokine stimulation of collagen accumulation. *J. Clin. Invest.* 58, 240-253.
- Kagan, E., Solomon, A., Cochrane, J.C., Beissner, E.I., Gluckman, J., Rocks, P.H. and Webster, I. (1977). Immunological studies of patients with asbestosis -- I. Studies of cell-mediated immunity. *Clin. Exp. Immunol.* 28, 261-267.
- Kang, A. (1978). Fibroblast activation. *J. Lab. Clin. Med.*, 92, 1-2.
- Kapanci, T., Assimacopoulos, A., Irle, G., Zwahlen, A., and Gabbiani, G. (1974). Contractile interstitial cells in pulmonary alveolar septa: a possible regulator of ventilation-perfusion ratio? Ultrastructural, immunofluorescence, and *in vitro* studies. *J. Cell Biol.*, 60, 375-392.
- Kapanci, Y., Weibel, E.R., Kaplan, H.P. and Robinson, F.R. (1969). Pathogenesis and reversibility of the pulmonary lesions of oxygen toxicity in monkeys. II. Ultrastructural and morphometric studies. *Lab. Invest.*, 20, 101-118.
- Karnovsky, M.L. and Lazdins, J.K. (1978). Biochemical criteria for activated macrophages. *J. Immunol.* 121, 809-813.
- Kawata, H., Myrvik, Q.N., and Leak, E.S. (1964). Dissociation of tuberculin hypersensitivity as a mediator for an accelerated pulmonary granulomatous response in rabbits. *J. Immunol.*, 93, 433-438.

- King, E.J., Yoganathan, M., Harrison, C.K. and Mitchison, D.A. (1957). Experimental infective pneumoconiosis. V. Massive fibrosis of the lungs produced by coal-mine dust and Mycobacterium tuberculosis var muris (Vole bacillus). A.M.A. Arch. Indust. Health. 16, 380-392.
- Kuhn, C. (1976). The cells of the lung and their organelles, In The Biochemical Basis of Pulmonary Function. Edited by R.G. Crystal, New York. Marcel Dekker, 3-48.
- Kulonen, E. (1970). Effects of SiO_2 - liberated macrophage factors with special reference to RNA. In Proceedings of the International Workshop on the in vitro Effects of Mineral Dusts, held at Cardiff, September 4-7 1979, in press.
- Laennec, R.T.H. (1834). A treatise on the diseases of the chest and on mediate auscultation. Translated by J. Forbes. 4th ed. London, Longman, Rees, ORME Publishers, 216-217.
- Langness, U. and Udenfriend, S. (1974). Collagen biosynthesis in nonfibroblastic cell lines. Proc. Natl. Acad. Sci., 71, 50-51.
- Laufer, A., Tal, C. and Behar, A.J. (1959). Effects of adjuvant (Freund's type) and its components on the organs of various animal species. A comparative study. Brit. J. Exp. Pathol., 40, 1-7.
- Laurenzi, G.A., Potter, R.T. and Kass, E.H. (1961). Bacteriological flora of the lower respiratory tract. N. Engl. J. Med., 265, 173-178.
- Lehtonen, E., Nordling, S. and Wartiovaara, J. (1973). Permeability and structure of ethanol sterilized nitro-cellulose (Millipore) filters. Exp. Cell. Res., 81, 169-174.

