PULMONARY SARCOIDOSIS: THE ROLE OF ALVEOLAR MACROPHAGES IN PATHOGENESIS, AND THEIR MODULATION WITH THERAPY.

by

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January, 1990
TO

MY PARENTS

&

DAVID

... who provided the pillars of strength when despair set in ...
ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr S W Clarke and Professor K M Spyer for presenting me with an opportunity to undertake this study, for offering me a free hand with the facilities in their departments, and for their total support and tremendous encouragement throughout this project.

I am most grateful to Dr L W Poulter, in whose laboratory this work was carried out, for his personal supervision and guidance.

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I owe an incalculable debt of gratitude to Miss M O'Malley for her excellent secretarial assistance, to all the volunteers and patients, and lastly to the nurses in the endoscopy unit (in particular Jan, Janet and Audrey), without whose help this study of alveolar macrophage in patients with sarcoidosis could not have been contemplated.
ABSTRACT

Pulmonary sarcoidosis is a clinical disorder that arises as a result of a granuloma-forming mechanism, driven by aberrant immunological reactions within the lung interstitium, in response to an as yet unknown stimulus. Extensive work identifying changes within the lymphocyte population and mediator release in sarcoidosis, does not fully explain the outcome of this granulomatous response; in particular its insidious progression to fibrosis, with ultimate irreversible tissue damage in ~ 15% of patients.

As it is now recognised that macrophages are intimately involved in T-cell stimulation and granuloma formation, the studies in this thesis test the hypothesis that aberrations within the alveolar macrophage (AM) population contribute to the pathogenesis of pulmonary sarcoidosis.

AM were obtained from the lungs of a total of 62 normal volunteers, and 115 biopsy-proven sarcoid patients by means of bronchoalveolar lavage (BAL), (the technique of which was standardized prior to formal studies). As the AM population is known to be heterogeneous, use was made of specific monoclonal antibody probes that discriminate subpopulations of macrophages.

Initial studies identified 3 phenotypically distinct macrophage subpopulations in both normal and sarcoid BAL. The latter consistently contained a high proportion of macrophages with the phenotype of dendritic cells. Much of this increase was accounted for by the emergence of AM expressing the surface phenotype of both dendritic cells and 'classic' macrophages.

To test the possibility that relative changes within these AM subsets may directly
influence lymphocyte inductive capacity, experiments were set up to isolate them from BAL. A combination of techniques involving cell adherence, metrizamide density gradients and antibody-conjugated magnetic beads were used. This enabled a detailed analysis of their individual phenotypic, physiological and functional characteristics. All 3 AM subsets isolated from normal volunteers displayed distinctive features. In addition, individual cell phenotype could be related to specific functional capacity. It was clearly demonstrated that within the AM pool, besides 'T-cell inducer-macrophages' and 'classic phagocytic macrophages', there existed a double phenotype macrophage subset capable of actively down-regulating T-cell responsiveness. With the advent of disease, changes in the proportion of these AM subpopulations were accompanied by sarcoid-related differences in phenotype, physiology and function. In particular, double phenotype AM from active sarcoid patients expressed a separate antigen, which identifies epithelioid cells, and showed an intense reaction for fibronectin. The T-cell suppressive action of this AM subset was also noted to be enhanced in sarcoidosis.

The above cellular aberrations in the lower respiratory tract of sarcoid patients do not seem to be pathognomonic to the disease itself. Current studies have shown that besides overlapping clinical features, both sarcoidosis and primary biliary cirrhosis share the same underlying mechanisms of macrophage-T-cell interaction, at least in the lung.

Finally, these local immune responses could be directly manipulated by therapeutic regimes. In an 18-month placebo-controlled study, it was seen that both inhaled and systemic corticosteroids were capable of altering the aberrant cellular profiles (including function), possibly through an influence on the AM population. Such immune modulation was accompanied by clinical improvement.
The information in this thesis bears testimony to the hypothesis that the changes observed within the AM population in the sarcoid lung are partly controlled by factors arising in the local milieu. Such changes are finely balanced, and as such are critical to the outcome of the cellular immune response in pulmonary sarcoidosis.
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SPITERI M A, POULTER L W
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Phenotypic and functional changes in alveolar macrophages contribute to the pathogenesis of pulmonary sarcoidosis

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Eur Resp J 1989; 2: 218 - 224

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Sarcoidosis 1989; 6: 107 -110

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The macrophage in sarcoid granuloma formation
Sarcoidosis 1989; 6: 12 - 14

SPITERI M A, JOHNSON M A, EPSTEIN O, SHERLOCK S, CLARKE S W, POULTER L W
Immunological features of lung lavage cells from patients with primary biliary cirrhosis may reflect those seen in pulmonary sarcoidosis
Gut 1990 (in press)

SPITERI M A, POULTER L W
Isolation of human alveolar macrophage subpopulations with distinctive phenotypic, physiological and functional characteristics.
(submitted for publication)

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The emergence of a 'suppressor' alveolar macrophage subpopulation may contribute to the pathogenesis of pulmonary sarcoidosis
(in preparation)

SPITERI M A, POULTER L W, CLARKE S W
The effect of long-term inhaled and systemic corticosteroid therapy on the local macrophage population in pulmonary sarcoidosis
(in preparation)
### Abbreviations

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<tr>
<td>AM</td>
<td>alveolar macrophage</td>
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<tr>
<td>AMDGF</td>
<td>alveolar macrophage-derived growth factor</td>
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<td>AMLR</td>
<td>autologous mixed lymphocyte reaction</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<td>BALM</td>
<td>mitomycin-treated bronchoalveolar lavage cells</td>
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<tr>
<td>BTTA</td>
<td>British Thoracic and Tuberculosis Association</td>
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<tr>
<td>cpm</td>
<td>counts per minute</td>
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<tr>
<td>CD</td>
<td>cluster designation</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<td>CT</td>
<td>computerized tomography</td>
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<tr>
<td>CXR</td>
<td>chest X-ray</td>
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<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td>ESR</td>
<td>erythrocyte sedimentation rate</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>forced vital capacity in 1 second</td>
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<td>Fig.</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FRC</td>
<td>functional residual capacity</td>
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<tr>
<td>FVC</td>
<td>forced vital capacity</td>
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<td>67Ga</td>
<td>gallium-67</td>
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<td>G-6-PD</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<tr>
<td>H. &amp; E.</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>HLA</td>
<td>histocompatible leukocyte antigen</td>
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<td>HMS</td>
<td>hexose monophosphate shunt</td>
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<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>³⁵H-Tdr</td>
<td>tritiated thymidine</td>
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<tr>
<td>IL-1</td>
<td>interleukin-1</td>
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<td>IL-2</td>
<td>interleukin-2</td>
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<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
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<tr>
<td>KCO</td>
<td>transfer coefficient</td>
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<td>kd</td>
<td>kilodalton</td>
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<tr>
<td>kPa</td>
<td>kilopascal</td>
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<td>ly</td>
<td>lymphocyte</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility gene complex</td>
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<td>mo</td>
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<td>monoclonal antibody</td>
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<td>MPC</td>
<td>magnetic particle concentrator</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<td>PBC</td>
<td>primary biliary cirrhosis</td>
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<tr>
<td>PEFR</td>
<td>peak expiratory flow rate</td>
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</table>
PBM peripheral blood mononuclear cells
PBMM mitomycin-treated peripheral blood mononuclear cells
PBS phosphate buffered saline
PBS/BSA phosphate buffered saline with bovine serum albumin
q.v. qua vide
RFH Royal Free Hospital
RFHSM Royal Free Hospital School of Medicine
RV residual volume
SACE serum angiotensin converting enzyme
S.E. standard error
S.I. stimulation index
SRBC sheep red blood cells
TLC total lung capacity
TLCO transfer factor for carbon monoxide
TRITC tetramethyl-rhodamine-isothiocyanate
UV ultraviolet

NOTE:
In tables 5.6 and 5.7, as well as Fig. 5.7 for clarity the AM subsets are represented as
D1=RFD1+D7- macrophages
D7=RFD1-D7+ macrophages
D1D7=RFD1+D7+ macrophages
CHAPTER ONE

INTRODUCTION

WHAT IS SARC OIDOSIS?

"It is a riddle wrapped in a mystery,
inside an enigma...."

W S Churchill, 1951.
PART ONE
Today, sarcoidosis is recognized as a multisystem granulomatous disorder in which the aetiology continues to be an enigma. This disease commonly affects young adults, and frequently presents with bilateral hilar lymphadenopathy with or without pulmonary infiltration, ocular and/or cutaneous lesions. The diagnosis is firmly established when well-recognized clinical and radiographic findings are supported by histological evidence of discrete non-caseating epithelioid cell granulomata in one or more organs. In addition, a positive Kveim-Siltzbach skin test and cutaneous anergy to tuberculoprotein are often present. Immunological features suggest aberrant cell-mediated reactions at the site of inflammation, in the presence of hypergammaglobulinaemia (James et al, 1976a). The disease is frequently self-limiting, with spontaneous resolution; however, in a few patients there is a progressive downhill course culminating in irreversible fibrosis and severe impairment of organ function (Richer & Clark, 1949).

The evolution of this description for sarcoidosis has occurred over a period of more than 100 years. During this time several workers have struggled to give a coherent working definition that would encompass the clinico-immunopathological nature of the disease, the cause of which remains unknown. Earlier reports on sarcoidosis just focused on a number of independently-described manifestations occurring in various tissues and organs. Later it became apparent that such clinical features not only shared a common histological pattern, but that they could also exist in the same patient, in various combinations. Such observations have given rise to today's concept of sarcoidosis as a systemic disorder (Scadding & Mitchell, 1985).

**HISTORICAL BACKGROUND**

Initial reports on sarcoidosis were largely descriptive, drawing attention to its cutaneous manifestations. The first recorded and well-illustrated examples of
erythema nodosum (a frequent cutaneous feature of acute sarcoidosis) were described in 1808 in Robert Willan's treatise 'On Cutaneous Diseases'. He described ".... large and rounded red patches.... on the fore parts of the legs.... seen mainly in females." In this paper, Willan also underlined the constitutional upset commonly observed before the onset of the rash, and which abated spontaneously on appearance of the erythema.

In contrast, Besnier in 1889 described a more chronic form of skin disease consisting of violaceous swellings of the nose, ears and fingers, and coined the term 'lupus pernio' (now commonly associated with a chronic form of sarcoidosis). Hutchinson (1898) also observed examples of such "..... multiple raised dusky-red patches" in his patients. He suggested that ".... these lesions belonged to the lupus family", yet they ".... differed widely from all other forms of lupus, both in features and in course.... in particular, the absence of all tendency to ulcerate or form crusts." In 1897 at the Christiania Medical Society in Oslo, Caesar Boeck presented his 34 year old police constable patient, who was suffering from skin lesions similar to those described by Besnier, but who in addition also had a generalised lymphadenopathy. The skin biopsies were reported to have shown histological evidence of ".... sarkoid (sic)* tissue, consisting of sharply defined non-caseating foci of epithelioid cells and some giant cells permeating the corium". Although the lymph nodes were not examined histologically, Boeck suggested that the cutaneous and glandular features were due to a ".... generalised disorder, allied in some way to tuberculosis". Just before his death, he published his detailed work on 24 patients with varying degrees of involvement of the lungs, conjunctiva, bone, lymph nodes, spleen and nasal mucosa. Such descriptions were among the first to suggest the potential multisystem nature of sarcoidosis. In 1904, Karl Kreibich described cystic changes in the bones of the hands in connection with lupus pernio. Such features were also reported by other

* derived from the Greek 'sarkos' meaning 'flesh'; hence the word 'sarcoid' for 'resembling flesh'.

independent workers (Rieder, 1910; Jungling, 1920). Indeed, in 1928 Jungling acknowledged the close association of such bone cysts with lupus pernio and Boeck's sarcoid in the same patients.

The involvement of the eye in acute sarcoidosis was initially described by Schumaker (1909) and Bering (1910). They also independently noted the simultaneous involvement of the parotid and submaxillary glands. While Heerfordt (1909) drew attention to 'fibrio uveoparotidea' in association with visceral malfunction, Waldenstrom (1937) finally established that this syndrome was yet another presentation of Boeck's sarcoidosis and distinct from tuberculosis. He emphasized that such patients had negative tuberculin skin tests, histological presence of granulomata in the affected tissues with absence of caseation, and persistent negative cultures for tubercle bacilli.

In 1915, Kuznitsky and Bittorf respectively drew attention to the pulmonary manifestations of sarcoidosis, while Jorgen Schaumann (1934) gave histological evidence linking the pulmonary lymphadenopathy with other presenting features of the disease, such as erythema nodosum and uveitis. He stressed the distinction from other granulomatous disorders. Later studies compared the clinical and radiological features of sarcoidosis with the histological manifestations of the disease in skin and lymph node biopsies (both of which showed evidence of sarcoidosis). Such observations were among the first to highlight the frequent association of erythema nodosum with bilateral hilar lymphadenopathy at initial presentation.

Soon this descriptive era of sarcoidosis gave way to the age of technology. In 1935 Salvesen remarked that "considerable hyperglobulinaemia" could be found in sarcoid patients. This paved the way to numerous reports on the biochemical derangement
possible in this disease, such as hypercalcuria with or without hypercalcaemia (Goldstein et al, 1971; Studdy et al, 1980). During this time a search was also in progress for a diagnostic tool to differentiate sarcoidosis from tuberculosis and other granulomatous disorders. Based on the hypothesis that sarcoidosis might be a viral disease, Williams and Nickenson (1935) obtained sarcoid tissue from a skin lesion of an affected patient, and suspended it in saline. Following heat sterilization, the preparation was inoculated intradermally into 4 patients with suspected sarcoidosis and 4 normal controls. Within 24 hours, a firm red papule was observed at the injection site in the first group. This cutaneous reaction persisted for a week. No such reaction was observed in the control group. In 1941, Kveim carried out similar experiments using an intracutaneous inoculation of heat-killed suspensions of sarcoid lymph nodes. He observed that within 1 to 4 weeks of injection, biopsies of these papules showed histological evidence of sarcoid tissue. Kveim noted that such lesions did not occur in patients with lupus vulgaris, thereby concluding that this skin test could be used to differentiate sarcoidosis from tuberculosis. In support Siltzbach (1961) showed that antigen prepared from the sarcoid spleen of a patient could serve as an effective test agent for detecting sarcoidosis world wide, despite patient differences in sex, race, clinical, radiological and immunological presentations.

By the beginning of this century, it had become acknowledged that 'Boeck's sarcoi'd' was indeed a distinct entity, with a mysterious aetiology, that could give rise to a wide spectrum of immunological and clinical expressions. In order to amalgamate the work of various sarcoid research groups throughout the world, the first world conference was held in 1958 at the Brompton Hospital, London (James & Jones Williams, 1985). Such meetings have continued to the present day. In particular, these conferences have witnessed the promotion of (a) the use of immunological techniques in diagnosis, and in monitoring the course of the disease; (b) the recognition of the importance of the cellular and non-cellular components of bronchoalveolar lavage (BAL) fluid in the pathogenesis; as well as (c) the advent of monoclonal antibody probes to investigate
immune effector cells in BAL and tissue biopsies.

These innovations have made a tremendous impact on the current understanding of the underlying immunopathological mechanisms operative in sarcoidosis. For although the cause of sarcoidosis remains unknown, they have provided fresh insights into this disease. It is now clear that sarcoidosis is not a disorder mediated by a generalized impaired cellular immunity as early reports tended to suggest; rather its protean clinical expressions are a reflection of localized aberrant immunological responses (involving both lymphoid and non-lymphoid cellular elements), to an as yet unknown stimulus or stimuli, which give rise to a granulomatous inflammation in one or more organs (q.v. below).

POSSIBLE AETIOLOGICAL FACTORS

Despite extensive research there is as yet no identifiable aetiological agent to account for the epithelioid/giant cell granuloma that characterizes sarcoidosis. Infectious agents, chemicals and drugs, allergy, autoimmunity and genetic factors have all been explored as potential causes of this disorder.

Most of the studies have focused on an infectious aetiology, possibly because of the superficial resemblance of sarcoid granulomata to those found in certain infections such as tuberculosis, leprosy and schistosomiasis. Many workers have tried to show that sarcoidosis is an aberrant form of tuberculosis (Buck & McKeesick, 1961a). In fact the relationship between sarcoidosis and tuberculosis has been extensively debated (Uderfeldt et al, 1982). However, the most complete summary available on the evidence for and against tuberculosis as an aetiological factor in sarcoidosis is that provided by Siltzbach (1968). He concluded that one disease is not a cause of the other; in particular tuberculosis did not appear to give rise to or to protect against sarcoidosis. Attempts to fulfill Koch’s postulates by isolating the mycobacteria from
sarcoid tissue samples (Nethercott & Strawbridge, 1956), or by inducing clinical tuberculosis with injection of sarcoid tissue into laboratory animal hosts (Bowman et al, 1972), have failed. A search for circulating antibodies to M. tuberculosis and to other mycobacteria in sarcoid patients has also proved inconclusive (Chapman & Speight, 1964). One study, on a limited number of patients with systemic sarcoidosis, observed the presence of acid-fast coccobacillary forms within the biopsy material obtained from such patients (Cantwell, 1982). It was postulated that the cell wall-deficient bacteria, possibly related to mycobacteria or corynebacteria, may be the causative agents in some sarcoid patients. However, although non-diphtheria corynebacteria may produce a recognizable clinical syndrome resembling sarcoidosis (Lipsky et al, 1982), such case reports remain only interesting incidental observations as they have not been, in general, successfully related to the aetiology of sarcoidosis.

Hanngren et al (1974) discussed the possibility that both mycobacterial and viral infections may interact to produce sarcoidosis. They pointed out that the T and B lymphocyte disturbance found in sarcoid patients might be due to the effects of a viral infection depressing T-cell function, and of simultaneous mycobacterial infection stimulating B-cell function. Other investigators have hypothesized that sarcoidosis could follow BCG vaccination or a tuberculous infection in a person with a virus-induced T-cell defect (Lofgren & Lundback, 1950; Lantrop et al, 1972). Sporadic reports have been made of virus isolation, such as mumps, influenza, parainfluenza, Newcastle agent and measles virus particles, in patients with sarcoidosis (Lofgren & Lundback, 1950; James & Jones Williams, 1985). These have however been subsequently dismissed as possible laboratory contaminants (Lundback & Lofgren, 1952). On the other hand, it has been suggested that organisms found intermittently in sarcoid tissues are able to survive there because of the altered immunity in such patients. Burnet (1959) postulated that in these subjects organisms such as tubercle
bacilli or low grade viruses might persist as intracellular parasites, multiplying only sufficiently to keep more or less in pace with the cells that contain them. High antibody titres to a variety of viruses eg. Epstein-Barr virus have also been found in sarcoid patients (Hirshault et al, 1970).

Overall, the idea that infectious micro-organisms could play a role in sarcoidosis remains largely speculative. Repeated tissue cultures and electron microscopy have failed to uncover any specific infectious agent in sarcoid patients, despite the above random claims. In addition a thorough examination of the suspended sarcoidal tissue used in the intracutaneous Kveim test has so far not revealed the nature of the responsible agent (Rocklin, 1983).

In view of the immunological features found in sarcoidosis (eg. raised serum immunoglobulins, involvement of T and B cells, circulating immune complexes), it has been hypothesized that sarcoidosis may represent a form of hypersensitivity to inhalation of organic antigens in the environment. Inhalation of pine pollen and peanut dust (Konig et al, 1981), clay soil (Buck & Sartwell, 1961b), talc (Farber et al, 1982) and secondary oxalosis (Fayemi & Ali, 1980) have all been incriminated as contributory regional factors in different areas. Exposures such as these are now recognized causes for silicate pneumoconiosis and hypersensitivity pneumonitis. However their role in relation to the pathogenesis of sarcoidosis remains unclear.

Exhaustive skin testing with metals and other inorganic substances in sarcoid patients and controls has not revealed any peculiar hypersensitivity to chemicals. Beryllium (Williams & Williams, 1983) and zirconium (Shelley & Hurley, 1958) are known to produce granulomata in the sensitized individual. Although such granulomata are found at the injection site one month after inoculation, and are histologically similar to those produced in the Kveim response in sarcoid patients, each skin test is specific for its own disorder, and there is no overlap. The common denominator to such dissimilar
stimuli appears to be susceptible individuals, with heightened local cellular-mediated immune responses to known (or unknown) antigen or antigens.

The occurrence of sarcoidosis in members of the same family has suggested that genetic factors might be involved (Prendiville et al, 1982; Priestley & Delaney, 1981), but no firm relationship has been demonstrated. Indeed, the exact mode of inheritance is not clearly known, although some workers have suggested an autosomal recessive mode of inheritance (James et al, 1974). Sarcoidosis has been reported to be more common in monozygotic, rather than heterozygotic, twins (Sharma et al, 1976). In addition, several investigators have indicated that various features of sarcoidosis may be associated with specific antigens of major histocompatibility loci. HLA-B8 has been associated with erythema nodosum (Guyatt et al, 1982) and arthritis (James & Neville, 1977); while HLA-B27 is found in a high percentage of patients with uveitis (Scharf & Zonis, 1980). Persson et al(1975) reported a statistically significant increase of HLA-B7 in 47 patients with negative reactivity to tuberculin. In contrast, the frequency of this allele was zero in the group with positive response to tuberculin. The most interesting HLA association in sarcoidosis has been that of B8, DR3 in patients with acute sarcoidosis (Hedfors & Lindstrom, 1983). Kremer(1986) has suggested that the B8, DR3 phenotype identifies a group of patients who are more likely to develop acute sarcoid arthritis and hilar adenopathy without developing the chronic disease. Other workers have shown an association of HLA-B8 with early resolution of the disease (Smith et al, 1981); while patients expressing HLA-B13 are more prone to have persistent disease (Neville et al, 1980). Despite such findings there is as yet no definitive evidence for the 'linkage' of the HLA associated alleles with disease susceptibility gene(s) in sarcoidosis.

In conclusion, the aetiology of sarcoidosis continues to remain elusive. It appears that genetic, environmental, nutritional and socio-economic factors could play a critical
governing role in the development of sarcoidosis in an immunologically susceptible human individual. Additional support for this view may be gained by the observation that nothing quite like sarcoidosis is seen in the veterinary world! Non-specific local sarcoid reactions are seen in some animals, but these appear to be different from the generalized multisystem disease seen in humans (Hall et al, 1969).
PART TWO
CLINICAL PRESENTATION

Sarcoidosis is a relatively common disease, occurring worldwide with varying incidence and prevalence. In Europe, its frequency may range from 3 to 500 cases per 100,000 population, usually affecting the 20 - 40 year age group (Levinsky et al, 1976). There is no sex predominance, although in a few studies more women are affected (Neville et al, 1983). Although certain geographic locations appear to have a high prevalence of the disease, there is no identifiable, aetiologically significant, geographic pattern (Sartwell, 1976). Despite this, certain studies do show that sarcoidosis is more prevalent and probably more severe in certain populations. The prevalence in the white population in the United States is about 5 per 100,000 in contrast to about 40 per 100,000 in the black population (Honeybourne, 1980). The disease also seems to be more severe, chronic and disabling in the black population than in the white population. In support of these observations is the clinical finding that, white patients with sarcoidosis are more likely to present with acute symptoms, which are often associated with a good prognosis (Johns et al, 1976).

Although the clinical features of sarcoidosis may be absent or confined to a single organ, it is a generalised disease that may involve almost any organ in the body in various combinations, thereby giving rise to many clinical syndromes. This is generally regarded as the clinical hallmark of sarcoidosis. However, these diverse presentations of the disease need not be accompanied by generalised clinical upset. Involvement of an organ may be great without clinical features, so that anatomical presence of the disease need not be associated with clinical dysfunction. The reported frequency of the mode of presentation and organ involvement in sarcoidosis appears to be influenced by other factors such as the country of origin of the patient, the routine use of chest X-rays (CXR) in medical clinics, as well as the speciality interest of the examining physician. The time of onset of sarcoidosis may be difficult to assess unless an acute event such as erythema nodosum or acute uveitis reflects the beginning of the
Approximately 20 - 40% of patients are symptom-free and their disease is usually discovered by routine CXR. Although the first awareness of sarcoidosis in these patients may be an abnormal routine CXR, this finding does not necessarily reflect the time of onset unless it can be determined from serial radiographs. Another 25% of patients seek medical advice because of cough and dyspnoea, while an additional 25% present with eye, skin, or nasal complaints. Constitutional symptoms such as fever, fatigue, malaise, anorexia and weight loss are usually absent or mild. Yet in 20 - 30% patients with sarcoidosis these systemic features are striking. In most patients, all manifestations of the disease disappear within a few months or years. In certain patients a chronic, benign course is characteristic. 10-15% of sarcoid patients have progressive disease, with ensuing major chronic disability. In this latter course, extrathoracic features are prominent. These may range from skin lesions to peripheral lymphadenopathy, parotid enlargement, central nervous system involvement, cardiac syndromes, hepato-splenomegaly, arthralgia, nephrocalcinosis and hypercalcaemia. These features appear to be more severe in elderly patients. (Katz, 1983; James et al, 1976b). The proportions of patients presenting with various manifestations are however likely to be affected by the way the epidemiological data is collected. It appears, that of the studies conducted worldwide, the BTTA survey of four areas in Great Britain (1969) gives the most reliable estimate of relative frequency of the various ways in which patients with sarcoidosis first come to medical notice.(Table 1.1)

The protean clinical manifestations and course of sarcoidosis lead to considerable inherent variability within the patient population as a whole, as well as in the same individual under evaluation. This raises the importance of selecting relatively homogeneous groups of patients, who appear to be in the same clinical stage of their process.
### TABLE 1.1

Percentage (%) of patients reported to show manifestations referable to various organs at first presentation. Results from the BTTA survey collected from four areas in Great Britain (1969).

<table>
<thead>
<tr>
<th>% presenting with:</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of cases</td>
<td>224</td>
<td>343</td>
</tr>
<tr>
<td>Abnormal CXR</td>
<td>35.7</td>
<td>28.0</td>
</tr>
<tr>
<td>Respiratory symptoms</td>
<td>25.9</td>
<td>18.1</td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>23.2</td>
<td>34.7</td>
</tr>
<tr>
<td>Febrile arthropathy</td>
<td>3.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Constitutional symptoms</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Ocular symptoms</td>
<td>2.7</td>
<td>5.5</td>
</tr>
<tr>
<td>Cutaneous sarcoid</td>
<td>4.0</td>
<td>8.5</td>
</tr>
<tr>
<td>Superficial lymphadenopathy</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Parotid gland enlargement</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Dactylitis</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Nervous system changes</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Myositis</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Cardiac symptoms</td>
<td>--</td>
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</tr>
</tbody>
</table>
Sarcoidosis, with all its diverse clinical presentations, shares many overlapping features with other multisystem, chronic granulomatous disorders, such as primary biliary cirrhosis (PBC) (Bass et al, 1982). Indeed, in some patients the distinction between sarcoidosis and PBC can prove impossible. Recently, these similarities were further emphasized by the finding of a subclinical, mononuclear inflammatory infiltrate in the lungs (termed 'alveolitis') of both sarcoid and PBC patients, who were clinically asymptomatic and had normal CXR (Wallaert et al, 1986). Such findings not only confirmed that both diseases were promoted by an immunological response, but also raised the possibility of an identical underlying granuloma-producing mechanism.

**PULMONARY MANIFESTATIONS**

The lung is the organ most commonly involved in sarcoidosis. It is the site most commonly associated with morbidity and (rarely) mortality. At least 90% of patients with sarcoidosis exhibit abnormalities on their CXR during the course of their disease (James et al, 1976b). 20-25% of these patients have a permanent loss of lung function; 5-10% die from complications of the disease (Mitchell & Scadding, 1974). Patients are commonly asymptomatic, or their respiratory symptoms may start insidiously with dry cough, progressive dyspnoea, exercise intolerance, and chest pain (Israel, 1970; Bacharach, 1961). Examination of the chest is often normal, but may reveal dry, late inspiratory crackles at the posterior bases. Any aspect of lung function may be abnormal in sarcoidosis. When present, this typically involves a decrease in diffusion capacity, with or without a loss in lung volume (Mitchell & Scadding, 1974). The type and degree of the physiological abnormality relate to the location and the extent of the pathology. For this reason, granulomatous infiltration of the bronchial wall may produce airflow limitation. This is usually common in those sarcoid patients who have diffuse parenchymal disease (Westcott & Noehren, 1973; Dines et al, 1978).
Although the precise pathogenic mechanisms of sarcoidosis are unknown, it is speculated that the lung is one of the first sites of involvement. The inflammatory process seems to extend through the lymphatics to the hilar and mediastinal nodes (over 85% of patients with pulmonary sarcoidosis have radiographically apparent mediastinal and hilar lymph node involvement). As in miliary tuberculosis, lymphohematogenous spread may then occur throughout the lung as well as to other favoured organs such as the liver (Katz, 1983).

The clinical course of the pulmonary sarcoidosis is related, at least in part, to the appearance of the disease on chest x-ray (Siltzbach et al., 1974; Wurm & Rosner, 1976; DeRemee, 1983). Patients with pulmonary sarcoidosis have been divided into 5 clinical groups according to the appearance of the chest film. Indeed by using a radiological staging, Siltzbach (1967) noted a clear relationship between the prognosis of sarcoidosis and the initial radiological appearance of the disorder, as well as associated extrathoracic features.

**Stage 0:** CXR is radiologically normal and only extrapulmonary disease is apparent in these patients. 5 -10% of patients with sarcoidosis have normal CXR at time of initial presentation (Dunbar, 1978). Such patients tend to have the more chronic features of the systemic sarcoidosis (q.v. above). This stage may represent either a late phase of the disease in which the intrathoracic manifestations originally present have cleared, leaving only the extrathoracic lesions; or the existence of an isolated form of extrapulmonary sarcoidosis with a different pathogenic mechanism.

**Stage 1:** CXR shows bilateral hilar lymphadenopathy (with or without paratracheal node involvement)(Fig. 1.1a). Many observers believe that this is the earliest clinically detectable form of sarcoidosis (Kirks et al., 1973). It is seen in about 40 - 60% of sarcoid patients. Patients with Stage I disease have minimal chest symptoms or are asymptomatic, unless there are other associated features. Over 85% of these
patients have erythema nodosum, and about 70% have polyarthralgia, usually occurring within two weeks before or after the onset of the skin lesions. There may be associated fever, malaise and fatigue. The constitutional, joint and skin features subside within an average of 3 weeks. Involvement of lymph nodes by sarcoidosis seems to be self-limiting. 60-80% of patients with Stage 1 disease show spontaneous resolution of lymphadenopathy within 6 months to 2 years (Dunbar, 1978). The resolution rate for the CXR approaches 90% for these patients who also have erythema nodosum. After returning to normal size, the lymph nodes rarely enlarge again (Schabel et al, 1978). The remaining Stage I patients either have a stationary radiographic appearance (10 - 20%), or may gradually advance to Stage II (10 - 15%) over a period as long as five years (James et al, 1976b).

Stage II: CXR shows hilar adenopathy and parenchymal infiltrates (Fig. 1.1b). This is noted at presentation in 25 - 35% of patients (James et al, 1976b). The pulmonary infiltrates may be localised and unilateral, but more often they are diffuse and symmetrical. Fine reticular radiations appear to extend into the lung fields from the enlarged hilar nodes. The middle lung zones and perihilar area show the most marked changes. Frequently as the parenchymal changes develop there is a regression of the lymph node enlargement, or at least a cessation of growth. There is no evidence to indicate that hilar node enlargement may develop subsequent to the pulmonary parenchymal disease. Patients are often asymptomatic or mildly symptomatic with low grade fever, cough, malaise, weight loss or tachypnoea. When pulmonary involvement is extensive, significant dyspnoea may be present. In 49 - 65% of Stage II patients, X-ray resolution occurs within two years of onset. Irreversible pulmonary fibrosis can develop in about 15 - 20% of patients, while the rest will show persistent infiltration without fibrosis, symptoms or signs (Katz, 1983).

Stage III: CXR shows clearing of intrathoracic lymph nodes with persistence or progression of the pulmonary infiltrates. This stage is observed at presentation in 5 - 15% of sarcoid patients. The commonest parenchymal abnormality is a
Fig. 1.1

Postero-anterior chest radiographs showing (a) bilateral symmetrical hilar lymphadenopathy: Stage I sarcoidosis; (b) bilateral hilar and paratracheal adenopathy, in addition to fluffy ill-defined shadowing, predominantly scattered in the mid- and lower zones: Stage II sarcoidosis.
reticuloniodular pattern, usually bilateral and less marked in the apices and bases (Kirks et al, 1973). Such parenchymal shadowing may show signs of resolution in about 20% of Stage III patients within 2 - 3 years of onset. It may however remain apparently non-progressive, with only minimal functional defects or symptoms, for long periods of time. There is however a distinct tendency towards fibrosis as the process ages (Fig. 1.2).

**Stage IV:** CXR shows diffuse, dense, progressive fibrosis with upward retraction of the hilar areas as well as honeycombing and bulla formation (Freundlich et al, 1970). The transition to interstitial fibrosis is subtle, so that the early changes of fibrosis may be difficult to appreciate radiologically. Clinically the diagnosis of this stage is usually prompted by the presence of linear opacities on CXR persisting unchanged for as long as 6 months, in the presence of decreased lung volumes. Such patients may develop pulmonary hypertension and right ventricular failure, as well as aspergillomas in the emphysematous cavities (Winterbauer & Kraemer, 1976). Dry cough and shortness of breath are common complaints in both Stage III and Stage IV patients. Extrathoracic manifestations in these patients are common; the appearance of which are are taken to indicate pulmonary chronicity, culminating in respiratory failure.

The above staging is arbitrary and based on radiological evaluation using routine postero-anterior and lateral views of the chest. The temporal course of events in progressive sarcoidosis has been suggested as the transition from Stage I to Stage IV (Scadding, 1961). It has been observed that sarcoid patients with radiological evidence of pulmonary disease, who do not appear to have recorded hilar adenopathy, have an increased tendency to develop fibrosis (Scadding, 1967). However, other workers have pointed out that these *classic* X-ray stages do not necessarily represent sequential progression, or indeed signify activity of the disease (De Labarthie & Chretien, 1981).
Fig. 1.2

Postero-anterior chest radiograph revealing diffuse parenchymal involvement with fibrotic and cystic changes, mainly in the upper lobes. In addition, there is also upward retraction of both hila, and apical volume loss. Stage IV sarcoidosis.
CORRELATIONS OF DIAGNOSTIC PARAMETERS

Prognostic signs and indicators of disease activity are necessary to determine the need for treatment. Such measures to date have included clinical, radiographic and pulmonary physiologic parameters. Unfortunately while general guidelines have been established, these measurements are often unreliable in predicting the course of individual patients. One major difficulty is that there is as yet no consensus as to the precise meaning of activity of sarcoidosis (Turner-Warwick, 1982; Junod, 1982).

Some reports have mistakenly interpreted activity in terms of the stage of the granuloma (recent or ancient), or the extent of the disease (De Labarthe & Chretien, 1981). The term activity has also been applied to reflect the evolution and outcome of the disease. However in practice it is seen that in particular groups of sarcoid patients, while the disease is often ascertained to be intense by certain indices, the majority of the patients recover spontaneously within a short time and without treatment (Valeyre et al, 1984). Another aspect to be considered is the multisystem involvement of the disease. In practice, patients who have an intense granulomatous inflammation within one organ, do not necessarily have serious general involvement (q.v. above). Indeed recent studies have shown that the old concept of a single or multiple extrathoracic organ involvement, as an indicator of poor prognosis, remains largely unconfirmed (Chretien et al, 1986).

Ideally the parameters for disease activity in sarcoid patients should not only be able to indicate the presence of disease in situ, but also to reflect the events involved in the maintenance and outcome of the granuloma formation. Such well-defined predictive criteria, as opposed to merely activity markers can then be used (1) to serve as reliable prognostic indicators early in the course of disease, so that those patients who are destined to deteriorate can be separated from those who will resolve spontaneously; (2) to serve in monitoring and planning of therapeutic regimes; (3) to identify the small group of patients who would reap the greatest benefit from
aggressive steroid treatment.

**CHEST X-RAY vs HISTOLOGY**

One constant index used in the follow-up of patients with sarcoidosis is the evaluation of serial CXRs. Studies have shown that the radiographic and histological findings in Stage I patients are not clearly related. Biopsy data have demonstrated that all Stage I patients have varying degrees of inflammation in their lung tissue (Rosen et al, 1977; Poe et al, 1979). In support, Winterbauer and Hutchinson (1980) found granulomata and interstitial pneumonitis in all lung biopsies of 19 patients with Stage I CXR.

Such observations indicate that although the lung fields may appear to be radiologically clear in sarcoidosis, parenchymal involvement is present histologically. In a long-term radiological follow-up of 95 patients, Kirks et al (1973) noted the development of an 'acinar pattern' during the course of the disease in 32 of these patients. This pattern was characterised radiologically by large segmental consolidation and hazy shadows with irregular borders. When correlated to pathological findings, it appeared that this acinar pattern reflected a secondary, non-specific response of the lung to the primary interstitial injury (Sahn et al, 1974). A third of these patients were observed to progress to fibrosis, which in some cases was rapid indicating very active disease. This was an important finding, as in general, radiological transition to fibrosis in sarcoid patients is subtle.

**CHEST X-RAY vs PULMONARY FUNCTION**

Studies have shown that Stage I patients frequently have abnormalities of pulmonary function (DeRemee & Andersen, 1974; Marshall & Karlish, 1971). However the abnormal physiological function detected in these early stages of sarcoidosis is usually not associated with significant disability and is rarely of consequence to the patient (Sharma et al, 1966). In contrast some patients with extensive x-ray changes may have few symptoms and normal lung function. It has been suggested that such discrepancies may reflect a form of sarcoidosis in which localised visible lesions are not associated with diffuse microscopic disease in other parts of the lung, and so
pulmonary function remains intact. Despite these exceptions, it is generally agreed that as the disease becomes more evident on CXR, impairment of diffusion capacity and vital capacity become more common (Kanagami et al, 1961; Kent & Spence, 1964). Miller et al (1976) observed a significant drop in these two indices in Stage II disease as compared to Stage I. However data into the correlation of duration of disease (as judged radiologically) and impairment of function remain controversial. It has been suggested that such a relationship is spurious, as in practice patients with mild disease and little functional upset may never be studied or followed up; whereas symptomatic patients with more severe illness and persistent radiographic abnormalities are more apt to receive continuing attention and to have repeated pulmonary function studies.

CHEST X-RAY vs CT SCAN

It has been suggested that computerised tomography (CT) of the lung could provide a more informative, initial assessment of the pulmonary changes in some sarcoid patients than just routine CXR. Nodular, cavitary and more important, early diffuse parenchymal disease can be easily visualised by CT scan. In addition, some workers have shown that findings from CT images correlate more closely with pulmonary function abnormalities than do those from standard radiographs (Putmann et al, 1977). CT scanning of the chest in these patients has also clarified interpretation of the plain film abnormalities eg. linear opacities on CXR have been verified to represent interstitial changes, based on the coarse honeycombed pattern seen on CT images (Solomon et al, 1979). Small isolated granulomata of the lung, not visualized radiologically, may be demonstrated by CT as round opacities 3-4 mm in diameter. In theory it seems that CT scan of the chest provides more reliable information about the lung parenchyma than the CXR, and allows more sensitive follow-up of established abnormalities.

CHEST X-RAY vs GALLIUM SCAN

Gallium scanning is a scintigraphic procedure which uses a cyclotron produced isotope (Gallium-67, $^{67}$Ga), that in the form of $^{67}$Ga-citrate is known to be taken up by activated macrophages, and to localize in regions of inflammation (Lavender et al,
Three days following an intravenous injection of 1.5 mCi of $^{67}$Ga citrate, simultaneous anterior and posterior scans of the entire thorax are done. The amount of uptake can be quantified by computerized methods (Van Unnik et al, 1983). Normal individuals show little $^{67}$Ga-uptake in pulmonary parenchyma; while two-thirds of patients with sarcoidosis demonstrate significant lung uptake (> 50 $^{67}$Ga index limits) (Line et al, 1978). The pattern of uptake on the scan is generally highly variable in distribution, intensity and extent of lung involvement. During the course of the disease, the pattern may change rapidly in character, throughout different regions of the lung. Gallium scans can not only be used to identify areas of pathology in the lung, but also to assess the spatial extent of the disease and the presence of radiologically unsuspected disease foci. Studies have found no correlation between CXR staging and activity of disease as quantified by $^{67}$Ga scans (Siemsen et al, 1976). It has been observed that normal $^{67}$Ga-uptake can occur in regions of stable pulmonary infiltrates in serial CXRs; while patients with normal or minimally abnormal CXRs may have intense diffuse $^{67}$Ga-uptake in the lung. In addition $^{67}$Ga scans have been reported to detect abnormal lung tissue before the appearance of radiographically discernible changes in Stage I patients. No significant differences have been shown for patients with Stage II or III disease (Line et al, 1978; Klech et al, 1982). The hypothesis put forward for such observations is that $^{67}$Ga scans may pick up the more acute patients rather than the chronic stable ones. In support the degree of $^{67}$Ga-uptake correlates well with histological evidence for active disease (Israel et al, 1986).

While data so far suggests that $^{67}$Ga scans are more sensitive than plain CXR films in estimating the extent and intensity of the acute inflammation in the alveoli, it appears that a positive gallium scan by itself appears to provide little information for therapeutic assessment of sarcoid patients (Klech et al, 1982; Lawrence et al, 1983).
It also seems from the above observations that a CXR is of limited usefulness in detecting the early signs of tissue involvement, unless the parenchymal abnormalities are large enough to be radiologically detectable. In comparison to the other parameters, conventional CXR films do not appear to provide enough information to adequately predict the patient's clinical course in sarcoidosis, or to distinguish areas of active inflammation from normal lung or irreversible interstitial fibrosis. Putmann and Hoeck (1986) suggested that this is because classic X-ray staging was purely based on a descriptive pattern of adenopathy or opacities. Their analyses utilized high KvP (130-140) films. In addition, they evaluated the CXR for the presence or absence of bronchograms, peribronchial cuffing and subpleural thickening to define areas of cellular infiltration. By so doing, they observed that the radiological appearances not only reflected the inflammatory changes in the lung, but they also correlated well with results obtained by other indices such as $^{67}$Ga scanning.

**PULMONARY FUNCTION vs HISTOLOGY**

Attempts have repeatedly been made to correlate functional abnormalities with morphological changes in the lung. In the study by Winterbauer and Hutchinson (1980), 13 of 19 Stage 1 patients with histological evidence of inflammation in the lung parenchyma had abnormal lung function. The degree of interstitial infiltration appears to be reflected well by overall functional impairment (Carrington et al, 1976). A most complete analysis of function-structure relationship in sarcoidosis was carried out by Huang et al (1979), who compared an extensive range of pulmonary function parameters with open lung biopsy findings in 81 subjects. While such physiological indices correlated well with the overall severity of the morphological findings, they could not distinguish between inflammatory infiltrates, established granulomata and fibrosis. Indeed, patho-physiological correlations may prove difficult: as the lung biopsies may not necessarily reflect the overall pathology present in the lung, the resulting data may only have a limited interpretation.
Nevertheless, there is general agreement that of all physiological parameters, the reduction in diffusion capacity is the best correlate to the degree of interstitial involvement in pulmonary sarcoidosis (Snider & Doctor, 1964; Young et al, 1967). The current view is that such impairment of diffusion in pulmonary sarcoidosis is not due to thickening of the alveolar wall-blood gas barrier as previously thought (Divertie et al, 1976), but rather reflects ventilation-perfusion inhomogeneities and reduction of the functioning alveolar-capillary units in the lungs of these patients.

**PULMONARY FUNCTION vs GALLIUM SCAN**

Niden et al (1976) showed that the more reactive the gallium scan, the greater the loss of lung function (such as vital capacity). No physiological deterioration was noted in those sarcoid patients in whom a normal scan was obtained and maintained. In contrast, other studies (Crystal et al, 1981a; Crystal et al, 1981b; Keogh et al, 1981) have shown that the extent of functional impairment (as defined by reduced total lung volume, reduced diffusion capacity and arterial hypoxaemia) bore little relationship to the degree of inflammation as measured by $^{67}$Ga scans. Such data might suggest that these physiological tests may not be specific enough for the extent of the parenchymal inflammation that can occur in sarcoidosis. It has therefore been suggested that pulmonary function parameters are more suitable to detect structural derangement as caused by granulomata and fibrosis, rather than the preceding alveolitis (Keogh & Crystal, 1980).