- Lewis, O.M. and Bunnell, R. (1975). Induction of fibrogenesis by lung antibody-treated macrophages. *Brit. J. Indust. Med.*, 33, 25-28.
- Lichtenstein, J.R., Martin, G.R., Koh, L.D. Byers, P.H. and McKusick, V. (1973). Defect in conversion of procollagen to collagen in a form of Ehlers-Danlos syndrome. *Science*, 182, 298-299.
- Liebow, A.A. (1975). Definition and classification of interstitial pneumonias in human pathology. In: Alveolar Interstitium of the Lung, Int. Symp., Paris 1974. *Prog. Respir. Res.*, 8, 1-33.
- Liebow, A.A., Steer, A. and Billingsley, J.G. (1965). Desquamative interstitial pneumonia. *Am. J. Med.*, 39, 369-404.
- Light, W.G. and Wei, E.T. (1979). Surface charge and in vitro activity of asbestos. In Proceedings of the International Workshop on the in vitro Effects of Mineral Dusts, held in Cardiff, September 4-7, in press.
- Livingstone, J.L., Lewis, J.G., Reid, L. and Jefferson, K.E. (1964). Diffuse interstitial pulmonary fibrosis: A clinical, radiological and pathological study based on 45 patients. *Quart. J. Med.*, New Series, 33, 71-103.
- Low, F.N. and Daniels (1952). Electron microscopy of the rat lung. *Anat. Rec.*, 113, 437-450.
- McDermott, M., Wagner, J.C., Tetley, T., Harwood, J. and Richards, R.J. (1977). The effects of inhaled silica and chrysotile on the elastic properties of rat lungs: physiological, physical and biochemical studies of lung surfactant. In Inhaled Particles IV. Edited by W.H. Walton, Oxford, Pergamon Press, 415-427.

- McCullough, B., Collins, J.F., Johason, W.G. and Grover, F.L. (1978). Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. *J. Clin. Invest.* 61, 79-88.
- Macklin, C.C. (1953). Osmiophilic bodies of two types in the peripheral lung tissue of normal and urethanized white mice, (a) in the granular pneumonocyte and (b) in the fibrocytes. *Anat. Rec.*, 115, 431 (Abstract of demonstration).
- Martinez, E. and Zucker (1960). *J. Phys. Chem.*, 64, 924.
- Mason, R.J., Williams, M.C., Greenleaf, R.D. and Clements, J.A. (1977). Isolation and properties of Type II alveolar cells from rat lung. *Am. Rev. Resp. Dis.*, 115, 1015-1026.
- Meyrick, B. and Reid, L. (1968). The alveolar brush cell in the rat - a third pneumonocyte. *J. Ultrastruct. Res.*, 23, 71-80.
- Meyrick, B. and Reid, L. (1970). The alveolar wall. *Brit. J. Dis. Chest*, 64, 121-140.
- Millard, M. (1966). Lung, pleura, and mediastinum, In Pathology. Vol. I, 5th ed. Edited by W.A.D. Anderson. St. Louis, C.V. Mosby Co., 686-766.
- Miller, K. and Kagan, E. (1976). The in vivo effects of asbestos on macrophage membrane structure and population characteristics of macrophages : a scanning electron microscope study. *J. Reticuloendothel. Soc.* 20, 159-171.
- Miller, K., Webster, I., Handfield, R.I.M. and Skikne, M.I. (1978). Ultrastructure of the lung in the rat following exposure to crocidolite asbestos and quartz. *J. Pathol.* 124, 39-44.

- Miyamoto, T., Kabe, J. and Noda, M. (1971). Physiologic and pathologic respiratory changes in delayed type hypersensitivity reaction in guinea-pigs. *Am. Rev. Respir. Dis.* 103, 509-515.
- Moore, V.L. and Myrvik, O.N. (1974). Relationship of BCG induced granuloma formation in rabbit lungs : effects of cortisone acetate. *Infect. Immun.* 7, 764-770.
- Morgagni, G.B. (1769). *De Sedibus et Causis Morborum* (The seats and causes of diseases) Translated by B. Alexander, London. A. Millar and T. Cadwell Publishers. Facsimile of the 1769 edition, New York, Hafner Publishing Co., 1960.
- Muller-Eberhard, H.J. (1975). Complement. *Ann. Rev. Biochem.*, 44, 697.
- Murphy, G.P., Brody, A.R. and Craighead, J.E. (1975). Monocyte migration across pulmonary membranes in mice infected with cytomegalovirus. *Exp. Mol. Pathol.*, 22, 35-44.
- Myllyla, R., Tuderman, L. and Kivirikko, K.I. (1977). Mechanism of the prolyl hydroxylase reaction . 2. Kinetic analysis of the reaction sequence. *Eur. J. Biochem.*, 80, 349-357.
- Nelson, D.S. and Boyden, S.V. (1963). The loss of macrophages from peritoneal exudates following the injection of antigen into guinea pigs with delayed-type hypersensitivity. *Immunology*, 6, 264-275.
- Newman, H.A.I., Saat, Y.A. and Hart, R.W. (1979). Inhibitory effect of chrysotile, crocidolite and amosite fibres on the more complex surface membrane glycolipids and glycoproteins. In Proceedings of the International Workshop on the in vitro Effects of Mineral Dusts, Cardiff,

September 4-7 1979, in press.