**BRONCHOALVEOLAR LAVAGE**

As it became recognized that the loss of alveolar-capillary units in pulmonary sarcoidosis may be due to an insidious alveolitis preceding the granuloma formation, it was suggested that analysis of the fluid in the lower respiratory tract obtained by BAL, would provide a more sensitive index of disease activity in a given patient (Daniele et al, 1985; Crystal et al, 1986; Rossi, 1986; Reynolds, 1987). Analysis has ranged from quantifying changes within the BAL cellular components to biochemical
A number of studies have shown that such a method may be superior to any clinical, radiological and physiological parameters in assessing the present status of the alveolitis and subsequent events in any individual patient (Keogh & Crystal, 1982; Hollinger et al, 1985; Costabel et al, 1986; Israel-Biet et al, 1986). These studies suggested that the degree of lymphocytosis in the lavage fluid was predictive of the disease activity and progression. As these observations have not been subsequently confirmed (Buchalter et al, 1986; Delaval et al, 1986; Turner-Warwick et al, 1986), it is now less widely accepted that a high initial lymphocyte count in BAL reflects a poor prognosis of the disease. While Bjerner et al (1988) agree with this latter view, they have also observed that a BAL lymphocytosis present on 2 successive investigations tends to correlate well with a deterioration of lung function. They therefore advocate that serial BAL measurements could be a more reliable estimate of disease activity. Ward et al (1989) have proposed that the observed disparity between studies could be partly due to genetic and racial differences (known to influence the mode of presentation: Scadding & Mitchell, 1985), as well as the heterogeneity that may exist within study populations. In addition, they found that an acute onset of sarcoidosis with high lymphocytosis in BAL offered a better prognosis, than disease of more insidious onset with low lavage lymphocyte counts. Recently, Foley et al (1989) concluded that the presence of a high BAL lymphocytosis could indicate a favourable prognosis for the outcome of lung function in pulmonary sarcoidosis, even in those patients with chronic disease. This suggests that the absence of a lymphocytosis in lavage does not necessarily indicate burnt out disease. In the study by Lin et al (1985), it was further suggested that in those patients with more extensive long-standing radiographic shadows, BAL neutrophils may be as important as lymphocytes in the assessment of disease activity, perhaps serving as an indicator to therapy.
Inconsistent correlations are found in the literature of the proportion of lavage T-cells as recovered by BAL and the amount of $^{67}$Ga- uptake by lung parenchyma (Gupta et al, 1979; Line et al, 1981; Beaumont et al, 1982). It has been suggested that this inconsistency may be due to the possibility that, while BAL fluid analysis reflected the alveolitis, gallium uptake reflected granuloma load: therefore the two parameters measured different factors. Of these two parameters, BAL is now believed to be more sensitive, and to yield more information; at least in the initial stages of the disease (Keogh & Crystal, 1980; Hunninghake et al, 1980a). In support, several studies have shown good agreement between the cellular findings in BAL fluid and those in lung biopsy specimens of the same patients (Campbell et al, 1985; Semenzato et al, 1985; Paradis et al, 1986). Data also suggests that the delineation of specific lavage lymphocyte and macrophage subpopulations found in the lavage fluid could serve as better parameters in assessing the current degree of lung inflammation, and in predicting subsequent events in a given patient. Indeed, a close relationship between the raised ratio of helper CD4+ : suppressor CD8+ T-lymphocytes and more progressive disease has been reported (Costabel et al, 1986). Recently, a correlation between phenotypically distinct macrophage subsets and radiographic staging was identified (Ainslie et al, 1989).

OTHER INDICES

Although various clinical criteria have been suggested to indicate activity of sarcoidosis (eg. weight loss, malaise, fever, cough and progressive dyspnoea on exercise), these features have been found to be insensitive and not specific for the inflammation in sarcoidosis (Crystal et al, 1981). Such features are likely to be influenced by the extent of the lung derangement as well as by disease in organs other than the lung. There is little evidence to show that blood test criteria, such as ESR, serum calcium, lysozyme and ACE*, as well as immune complexes and

* serum angiotensin converting enzyme (SACE)
immunoglobulins, can reflect changes in the density and activation of the effector cells within the alveolar structures as judged by BAL. In support, both false positives with high SACE levels in the presence of normal proportions of lavage cells, and false negatives with normal SACE levels in the presence of marked BAL cellular changes have been observed (Schoenberger et al, 1981; Schoenberger et al, 1982; Radermecker et al, 1984; Cohen et al, 1985). In a 2-year study by Choudat et al (1983) comparing the prognostic value of SACE to that of CXR, it was observed that SACE was more sensitive as an indicator of immediate disease activity in sarcoid patients. Indeed 23 of 28 patients with active sarcoidosis were found to have high SACE levels. In contrast, those patients in whom the sarcoidosis was clinically in remission had normal SACE. These findings have been supported by other workers (Lieberman et al, 1979; DeRemee & Rohrbach, 1980; Romer, 1981).

Overall the evidence available suggests that while SACE may be useful in the diagnosis of sarcoidosis, it is not a reliable prognostic index. SACE therefore is not helpful in monitoring the patient's clinical status, as it does not accurately reflect the intensity of the pulmonary inflammation in sarcoidosis.
PART THREE
THE IMMUNE RESPONSE IN SARCOIDOSIS

Much of the investigations into the pathogenesis of sarcoidosis, have aimed at determining how well the immune aberrations found in this disease fit the current model of immune stimulation. Such immune phenomena have been observed both systemically, and locally in the lung (Fig. 1.3).

LUNG CELLULAR IMMUNE ABNORMALITIES

It has been a consistent finding from BAL analysis, that early disease in patients with sarcoidosis is characterized by a mononuclear cell infiltrate comprising of macrophages and T-lymphocytes (Hunninghake et al,1979; Hunninghake et al,1981). The lymphocytic presence distinguishes sarcoidosis from some other interstitial lung diseases where polymorphonuclear cells usually predominate (Hunninghake et al,1979). In addition, the presence of such a mononuclear cell infiltrate is an important clue that immune mechanisms are involved.

The type and proportion of cells recovered in BAL fluid in sarcoidosis have been reproducibly observed by different workers to be similar to those found in lung biopsy specimens from the same patients (Campbell et al,1985; Semenzato et al,1985; Paradis et al,1986). The lymphocytes associated with the granulomata are larger than usual, and have the morphological features of activated cells (q.v. below). The accumulation of lymphocytes around the granulomata is more prominent within fresh active lesions, and tends to diminish as the granulomata mature. These lymphocytes are mainly of the helper T-cell type, which express CD4+ surface antigens. As the lesions become less active these cells decrease, and suppressor CD8+ T-cells predominate (Paradis et al, 1986). A similar switch in immunoregulatory T-cells has also been seen in animal models of granulomatous lung disease. It is thought that this process may modulate granuloma formation (Chensue et al,1980; Chensue et al,1981).
Fig. 1.3

A simplified model for macrophage-T-lymphocyte interaction in the cellular-mediated immune response

MO = macrophage
IL-1 = interleukin 1
IL-2 = interleukin 2
IL-2R = interleukin 2 receptor
Y-IF = gamma interferon
Antigen Presentation and recognition

T-cell activation
- IL-2R expression
- IL-2 release

T-cell proliferation and differentiation
- IL2/IL2R binding

Production of lymphokine

Diagram:
- Antigen
- Class 2 MHC antigen
- Processed antigen
- T1-T3 receptor
- IL-1
- MHC receptor
- IL-2
- IL-2 receptors
- DR antigen
- Soluble IL-2R release
- Production of lymphokine
- M\(\phi\) chemotactic factors
- Y-IF
- B-cell growth factor
Such changes within the lung interstitium are accompanied by an increase in the proportion and total number of lymphocytes in BAL fluid. This increase consists mainly of T-helper cells. The ratio of helper: suppressor T-lymphocyte ratio in active sarcoid lavage is 4-10 times greater than that found in normal BAL (Hunninghake & Crystal, 1981a). The finding of an increased helper to suppressor T-cell ratio suggests the presence of an immuno-regulatory imbalance in sarcoidosis. Functional studies using these two lymphocyte subsets have been shown to agree with their phenotypic expression (Rocklin, 1983).

The lavage T-lymphocytes obtained from patients with active sarcoidosis also appear to be activated both morphologically and functionally. These cells show an augmented response to external stimuli such as lectins and antigens (Daniele et al, 1980). They exhibit active proliferation and spontaneously release various biologically active substances called lymphokines, which influence the migration and function of macrophages as well as other target cells. These T-cells also possess markers of cell activation including the capacity to form stable E-rosettes at 37 °C, the expression of class II MHC antigens on their surface, and the acquisition of the receptors for interleukin-2 (IL-2 or Tac antigen) (Thomas & Hunninghake, 1987). Activation of such T-cells is further supported by flow cytometry analysis of DNA content, which shows a subpopulation of T-lymphocytes in the proliferative phase of their cell cycle (S/G2) (Mornex et al, 1985).

Separate in vitro studies (Hunninghake et al, 1983; Pinkston et al, 1983) have demonstrated that lung T-lymphocytes from active sarcoid patients spontaneously secrete IL-2, the glycoprotein that stimulates proliferation in responsive T-cells. IL-2 is a central mediator, which has been postulated to be involved in at least 3 inter-related events in the pathogenesis of sarcoidosis: (a) the stimulation of T-cell
proliferation, and the expansion of the number of T-cells at the sites of inflammation; (b) the differentiation of T-lymphocytes into effector cells that produce additional lymphokines; and (c) the recruitment of additional helper T-cells from the peripheral blood. In support, T-cells within inflamed tissue in sarcoidosis have been shown to replicate faster than normal. In addition, raised levels of IL-2 have been found in active sarcoid BAL, and found to be chemotactic for T-cells (Alvarez et al, 1979; Watson & Mochizuki, 1980; Hunninghake et al, 1983). Such findings may partly explain the expansion in number of activated T-cells observed within the lung parenchyma and BAL in active sarcoidosis. It is postulated that these cells are deployed to amplify the immune inflammatory response within the lung interstitium.

T-lymphocytes recovered from active sarcoid BAL, have also been observed to spontaneously release monocyte chemotactic factor (MCF), and monocyte migration inhibition factor (MIF) (Hunninghake et al, 1980b). It has been demonstrated that lung T-cells release approximately 25 times more MCF on a per cell basis than do blood T-cells from these same patients. This observation, together with findings that there are increased numbers of T-cells in the lung and decreased numbers of T-cells in peripheral blood (q.v. below) suggests that a gradient of monocyte chemotactic activity exists between the lung and blood, the purpose of which is to attract monocytes to sites of disease. Such a recruitment of monocytes to the pulmonary interstitium may be an initial step in the assembly of granulomata (q.v. below). Other distinct lymphokines secreted by the same or different subpopulation of T-cells, may then be responsible for the activation and differentiation of recruited monocytes into activated macrophages, giant cells and epithelioid cells (all constituents of sarcoid granulomata). One of these mediators, interferon gamma (IFN-γ), is a potent activator of macrophages, and has been shown to be liberated by lung T-lymphocytes (and alveolar macrophages) in patients with active sarcoidosis (Robinson et al, 1985).
SYSTEMIC CELLULAR IMMUNE ABNORMALITIES

Although lung T-cells appear activated in sarcoidosis, the opposite is seen in the peripheral blood of the same patients. Here there is an absolute decrease in the number of circulating T-cells. Functionally the response of peripheral lymphocytes to mitogen and recall antigen is partially impaired, and B-cell function (in vitro) is consequently depressed. Such findings in the peripheral blood are also accompanied by a depression in certain cutaneous delayed hypersensitivity responses (Daniele et al, 1980).

Many active sarcoid patients seem to have a partial to complete anergy to tuberculin PPD. This was one of the earliest immunological abnormalities to be observed in sarcoidosis. Such skin anergy also extended to certain antigens eg. trichophyton, mumps virus, streptokinase / streptodornase, candida, as well as dinitrochlorobenzene. The incidence of anergy to tuberculoprotein and other antigens in sarcoid patients varies from 30 - 70% depending on the number of antigens used (Broom & MacLaurin, 1973; Mitchell & Scadding, 1974; Siltzbach et al, 1974). This anergy also appears to parallel disease severity, being especially marked when the sarcoidosis is clinically active, and disappearing months to years after resolution of the disease. For a time, therefore, there appeared a puzzling paradox: as such, cutaneous anergy is usually associated with depressed cellular immunity. Yet, lung studies have shown the presence of an active, if not overactive, immune response. The nature of such cellular anergy, like the aetiology of the disease itself, remains an enigma.

To add to this paradox, while sarcoid patients do not respond to the above cutaneous antigens, they are uniquely capable of mounting a cutaneous response to Kveim preparation (Siltzbach et al, 1974). These observations have therefore raised the question of whether the proposed defect in cellular mediated immunity in sarcoidosis is
in fact real or apparent.

First, the partial skin anergy observed in sarcoid patients is frequently associated with a reduction in the number and function of circulating T-lymphocytes (Daniele et al, 1985). It has been suggested therefore that the reduction of delayed type skin test reactivity could reflect a depletion of the population of immunoreactive cells being sampled from the skin. In addition studies of animal models of granulomatous disease suggest that there is an altered circulating pattern for T-lymphocytes. In particular, T-helper cells become sequestrated in central lymphoid organs and sites of inflammation; thereby leaving a proportionately increased number of suppressor T-cells in the peripheral blood (Rocklin, 1983). In sarcoid patients, the ratio of helper: suppressor T-cells in the blood may be normal or slightly decreased (Rocklin, 1983). Other studies have also noted serum inhibitory factors, that are partly derived from monocytes, in the blood of active sarcoid patients. These factors have been shown to inhibit the in vitro response of normal lymphocytes to mitogens and antigens. Such circumstantial evidence has also been postulated to account for the observed suppressed function of sarcoid peripheral blood lymphocytes in vitro (Mangi et al, 1974; Goodwin et al, 1979).

Secondly, the mechanics of the Kveim response are not entirely clear. Although, Kveim test lesions exhibit histopathologic similarities to sarcoid granulomata, there are marked differences between the Kveim reaction and the classic delayed type hypersensitivity skin reaction. The most obvious difference is in the kinetics of the two reactions. The delayed-type hypersensitivity reaction begins at 8 hours, peaks at 24 - 48 hours, and resolves by 96 - 120 hours. By contrast, the Kveim reaction takes 4 - 6 weeks to develop. Furthermore no specific antigen in the Kveim preparation has been identified as the inducing agent in this response, and no convincing data have been generated to show reproducibly that sarcoid lymphocytes are actively sensitized to the soluble or insoluble products in the Kveim material.
Attempts to induce blood lymphocytes from sarcoid patients to proliferate or secrete lymphokines, after in vitro exposure to the Kveim material, have been unrewarding (Daniele, 1988). Thus the nature of the stimulus and the reaction itself in the Kveim test remain the subjects of much controversy.

Finally, this apparent impairment of cellular immune function (in blood and skin) in sarcoid patients was noted to be similar but less profound than that in Hodgkin's disease or other lymphomas. However, unlike with the latter diseases, patients with sarcoidosis do not seem to be predisposed to opportunistic infections or to develop cancer (Winterbauer & Kraemer, 1976).

**HUMORAL ABNORMALITIES**

There appears to be a hyper-reactivity of the humoral immune system in sarcoidosis (Hunninghake & Crystal, 1981b). This is expressed mainly as an increased response to exogenous antigens, and the development of auto-antibodies to host antigens (eg. rheumatoid factor, antinuclear antibody, auto-antibodies to T-cells) (Oreskes & Siltzbach, 1968; Veien et al, 1978; Lobop & Suratt, 1979). One of the first observed humoral immune abnormalities, was a polyclonal elevation in gamma-globulins. This has been reported to be present in the blood of 70% of active sarcoid patients (Siltzbach et al, 1974). These abnormalities are often associated with an exaggerated response to certain common antigens (eg. mycoplasma and respiratory viruses) (Byrne et al, 1974), and the presence of immune complexes (q.v. below). A polyclonal hypergammaglobulinaemia is also found in active sarcoid BAL (Rankin et al, 1986). Early reports suggested that the high titres of antibodies directed against specific antigen or antigens might have initiated the inflammatory process (Veien et al, 1978; Lobop & Suratt, 1979). There is however no convincing evidence to support these views. Available data suggests that immunoglobulin formation in sarcoid patients is non-specific, and merely a by-product of the presence of large numbers of activated T-cells at site of granuloma formation. Lung CD4+ T-cells, but not blood T-
lymphocytes, from patients with active sarcoidosis are capable of polyclonally activating normal B-cells (without added antigen or mitogen) which differentiate into immunoglobulin-secreting cells (Hunninghake & Crystal, 1981b). In contrast, lung T-lymphocytes from normal subjects or patients with idiopathic pulmonary fibrosis do not exhibit such functional features. Consistent with such *in vitro* observations, *in vivo* studies have demonstrated that immunoglobulins are produced primarily at sites of granuloma formation in sarcoid patients (Lawrence et al, 1980), and that the numbers of immunoglobulin secreting cells at these sites are directly correlated with the numbers of activated T-helper cells present therein (Hunninghake & Crystal, 1981b). However, no significant numbers of B-cells are found in sarcoid BAL (Campbell et al, 1985). While it is clear that lung B-lymphocytes are actively producing immunoglobulin in sarcoidosis, the relevance to pathogenesis of this disease is unknown. It would seem that the observed hypergammaglobulinaemia reflects an *epiphenomenon*, unrelated to the morphological derangements of this disease. Suggestions on the pathogenesis of the humoral abnormalities in sarcoidosis are incomplete, and largely speculative. The elevated levels of immunoglobulin found in BAL and serum of sarcoid patients could reflect a defect in T-cell regulation of B-cell function, with consequent immunoglobulin synthesis by lung B-cells at sites of inflammation and subsequent diffusion into the blood (Daniele, 1983).

The prevalence of immune complexes ranges from 23% to 70% of sarcoid patients (Hedfors & Norberg, 1974). It has been postulated that such variability in incidence could either signify the degree of disease severity, or the fact that different techniques are employed by different laboratories to identify the immune complexes. The latter, when present, appear to persist in active disease but disappear with resolution. A high prevalence of immune complexes is noted in sarcoid patients presenting with erythema nodosum and arthritis. It is however unclear what role they play in the evolution of the disease, as there is no apparent association between presence of
immune complexes and stage of disease or presence of other extrapulmonary features (Gupta et al, 1977; Daniele et al, 1978). In addition, some of the serious clinical effects of immune complexes such as renal disease seen in other diseases (e.g. SLE*), do not occur in sarcoidosis.

In conclusion, there is now firm evidence available to support the notion that sarcoidosis is primarily an immunological disorder. Available data suggests that, sarcoidosis does not represent a generalized depression of the immune system, but a heightened inflammatory reaction at sites of disease activity. This has led to further speculation on the possible histopathological sequence of events occurring in the lung interstitium. In this regard, BAL has been instrumental in showing that the initial lesion in the lungs of sarcoid patients arises as a result of an exaggerated local cellular mediated immune response, to unknown stimulus or stimuli. Such a reaction involves the accumulation and compartmentalization of mononuclear cells in the lung parenchyma, thereby providing the appropriate setting for granuloma formation.

Available evidence suggests that an inverse relationship between the number of granulomata and the total number of inflammatory cells in sarcoid lung biopsies (Garrett et al, 1984). In patients with early disease there is a tendency towards greater numbers of inflammatory cells and fewer granulomata. By comparison, patients with advanced disease tend to have a greater proportion of granulomata and a less prominent alveolitis. Such findings are consistent with experimental models of granulomatous lung disease. These demonstrate that an accumulation of mononuclear cells in the lung precedes granuloma formation (Adams, 1976; Boros, 1978). Such observations support the hypothesis that the presence of an initial cellular inflammatory process is essential to the development of the granulomatous lesion.

* systemic lupus erythematosus
THE SARCOID GRANULOMA

Over 90% of patients exhibit histological evidence of non-caseating granulomata in the lung interstitium, or intrathoracic lymphoid tissues or both. These granulomata can be found in the peribronchial, subpleural and interlobular septa, as well as the perivascular connective tissue. They can also at times occur in the submucosa of the bronchus and bronchial wall.

The granulomata in biopsies from clinically active sarcoid patients, consists of a tightly packed central follicle of macrophages, epithelioid cells and multinucleated giant cells, intermingled with a few lymphocytes. This is surrounded by a mantle of loosely arranged lymphocytes, cells of the monocyte-macrophage lineage, and in some by fibroblasts (Fig 1.4). A few lymphocytes may be present in the central core. Monocytes are the building blocks of the central follicle of the granulomata, the maintenance of which requires a constant recruitment of circulating blood monocytes, which are able to differentiate into macrophages, epithelioid cells, and multinucleated giant cells. The latter are commonly seen in more mature granulomata, which contain areas of fibrosis and are most commonly of the Langhans type, with multiple peripherally located irregular nuclei. Epithelioid cells are believed to be the progenitors of giant cells in the granulomata. Support for this view is provided by the identical cytoplasm and nuclear features seen in both cell types (Soler & Basset, 1976), and by the ability of epithelioid cells to transform into giant cells, as seen in vitro (Black & Epstein, 1974). Necrosis during early granuloma formation is common, and is characterised by the presence of small foci of nuclear debris within the centre of the granulomata. True caseous necrosis is however not a feature of sarcoidosis. As the granulomata mature, collagen is laid down at the periphery by an ingrowth of fibroblasts.

The lung parenchyma between the granulomatous nodules is usually normal, so that
Fig. 1.4

Transbronchial biopsy from a 26-year old female patient presenting with Stage III pulmonary sarcoidosis. H. & E.
(a) Low power, well-formed distinct non-caseating epithelioid cell granulomata within the lung parenchyma
(b) High power, a close-up view of one of the above granulomata (arrow), showing its cellular components: epithelioid cells, multinucleated giant cells, macrophages, and lymphocytes
alveolar ventilation and perfusion is not usually compromised. Conglomeration of individual deposits results in extensive distortion of the lung architecture, and in odd cases, airway obstruction. The sarcoid granulomata may appear fresh for months to years; ultimately they either resolve completely (ranging from no scars, to focal pulmonary scars without evidence of the initial granulomatous inflammation); or they undergo an obliterative fibrosis, the end result of which is widespread scar formation with adjacent bronchiectasis and distortion of lung architecture (McCort & Pare, 1954; Rosen et al, 1979; Scadding & Mitchell, 1985).