Okada, Y. and Genka, K. (1964). Histological and electron microscopic observations on the adenomatous hyperplasia of the alveolar epithelial cell in human lung. *Acta Tuberc. Jpn.*, 48-56.

Palmer, K.C., Snider, G.L. and Hayes, J.A. (1976). An association between alveolar cell proliferation and interstitial fibrosis following acute lung injury. *Chest*, 69, suppl. 2, 307-309.

Pearse, A.G.E. (1968). In Histochemistry: Theoretical and Applied. Third edition, Edinburgh, Churchill Livingstone, 214-230.

Peterkowsky, B. and Udenfriend, S. (1963). Conversion of proline to collagen hydroxyproline in a cell-free system from chick embryo. *J. Biol. Chem.*, 238, 3966-3977.

Pickrell, J.A., Harris, D.V., Mauderly, J.L. and Hahn, F.F. (1976). Altered collagen metabolism in radiation-induced interstitial pulmonary fibrosis. *Chest*, 69, suppl. 2 311-316.

Pierce, J.A. and Ebert, R.V. (1965). Fibrous network of the lung and its change with age. *Thorax*, 20, 469-476.

Piez, K.A. (1976). Primary structure. In Biochemistry of Collagen. Edited by G.N. Ramachandran and A.H. Reddi. New York, Plenum Press, 1-44.

Policaud, A., Collet, A., Ralyte, G. and Renet, C. (1954). L'alveole pulmonaire au microscope electronique. *Presse Med.*, 62, 1775-1782.

- Popenoe, E.A. and Aronson, R.B. (1972). Partial purification and properties of collagen lysine hydroxylase from chick embryos. *Biochim. Biophys. Acta*, 258, 380-386.
- Porter, R.R. and Reid, K.B.M. (1978). The biochemistry of complement. *Nature*, 275, 699-704.
- Postlethwaite, A.E., Seyer, J.M. and Kang, A.H. (1978). Induction of fibroblast chemotaxis by type I, II and III collagens, and collagen-degradation peptides. *Proc. Natl. Acad. Sci. USA*, 75, 871-875.
- Postlethwaite, A.E., Snyderman, R. and Kang, A.H. (1976). The chemotactic attraction of human fibroblasts to a lymphocyte-derived factor. *J. Exp. Med.* 144, 1188-1203.
- Postlethwaite, A.E., Snyderman, R. and Kang, A.H. (1977). The chemo-attraction of human fibroblasts to a fragment from the fifth component of complement. *Arthritis Rheum.*, 20, 131 (abstract).
- Powell, R. Douglas and Hartley, P. Horton-Smith (1911). On diseases of the lungs and pleurae including tuberculosis and mediastinal growths. 5th ed., London, H.K. Lewis 296-301.
- Powell, R. Douglas and Hartley, P. Horton-Smith (1921). On diseases of the lungs and pleurae including tuberculosis and mediastinal growths. 6th ed., London, H.K. Lewis, 335-343.
- Pras, M. and Glynn, L.E. (1973). Isolation of a non-collagenous reticulin component and its primary characterization. *Brit. J. Exp. Pathol.*, 54, 449-456.

- Pras, M. and Glynn, L.E. (1975). Isolation of a non-collagenous reticulin component and its primary characterization. *Brit. J. Exp. Pathol.*, 54, 449-456.
- Prockop, D.J., Berg, R.A., Kivirikko, K.I. and Uitto, J. (1976). Intracellular steps in the biosynthesis of collagen. Edited by G.N. Ramachandran and A.H. Reddi, New York, Plenum Press, 163-273.
- Prockop, D.J., Kivirikko, K.I. Tuderman, L. and Guzman, L. (1979). The biosynthesis of collagen and its disorders. *N. Engl. J. Med.*, 301, 13-23, 77-85.
- Prockop, D.J. and Udenfriend, S. (1960). A specific method for the analysis of hydroxyproline in tissue and urine. *Anal. Biochem.* 1, 228-239.
- Reid, L. (1967). The pathology of emphysema. London, Lloyd-Lake Medical Books Ltd., 319-361.
- Richards, R.J. and Jacoby, F. (1976). Light microscope studies on the effects of chrysotile asbestos and fibre glass on the morphology and reticulin formation of cultured lung fibroblasts. *Environ. Res.* 11, 112-121.
- Richards, R.J. and Wusteman, F.S. (1974). The effects of silica dust and alveolar macrophages on lung fibroblast grown in vitro. *Life Science*, 14, 355-364.
- Richards, R.J., Wusteman, F.S. and Dodgson, K.S. (1971). The direct effect of dusts on lung fibroblasts grown in vitro. *Life Science*, 10, 1149-1159.
- Richerson, H.B., Cheng, F.H.F. and Bauserman (1971). Acute experimental hypersensitivity pneumonitis in rabbits. *Am. Rev. Respir. Dis.* 104, 568-575.

- Richerson, H.B., Seidenfeld, J.J., Ratajczak, H.V. and Edwards, D.W. (1978). Chronic experimental interstitial pneumonitis in the rabbit. *Am. Rev. Respir. Dis.*, 117, 5-13.
- Richerson, H.B., Seidenfeld, J.J., Ratajczak, H.V., Richards, D., Butler, J.E. and Swanson, P. (1979). Models of pulmonary fibrosis - misadventures and ramifications. *Chest*, Suppl. 2, 75, 267-269.
- Rosenberry, T.L. and Richardson, J.M. (1977). Structure of 18s and 14s acetylcholinesterase: identification of collagen-like subunits that are linked by disulfide bonds to catalytic subunits. *Biochemistry*, 16, 3550-3558.
- Ross, R. (1973). The elastic fibre: a review. *J. Histochem. Cytochem.* 21, 199-208.
- Rupp, J.C., Moore, R.D. and Schoenberg, M.D. (1960). Stimulation of the reticuloendothelial system in the rabbit by Freund's adjuvant. *Arch. Pathol.* 70, 43-49.
- Rutherford, R.B. and Ross, R. (1976). Platelet factors stimulate fibroblast and smooth muscle cells quiescent in plasma serum to proliferate. *J. Cell. Biol.*, 69, 196.
- Ryan, S.F. (1972). Experimental fibrosing alveolitis. *Am. Rev. Respir. Dis.*, 105, 776-791.
- Saint-Remy J. (1979). Personal communication.
- Salvin, S.B., Younger, J.S. and Lederer, W.H.¹ (1973). Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *Infect. and Immun.* 1, 68-72.

- Scadding, J.G. (1964). Fibrosing alveolitis. Brit. Med. J., 2, 686 & 941.
- Scadding, J.G. (1974). Diffuse pulmonary alveolar fibrosis. Thorax, 29, 271-281.
- Scadding, J.G. and Hinson, K.F.W. (1967). Diffuse fibrosing alveolitis (diffuse interstitial fibrosis of the lung). Thorax, 22, 291-304.
- Selikoff, I.J., Bader, R.A., Bader, M.E., Churg, J. and Hammond, E.C. (1967). Asbestosis and neoplasia, Am. J. Med., 42, 487-496 (editorial).
- Selikoff, I.J. and Lee, D.H., (1978). Asbestos and disease. London, Academic Press, 143-187.
- Seifter, S. and Harper, E. (1971). The collagenases. In The Enzymes, III. Hydrolysis: Peptide Bonds. Edited by P.D. Boyer, New York, academic Press, 649-666.
- Seyer, J.M., Hutcheson, E.T. and Kang, A. (1976). Collagen polymorphism in idiopathic chronic pulmonary fibrosis. J. Clin. Invest., 57, 1498-1507.
- Silbert, J.E. (1973). Biosynthesis of mucopolysaccharides and proteoglycans. In Molecular Pathology of Connective Tissues. Edited by R. Perez-Tamayo, and M. Rojkind, New York, Marcel Dekker, 323-353.
- Smith, T.M., McIntosh, G.H. and Morris, B. (1970). The migration of cells through chemically inflamed tissues. J. Pathol., 100, 21-29.