**ROLE OF MACROPHAGES IN SARCOIDOSIS**

A major cellular component in cellular immune responses is the macrophage population. In normal BAL fluid, over 80% of the cells are macrophages (Reynolds & Newball, 1974). The principal role of the alveolar macrophage (AM) is the primary defence against inhaled micro-organisms and inert particles. AM differ in several ways from macrophages in other tissues. They reside in an air-tissue interface with direct exposure to inhaled micro-organisms and environmental toxins. In view of such an aerobic environment, such cells have developed special metabolic adaptations. In certain animal models, the turnover time for AM has been estimated to be about 21 to 28 days. Although, it has been speculated that the turnover time in human lungs is longer, there are few convincing studies. In comparison with other macrophages, AM have large numbers of mitochondria and an increased content of lysozomal hydrolytic enzymes (Gil & Daniele, 1988). In addition, it has been recently suggested that in view of their environment, their cell surface receptor expression may differ from other macrophages eg. C3b and Fc receptors (Coonrod & Yoneda, 1983).

The AM forms part of the dynamic 'mononuclear-phagocyte' family, originating in the bone marrow as a common committed progenitor cell for the granulocyte and monocyte- macrophage pathways. The transit time in the bone marrow from first
monocyte precursor to mature monocyte is believed to be about 6 days (Groopman & Golde, 1981). Migration of monocytes into the different tissues appears to be a random phenomenon, in the absence of localized inflammation. Once in the tissues, monocytes have not been shown to re-enter the circulation (Van Furth et al, 1979). Rather, they undergo transformation into tissue macrophages with morphologic and functional properties that are characteristic for the tissue in which they reside. Such transformation is assumed to be in response to tissue-specific stimuli (Riches & Henson, 1986). The terminal stage of development in the mononuclear-phagocyte line is the multinucleated giant cells, which characterize granulomatous inflammatory disorders, such as tuberculosis and sarcoidosis. Both monocytes and macrophages appear in the lesions of these diseases before the giant cells, and they are thought to be the precursors of the multinucleated cells (Murray et al, 1987). Monocytes kept in culture for 3-10 days develop the general features of tissue macrophages, and some of them are fused into large multinucleated cells (Schlesinger et al, 1984). A soluble factor released from stimulated human T-lymphocytes (IFN-γ) has been shown to promote giant-cell formation when added to cultured macrophages (Weinburg et al, 1984).

Macrophage-lymphocyte interaction is essential for the initiation and expression of acquired immune responses (Unanue, 1984). The role of the macrophage in resistance to many infectious agents as well as tumours is now widely recognized, both in the presence or absence of specific immune responses. Macrophages can therefore be involved in the recognition of antigens and in antibody formation, as well as in the formation and release of a wide range of immuno-regulatory substances (e.g., enzymes and kinins) that influence the immune response (Friedman, 1978).

Evidence suggests that the immune response begins with antigen processing by the macrophage (Unanue & Allen, 1987). This involves the uptake, localization, and
degradation of the foreign antigen by the macrophage. Phagocytosis, one of the oldest recognized functional activities of macrophages, is thus invaluable at this stage. These initial preparative steps are necessary in order to promote the optimal presentation of the antigen in a biochemically modified (and thus more reactive) form to lymphocytes. An essential step in lymphocyte recognition of antigen is that the material processed by macrophages must be presented to T-cells in conjunction with MHC* class II molecules (Ia molecules in animals). These molecules are heterodimeric, made of 34- and 28-KD chains, and found mainly on the surface of macrophages, B-cells and the Langerhans-dendritic cells of the skin and lymphoid organs. Data suggests that MHC Class II molecules expressed by antigen-presenting macrophages, are the key molecules that regulate the capacity to make a cellular response against protein (Rosenthal & Schevach, 1973). Studies have shown that CD4+ T-cells recognize proteins only when the proteins are presented to them by an antigen-presenting cell that bears an MHC Class II molecule. Since MHC Class II molecules are expressed on only a few cells (such as macrophages), this limits immune recognition and prevents recognition of proteins, including autologous ones, on cell types that do not bear an MHC Class II molecule (i.e. on most of the cells of the body). Other studies have shown that the expression of interleukin-1 (IL-1) receptors by the macrophage is a also pre-requisite for macrophage-lymphocyte interaction (Lowenthal et al, 1986). It has been recently postulated that when protein is processed by the macrophage, and the immunogen subsequently displayed on the cell surface together with ample expression of MHC Class II molecules, the T-cell recognizes the complex and then induces IL-1 on the macrophage. Depending on the amounts of antigen and MHC Class II molecules displayed, the macrophage is induced to produce IL-1 on its surface within a few hours (Kurt-Jones et al, 1985; Weaver & Unanue, 1986). For this to happen however, these workers have demonstrated that

*MHC, stands for Major Histocompatibility Gene Complex. Class II MHC products in humans comprise the HLA-D region (HLA-DR and HLA-DQ molecules).
both cell-to-cell contact and a T-cell secreted product are required. This T-cell product is as yet unidentified. It is now also recognized that other activating signals such as IL-6, LFA-3, and LFA-1, may be crucial when antigen-presenting macrophages present antigen in a recognisable, immunogenic form to CD4+ T-lymphocytes (Asherson & Colizzi, 1989).

Antigen interaction with the T-cell system usually leads to lymphocyte proliferation. IL-1 secretion is believed to be an important step in T-cell activation, by inducing a subpopulation of T-cells to express specific receptors (Tac antigen) for another soluble factor, IL-2 (q.v. Fig. 1.1). In support, CD4+ lymphocytes have been shown to be activated by antigen or mitogen, in the presence of IL-1, and to acquire receptors for IL-2 as well as secrete IL-2 (Unanue & Allen, 1987). Such lymphocyte activation is thought to occur in a cascade of steps *in vivo*. These sustain the inflammatory response, and result in the secretion of products that act back on the mononuclear phagocyte system.

The first step involves the development of a receptor for IL-2 (IL-2R), and the production of IL-2. The latter then causes the proliferation of T-cells, as well as the upregulation of its own receptor. Studies by Muller-Quernheim et al (1989) have shown that during the course of T-cell activation an orchestrated transcriptional activation of genes involved in the progression of the cell cycle takes place. In this way, the transcription of the IL-2 gene is intimately linked to the transcript of the gene of IL-2R. This results in an increase in the number of local CD4+ T-cells, as well as an increase in the CD4+helper to CD8+suppressor ratio (Semenzato et al, 1982).

The secreted macrophage-activating lymphokines include macrophage activity factor
(which draws monocyte-macrophage cells specifically to the site of the T-cell macrophage interaction), macrophage inhibition factor (which ensures that locally resident macrophages stay at site of inflammation), and IFN-Y (which has been observed to increase the expression of MHC Class II molecules on macrophages) (Hunninghake et al, 1984). This macrophage activation further equips this cell with enhanced bacteriostatic and cytocidal effects. Studies have shown that once activated, macrophage receptors e.g. C3b and Fc receptors also increase (Johnston, 1988).

This macrophage-T-lymphocyte interaction constitutes the basis of cell-mediated immunity (Fig. 1.3). Data from BAL fluid analysis, suggests that the cellular events leading to granuloma formation in sarcoidosis occur as a result of macrophage-T-cell interaction (Daniele et al,1980; Crystal et al,1981a; Daniele et al,1986). BAL samples as well as lung biopsies have recorded an increase in the total number of macrophages present in active sarcoid patients (Daniele et al, 1980). Studies suggest that these AM might serve as antigen-presenting cells, and initiate the alveolitis seen in pulmonary sarcoidosis by presenting some as yet unidentified antigen(s) to locally resident and recruited T-lymphocytes (Daniele et al, 1986). In support, two independant studies have shown that AM from active sarcoid patients have enhanced capacity to present recall antigen to T-cells in vitro (Venet et al,1985; Toews et al,1986). Furthermore, sarcoid AM have an enhanced expression of HLA-D region molecules, as well as increased HLA-DR membrane determinants (Razma et al,1984; Campbell et al,1986a). The mechanism for enhanced antigen presentation by AM in pulmonary sarcoidosis is unclear. Normal AM have been shown to suppress T-cell proliferative responses in vivo (Toews et al, 1984). It has been suggested that the observed enhancement in sarcoid AM may reflect an absence of suppression in these patients; or an alteration in the differentiation of sarcoid AM (consequent on local environmental factors), which allows the emergence of macrophage-like cells that would support T-cell activity (Toews et al, 1986). Indeed in sarcoidosis following
activation, T-lymphocytes have been shown to release IFN-Y. Increased levels of this substance have also been shown in the BAL of active sarcoid patients (q.v. above). This substance is a potent activator of macrophages, and has been shown in vitro to increase HLA-DR expression on sarcoid AM (Robinson et al, 1985). Other workers believe that the enhancement seen in antigen presentation by sarcoid AM could be the result of an increase in the number of newly recruited monocytes, which have retained their antigen-presenting capability (Toews et al, 1986). However, data has revealed that in contrast to normal blood monocytes, sarcoid peripheral blood mononuclear cells do not support T-cell responsiveness (Daniele et al, 1980).

Sarcoid AM have been reported to spontaneously secrete IL-1, which further stimulates the local T-cell population (Hunninghake, 1984). In addition, AM from active sarcoid patients have been shown to have increased phagocytosis, as well as increased Fc receptor expression (Schyler & Steinberg, 1982; Saltini et al, 1984a). Recent reports have also observed increased IL-2 expression on sarcoid AM, and have postulated that this could be involved in macrophage activation (Hancock et al, 1986).

These macrophages also spontaneously liberate soluble mediators capable of mobilizing, and stimulating other cell types (including monocytes and fibroblasts), which participate in the maintenance and progression of the chronic inflammatory response. Active sarcoid AM have been observed to produce large amounts of a growth factor, that can stimulate the proliferation of human fibroblasts (Bitterman et al, 1983a). In addition sarcoid AM also secrete fibronectin, a large 220,000 dalton adhesive glycoprotein, that modulates fibroblast attachment to the extracellular matrix and serves as a chemoattractant for fibroblasts (Rennard & Crystal, 1981).

In conclusion, it appears that within the AM population of sarcoid patients can be found: (1) those macrophages capable of presenting antigen to and stimulating T-lymphocytes, and thereby initiating granuloma formation, as well as (2) other
effector macrophages, which through the release of a series of biologically active molecules, can act as critical modulators of granuloma formation and its possible progression to fibrosis.

**ALVEOLAR MACROPHAGE HETEROGENEITY**

It appears that the AM population possesses a versatile character, that enables it to respond to the local micro-environment, with which it interacts. Such adaptibility is a pre-requisite, if AM are to play a role in the initiation and maintenance of immunological reactions.

Investigation into the functional role of macrophages has spanned over the past century. In 1882, Metchnikoff observed macrophages in starfish larvae; and concluded that macrophages were scavenging, phagocytic cells important as the host's first line of defence against invading organisms. Ebert and Florey (1939) demonstrated that blood monocytes could differentiate into tissue macrophages in the living animal. Using rabbit ear chambers, they showed that monocytes containing phagocytozed carbon particles migrated from the blood vessels into the connective tissue, and transformed into mature macrophages. One of the early studies on the role of macrophages in antigen presentation came from Gordon in 1974. He described the importance of phagocytosis in the initial processing stage. Rosenthal and Schevach (1973) were among the first to show that the MHC was involved in the interaction between macrophages and lymphocytes. Other workers demonstrated the secretory function of macrophages, which included the production of proteases, complement proteins, growth regulatory factors, and arachidonic acid derivatives (McLennan & DeYoung, 1984).

This heterogeneity in macrophage function led to the question as to whether all these different functions were being performed by one cell type, or whether there existed
functional subpopulations within the macrophage-cell lineage. The development of a technique for the production of monoclonal antibody probes, that could be directed against specific membrane determinants was an important step in understanding the pleuripotential nature of macrophages. By combining the nuclei of normal antibody-forming cells with those of their malignant counterparts, Kohler and Milstein (1975) developed an unprecedented technique of analyzing and purifying individual molecules within the enormously complex mixtures encountered in biological material. This achievement was the culmination of many seemingly unrelated discoveries. Of particular importance were, the proof of the clonal selection theory (Nossal and Lederberg, 1958), the development of cell fusion techniques (Okada, 1962; Littlefield, 1964), the artificial induction of plasmacytomas (Potter and Boyce, 1962), and their adaption to tissue culture (Horibata and Harris, 1970). Finally, the demonstration that it was possible to fuse two different plasma cell tumour lines with retention of both antibody products (Cotton and Milstein, 1973) paved the way for subsequent developments.

The use of monoclonal antibodies to identify specific populations of cells has been of considerable value in determining lymphoid as well as macrophage subsets. Studies using such probes, particularly on tissue sections, have led to a clearer understanding of the in situ cellular micro-environmental inter-relationships. Indeed, morphological and functional distinctions have been reported between classic phagocytic macrophages, and populations of macrophage-like cells that are poorly phagocytic, but exhibit antigen-presenting capabilities. Springer et al (1979) were the first to describe a monoclonal antibody directed specifically against mouse macrophage membrane determinants, that were expressed during monocyte maturation. In 1980, Raff developed a monoclonal antibody which was able to detect a 120 kd determinant on about a third of the peripheral blood monocytes. This antibody was able to isolate the macrophages into two broad subpopulations: those bearing both
the 120 kd determinant and the HLA-DR, and those displaying only HLA-DR. Functional studies showed that only those macrophages with the 120 kd determinant plus HLA-DR were able to act as antigen-presenting cells. Despite such findings, the authors were unable to prove the existence of two phenotypically distinct macrophage subpopulations, because of the constraint imposed by the sensitivity of their techniques. Sun et al (1982) were able to characterize three different macrophage subpopulations by producing rat anti-mouse macrophage monoclonal antibodies. One macrophage subset was found to be responsible for natural killer activity, one for lymphokine induced macrophage-mediated cytotoxicity, and a third one with neither of these functions. Such observations together with those of others, pointed to the existence of different macrophage subpopulations which could be discriminated by their surface membrane components, and possibly each with different functions. Following these results, other workers (Dimitru-Bona et al,1983; Hofman et al,1984) described a variety of monoclonal antibodies that could differentiate between subpopulations of macrophages in various tissues in animal and man. They emphasized that while such cells shared a common bone marrow origin, their phenotypic diversity suggested the existence of an intricate differentiation programme within the macrophage family that was associated with the expression of various gene products.

In order to investigate macrophage heterogeneity in the lung, AM from the lower respiratory tract were obtained by BAL. This was first described in animals by Myvick(1961), and later adopted to human studies by Reynolds (1974). Indeed the AM family was also observed to be heterogeneous in terms of cell size (Cohen & Cline, 1971), surface receptor activity and immunologic function (Branner & Chandler,1988 ). Subfractions of AM were isolated on the basis of cell density (Zwilling et al, 1982), and these were shown to possess different capacities for suppression of lymphocyte proliferation and for production of IL-1( Shellito & Kaltreider, 1984). This AM heterogeneity has been described in both animal and
human studies (Cohen & Cline, 1971; Barsoum et al, 1979; Shellito & Kaltreider, 1984).

In previous sections it has been seen that through an interaction with lymphocytes, macrophages are intimately involved in the expression and modulation of inflammatory reactions, such as occur in sarcoidosis. Significant variations from the normal in AM phenotype have been described in association with sarcoidosis (Campbell et al, 1985). In addition, Ainslie et al (1989) found that the increase in the proportions of certain phenotypically distinct AM subpopulations correlated well with disease activity (as determined by clinical criteria).

The elucidation of AM subpopulations in vivo could therefore provide insight into the underlying mechanisms that govern the outcome of the immune responses involved in pulmonary sarcoidosis: a disease characterized by an unknown aetiology, a variable course and prognosis, in the absence of a reproducibly adequate index to predict its clinical course or monitor the effect of therapeutic regimes.
THESIS OBJECTIVES

The extensive work on the lymphocyte population and released cytokines described in this introduction, has made a valuable contribution to the understanding of cell-to-cell interaction in the inflammatory process of sarcoidosis. However, there is as yet no clear explanation as to the persistence of granulomata, or more important the insidious drift to fibrosis with irreversible damage in -15% of sarcoid patients. Data has also been presented which shows that macrophages are intimately involved in T-cell stimulation and granuloma formation.

In the light of this, the present thesis tests the hypothesis that aberrations within the AM population are important in critically modulating the pathological and clinical course of pulmonary sarcoidosis. Studies were therefore designed:

1. To determine the extent of aberrations within the AM population, that may be associated with the immunopathogenesis of pulmonary sarcoidosis;
2. To identify the emergence of particular AM subpopulations in BAL of clinically active sarcoid patients, by using monoclonal antibody probes directed against specific macrophage determinants;
3. To isolate these AM subsets from the lavage of normal subjects and sarcoid patients;
4. To characterize their phenotypic, physiological and functional features, in order to reveal any sarcoid-related differences arising in response to changes in the immediate micro-environment;
5. To evaluate the effect of targetted corticosteroid regimens on the observed AM aberrations; and
5. To correlate their local cellular effects with clinical efficacy;
6. To investigate the immunological abnormalities present in the lungs of patients with PBC, in order to delineate any similarities to the events that occur in the lungs of sarcoid patients, and hence to determine whether the ensuing clinical expression of the disease is partly controlled by the environment of the patient.
CHAPTER TWO

GENERAL ASPECTS RELATING TO STUDIES:

subjects, selection criteria and assessment
SUBJECT SELECTION

All 62 normal subjects were healthy volunteers recruited from hospital laboratory staff and medical students. Prior to enrollment, the subjects were questioned about their health and smoking habits. They were excluded from a study if they had smoked within the last year prior to the test, if they had suffered from an upper respiratory tract infection within two weeks of the investigation, or if they ever had a history of recurrent chest infections, asthma, pulmonary tuberculosis or heart disease. Each recruited subject had to have normal chest X-ray and pulmonary function.

All 115 patients with sarcoidosis were recruited from the Sarcoidosis Clinic at the Royal Free Hospital (RFH); each one had to fulfil specific criteria. The diagnosis of sarcoidosis had to be biopsy-proven; the biopsies revealing the presence of non-caseating epithelioid granulomata, and morphological features consistent with sarcoidosis. In addition, no evidence of any fungal, mycobacterial, bacterial or parasitic infection was present. All subjects were asked for any known history of exposure to materials recognized to cause granulomatous diseases (Chapter 1). The enrolled patients were caucasian, and all were non-smokers*. None had received any systemic or inhaled corticosteroids for at least 18 months prior to investigation (except for 20 of the patients selected for the pilot studies in Chapter 3).

As far as possible, the sarcoid patients represented a homogeneous group on clinical grounds. They were all symptomatic with cough and/or shortness of breath on exertion at the time of study, and had unequivocal bilateral parenchymal shadowing on chest x-ray with or without minimal hilar lymphoadenopathy. Each patient had some degree of abnormal ventilatory function. None of the patients had suffered any recent respiratory tract infection or had a past history of any other relevant medical disorder. All patients underwent a detailed baseline assessment prior to each study to

* except for one patient in Chapter 6
determine the degree of their pulmonary condition, as well as the extent of any extrapulmonary activity. The evaluation included a full general medical and ophthalmological examination and laboratory measurements. The latter involved blood tests for erythrocyte sedimentation rate (ESR), SACE, liver function tests, serum calcium and immunoglobulins; 24-hour urinary specimens for calcium, electrocardiograms and echocardiograms.

Using these objective measurements, none of the recruited patients had clinical evidence of extra-pulmonary sarcoid activity at the time of each test. All had a negative intradermal tuberculin test of 1:1,000 dilution. Specific details of the patients' sex, age, lung function defects, radiographic appearances, and serological tests (where relevant) are given for each study in Chapters 3 - 7.

Only 21 of the 115 patients had had Kveim tests performed at initial presentation. All of these had been shown to be positive.

**CHEST RADIOGRAPHS**

The chest radiographs in all normal and sarcoid subjects were performed with a standardised technique in routine use at RFH. This utilized a penetration beam power of 75 KvP, a tube current of 600 mA and an exposure time of 0.02 seconds. All X-rays were taken at total lung capacity with each subject in deep inspiration, and in both postero-anterior and right lateral chest views. Two independent radiologists reviewed all initial and follow-up films, and these were reported in terms of the classic radiographic staging of sarcoidosis, described in Chapter 1. Ninety-seven of the 115 enrolled sarcoid patients had evidence of bilateral interstitial infiltrates only at the start of each study (Grade III stage), while hilar lymph node enlargement was also noted in the remaining 18 patients (Grade II stage).
PULMONARY FUNCTION TESTS

Both dynamic and static lung volumes were performed on all subjects to assess their respiratory function. Forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁) were determined using a dry bellows spirometer (Vitalograph). Peak expiratory flow rates (PEFR) were measured using a Wright peak flow meter. These tests were performed in triplicate, and the highest value taken as a true index of lung function (Clarke, 1976). Measurements of residual volume (RV), functional residual capacity (FRC) and total lung capacity (TLC) were derived from the dilution of a single breath of helium, used to estimate lung volume during the measurement of transfer factor for carbon monoxide (TLCO). This single breath test method utilised a Resparameter Mark IV (P K Morgan, Chatham, Kent), which consists of a spirometer, a bag for the test gas and an automatic method for sampling the mid-expired gas. The test gas on each occasion consisted of 0.03% carbon monoxide, 14% helium, and air. The measurements on this machine were repeated three times on each subject, with five minutes between each analysis in order to allow washout of carbon monoxide. The mean of three congruent readings was then taken.

In all studies, the same experienced technician was responsible for carefully calibrating the equipment, and performing all physiological measurements at the same time of day (9.00 am), (McDermott, 1966; Cinkotai & Thomson, 1966), with each subject sitting in the upright position. All measurements were standardised to body temperature, and barometric pressure saturated with water vapour. The results were expressed as percentages of predicted values for each subject (based on age, sex, and height) (Cotes, 1979; Knudson et al, 1983). The transfer factor was also expressed per litre of alveolar volume as the transfer coefficient or Krogh factor (KCO).
If pulmonary function analysis showed a reduction in TLCO of 20% or more of the reference value, this was taken to reflect a significant loss of functioning alveolar-capillary units. If such a reduction in TLCO was accompanied by a decrease in lung volumes (namely FVC and TLC) below 80% of the predicted value, the defect was then also considered to be restrictive in pattern.

OTHER MEASUREMENTS
Arterial blood gases were performed at rest on all sarcoid patients prior to bronchoalveolar lavage (Chapter 3), without supplemental oxygen. Bubble-free arterial blood was drawn into sealed glass heparinized syringes and tested forthwith. In all tests, the Corning 178 blood gas analyzer was used (Medfield MA, USA). The results were recorded in terms of KPa.

ETHICAL CONSIDERATIONS
Each study involving both normal volunteers and sarcoid patients had received prior approval by the Royal Free Hospital Ethical Practices Committee, and where relevant, the Administration of Radioactive Substances Advisory Committee of the Department of Health and Social Security. All protocols were explained in full to each subject, prior to recruitment to each study. All subjects had to give formal written consent before each investigation.

STATISTICAL ANALYSIS
The statistical methods used in this thesis are those in standard practice for the calculation of the mean, standard error of the mean, and correlation coefficient. The Student's t-test was used to determine the significance of the difference, for paired and unpaired observations, when the observations under study were assumed to be drawn from normally distributed populations. Wherever this assumption could not be made, the Wilcoxon matched pair signed-ranks test was made. In some studies, a value for t was also determined to assess the significance of the correlation coefficient,
obtained from the regression analysis. A probability value less than or equal to 0.05 was taken to indicate statistical significance.
CHAPTER THREE

BRONCHOALVEOLAR LAVAGE
Bronchoalveolar lavage (BAL) is a relatively simple extension of routine fibreoptic bronchoscopy. The principal objective of this technique is to instil small quantities of sterile saline into the distal airways, with subsequent recovery of the aspirate for analysis of its cellular and non-cellular constituents. It is the principal method used in all the studies in this thesis (Chapters 4 - 7), to obtain a representative sample of the cell populations in the lower respiratory tract of both normal volunteers and sarcoid patients.

**HISTORICAL BACKGROUND**

Indications for the use of bronchial lavage were first introduced by the pioneer bronchoscopist Chevalier Jackson in a paper delivered to the Massachusetts Medical Society in 1928. The procedure was carried out using a modified version of a rigid bronchoscope* incorporating a suction tube as well as tip-illumination. Large volumes (> 500 ml) of fluid were introduced into the bronchi in order to irrigate the airways, and remove purulent secretions that were predominantly found in bronchiectatic patients. The rigid bronchoscope continued to be used as a vehicle for lung lavage until the mid-1970's, when it was replaced by the flexible fibreoptic bronchoscope. This instrument was conceived by Ikeda in the early 1960's (Ikeda, 1970; Ikeda, 1974), but not until 1973 did its routine use become widespread. By selectively catheterising one of the major bronchi with this flexible instrument, large volume lavages continued to be used therapeutically, to remove excessive secretions and impacted mucus in patients with bronchiectasis, cystic fibrosis, or severe asthma. Although this form of therapy has its occasional exponents today, it has not achieved an established place in the management of any of these conditions. However, whole lung controlled-volume lavage under general

* Gustav Killian (1860 - 1921), Freiburg has been quoted as the father of bronchoscopy.
anaesthesia continues to remain the established treatment for alveolar proteinosis (Selecky et al, 1977, du Bois et al, 1983).

Since the mid-1970's such massive whole lung irrigation has been largely superceded by BAL. This involves the use of smaller volumes of fluid instilled (under local anaesthesia) into a bronchial segment or subsegment of the lower respiratory tract, with subsequent recovery of the aspirate for analysis of cells and secretions (Reynolds and Newball, 1974). This technique has enabled the exploration of previously inaccessible distal airways.

Recent work by Kelly et al (1987), using a radio-opaque dye and a digital subtraction imaging technique, has elegantly shown that lavage fluid does indeed sample the periphery of a lung segment (distal airways and alveoli), provided a fluid volume of 120 ml or greater has been instilled. BAL samples are therefore recognized to contain the inflammatory and immune effector cells, and their mediators in the fluid lining the epithelium of the alveolar structures.

The availability of BAL has opened up a field of enquiry into the immunopathogenesis and cell biology of various pulmonary diseases. Early studies largely examined the immunologic aspects of the normal human lung. Cantrell et al (1973) were among the first to perform BAL in healthy volunteers in order to retrieve sufficient macrophages from smokers and non-smokers, and to allow comparative measurements of cytosol enzyme levels. Thereafter, there appeared in quick succession voluminous reports on the analysis of the cells and proteins found in BAL fluid from normal volunteers (Waldmann et al, 1973; Daniele et al, 1975; Warr et al, 1977; Low et al, 1978). These observations have led to a considerable increase in the knowledge of the basic defence mechanisms of the human lung. More recently, data has emerged showing that cytological examination of BAL cells and other components may be of clinical value in the differential diagnosis of diffuse interstitial lung disorders, and of potential value in their management.
(Reynolds et al, 1977; Davis et al, 1978; Crystal et al, 1984). It has become apparent that those conditions associated with interstitial fibrosis (e.g., cryptogenic fibrosing alveolitis, asbestosis) are associated with aberrant cell profiles that are distinctly different from other conditions (such as sarcoidosis, tuberculosis, extrinsic allergic alveolitis).

Today, the indications for patients undergoing BAL continue to grow; and have incorporated immunocompromised patients with radiographic pulmonary shadows (e.g., post-bone marrow transplantation patients and AIDS sufferers) (Stover et al, 1984), as well as patients exposed to inorganic dusts (e.g., heavy metal disease) and noxious gases (Johnson et al, 1986).

**VALIDITY OF ASSESSING PULMONARY SARCOIDOSIS BY BAL**

To date segmental BAL has been extensively used to study the diffuse inflammatory responses of the lung interstitium in sarcoidosis. Before the widespread use of BAL, an understanding of the inflammatory processes occurring in this disease was based on a paucity of pathological material, coming from open lung samples and post-mortem specimens. With the advent of BAL, several lines of evidence have supported the view that the cells and soluble products recovered by segmental lavage may be used to assess the inflammatory events occurring in the lung parenchyma in this disorder.

First, different workers have repeatedly shown that the types and numbers of the cells retrieved by BAL are similar to those found in biopsy specimens of the lung of the same sarcoid patients (Campbell et al, 1985; Semenzato et al, 1985; Paradis et al, 1986). In addition, other studies have shown that BAL constituents generally reflect the inflammatory changes that parallel the severity of the acute illness in sarcoidosis, and these resolve as the disease abates or responds to treatment (q.v. Chapter 1). However, this does not discount the possibility, that the inflammatory response in the alveolar air-
space may not be in phase with that in the interstitium.

Secondly, the cellular constituents obtained by BAL from sarcoid patients have been shown to be "immunocompetent" in terms of proliferation to mitogenic and antigenic stimulation, as well as the production of immunologically active mediators (q.v. Chapter 1). As such therefore, these cells appear to be part of the local immune apparatus of the lung.

Finally, the lung in sarcoidosis appears to be the site of a compartmentalised inflammatory response that is not reflected in the peripheral blood (Hudspith et al, 1987). Traditional blood cell and serum or plasma results may not reflect events occurring in the lower respiratory tract. Consequently, an air space lavage specimen may capture changes indicative of the inflammation present in the lower respiratory tract (as reflected by an intra-alveolar accumulation of cells). Critics of this technique however, maintain that as lavage cannot sample all of the lung tissue, segmental BAL findings (at best) can only be regarded as a partial representation of the actual disease pattern (Reynolds, 1988).

PILOT STUDIES

One of the main recurring obstacles for the universal acceptance of BAL in the management of interstitial lung disease, appears to be the widespread reported differences in technical procedure (Davis et al, 1982; Cantin et al, 1983; Crystal et al, 1986; Walters et al, 1989). Preliminary studies were therefore undertaken to address some of these controversial issues, in an attempt to obtain a standardized technique for performing BAL throughout all studies in this thesis.

OBJECTIVES

The specific aims of these initial studies were:

1. To evaluate the adjunct use of anticholinergic agents such as atropine in the pre-medication drug regime prior to BAL.
2 To assess the value of the pre-warming the fluid to be instilled during BAL to 37 °C.
3 To determine the effect of high suction pressures during aspiration of BAL fluid.
4 To investigate the effect of BAL on the subjects' arterial oxygen saturation.

SUBJECTS AND METHODS
Thirty patients with biopsy-proven sarcoidosis were recruited from the Sarcoidosis Outpatient Clinic at RFH: 24 males, 6 females; mean±SE age 40.5 ± 2.72 years; all non-smokers. Ten of the patients had not received any prior corticosteroid treatment; while 20 patients were currently receiving systemic corticosteroids in varying dose regimes. Twenty-two patients showed radiological evidence of bilateral parenchymal shadowing in their lung fields; 8 patients had superadded hilar lymphadenopathy in addition to their interstitial lung disease. None of the patients gave a past or current history of any other medical condition. Patients were selected only if they had significant impairment of their lung function (so that no bias would be introduced from differences in pulmonary function). All had an FEV1 > 1L, and a mean PaO2 at rest (without supplemental O2) of 10.24 KPa.

The patients were randomly divided into five groups with 6 patients in each. Specific details of the patients in each group are given in Table 3.1. The variables tested within each group included: type of premedication; the temperature and volume of fluid instilled; and the suction pressure during the lavage (Table 3.2).

All groups received 5 - 10 mg midazolam intravenously (relative dose depending on individual characteristics of age, weight and clinical assessment) (Shelley et al, 1989), 15 minutes prior to the procedure. The BAL in each patient was performed by the same person (author), using a fibre-optic bronchoscope (Olympus model BT-IT20D) inserted transnasally under topical anaesthesia (2 - 4% lignocaine hydrochloride). 20 ml aliquots of 0.9% normal saline, to a total of 180 ml (except in group 5, q.v. Table 3.2) were instilled into the right middle lobe. The lavage fluid was aspirated after each aliquot, and
TABLE 3.1

CHARACTERISTICS OF PATIENTS WITHIN EACH GROUP

<table>
<thead>
<tr>
<th></th>
<th>GROUP 1</th>
<th>GROUP 2</th>
<th>GROUP 3</th>
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</thead>
<tbody>
<tr>
<td>No of patients</td>
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<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Sex</td>
<td>4M, 2F</td>
<td>5M, 1F</td>
<td>4M, 2F</td>
</tr>
<tr>
<td>Age*</td>
<td>35 ± 2.1</td>
<td>43 ± 1.0</td>
<td>45 ± 1.6</td>
</tr>
<tr>
<td>CXR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHL + infiltrates</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Infiltrates alone</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Lung function *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ L</td>
<td>1.91 ± 0.30</td>
<td>1.72 ± 0.50</td>
<td>1.89 ± 0.91</td>
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<tr>
<td>FVC L</td>
<td>3.83 ± 0.51</td>
<td>2.90 ± 0.23</td>
<td>3.98 ± 0.34</td>
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<td>PEFR L/min</td>
<td>290 ± 15.6</td>
<td>200 ± 17.0</td>
<td>300 ± 11.2</td>
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<tr>
<td>TLC L</td>
<td>5.28 ± 0.91</td>
<td>4.87 ± 0.12</td>
<td>5.05 ± 0.84</td>
</tr>
<tr>
<td>RV L</td>
<td>1.53 ± 0.21</td>
<td>1.40 ± 0.27</td>
<td>1.30 ± 0.21</td>
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<tr>
<td>TLCO</td>
<td>7.93 ± 1.9</td>
<td>7.12 ± 0.9</td>
<td>7.69 ± 1.2</td>
</tr>
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<td>mmol/min/kPa</td>
<td>1.84 ± 0.9</td>
<td>1.84 ± 0.3</td>
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<tr>
<td>KCO</td>
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<td>mmol/min/kPa/L</td>
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<tr>
<td>Blood Gases *</td>
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</tr>
<tr>
<td>pH</td>
<td>7.42 ± 0.01</td>
<td>7.41 ± 0.01</td>
<td>7.46 ± 0.02</td>
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<tr>
<td>PCO₂</td>
<td>5.19 ± 0.10</td>
<td>5.67 ± 0.13</td>
<td>4.85 ± 0.10</td>
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<td>PO₂</td>
<td>10.9 ± 0.31</td>
<td>10.0 ± 0.21</td>
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<td>HCO₃</td>
<td>25.7 ± 0.17</td>
<td>25.9 ± 0.20</td>
<td>25.9 ± 0.16</td>
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<tr>
<td>BE</td>
<td>2.1 ± 0.02</td>
<td>2.9 ± 0.07</td>
<td>3.1 ± 0.04</td>
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<tr>
<td>SaO₂</td>
<td>96.0 ± 1.0</td>
<td>94.0 ± 1.2</td>
<td>95.0 ± 1.0</td>
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* The data is expressed as mean ± SE
<table>
<thead>
<tr>
<th>CHARACTERISTICS OF PATIENTS WITHIN EACH GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 4</td>
</tr>
<tr>
<td>No of patients</td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Age*</td>
</tr>
<tr>
<td>CXR</td>
</tr>
<tr>
<td>BHL + infiltrates</td>
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<tr>
<td>Infiltrates alone</td>
</tr>
<tr>
<td>Lung function *</td>
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<tr>
<td>FEV1 L</td>
</tr>
<tr>
<td>FVC L</td>
</tr>
<tr>
<td>PEFR l/mim</td>
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<td>TLC L</td>
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<tr>
<td>RV L</td>
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<tr>
<td>TLCO</td>
</tr>
<tr>
<td>mmol/min/kPa</td>
</tr>
<tr>
<td>KCO</td>
</tr>
<tr>
<td>mmol/min/kPa/L</td>
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<tr>
<td>Blood gases*</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>PCO₂</td>
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<tr>
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<tr>
<td>SaO₂</td>
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* The data is expressed as mean ± SE
TABLE 3.2
DETAILS OF THE VARIABLES EXAMINED IN EACH PATIENT GROUP UNDERGOING BAL

<table>
<thead>
<tr>
<th>PATIENT GROUP*</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Pre-medication</td>
<td>Midazolam</td>
<td>Midazolam</td>
<td>Midazolam</td>
<td>Midazolam</td>
<td>Midazolam</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>+ Atropine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature of instilled fluid °C</td>
<td>37</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Suction pressure during BAL mmHg</td>
<td>70-80</td>
<td>70-80</td>
<td>140-160</td>
<td>70-80</td>
<td>70-80</td>
</tr>
<tr>
<td>Total volume of fluid instilled in ml</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>180</td>
<td>240</td>
</tr>
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</table>

* The number of patients examined in each group was six
collected into a sterile siliconized glass bottle maintained at 4 °C. The negative suction pressures used during BAL retrieval were recorded for each patient. The procedure was carried out with the patients supine at an angle of 45°. Supplemental oxygen (O₂) was not used; an OHMEDA BIOX 3700 oximeter with a finger probe for monitoring oxygen saturation was used for continuous recording. Baseline O₂ saturation values and heart rate were noted prior to pre-medication; these values continued to be recorded on insertion of the bronchoscope, during BAL and for at least 2 hours after examination was completed. Baseline temperature measurements were taken from each patient before BAL, and subsequently at hourly intervals for the next 12 hours. The total volume of lavage fluid obtained was noted for each patient, and total BAL cell count taken before the fluid was filtered and washed.

RESULTS
These are summarised in Table 3.3

BAL RETURN
The mean volume of lavage fluid retrieved in Groups 2 and 5 (63.6% and 66.5% respectively) was significantly higher than that obtained from the other patient groups (all < 60%) (p < 0.001). The lowest lavage return was observed in Group 3 patients. The change in temperature of instilled fluid had little effect on the amount of fluid obtained (groups 1 and 4).

TOTAL CELL RECOVERY
As would be expected the total BAL cell yield was generally comparable to the retrieved lavage volume in each case. It was therefore highest in Groups 2 and 5 (p < 0.002 and p <0.003 respectively), and lowest in Group 3 (p < 0.001) .
TABLE 3.3 COMPARISON OF THE CLINICAL, PHYSIOLOGICAL AND LAVAGE ANALYSIS OBTAINED FROM EACH PATIENT GROUP

| PATIENT GROUP |
|---------------|---------------|---------------|---------------|---------------|---------------|
|               | 1             | 2             | 3             | 4             | 5             |
| % BAL return  | X 58.3        | 63.6          | 50.4          | 57.3          | 66.5          |
|               | SE 0.56       | 0.63          | 0.61          | 0.47          | 0.60          |

Total cell recovery x $10^6$
| X 11.1        | 14.6          | 9.2           | 10.2          | 15.7          |
| SE 1.1        | 1.0           | 0.8           | 0.6           | 1.0           |

% RBC in BAL
| < 1           | < 1           | 8             | <1            | <1            |

% O2 saturation:

| Baseline  | X 96          | 94            | 95            | 95            | 96            |
|           | SE 0.75       | 0.62          | 0.40          | 0.12          | 0.23          |
| During BAL (at 2 mins) | X 92          | 89            | 82            | 92            | 85            |
|           | SE 0.81       | 0.91          | 0.64          | 0.34          | 0.42          |

Heart Rate beats per min:

| Baseline  | X 80          | 87            | 85            | 82            | 85            |
|           | SE 4.1        | 3.2           | 4.5           | 1.2           | 1.6           |
| During BAL | X 84          | 89            | 100           | 85            | 96            |
|           | SE 3.2        | 4.6           | 6.2           | 2.4           | 3.2           |

Symptoms:

| During BAL | -- | -- | coughing | coughing | coughing |
|           |    |    | wheeze   |          |          |
| Post BAL  | -- | -- | Chest pain | coughing | chest pain |
|           |    |    | pyrexia |          | pyrexia |
PRESENCE OF RED BLOOD CELLS (RBCs) IN RECOVERED FLUID

There was a marked increase in RBCs in Group 3 patients, where the suction pressure during BAL was greater than 140 mm Hg. Endobronchially, this corresponded to overt in situ mucosal bleeding in the right middle lobe. This was not seen in the other patient groups.

OXYGEN SATURATION AND HEART RATE

Following insertion of the bronchoscope, all patients had a 0.2 - 0.6% rise in O₂ saturation, but no significant change in heart rate. Instillation of BAL fluid produced a significant gradual decline in O₂ saturation in all groups (p < 0.001). A desaturation of less than 5% was observed in Groups 1, 2, and 4 patients; this was not associated with any significant patient discomfort. Much higher drops in O₂ saturation were noted in patients in Groups 3 and 5 (13% and11% respectively); these patients also appeared to be in some discomfort during the procedure (qv. below). These results cannot be attributed to baseline differences in lung function amongst the groups tested, as strict selection criteria were adhered to (qv. above; table 3.1). Changes in oxygen saturation were accompanied by significant increases in heart rate in all groups (p < 0.002), more so in Groups 3 and 5. A return towards baseline values in both O₂ saturation and heart rate were noted in all groups, within 15 minutes of the completion of the procedure.

SYMPTOMS

The fall in O₂ desaturation did not produce any discomfort to patients from Group 1, 2 and 4. However, patients in Groups 3 and 5 were observed to have increased coughing and audible wheezing during the procedure; and therefore tolerated the BAL less well. Furthermore, following BAL, these patients were noted to have a 2°C rise in temperature within 2 hours of the procedure (pyrexia in these studies was defined as a ≥ 1 °C rise in temperature). Four of the 6 patients in Group 4 were also noted to have increased coughing during the instillation of BAL fluid. All patients in Group 5 complained of right-
sided anterior pleuritic chest pain immediately post-lavage. All patients recovered from their symptoms within 6 hours of lavage.

**CONCLUSIONS**

1. It appears that the use of atropine in the pre-medication drug regime increases the volume of lavage retrieved. This is in accordance with the observations made by other workers (Neuhaus et al, 1978; Pirozynski et al, 1988; Zavala, 1988). They suggest that atropine may also minimise any vasovagal induced bradycardia, decrease airway secretions, as well as neutralize the bronchospastic effects of topical anaesthesia and instrumentation.

2. Pre-warming of the fluid to be instilled during BAL may increase cell recovery and also reduce the incidence of cough and bronchospasm during the procedure (Pingleton et al, 1983).

3. Negative pressures in excess of 100 mm Hg during the suction of instilled BAL fluid may collapse the smaller airways beyond the tip of the bronchoscope, thereby impeding fluid return (Reynolds, 1988). This may also cause trauma to the mucosal surface of the bronchi, with subsequent bleeding into the lavage fluid.

4. The effect of BAL itself and the total volume of BAL fluid instilled on arterial oxygen saturation has been previously observed by independent workers (Albertini et al, 1974; Pirozynski et al, 1988), whose findings are similar to ours. Albertini et al (1974) have suggested that the hypoxaemia produced during BAL may be related to the procedure time. In all our patients BAL was not longer than 4 minutes. The effect of BAL on lung function has also been previously noted. The audible wheeze heard in some of our patients during the lavage may be due to increased small airway resistance as a result of the instilled fluid (Goeree et al, 1987), as well as an increase in bronchial responsiveness (Kelly et al, 1988a). In this respect the temperature of instilled fluid may therefore be critical to the incidence of bronchospasm (Burns et al, 1983). Pirozynski (1988) has postulated that local airway irritation by lavage fluid may aggravate, through a reflex action, the local ventilation and perfusion of the lavaged segment resulting in a profound fall in
saturation. However, no study has ever shown that the degree of saturation produced during BAL is detrimental to the patient.

**BAL PROTOCOL USED IN THIS THESIS**

The above preliminary studies enabled a standard protocol to be set for BAL, and to be followed throughout all studies in this thesis.

**PREPARATION**

All BAL procedures were performed at 8.30 am. The subjects were asked to have nothing to eat or drink for at least four hours prior to the procedure. BAL was performed by the same person (author) in a standard fashion throughout this thesis on a total of 62 healthy non-smoking volunteers and 85 sarcoid patients (Chapters 4-7). None of the patients suffered from unstable heart disease; all had an FEV₁ > 1 litre, and PaO₂ > 10.0 KPa (without supplemental O₂) and normal PaCO₂ at rest (qv Chapter 2).