- Snider, G.L., Celli, B.R., Goldstein, R.H., O'Brien, J.J., and Lucey, E.C. (1978). Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin--lung volumes, volume pressure relations, carbon monoxide uptake and arterial blood gas studies. *Am. Rev. Respir. Dis.*, 117, 289-297.
- Snyderman, R. and Mergenhagen, S.E. (1976). Chemotaxis of macrophages. In Immunobiology of the Macrophage. Edited by D.S. Nelson, New York, Academic Press, 323.
- Sorokin, S.P. (1970). The cells of the lung. In Morphology of Experimental Respiratory Carcinogenesis. Edited by P. Nettsheim, M.G. Hanna, Jr., and J.W. Deatherage, Jr. Oak Ridge, Tennessee, U.S. Atomic Energy Commission, 3-14.
- Spector, W.G. (1973). The fibroblast and inflammation, In Biology of Fibroblast, Fourth Sigrid Juselius Foundation Symposium, Turku, Finland, August, 1972. Edited by E. Kulonen and J. Pikkarainen. London, Academic Press, 525-528.
- Spector, W.G., Heesom, N., and Stevens, J.E. (1968). Factors influencing chronicity in inflammation of rat skin. *J. Pathol.*, 96, 203-213.
- Spector, W.G. and Mariano, M. (1975). Macrophage behavior in experimental granulomas. In Mononuclear Phagocytes in Immunity, Infection and Pathology. Edited by R. van Furth, Oxford, Blackwell Scientific Publications 927-942.
- Spector, W.G., Reichhold, N. and Ryan, G.B. (1970). Degradation of granuloma-induced micro-organisms by macrophages. *J. Pathol.*, 101, 339-354.

- Spencer, H. (1968). Pathology of the lung (excluding pulmonary tuberculosis). 2nd edition, London, Pergamon Press, 472-488.
- Stack, B.H.R., Grant, I.W.B., Irvine, W.J. and Moffat, M.A.J. (1965). Idiopathic diffuse interstitial lung disease. *Am. Rev. Respir. Dis.*, 92, 939-948.
- Starcher, B.C., Kuhn, C. and Overton, J.E. (1978). Increased elastin and collagen content in the lungs of hamsters receiving an intratracheal injection of bleomycin. *Am. Rev. Respir. Dis.*, 117, 299-305.
- Steiner, J.W., Langer, B. and Schatz, D.L. (1960). The local and systemic effect of Freund's adjuvant and its fractions. *Arch. Pathol.*, 70, 424-434.
- Stokes, W. (1882). A treatise on the diagnosis and treatment of diseases of the chest. Part I. Diseases of the lung and windpipe. Edited by Alfred Hudson, London. The New Sydenham Society. 332-333.
- Tainer, J.A., Turner, S.R. and Lynn, W.S. (1975). New aspects of chemotaxis. Specific target-cell attraction by lipid and lipoprotein fractions of Escherichia coli chemotactic factor. *Am. J. Pathol.*, 81, 401-408.
- Tanzer, M.L. (1976). Cross-linking. In Biochemistry of Collagen. Edited by G.N. Ramachandran and A.H. Reddi, New York, Plenum Press, 137-162.
- Tennant, J.R. (1964). Evaluation of trypan blue technique for determination of cell viability. *Transplantation* 2, 685-694.