All subjects undergoing BAL received premedication with 0.6 mg of atropine sulphate IV and 5 - 10 mg of midazolam IV (the relative dose depending on individual characteristics age, weight and lung function) 15 minutes prior to the procedure. Topical anaesthesia was applied to the naso-oropharynx with 2% lignocaine hydrochloride spray aerosol.

**PROCEDURE**

The trolley as laid out for the procedure is shown in Fig 3.1

BAL was performed via a 6 mm fibreoptic flexible bronchoscope (Olympus model BT-IT20D) inserted transnasally and passed to the level of the hypopharynx where two successive 2 ml volumes of 4% lignocaine hydrochloride solution were gently instilled to anaesthetize the vocal cords before the scope was passed into the trachea. Nasal access proved difficult in 2 of the normal volunteers and 4 of the sarcoid patients; the oral route was therefore used, with the scope inserted via a plastic mouthpiece. Two ml bolus doses
Fig. 3.1

A view of the bronchoscopy trolley as laid out prior to use.
of 2% lignocaine hydrochloride* were used as appropriate (never exceeding three in total) to obtain mucosal anaesthesia and reduce coughing as the bronchoscope was advanced through the tracheobronchial tree to the level of the right middle lobe opening. This amount of lignocaine (total dose never > 10ml) in the airways has not been found to contaminate the lavage fluid appreciably or to interfere with cellular activity.

The tip of the bronchoscope was wedged into one of the subsegmental bronchi of the right middle lobe (at the level of the third or fourth generation bronchus) so as to obtain a seal with the bronchial wall. The adequacy of the wedge was verified by gentle suctioning and consequent immediate collapse of the distal airway. With the bronchoscope correctly wedged throughout the whole procedure, the subject experienced no coughing during the lavage as no fluid escaped proximal to the wedged scope. Sterile buffered 0.9% isotonic saline was then instilled through the working channel of the instrument in 20 ml aliquots at the rate of 5 ml per second to a total fluid volume of 180 ml. The saline had been previously corrected to a pH 7.0 by the addition of a calculated volume of sodium bicarbonate (B.P. 8.4% 275 ul per 500 ml of standard 0.9% physiological IV saline), and warmed to 37 °C.

Following each instillation, the fluid was recovered with the help of a suction trap to which not greater than 80 mmHg negative pressure was applied. All subjects were encouraged to take a few deep breaths during each aspiration so as to help the flow of fluid and increase the return volumes. The recovered fluid was collected into a sterile siliconized glass bottle maintained on ice, to prevent adhesion of macrophages to the glass and cellular loss.

* To avoid toxic blood levels of lignocaine (5-6 ug/ml), this local anaesthetic was always instilled in small doses. In every procedure, the total dosage of lignocaine used was kept under 400mg.
The time required to perform BAL in each case did not exceed 3 minutes. The percentage recoveries of lavage fluid instilled are given in each study (Chapters 4-7).

**POST-BAL**

At the completion of the lavage, the suction trap was disconnected from the collecting bottle and the bronchoscope gently withdrawn proximally. Each subject was asked to give a few gentle coughs to help empty the residual fluid, which was suctioned but not included for analysis. All subjects were observed for 4 hours post-lavage until the effects of local anaesthesia resolved and then allowed to go home. Complications from the procedure were infrequent, and when they occurred they consisted of a delayed transient febrile response (defined in our studies as an increase in oral temperature $\geq 1 ^\circ C$) 6 - 8 hours post BAL. This occurred in 2 out of 55 normal volunteers and 5 out of 75 sarcoid patients. All febrile episodes resolved spontaneously and uneventfully. Arterial oxygen and carbon dioxide tensions were not routinely measured during and following the lavage procedure. By virtue of the strict patient selection criteria, and standardised lavage technique in each study, any profound hypoxaemia during BAL was avoided.

**PROCESSING OF BAL SAMPLES**

The lavage fluid was filtered through a sterile single layer of loose cotton gauze to trap any gross mucus particles, and then centrifuged at 480 g, 4 $^\circ C$ for 5 minutes. The cell pellet was then washed twice in RPMI 1640 medium (Flow Laboratories) and counted in a modified Neubauer haemocytometer. The latter was preferred to the automated Coulter counter, which has been reported to underestimate the number of cells (Mordelet-Dambrine et al,1984; Saltini et al,1984b); the size of the macrophages may be outside of the "window" settings used. The cell viability was assessed by cellular exclusion of trypan blue.

A portion of the cell suspension was adjusted to a concentration of $1 \times 10^6$ cells/ml using
supplemented RPMI 1640 (containing 1.25% 200mM L-glutamine, 10% heat inactivated fetal calf serum (FCS; Sigma Chemical Co), 100 µg/ml streptomycin (GIBCO), and 100 IU/ml penicillin (GIBCO)). This cell suspension was kept on ice until required.

**CYTOSPIN PREPARATION**

The remaining cells were suspended in serum-free RPMI at a concentration of $2 \times 10^5$ cells/ml, of which 100 µl aliquots were used to prepare cytospins on a Shandon Cytospin 2. The presence of serum in the medium has been shown to reduce the percentage proportion of lymphocytes by as much as 5% on cytospin preparations in otherwise identical cytospin conditions (Saltini et al, 1984b).

One cytospin from each sample was stained for morphology (qv below), while the remainder were air-dried for 1 hour at room temperature, fixed in 1:1 mixture of chloroform-acetone for 10 minutes, wrapped in plastic film and stored unstained at -20°C until used for immunocytological analysis (q.v. below).

**CELL DIFFERENTIAL COUNTS**

Morphology was ascertained using a Diff-Quick (Dade Diagnostic) differential white cell stain (Fig 3.2) and viewed with a light microscope, using a x60 objective. At least 300 cells were counted (random field counting method) in order to reach a good reproducibility. Ciliated or squamous epithelial cells were registered but not included in the differential cell count. All samples in the studies had less than 1% epithelial cells, indicating that there was hardly any contamination of the alveolar samples by upper bronchial airway cells. All slides were also viewed for the presence of any unusual morphologic features such as inclusions or micro-organisms.
Fig. 3.2
Cytospins of unfractionated lavage cells stained for morphology
(a) normal volunteer, (b) sarcoid patient. Original magnification X600.
Note the increased number of lymphocytes (arrow) present in sarcoid lavage
IMMUNOCYTOLOGICAL ANALYSIS

Immunocytochemical and immunofluorescent techniques are used in this thesis to provide information regarding the phenotypic characteristics of the cellular components found in the retrieved BAL fluid.

ANALYSIS BY IMMUNOCYTOCHEMISTRY

A standard protocol for the indirect immunoperoxidase method was used to identify individual cell surface antigens. This procedure makes use of an unconjugated antibody which binds to the antigen in the specimen. This attachment is localized by a peroxidase conjugated antibody. A substrate is then added in order that the reaction can be visualized (Mason et al, 1983).

Cytospin preparations were removed from -20 °C storage, and allowed to equilibrate to room temperature. During the whole staining procedure all incubations were carried out at room temperature. The cytospins were first incubated with the appropriate MoAb at an optimal dilution for 1 hour. After washing in phosphate buffered saline (PBS), these specimens were further incubated with peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO, Copenhagen) for an hour. The specimens were then washed in PBS and developed for 5 - 15 minutes in freshly made diaminobenzidine (DAB) and hydrogen peroxide ($\text{H}_2\text{O}_2$) solution (Fig 3.3).

During the whole staining procedure all incubations were carried out at room temperature in a humidified chamber to prevent both drying of the cells and evaporation of antibody. Simultaneous negative controls were set up on which the MoAb was omitted, and positive controls were prepared using sections of human palatine tonsil.

The immunoperoxidase staining was read using an Olympus light microscope with high magnification (x600). Background staining was identified by comparison with the
Fig. 3.3
The schematic diagram illustrates the main steps involved in the indirect immunoperoxidase method used in throughout this thesis.
Cytospin thawed to room temperature

Specimens circled with Polysiloxane

Placed in humidity chamber

Application of 50 ult 1:100 dilution NRS to cytospin, left for 10 mins

50 ult of MoAb incubated for 60 mins

Rinsed twice in PBS

50 ult of 2nd layer Ab (1:200 P161 & 1:100 NRS) applied for 60 mins

Rinsed twice in PBS

50 ult of developing soln. (0.125 ml DAB & 5ult 3% H₂O₂ in 5 ml, pH 7.6, 0.05M Tris HCl) for 10 mins

Rinsed thoroughly in tap water

Counterstained with Haematoxylin

Dehydrated through graded alcohols

Mounted in DPX
negative control cytospins. Staining of any lysed or crushed cells was disregarded.

At least 300 cells were counted in successive high power fields, and assessed as being positive or negative (background staining only) for each antibody. The results were expressed as the percentage of cells that were positive. (Fig 3.4).

**ANALYSIS BY IMMUNOFLUORESCENCE**

The simultaneous expression of two different determinants on one single cell was identified by the double fluorescence technique, which using two different fluorochromes (fluorescein-isothiocyanate, FITC and tetramethyl-rhodamine-isothiocyanate, TRITC). Both these fluorochromes are excited by UV radiation but they display a different spectrum of light emission (green for FITC and red for TRITC) (Poulter et al, 1983). Cytospin preparations were allowed to equilibrate to room temperature. 50 ul of each selected MOAb were mixed and incubated with the specimens for 1 hour. After washing in PBS, a 50 ul mixture of goat anti-mouse immunoglobulin M TRITC, and goat anti-mouse immunoglobulin G FITC (Southern Biotechnology Associates) was added as a second layer, for 45 minutes (Fig 3.5). Goat Ig second layer reagents were used in view of previous reports of binding of aggregated immunoglobulin (Ig) to Fc receptors on human viable cells during such tests. Goat Ig is known to bind inefficiently to human Fc receptors and thus gives cleaner results.

A Zeiss fluorescence microscope, equipped with epi-illumination and narrow band barrier filters appropriate for FITC and TRITC was used for recording the doubly-labelled cells. Background fluorescence was identified using positive and negative controls as used in the immunoperoxidase method. Great care was taken to avoid spurious results that could arise as a result of interference with auto-fluorescence of macrophages. Morphological details of the cells under study were counter-checked using phase-contrast microscopy.
Fig. 3.4
Cytospins of unfractionated lavage cells, showing macrophages stained with monoclonal antibody probes, using immunoperoxidase method. The probes used here are RFD1 and RFD7. Original magnification X400
A positive reaction is recorded in the presence of (a) brownish cytoplasmic staining for RFD7, (b) dark brown granular staining of the cell membrane for RFD1
Fig. 3.5

The schematic diagram illustrates the main steps involved in the immuno-fluorescence method used throughout this thesis.
50 ult of MoAb applied to specimen, left for 60 mins

Rinsed twice in PBS

50 ult of goat Ig class-specific 2nd layer reagents conjugated with FITC & TRITC respectively

Rinsed twice in PBS

Mounted in 10% PBS/ 90% glycerol
At least 200 cells were counted in successive high power fields. The cells fluorescing either red only, green only, or both were counted, and the number expressed as a percentage of the total cells (Fig 3.6).

**INTERPRETATION AND EXPRESSION OF LAVAGE DATA**

Interpretation of lavage date can be complicated by a number of factors.

First, the number of cells recovered by lavage may vary from one procedure to the next, depending upon the volume of fluid instilled and then recovered. The total number of recovered cells has been shown to correlate well with the volume of recovered lavage fluid in both non-smoking healthy volunteers and patients (Davis et al,1982; Daniele et al,1985). The average volume of recovered fluid is also dependant on the site of BAL, the force at which the fluid instilled, the length of time it is left in the distal lung and the force of the vaccum used for aspiration, as well as the disease state (Pingleton et al,1983; Martin et al,1985; Ettensohn et al,1988; Kelly et al,1988b). In order to minimise such factors, in each study all lavages and subsequent analysis were carried out by the same person (author) under identical conditions using the above standarised protocol. In addition, for each study, the selected sarcoid patients were a relatively homogeneous group in terms of their clinical parameters.

Secondly, in presenting the results of BAL data in each study, both the differential cell counts and total cell counts are reported. Several workers have observed that the percentage analysis gives an accurate view of the relative distribution of cell types in the lower respiratory tract, thereby revealing any shift toward a particular effector cell type; while the total cell count gives insight into the cell density prevalent in the alveolar structures (Rossi, 1986).

Thirdly, washing out constituents from the lung may tend to homogenise *in situ*
Fig. 3.6
A cytospin stained for co-expression of antigens on single cells using immunofluorescence. Original magnification X400
With FITC and TRITC as second layer antibodies, the positive double immunofluorescence is seen as a bright orange staining of the cell membrane.
relationships and give a spurious picture of reality (Yeager et al, 1976). Using the above technique, the lavaged area is 100 times more alveolar surface than conducting airway surface. However, lavage may artificially mix cells and even discrete proteins that are not in actual proximity to the alveolar surface. Thus in a BAL sample, certain cell intimacy that seems apparent might be due to telescoping spatial relationships. For example, in Fig 3.7 a cytospin from the BAL of an active sarcoid patient has been stained for morphology and shows so-called spontaneous lymphocyte-alveolar macrophage rosettes. These might have been artificially created and do not necessarily represent previously reported \textit{in situ} physical contact between activated T-cells and activated macrophages (Yeager et al, 1976; Herscowitz, 1985). Appropriate caution was therefore taken not to over-interpret such lavage findings in Chapters 4 - 7.

Finally, all studies in this thesis are concerned with the cellular components of the BAL fluid, and therefore no mention is made of the soluble substances (e.g., immunoglobulins or cytokines) in the lavage fluid or their standardisation to albumin.

**SUMMARY**

BAL is a simple extension of routine fibreoptic bronchoscope. It permits a repeatable, safe, and quantitative evaluation of the inflammatory and immune processes in the alveolar structures. Provided appropriate precautions are taken during the procedure as well as in subsequent interpretation of the results, analysis of the cellular constituents in BAL fluid can provide a dynamic and kinetic impression of what is present or developing \textit{in situ} on the gas exchange surface in both normal subjects and sarcoid patients.
Fig. 3.7

A cytospin of unfractionated lavage cells from a sarcoid patient stained for morphology. Original magnification X 600

Lymphocyte-macrophage rosettes are seen (arrow)
CHAPTER FOUR

PHENOTYPIC AND FUNCTIONAL ANALYSIS OF UNFRACTIONATED ALVEOLAR MACROPHAGES FROM PATIENTS WITH ACTIVE PULMONARY SARCOIDOSIS
INTRODUCTION

Evidence has been provided in Chapter 1 that the inflammation associated with pulmonary sarcoidosis is initiated by a CD4 helper/inducer T cell lymphocytic alveolitis driven by aberrant immunological reactivity (Crystal et al, 1984; Campbell et al, 1985; Venet et al, 1985). Studies of such cells in sarcoid BAL, have shown them to release spontaneously a variety of biochemical active mediators (Hunninghake et al, 1980b; Keogh et al, 1983; Robinson et al, 1985). These substances appear to activate the AM population (Hunninghake et al, 1984). Although it is generally accepted that this T-lymphocyte-macrophage interaction generates the granulomatous inflammation within the lung parenchyma of sarcoid patients, the cellular interactions that determine the subsequent course of pulmonary sarcoidosis are less clear cut.

Clinically, in up to 80% of sarcoid patients, remission occurs either spontaneously or following steroid therapy; yet in the remaining 15% of patients there is a progression to fibrosis with considerable morbidity and mortality (James & Williams, 1985). It would seem possible that the persistence of granulomata, and development of fibrosis in this group of patients, are features that may be determined by the interaction of macrophage-like cells and T-lymphocytes.

It is recognised that non-lymphoid accessory cells such as macrophages are necessary to initiate and maintain T-cell mediated immune processes (Unanue, 1984). Several workers have shown that AM are increased in the BAL fluid of patients with active sarcoidosis (Keogh et al, 1983). In one study AM from certain patients with active sarcoidosis were shown to a release a growth factor which stimulated the proliferation of human fibroblasts (Bitterman et al, 1983a). In addition these macrophages spontaneously released mediators such as fibronectin which stimulate fibroblast recruitment and proliferation, potentially causing the development of tissue fibrosis.
These findings suggest at least one mechanism for the increase in fibroblast number and biological activity seen in a proportion of sarcoid patients. However, current data does not explain why the majority of patients with sarcoidosis have spontaneous remission of their disease, and do not undergo progression to fibrosis. Work that may help answer this question arises from observations that lung mononuclear cells can spontaneously release a factor that inhibits fibroblast growth (Elias et al, 1984). Interestingly those patients exhibiting this phenomenon were shown to have fewer pulmonary function abnormalities than the patients whose AM lacked this spontaneous inhibitory activity.

It therefore appears that aberrations in macrophage function may play a crucial role in both the induction and expression of the immune processes occurring in the lungs of patients with pulmonary sarcoidosis (q.v. Chapter 1). Detailed analysis of the local macrophage population in the lungs of sarcoid patients may thus shed more light into the pathogenic course of the disease, and help distinguish those patients who will have resolution of their disease (with or without treatment), from those who are destined to progress to irreversible fibrosis.

By using monoclonal antibody (MoAb) probes specific for macrophage surface determinants, Campbell et al (1986b) showed that AM in normal lavage are a heterogeneous population. Phenotypically distinct subpopulations of AM have been identified using MoAbs RFD1 and RFD7. These reagents distinguish dendritic cells and mature macrophages respectively in normal tissue (Poulter et al, 1986). Proportional differences have been shown to occur in macrophage subsets in sarcoidosis (Hance et al, 1985; Campbell et al, 1986b), and these reflect disease activity (Ainslie et al, 1989). Within such AM subsets, there would be expected to exist those macrophages responsible for T-lymphocyte stimulation, granuloma formation and fibrosis. Any change in the functional capacity of these cells could
therefore be crucial to the progression and outcome of pulmonary sarcoidosis.

This study was therefore designed to compare the phenotype and functional capacity of AM obtained by BAL from clinically homogeneous groups of healthy volunteers and untreated patients with active pulmonary sarcoidosis.

MATERIALS AND METHODS

SUBJECTS
The study population consisted of 10 patients all Caucasian with biopsy-proven sarcoidosis (diagnosis over one year): 7 males, 3 females; mean± S.E. age 38.3±2.06 years; all non-smokers. None of the patients had received any prior corticosteroid treatment. The 10 patients represented a homogeneous group on all clinical grounds. They were symptomatic with dry cough and/or shortness of breath on strenuous exercise. Each had unequivocal bilateral parenchymal shadowing (Grade III CXR), and restrictive ventilatory function (Table 4.1). There was no clinical or biochemical evidence of extrathoracic sarcoidosis. A population of 10 healthy controls (all non-smokers; 9 males, 1 female; mean ±S.E. age 22.6±0.12 years) were also recruited; all satisfied the selection criteria outlined in Chapter 2.

BRONCHOALVEOLAR LAVAGE
BAL was performed, and all samples processed using the standard procedure described in Chapter 3.

PERIPHERAL BLOOD MONONUCLEAR CELLS
All subjects had 20 ml of peripheral blood taken by venepuncture at the time of the BAL. Peripheral blood mononuclear cells (PBM) were separated on a Ficoll-Hypaque gradient, washed twice in Hanks' balanced salt solution and then resuspended in 10 ml supplemented RPM1 1640. The PBM suspensions were counted in a modified
### TABLE 4.1

Characteristics* of patients in this study

**Blood**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tr>
<td>ESR mm in 1 hour</td>
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<tr>
<td>SACE mmol/min/ml</td>
<td>85.3 ± 5.13</td>
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</table>

**Lung function**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Observed Value</th>
<th>% predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV₁ L</td>
<td>2.5 ± 0.4</td>
<td>83 ± 0.2</td>
</tr>
<tr>
<td>FVCL</td>
<td>3.22 ± 0.60</td>
<td>84 ± 0.32</td>
</tr>
<tr>
<td>PEFR L/min</td>
<td>500 ± 21.0</td>
<td>115 ± 5.16</td>
</tr>
<tr>
<td>FRC L</td>
<td>2.43 ± 0.12</td>
<td>80 ± 0.26</td>
</tr>
<tr>
<td>RV L</td>
<td>2.11 ± 0.14</td>
<td>105 ± 0.10</td>
</tr>
<tr>
<td>TLC L</td>
<td>5.31 ± 0.44</td>
<td>91 ± 0.26</td>
</tr>
<tr>
<td>TLCO mmol/min/kPa</td>
<td>15.9 ± 3.43</td>
<td>60 ± 2.40</td>
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<tr>
<td>KCO mmol/min/kPa/L</td>
<td>3.25 ± 1.51</td>
<td>59 ± 0.55</td>
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</table>

**Arterial Blood Gases**

<table>
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<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
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<td>pH</td>
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</tr>
<tr>
<td>PCO₂ kPa</td>
<td>4.83 ± 0.14</td>
</tr>
<tr>
<td>PO₂ kPa</td>
<td>11.0 ± 0.60</td>
</tr>
</tbody>
</table>

*The data is expressed as mean ± SE
Neubauer haemocytometer, and viability assessed by cellular exclusion of trypan blue. The cell concentration in each case was then adjusted to 1 X 10^6 cells/ml.

**IMMUNOCYTOLOGICAL ANALYSIS**

Cytospins were prepared from BAL, and the cells analysed for single or double antigen expression, using the standard techniques described in Chapter 3. In this study the MoAb probes used were RFD1 and RFD7 (Table 4.2).

**AUTOLOGOUS MIXED LYMPHOCYTE REACTIONS (AMLR)**

Mitomycin C was used to stop cell division in selected cell samples and to create one-way AMLR when two cell populations were admixed. Separate aliquots of BAL and PBM (each at a concentration of 1 x 10^6 cells/ml) were therefore initially incubated in the presence of mitomycin C (25 ul/ml) for 45 min at 37°C, 5% humidified CO₂, after which the treated samples were washed three times in RPMI. AMLR cultures were then set up in triplicate, in round bottomed microtitre wells, in 200 ul of supplemented medium containing the following populations: (a) BAL alone (1 x 10^5 cells/well); (b) PMB alone 1 x 10^5 cells/well); (c) mitomycin treated BAL admixed with PBM (2 x 10^5 cells/well).

Separate triplicate control wells of mitomycin treated BAL and PBM were also set up. In all, a total of five triplicate sets of cultures for each sample were incubated at 37 °C in an atmosphere of 5% humidified CO₂ for 4 days. After this period each well was pulsed with 2 uCi ^3^H-thymidine (^3^H-Tdr) (Amersham, 5 Ci/mmol) and the cultures incubated for a further 18 hours. The cultures were then harvested using a semi-automatic cell harvester (Titertek-Flow, Laborat Inc, McLean VA) and the amount of incorporated radioactivity was measured in a liquid scintillation counter and expressed as average counts per minute (cpm) of triplicate cultures. The measurement of DNA synthetic rate by the incorporation of tritiated thymidine has
TABLE 4.2

Monoclonal antibodies used in this study

**RFD1** - Recognises a unique epitope within the framework of the Class II HLA-DR molecule (28 - 33kd)

**RFD7** - Identifies a 77 kd antigen in mature phagocytic macrophages

**RFD1D7** - represents co-expression of the above antigens on a single cell

RF= Royal Free
become the standard method for the measurement of lymphocyte proliferation in MLR (Dupont & Hanson, 1976). The results were recorded as stimulation index (SI), defined in this study, as the factor by which (cpm $^3$H-Tdr) of cells was increased over the (cpm $^3$H-Tdr) of the same cells treated with the mitomycin. Blank wells containing medium only, but no cells, were set up as controls.

**SELECTED CELL CONCENTRATIONS IN AMLR**

In a separate experiment PBM and BAL cells were obtained from normal volunteers and processed as described above. The PBM were plated onto sterile glass culture dishes, and incubated for 1 hour at 37 °C in 5% humidified CO$_2$. The non-adherent PBM cells (98% lymphocytes) were then collected and counted. The BAL cell suspensions each contained over 95% macrophage-like cells, the remainder being lymphocytes. AMLRs were then set up using a titration of PBM lymphocytes (0.5 - 8.0 x 10$^5$/ml) against a constant proportion of BAL macrophages (6.0 x 10$^5$/ml); the selected range of lymphocyte concentration covering the extremes previously observed in the test BAL samples.

**STATISTICAL ANALYSIS**

Wherever relevant, quantitative data was analysed as outlined in Chapter 2, and expressed as mean±S.E..

**RESULTS**

**BRONCHOSCOPIC FINDINGS**

No evidence of any endobronchial infection or inflammation was found in any sarcoid patient, or in any normal volunteer. The mean percentage return of lavage fluid was 71.2±0.13% of the instilled volume in the sarcoid group, and 72.5±3.5% in the normal group.
Fig. 4.1

The mean total number of cells and absolute proportions of morphologically defined cells in normal and sarcoid lavage (a).

The mean percentage of these cells in total lavage in both groups (b).

T = total cells
M = macrophages
L = lymphocytes
N = neutrophils
Fig. 4.2
The mean percentage of total morphologically identifiable macrophages in 10 normal subjects and 10 sarcoid patients, that express positivity with monoclonal antibodies RFD1, RFD7 and RFD1D7.
CELL COUNTS
The total BAL cell yield in the sarcoid group (mean ± SE 14.9 ± 3.01 x 10^6) was higher than that of the normal group (9.71 ± 1.90 x 10^6 cells) (Fig 4.1a) (p <0.006). The sarcoid patients had a greater percentage of lymphocytes in lavage (29.2 ± 4.51) than the normal control population (7.11 ± 3.02) (Fig 4.1b) (p <0.001). The absolute number of alveolar macrophages in the sarcoid patients (10.31 ± 2.23 x 10^6 cells) was also observed to be significantly higher than the normal (8.82 ± 1.93 x10^6 cells) (Fig 4.1a) (p<0.001). The viability of the cells in all BAL and PBM suspensions was >95%.

IMMUNOCYTOLOGICAL FINDINGS
In normal volunteers, 12.3±4.04% macrophages were positive for RFD1 and 20.3 ± 3.61% for RFD7. In contrast, 44.7 ± 10.3% of the macrophage-like population in sarcoid BAL expressed positivity for RFD1 (p<0.002), and 29.9 ± 7.60% for RFD7 (p<0.36). Interestingly, in patients with sarcoidosis, 27.2 ± 6.13% of the total macrophage population in BAL expressed both surface markers, compared to only 7.32 ± 2.04% in normal volunteers (p<0.003) (Fig 4.2). The proportions of macrophages expressing RFD1, RFD7, RFD1D7 add up to greater than 100%. Much of this 'surfeit' of cells can be explained by the double expression of RFD1 and RFD7. It is also true that there must be cells that are not identified by either of the macrophage markers used.

AMLR ANALYSIS
The SI of peripheral blood in the sarcoid group (2.48 ± 0.45) was markedly reduced when compared to the normal (7.33±0.80) (p<0.001). There was no difference in lavage cell activity between the normal (SI=1.20±0.24) and sarcoid (SI=1.20 ± 0.35) groups (p<1.0). The reactivity of peripheral blood from healthy volunteers
Fig. 4.3
The autologous mixed lymphocyte reactivity expressed as mean stimulation index is
given for various cell combinations in the two study groups.
Cells tested were bronchoalveolar lavage cells (a); peripheral blood mononuclear
cells (b); and a mixture of these two (c).
The mean and standard error for each result is given in the text.
Fig. 4.4
A constant proportion of macrophages from BAL was admixed with a titration of autologous lymphocytes (0.5-8.0 x 10^5/ml).

The graph demonstrates the direct proportionality of autologous mixed lymphocyte reactivity in cpm resulting from the above cultures, to the concentration of lymphocytes (within the range tested).
TABLE 4.3
Reproducibility of autologous mixed lymphocyte reactions

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>BAL (cpm)</th>
<th>BALM (cpm)</th>
<th>SI</th>
<th>PBM (cpm)</th>
<th>PBMM (cpm)</th>
<th>SI</th>
<th>BALM</th>
<th>PBM</th>
<th>SI</th>
<th>Background (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>150</td>
<td>66</td>
<td>1103</td>
<td>103</td>
<td>303</td>
<td>10</td>
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<td>138</td>
<td>78</td>
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<td>83</td>
<td>984</td>
<td>144</td>
<td>294</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>144±3.5*</td>
<td>74±5.0*</td>
<td>1.95</td>
<td>1104±69.8*</td>
<td>145±24.6*</td>
<td>7.61</td>
<td>289±10.2*</td>
<td>1.32</td>
<td>12±1.1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>122</td>
<td>132</td>
<td>1312</td>
<td>106</td>
<td>670</td>
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<td>666</td>
<td>9</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>100±11.0*</td>
<td>121±10.58</td>
<td>10</td>
<td>1185±1288</td>
<td>132±17.0*</td>
<td>8.98</td>
<td>660±8.1*</td>
<td>2.6</td>
<td>7.3±1.2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>174</td>
<td>178</td>
<td>1896</td>
<td>193</td>
<td>402</td>
<td>13</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>171±19.6*</td>
<td>132±18.8*</td>
<td>1.30</td>
<td>1602±1278</td>
<td>195±32.2*</td>
<td>8.20</td>
<td>407±4.7</td>
<td>1.24</td>
<td>14.6±1.2</td>
<td></td>
</tr>
</tbody>
</table>

Three examples of AMLR results in normal subjects are presented. The background values represent the amount of radioactivity in wells containing culture medium alone.

* The values represent the mean ± SE of triplicate wells.
TABLE 4.4

Comparison of predicted and actual isotopic incorporation in BAL and peripheral blood lymphocytes from sarcoid patients and normal subjects in autologous mixed lymphocyte reactions

<table>
<thead>
<tr>
<th></th>
<th>PREDICTED*</th>
<th>ACTUAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SARCOID</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>375 ± 35</td>
<td>233 ± 46#</td>
</tr>
<tr>
<td>PBM</td>
<td>500 ± 50</td>
<td>290 ± 37#</td>
</tr>
<tr>
<td>NORMAL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAL</td>
<td>150 ± 15</td>
<td>178 ± 23</td>
</tr>
<tr>
<td>PBM</td>
<td>900 ± 100</td>
<td>1100 ± 200</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE counts per minute

* predicted values from 10 sarcoid patients and 10 normal subjects calculated from results in Fig 4

# \( P = < 0.01 \) compared to predicted values
was suppressed on admixture with autologous mitomycin-treated BAL cells (SI = 1.44 ± 0.36). In contrast when mitomycin-treated unfractionated BAL cells were used to stimulate PBM in sarcoid patients, the AMLR was completely abolished (SI = 0.87 ± 0.33). (Fig 4.3). Control wells in each experiment consistently gave recordings of less than 20 cpm. Table 4.3 illustrates the reproducibility of the results in each group.

It could be argued that the above observations were influenced by gross differences in lymphocyte proportions in the test samples: lymphocytosis is generally present in sarcoid BAL compared to normal, and proportionately more lymphocytes are present in normal PBM. However, in admixing a constant proportion of macrophages with a titration of autologous lymphocytes, it was observed that the AMLR was directly proportional to the number of lymphocytes present within the range tested (Fig 4.4). Using this information, the SI in AMLRs of the test cultures could be predicted and compared with the actual results obtained. As the data in Table 4.4 shows, the actual results obtained using normal PBM and BAL cells matched the predicted values. In contrast, in the sarcoid test samples, the actual results obtained were repeatedly significantly lower than the predicted values.

**DISCUSSION**

The results in this chapter show that consistent phenotypic and functional aberrations exist within the AM population in the BAL of patients with active pulmonary sarcoidosis. Significant increases over normal in the proportions of certain phenotypically distinct macrophage subsets (recognized by the MoAbs RFD1 and RFD7) were a consistent feature in the lavage of sarcoid patients in this study.

RFD1 recognizes a unique class II MHC cell membrane antigen related to HLA-DR (Poulter et al, 1986). This observation is substantiated by its normal tissue
distribution, which is limited within the macrophage-like cell family, to the dendritic interdigitating cells of the T-cell zones of secondary lymphoid tissue. While all RFD1+ cells are HLA-DR positive, not all HLA-DR cells coexpress RFD1 (eg. Langerhans' cells). This suggests that the RFD1 antibody recognizes an epitope with restricted expression, or an associated but distinct class II antigen as yet ill-defined. The functional role of the RFD1 epitope in antigen presentation is supported by studies in which, the addition of the RFD1 MoAb inhibited increase in lymphocyte reactivity (Poulter & Duke, 1983).

RFD7 identifies a 77kd cytoplasmic antigen, that is found in mature, acid phosphatase positive, phagocytic, tissue macrophages. This antigen is not present in dendritic cells (Poulter et al, 1986). Studies of fetal tissue reveal that RFD7+ macrophage-like cells appearing in the yolk sac (Janossy et al, 1986), also react with the MoAb UCHM1 (described as a monocyte marker) (Hogg et al, 1984). In contrast, when RFD7+ cells appear in the fetal liver at 9-12 weeks, these more mature cells are UCHM1- (Janossy et al, 1986). Separate studies have shown that the RFD1 and RFD7 antigens are separate in the developing fetus (Janossy et al, 1986), and are mutually exclusive on cells in normal secondary lymphoid tissue, skin and cultured peripheral blood monocytes. Studies have shown that when peripheral blood monocytes(RFD1-D7-) are cultured in vitro, two distinct populations of RFD1+D7- and RFD1-D7+ cells emerge (Poulter et al, 1986).

A very small proportion of macrophages in normal BAL appear to express both antigens. In addition, the present study shows that high proportions of these RFD1+D7+ macrophages are found in the lavage of clinically active sarcoid patients. These results differ from data obtained from similar studies of BAL from patients with extrinsic allergic alveolitis (Johnson et al., 1989), and cryptogenic fibrosing alveolitis (Noble et al, submitted 1989), where greater increases in RFD1+D7- cells
are seen. The emergence of RFD1+D7+ macrophages in sarcoid BAL may indicate that such cells are immature, and have not yet developed along separate pathways to express one or other antigens as mature cells. This possibility is however not supported by the fetal studies (q.v. above). An alternative possibility is that dual antigen expression is the production of the local immune environment, influenced by lymphocyte interaction, and/or soluble mediator production. In support, Langerhans cells have been shown to become RFD1+ when included in lesions of atopic eczema (Alegre et al, 1986). Poulter et al (1987) have demonstrated that gamma-interferon can induce an increase in the proportion of RFD1+ cells developing from PBM with concomitant suppression of RFD7+ expression. It is however not known whether such changes also result in altered function. Indeed the functional significance of dual antigen expression on macrophages in normal BAL is unknown.

The MLR is an *in vitro* T-cell mediated response representing the recognition of cell-bound alloantigens (Dupont & Hanson, 1976). The cells responding by proliferation in the mixed lymphocyte culture are T-cells (Andersson et al, 1973; Blomgren, 1977). These responder T-cells have been shown to react with human HLA-D region antigens (Dupont & Hanson, 1976), and gene products on macrophages in the stimulator population (Huber et al, 1981). If disparity exists between the responder and stimulator populations at the HLA-D regions, proliferation of the responder cells takes place. It is recognized that the stimulating cells in the MLR are the macrophages (Rode & Gordon, 1974). In fact, cells of the monocyte-macrophage lineage must be present during the course of the reaction for a significant response to be generated (Berlinger et al, 1976; Huber et al, 1981). The use of mitomycin C effectively blocks the proliferation of the lymphocyte population in a selected sample, resulting in a one-way MLR (Beale et al, 1980). The AMLR is seen to be a proliferative reaction between T-cells and autologous mitomycin C-treated cells of the monocyte-macrophage lineage (Beale et al, 1980). Using such cultures, the data in this study suggests that the lavage lymphocytes isolated from sarcoid patients do not express increased AMLR
activity. This observation is in contrast to a degree with the findings of Semenzato et al (1986). Their study demonstrated increased CD4+ T lymphocyte activity in BAL from a heterogeneous group of patients with active sarcoidosis. This T cell hyperactivity was seen in response to mitogenic and antigenic stimulation. The difference in these observations can be accounted for, by two facts. First, in view of the variable nature of sarcoidosis (James & Williams, 1985) any group of sarcoid patients under study is already potentially heterogeneous as regards their immune and clinical status. A homogeneous group of patients, appearing consistent in terms of clinical status, pulmonary function and radiological grading was therefore enrolled in the present study (no such heterogeneity was reported in the work by Semenzato et al, above). Secondly, any exogeneous mitogenic or antigenic manipulation of cell samples from these patients would only imply what these cells 'could potentially do' and not necessarily be representative of what they are functionally doing in vivo. In this regard the 'standard lymphocyte graph' (Fig 4.4) confirmed the initial observations on lavage that AMLR activity did not identify lymphocyte activation in the lungs of this homogeneous group of sarcoid patients in vivo, and that AMLR in these patients appears suppressed both systemically and locally in the lung. Clearly this may only be true of Grade III CXR progressive sarcoid patients, and quite different results might be obtained if earlier presenting patients only with BHL and a lymphocytic alveolitis were studied. Indeed there is little doubt that the lavage lymphocytes in sarcoidosis are activated as described previously in Chapter 1.

The observations that HLA-DR+ alveolar macrophages from normal volunteers fail to stimulate the proliferation of peripheral blood T-lymphocytes confirm the work of Lipscomb et al (1986) and Lyons et al (1986). The results presented in this study go further, in showing that BAL populations when mixed with autologous PBM suppress the latter's AMLR reactivity, and in the case of the sarcoid patients investigated, abolish the AMLR completely. This is in conflict with the previous observations of
Lem et al (1986) and Venet et al (1985), who demonstrated that BAL cells from sarcoid patients enhanced antigen presentation, by supporting in vitro antigen-induced T-cell proliferation. Again the difference in results may arise due to varying patient groups in different stages of their disease. Workers have suggested that the enhanced T-cell activity by unfractionated sarcoid BAL macrophages may be a result of an increase in the number of newly recruited monocytes to the lung, retaining their antigen-presenting ability. Yet this study clearly shows that the intrinsic AMLR of sarcoid PBM is much lower than that of normal PBM. As the relative proportions of these subpopulations could be influenced by local stimuli, they may therefore vary in different patient groups, depending on the current immune status and/or level of disease activity. In support of this suggestion, Ainslie et al (1989) produced evidence that the proportions of phenotypically distinct AM subpopulations change in relation to clinical activity. In that study, the proportion of RFD1+D7+ cells was seen to increase in parallel to increasing radiological parenchymal involvement. The observations in the current study, of a significant increase in RFD1+D7+ macrophages, together with a suppression of peripheral blood lymphocyte reactivity by unfractionated BAL cells (that contain these macrophages), raise the possibility of a cause and effect relationship.

In conclusion, the results in this chapter suggest that in a homogeneous group of patients with type III chest X-ray, unmanipulated lavage lymphocytes showed suppressed rather than enhanced AMLR activity. The basis for this suppressed response may be associated with phenotypic and functional changes within the local macrophage populations.
SUMMARY
Bronchoalveolar lavage (BAL) was performed on 10 patients with active pulmonary sarcoidosis, and 10 healthy normal volunteers. In each case aliquots of the lavage were used to prepare cytospins, on which differential cell counts were performed. Immunocytological methods using monoclonal antibodies RFD1 and RFD7 (identifying dendritic cells and mature macrophages in normal tissues) were performed to identify macrophage subsets. Sarcoid BAL contained a significantly higher proportion of RFD1+ cells than did normal lavage. Much of this increase was accounted for by a highly significant rise in the proportion of cells with the double phenotype RFD1+D7+. Suspensions of unfractionated sarcoid and normal BAL cells were also studied in autologous mixed lymphocyte reactions (AMLR) using autologous PBM as the responder population. In each experiment reactivity was compared to mitomycin treated controls. These functional studies revealed that sarcoid PBM expressed markedly reduced AMLR reactivity when compared to normal. However, both sarcoid and normal unfractionated BAL cells were relatively unreactive. BAL mixed with PBM suppressed peripheral blood AMLR reactivity in the normal group. In sarcoid patients BAL mixed with PBM abolished AMLR completely. The possibility is raised that within the lung, there may exist a cause and effect relationship between changes in macrophage phenotype and function, and reduced T-cell responsiveness.
CHAPTER FIVE

A COMPARATIVE STUDY OF THE PHENOTYPIC, PHYSIOLOGICAL, AND FUNCTIONAL CHARACTERISTICS OF ISOLATED AM SUBPOPULATIONS FROM NORMAL AND SARCOID BAL
INTRODUCTION

Local defence mechanisms in the lung rely heavily on alveolar macrophages (AM) to remove inhaled particulate material, to promote acquired immune responses (North, 1978; Unanue et al, 1987), and to secrete mediators central to the control of local cellular interactions (Takemura & Weib, 1984). In the light of this, there have been several reports demonstrating a heterogeneity within the AM population. Separate subsets have been identified in terms of morphology (Nakstad et al, 1989), cell density (Zwilling et al, 1982), and surface antigen phenotype (Baumgartner et al, 1988). Cell populations isolated by colloidal silica gradients from animal and human BAL and lung tissue digests also show heterogeneity in terms of motile capacity and immunologic function (Shellito & Kaltreider, 1984; Brannen & Chandler, 1988; Holt et al, 1988). Of importance in human studies, have been the findings that while the majority of density-fractionated macrophage-like cells functioned as stimulators of mixed lymphocyte reactions (MLR), some fractions were found to be inhibitory (Sandron et al, 1986; Nicod et al, 1987). This observation is in keeping with the results of investigations into the T-cell inducing capacity of AM in rats (Holt et al, 1985). Such data therefore strongly suggests that within the AM pool, exist subsets of cells that regulate the induction and strength of local T-cell mediated immune responses.

The use of density gradients alone to separate sub-populations has been shown to produce anything from three (Zeidler et al, 1987) to eighteen bands of cells (Chandler et al, 1986). However, with the possible exception of the relationship between so-called dendritic cells and mature phagocytes in light-density and dense bands respectively (Holt et al, 1988) no established correlations exist between cell density and function. Furthermore it is clearly impossible to use density alone to discriminate cells in situ. On the other hand, correlations between surface antigen expression (phenotype) and function in cells of the monocyte-macrophage lineage are

By using MoAbs that discriminate functionally distinct macrophage-like cells in normal tissues (Poulter et al, 1986), the study in Chapter 4 identified three subpopulations within the AM pool obtained by BAL from human subjects and active sarcoid patients. The possible significance of these subsets was revealed by the observation that their proportions within BAL altered dramatically with the advent of disease, and that T-cell responsiveness might be influenced by relative changes within this heterogeneous AM population. In addition other studies have shown that such differences in AM subsets in sarcoid BAL may reflect disease severity (Ainslie et al, 1989).

A study was therefore set up to identify and isolate the three phenotypically distinct macrophage subpopulations from normal and sarcoid BAL, using macrophage-specific MoAbs in conjunction with antibody-coated magnetic beads and density separation techniques. This would then allow a detailed comparative characterization of each AM subpopulation obtained from the two groups. Such data would determine whether differences in macrophage subset proportion in sarcoid BAL were simultaneously accompanied by changes in surface receptor expression, physiology, and functional capacity consequent on the advent of sarcoid inflammation in the local microenvironment.

MATERIAL AND METHODS

SUBJECTS

A total of 30 patients with clinically active sarcoidosis were recruited: all non-smokers, 25 males, 5 females; mean ± S.E. age 35.0 ± 4.71 years. None of the patients were receiving any treatment at the time of the study. All had bilateral interstitial
shadowing on CXR. Their other details, including physiological function, are given in Table 5.1. The control population consisted of 32 healthy controls: all non-smokers, 25 males, 7 females; mean ±S.E. age 23.4 ±1.40 years, with normal chest radiographs and pulmonary function. None had a past history of lung disease or any viral illness in the preceding two weeks.

**BRONCHOALVEOLAR LAVAGE**

BAL was performed on all recruited subjects, and all samples processed with the standard procedure described in Chapter 3. The final cell concentration in each sample was adjusted to 1 x 10^6 cells/ml using supplemented RPMI 1640.

**SEPARATION OF MACROPHAGE SUBPOPULATIONS**

A schematic diagram of the methods involved in separation of the macrophage subpopulations is shown in Fig 5.1.