- Tetley, T.D., Hext, P.M., Richards, R.J. and McDermott, M. (1976). Chrysotile-induced asbestosis: changes in the free cell population, pulmonary surfactant and whole lung tissue of rats. *Brit. J. Exp. Pathol.*, 57, 505-514.
- Thurlbeck, W.M. and Thurlbeck, S.M. (1976). Pulmonary effects of paraquat poisoning. *Chest*, 69, suppl. 2, 276-280.
- Truitt, G.L. and Mackaness, G.B. (1971). Cell-mediated resistance to aerogenic infection of the lung. *Am. Rev. Respir. Dis.*, 104, 829-843.
- Turner-Warwick, M. (1974). A perspective view on widespread pulmonary fibrosis. *Brit. Med. J.*, 2, 371-376.
- Unanue, E.R. (1976). Secretory function of mononuclear phagocytes - A review. *Am. J. Pathol.*, 83, 396-417.
- Van oud Alblas, A. Blusse and Van Furth, R. (1979). Origin, kinetics, and characteristics of pulmonary macrophages in the normal steady state. *J. Exp. Med.*, 149, 1504-1518.
- Veis, A., Anesey, J., Yuan, L. and Levy, S.J. (1973). Evidence for an aminoterminal extension in high-molecular weight collagens from mature bovine skin. *Proc. Natl. Acad. Sci.*, 70, 1464-1467.
- Vijayarathnam, G.S. and Corrin, B. (1972). Origin of the pulmonary alveolar macrophage studied in the iprindole-treated rat. *J. Pathol.*, 108, 115-118.
- Visvanathan, P.N., Dogra, R.K.S. and Zaidi, S.H. (1973). Pulmonary fibrogenic response of guinea pigs to amosite dust. *Int. Arch. Arbeitsmed.*, 31, 51-59.

- Vorwald, A.J., Durkan, T.M. and Pratt, P.C. (1951). Experimental studies of asbestosis. Arch. Indust. Hyg., 3, 1-43.
- Wachsmuth, E.D. (1975). Aminopeptidase as a marker for macrophage activation. Exp. Cell Res., 96, 409-412.
- Wagner, J.C. (1963). Asbestosis in experimental animals. Brit. J. Indust. Med., 20, 1-12.
- Wagner, J.C. (1979). Personal communication.
- Wahl, S.M., Wahl, L.M. and McCarthy, J.B. (1978). Lymphocyte-mediated activation of fibroblasts proliferation and collagen production. J. Immunol., 121, 942-946.
- Wahl, L.M., Wahl, S.M., Mergenhagen, S.E. and Martin, G.E. (1975). Collagenase production by lymphokine-activated macrophages. Science, 187, 261-263.
- Waksman, B.H. (1979). Adjuvants and immune regulation by lymphoid cells. Springer Semin. Immunopathol., 2, 5-23.
- Ward, P.A., Remold, H.G. and David, J.R. (1969). Leukotactic factor produced by sensitized lymphocytes. Science, 163, 1079-1081.
- Weibel, E.R., Gehr, P., Haies, D., Gil, J. and Bachofen, M. (1976). The cell population of the normal lung. In Lung Cells In Disease, Proceedings of a Brook Lodge Conference, Augusta, Michigan, April 21-23, 1976. Edited by A. Bouhuys, Oxford, North-Holland Publishing Co. 3-16.
- Werb, Z., Burleigh, M.C., Barrett, A.J. and Starkey, P.M. (1974). The interaction of γ 2 macroglobulin with proteinases, binding and inhibition of mammalian collagenases and other metal proteinases. Biochem. J., 139, 359-368.

Werb, Z. and Gordon, S. (1975). Secretion of a specific collagenase by stimulated macrophages. *J. Exp. Med.*, 142, 346-360.

Willoughby, W.F., Barbaras, J. and Wheelis, R. (1976). Immunologic mechanisms in experimental interstitial pneumonitis. *Chest*, 69, suppl. 2, 290-294.

Yajima, T. (1977). Phagocytosis of collagen by human gingival fibroblasts. *J. Dent. Res.*, 56(10), 1271-1277.

Zaidi, S.H., Harrison, C.V., King, E.J. and Mitchison, D.A. (1955). Experimental infective pneumoconiosis. II. Coal-mine dust with attenuated tubercle bacillus (BCG) in the lungs of immunised guinea-pigs. *Brit. J. Exp. Pathol.*, 36, 541-559.