The above cell suspension was plated onto sterile plastic 85 mm - diameter tissue culture grade petri dishes (Nunc, Denmark), with no more than a total of 6.0 x 10^6 cells on each with a medium depth of 3 mm. These were incubated for 2 hours at 37 °C in an atmosphere of 5 % humidified CO₂. The supernatant containing the nonadherent cell population was then collected and the plate washed three times with medium to remove any further non-adherent cells. The adherent cells were gently scraped off the plates using a sterile rubber policeman. The adherent and non-adherent cell populations were centrifuged at 480 g, 4 °C for 5 minutes, resuspended in medium at 1 x 10^6 cells/ml and 2 x 10^6 cells/ml respectively and kept on ice until required.
TABLE 5.1

Characteristics* of patients in this study

**BLOOD**

- ESR mm in 1 hour: $24 \pm 2.24$
- SACE mmol/min/ml: $79 \pm 8.12$

<table>
<thead>
<tr>
<th>Lung function Test</th>
<th>Observed Value</th>
<th>% predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEFR L/min</td>
<td>$430 \pm 10.4$</td>
<td>$71 \pm 5.41$</td>
</tr>
<tr>
<td>FEV$_1$ L</td>
<td>$2.61 \pm 0.11$</td>
<td>$67 \pm 0.22$</td>
</tr>
<tr>
<td>FVC L</td>
<td>$4.04 \pm 0.64$</td>
<td>$80 \pm 0.43$</td>
</tr>
<tr>
<td>FRC L</td>
<td>$3.41 \pm 0.42$</td>
<td>$87 \pm 0.61$</td>
</tr>
<tr>
<td>RV L</td>
<td>$6.22 \pm 0.61$</td>
<td>$83 \pm 0.60$</td>
</tr>
<tr>
<td>TLC L</td>
<td>$2.23 \pm 0.21$</td>
<td>$96 \pm 0.34$</td>
</tr>
<tr>
<td>TLCO mmol/min/kPa</td>
<td>$26.2 \pm 1.43$</td>
<td>$78 \pm 0.91$</td>
</tr>
<tr>
<td>KCO mmol/min/kPa/L</td>
<td>$4.41 \pm 0.81$</td>
<td>$92 \pm 0.50$</td>
</tr>
</tbody>
</table>

**Arterial Blood Gases**

- pH: $7.50 \pm 0.41$
- PCO$_2$ KPa: $4.95 \pm 0.64$
- PO$_2$ KPa: $10.8 \pm 0.45$

*The data is expressed as mean ± SE
1 Neuraminidase treatment of sheep red blood cells (SRBC)

SRBC were used in these studies for T-cell rosetting, which was facilitated by prior treatment of the SRBC with neuraminidase that sloughs off any sialic acid residues present on these cells (Warren, 1959). Neuraminidase, supplied as a salt-fractionated, dialyzed and lyophilized powder (5.6 ml solid, Sigma Chemical Company) was dissolved in 10 ml distilled water. The solution was then divided into 0.3 ml aliquots and stored at -20 °C until further use. Four ml of 25% SRBC (Tissue Culture Service, Berkshire, UK) were washed three times in Hanks buffered saline solution. Following the last wash, the red cell pellet (about 1 ml packed volume) was resuspended in 10 ml RPMI 1640 to give a 10% SRBC solution; to which 0.3 ml of neuraminidase were added. This mixture was incubated for 45 minutes at 37 °C, after which the cells were washed three times and finally resuspended in RPMI 1640 to obtain the original 10% SRBC concentration. The cells were then stored at 4 °C (not more than one week) until use. Before each experiment the supernatant of the SRBC suspension was checked for lysis and the viability of the cells ascertained with trypan blue.

2 Conjugation of magnetic beads

The MoAb RFD1 (Ig Class) was conjugated onto M450 uncoated non-activated magnetic polystyrene beads (Dynal) by physical adsorption. The RFD1 coated beads were then used for specific separation and rosetting of any RFD1 positive cells from the heterogeneous AM suspension. The purified boric acid precipitated RFD1 antibody was dissolved in 0.2 M Sorenson phosphate buffer (prepared by adding 2.72 g KH$_2$PO$_4$ dissolved in 100 ml distilled water, to a 100 ml solution of 2.84 g Na$_2$HP0$_4$, until a

*Sialidase, N-acetyl neuraminate glycohydrolase from Clostridium perfringens.
pH of 7.5 was reached) in a concentration of 150 ug antibody per ml of buffer. 500 ul of this solution was then added to an equal volume of a homogeneous suspension of M450 uncoated magnetic beads in a sterile Nunc freezing tube and mixed well. This mixture achieved a ratio of 75 ug antibody per 15 mg of beads, which was incubated for 24 hours at room temperature by slow end over end rotation. The suspension was then transferred to a 10 ml conical tube which was placed for 10 minutes in a Dynal Magnetic Particle Concentrator (MPC). With the tube still in the MPC, the supernatant was decanted off and discarded, while the MoAb coated beads were collected and washed four times at 4 °C in a buffer made up of 0.1% (W/V) phosphate buffered saline with bovine serum albumin (PBS/BSA). The first two washings were performed by end over end rotation for five minutes each, while the final two were done over 30 minutes. The RFD1 coated beads were finally resuspended in 0.1% PBS/BSA to a concentration of 2 x 10^8 beads/ml (30 mg /ml) and stored at 4 °C. For storage periods of greater than 2 weeks, 0.2% sodium azide was added as a bacteriostatic agent. Adequate binding of the MoAb to the beads was tested prior to use by a modified indirect immunoperoxidase method. Cytospins of the MoAb-bead suspension were incubated for one hour with a peroxidase conjugated rabbit antimouse immunoglobulin only (q.v. Chapter 3). Presence of adequate MoAb conjugation to the beads was indicated by clear dark brownish staining at the bead surface.

3 Preparation of Metrizamide

Metrizamide (Nycomed AS, Diagnostics, Norway) is a tri-iodinated benzamide-derivative of glucose (molecular weight 789, density 2.17g/cm^3) (Rickwood et al 1974). Its value lies in its capacity to act as an in situ density gradient medium for the separation of cells by isopycnic centrifugation. 14.5g of metrizamide were dissolved in 100 ml Dutch modified RPMI 1640 (20 mM hepes buffer, 1.0 g/l sodium bicarbonate and 6.5 g/l sodium chloride supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 ug/ml streptomycin and 10% FCS). The suspension was then
divided into 2 ml aliquots in bijoux bottles, and stored frozen at -20 °C until use.

**ISOLATION OF DISTINCT MACROPHAGE SUBSETS**

To each volume of the 2 x 10^6 cells/ml non-adherent cell suspension, an equal volume of 1 % (v/v) neuraminidase treated sheep red blood cells (Tissue Culture Services, Berkshire UK) was added. This suspension was supplemented with 2.5% FCS (Sigma Chemical Co, St Louis USA). This was then incubated for 30 mins at 37 °C in 5 % humidified CO_2. Following incubation, the cell suspension was centrifuged at 450 g at 4 °C for 5 mins and then left on ice at 4 °C with the supernatant intact for one hour. The cell pellet was then very gently resuspended and each 20 ml of the cell suspension carefully underlayed with 14 ml of a Ficoll-Hypaque gradient (Nycomed, Norway) using a large bore pipette. The preparation was centrifuged (650 g) at room temperature for 15 mins, the resulting cell interface (free of E-rosetting cells) was harvested, washed twice, and finally resuspended in supplemented RPMI 1640 medium, labelled and kept on ice.

The adherent cell population was divided into two separate cell suspensions, each adjusted to 1 x 10^6 cells/ml. To one cell suspension was added 50 ul magnetic beads (Dynabeads, Norway) conjugated to monoclonal antibody RFD1(Table 5.2) at 2 x 10^8 beads per ml giving a bead: cell ratio of 100:1. This bead/cell suspension was then made up to a volume of 5 ml with phosphate buffered saline containing 0.1% bovine serum albumin (v/v). This was allowed to stand on ice for 30 mins (being gently agitated every 10 mins). A magnet was then applied to the side of the tube for 10 mins to separate out any cells bound to the antibody-conjugated beads. The supernatant was then carefully decanted into a clean tube, a further 50 ul of beads added and the incubation/ magnetization steps repeated. The cells obtained in the supernatant were washed twice in medium, resuspended in supplemented RPMI 1640 and kept on ice.
TABLE 9.2

PANEL OF MONOCLONAL ANTIBODIES USED IN THIS STUDY

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>MOL.WT</th>
<th>SPECIFICITY in NORMAL TISSUE</th>
<th>SOURCE</th>
<th>REFERENCE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFDR1</td>
<td>28/33 kd</td>
<td>Identifies a framework epitope on the HLA-DR molecule</td>
<td>RFHSM</td>
<td>Janossy, 1986</td>
</tr>
<tr>
<td>RFD1</td>
<td>28/33 kd</td>
<td>Identifies interdigitating cells &amp; a small proportion of B-cells</td>
<td>RFHSM</td>
<td>Poulter, 1986</td>
</tr>
<tr>
<td>RFD7</td>
<td>77 kd</td>
<td>Identifies mature phagocytic macrophages</td>
<td>RFHSM</td>
<td>Poulter, 1986</td>
</tr>
<tr>
<td>RFD9</td>
<td>-</td>
<td>Identifies epithelioid cells &amp; tingible body macrophages</td>
<td>RFHSM</td>
<td>Munro, 1987</td>
</tr>
<tr>
<td>EMB11</td>
<td>110 kd</td>
<td>Identifies all cells of the monocyte - macrophage lineage</td>
<td>DAKOPATTS DENMARK</td>
<td>Kelly, 1988</td>
</tr>
<tr>
<td>[CD68]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCHMI</td>
<td>52 kd</td>
<td>Identifies antigen present on the majority of blood monocytes</td>
<td>PCL Beverley</td>
<td>Hogg, 1984</td>
</tr>
<tr>
<td>[CD14]</td>
<td></td>
<td></td>
<td>University College London</td>
<td></td>
</tr>
<tr>
<td>Anti C3b</td>
<td>205 kd</td>
<td>Reacts with the receptor for the third component of human complement</td>
<td>DAKOPATTS Denmark</td>
<td>Gerdes, 1982</td>
</tr>
<tr>
<td>receptor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti Fibronectin</td>
<td>-</td>
<td>Reacts with fibronectin in human cells</td>
<td>DAKOPATTS Denmark</td>
<td>Kradin, 1986</td>
</tr>
<tr>
<td>MoAb 10.1</td>
<td>71 kd</td>
<td>Reacts with Fc receptors FcRI human mononuclear cells</td>
<td>N Hogg Imperial Cancer Research Laboratories London</td>
<td>Dougherty, 1987</td>
</tr>
</tbody>
</table>

RFHSM = Royal Free Hospital School of Medicine
CD= cluster designation
* = only first author is given for clarity of figure
Using 10 ml conical centrifuge tubes, 5 ml aliquots of the second adherent cell suspension (1 x 10^6 cells/ml) were layered on a 2 ml metrizamide gradient (frozen metrizamide suspension was removed from -20°C prior to use, and allowed to equilibrate to room temperature unaided). This was then spun at 650 g for 10 minutes at room temperature. The light density fraction was then removed washed twice in medium resuspended in supplemented RPMI and kept on ice at 4°C.

**PHENOTYPE OF SEPARATED CELL POPULATIONS**

The homogeneity of each macrophage subpopulation obtained by the above techniques was assessed using monoclonal antibodies RFD1 and RFD7 (Poulter et al, 1986), and analysed by immunocytological methods (q.v. Chapter 3). Any separated macrophage sample that was less than 85% homogeneous for its subset phenotype, was discarded. The viability of the cells recovered by the above methods was assessed by trypan blue exclusion.

Cytospins were prepared on a Shandon Cytospin 2 using 100ul aliquots of each of the above cell suspensions(2x10^5 cells/ml). One cytospin from each sample was stained for morphology, while the remainder were air-dried for one hour at room temperature, fixed in 1:1 mixture of chloroform-acetone for 10 mins, wrapped in plastic film and stored at - 20°C until use.

**IMMUNOCYTOLOGICAL ANALYSIS**

The proportion of the three macrophage subsets in all unfractionated normal and sarcoid BAL samples was assessed using MoAbs RFD1 and RFD7. Other MoAb probes (Table 5.2) were used to characterise the three macrophage subpopulations in both study groups. Immunocytological analysis was carried out using the standard techniques described in Chapter 3. In addition, the proportion of RFD1+D7+ cells in each BAL sample was related to the number of lymphocytes therein.
HISTOCHEMICAL ANALYSIS

The experiments in this section were performed on cytospins from lavage samples of 10 of the above sarcoid patients, and 10 of the normal volunteers.

1. Lyzosomal Enzyme Activity

Lyzosomal enzyme activity in unfractionated normal and sarcoid AM, as well as in each of the subsequent isolated macrophage subpopulations was investigated using a standard histochemical reaction for acid phosphatase (Lojda et al., 1964). The reaction mixtures were prepared immediately prior to use. Two ml of 4% sodium nitrate in distilled water was mixed with 2 ml hexa3 pararosaniline. Of this mixture 2 ml was added to 0.1 M acetate buffer at pH 5.0 and the solution then gently stirred into 0.5 ml naphthol AS-BI phosphate. The appropriate cytospins were then incubated in the above mixture at 37 °C for one hour and counterstained with haematoxylin. The percentage of total morphologically identifiable macrophages that gave a positive result in each test sample was read using an Olympus light microscope with high magnification (x600).

2. Hexose Monophosphate Shunt Activity

The level of hexose monophosphate shunt activity in unfractionated and separated AM from both study groups was determined by a histochemical method that identified glucose-6-phosphate dehydrogenase(G-6-PD) (Altman, 1969). The reaction mixtures were prepared immediately prior to use. To 5 ml of glycyl glycine buffer (0.1 M; pH 8.0; warmed to 37 °C) was added 15 mg glucose-6-phosphate disodium, 7.5 mg NADP and 15 mg of nitroblue tetrazolium (NBT). A negative control mixture consisting of 3 mg NADP and 6 mg NBT measured into 2 ml glycyl glycine buffer was also prepared. Unfixed cytospins of each macrophage subpopulation were incubated with 50 ul of the reaction mixture and control mixture respectively for 90 minutes at 37 °C.
QUANTIFICATION OF G-6-PD REACTIVITY. The density of G-6-PD reactivity in each cell is reflected in the 'reaction product' (i.e. the insoluble formazan) that precipitates following the reduction of the NBT salt (which acts as an H+-acceptor). To facilitate the quantification of the amount of formazan deposited, use was made of a Vickers M85 scanning and integrating microdensitometer set at 585 nm [the isobestic wavelength of the formazans of NBT (Butcher, 1972)] and a x40 objective. The mask was positioned so that only one cell was within the masked area at each reading. 100 consecutive readings of individual AM were made in each cytospin by a single observer (author) who completed all the recordings in the study; repeat sets of measurements were conducted to a total of 6 cytospins from each separated macrophage subset. Each reading was recorded as relative density of 'reaction product' per unit area of cell. The area of each cell was determined simultaneously by setting the threshold value of the microdensitometer to the background absorbance value.

PHAGOCYTOSIS

Macrophages from each separated subpopulation (1 x 10^5 cells), obtained from 5 normal subjects and 5 sarcoid patients, were incubated with fluorescein-coated latex beads of 1.0um diameter (Polysciences, Northampton England) in a concentration of 100 beads per cell for 2 hours at 37 °C in an atmosphere of 5% of humidified CO₂. Control plates were set up with the inclusion of cytochalasin B (Imperial Chemical Industries Ltd, Alderly Park, England) in a concentration of 2.0 x 10⁻⁵ M prepared fresh in a solution of 0.2% dimethylsulfoxide to block phagocytosis (Axline & Reaven, 1974). This would facilitate differentiation of the cells that have actually phagocytosed the beads, from those in which the beads are just stuck to their surface. Following incubation, each cell suspension was washed twice to remove excess beads. Cytospin preparations were made and latex beads within the cells were identified by the presence of fluorescein on their surface using a Zeiss standard microscope fitted with epi-illumination and appropriate filters for FITC. A minimum of 100 cells were
counted on each cytospin preparation and the presence of 5 or more latex beads in a cell was considered to constitute phagocytosis.

**ALLOGENEIC MLR**

Functional experiments using allogeneic MLR were performed in parallel with macrophage subpopulations obtained from 7 normal subjects, and 10 sarcoid patients. In these experiments, aliquots of $1 \times 10^4$ unfractionated BAL cells and similar aliquots of each of the three separated cell subsets were respectively co-cultured in triplicated wells with a standard population of $1 \times 10^5$ allogeneic normal peripheral blood mononuclear cells (PBM) from a constant donor as a responder population (the PBM having first been processed using the same technique as in Chapter 4).

In subsequent studies, varying concentrations of selected subsets of AM were incubated with a constant number of the allogeneic PBM responder population. In certain experiments (performed on only 5 of the normal subjects) the proportions of one macrophage subset remained constant while a second varied from ratios of 1:10 to 1:1. In other experiments (using samples from another 5 normal volunteers, and 5 sarcoid patients) the proportions of both cell populations varied to maintain a constant number of stimulating cells within each well. Details of each culture situation are described in appropriate places in the results section.

All cultures were incubated for 5 days at 37 °C in 5% humidified CO₂. The cultures were then pulsed with tritiated thymidine for 18 hours and harvested using a semi-automatic cell harvester (Titerek-Flow, Laborat. Inc, McLean Va). The amount of incorporated reactivity was measured in a liquid scintillation counter and expressed as average counts per minute (cpm) of triplicate cultures.
STATISTICS
Quantitative data were expressed as the mean± standard error (S.E.). Wherever relevant, appropriate significance between results was determined as outlined in Chapter 2.

RESULTS
BRONCHOSCOPIC FINDINGS
No evidence of any bronchial infection or inflammation was found in any normal subject. Signs of endobronchial mucosal inflammation was observed in 6 out of 30 active sarcoid patients. In this study, the mean percentage return of lavage fluid was 72.0 ± 11.0 % of the instilled volume in the normal group, and 70.0±9.5% in the sarcoid group.

DIFFERENTIAL CELL COUNTS
The mean ± SE total BAL cell yield in the normal volunteers was 9.0± 1.4 x 10^6 cells, of which the absolute number of AM was 8.3 ± 1.0 x 10^6 cells. In contrast, the mean +SE total BAL cell yield in 30 sarcoid patients was 16.5 ±2.5 x10^6, of which the absolute number of AM was 11.2±0.9 x10^6. The mean proportion of lymphocytes and polymorphonuclear cells in sarcoid BAL was 35% and 5% respectively; while that in normal lavage was 9% and 2% respectively. The viability of unfractionated macrophage-like cells and separated AM subsets in both normal and sarcoid samples was >90% by trypan blue exclusion. All cells in the separated populations were morphologically identified as macrophages.

IMMUNOCYTOLOGICAL ANALYSIS
In unfractionated normal lavage, 13.2±3.5% of macrophage-like cells expressed positivity for RFD1 and 28.2±10.6% for RFD7. Only 6.0±1.5% of normal lavage macrophages expressed both these surface markers. In contrast, of the unfractionated
<table>
<thead>
<tr>
<th>SUBSET</th>
<th>RFD1+D7-</th>
<th>RFD1-D7+</th>
<th>RFD1+D7+</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL</td>
<td>97.0 ± 2.41</td>
<td>90.5 ± 4.16</td>
<td>97.4 ± 1.60</td>
</tr>
<tr>
<td>SARCOID</td>
<td>96.4 ± 1.40</td>
<td>89.1 ± 3.51</td>
<td>98.2 ± 1.41</td>
</tr>
</tbody>
</table>

*The homogeneity is expressed as the mean ± SE percentage of macrophage-like cells (within each isolated sample) that express positivity to the MoAb probe/s that identify the appropriate AM subpopulation.
lavage macrophage population in active sarcoid BAL, 50.4±8.6% were positive for RFD1 (p< 0.001) and 35.4±6.4% for RFD7 (p< 0.001). Much of the increase in these cell populations was due to the increased emergence of doubly labelled macrophages (30.4±4.0%) in sarcoid lavage (p< 0.001). In both normal and sarcoid groups, the remaining AM did not express positivity to either of these specific probes. While they all reacted with EBM11, only 5% of unfractionated AM were UCHM1 positive in normal BAL, and 7% in active sarcoid BAL.

The mean phenotypic homogeneity in each macrophage subset was over 85% in each case (Table 5.3).

Analysis of the phenotypic features of the AM subpopulations in normal and sarcoid groups are summarized in Tables 5.4 and 5.5 respectively. The 3 isolated AM subsets exhibited distinctive phenotypic features. In addition, sarcoid-related differences were observed. Over 95% of each AM subpopulation in both normal subjects and active sarcoid patients are RFD1 and EBM11 positive; but UCHM1 negative. The intensity of RFD1 on RFD1+D7+ macrophages was much stronger in sarcoid patients. An increased proportion of RFD1+D7+ AM in active sarcoid BAL showed expression of a separate antigen RFD9 (p< 0.001). Both normal and sarcoid RFD1+D7- cells had poor C3b and Fc receptor expression, and low fibronectin content, in contrast to the other two AM subsets. Fibronectin content was positively stronger in RFD1+D7+ and RFD1-D7+ AM from active sarcoid patients.

**CORRELATION OF BAL LYMPHOCYTOSIS WITH RFD1+D7+ AM CONCENTRATION**

Sarcoid patients had significantly higher proportions of lymphocytes in their BAL (35.3±9.1%), than normal subjects (9.4±3.1%) (p <0.001). Similarly, greater numbers of RFD1+D7+ cells were isolated from sarcoid lavage than normal (p< 0.001). In both groups the concentration of RFD1+D7+ AM in the samples was noted
TABLE 5.4

PHENOTYPIC FEATURES OF MACROPHAGE SUBSETS ISOLATED FROM 10 NORMAL VOLUNTEERS

<table>
<thead>
<tr>
<th>AM SUBSET#</th>
<th>RFDR1</th>
<th>RFD9</th>
<th>EBM11</th>
<th>UCHM1</th>
<th>ANTI c3b</th>
<th>ANTI Fc</th>
<th>ANTI-F.NECTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>98.3</td>
<td>&lt;1</td>
<td>95.4</td>
<td>&lt;1</td>
<td>20.3</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>1.21</td>
<td>0.61</td>
<td></td>
<td></td>
<td>1.21</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>D7</td>
<td>95.7</td>
<td>&lt;1</td>
<td>98.2</td>
<td>&lt;1</td>
<td>65.3</td>
<td>63.4</td>
<td>90.5</td>
</tr>
<tr>
<td>SE</td>
<td>4.21</td>
<td>0.14</td>
<td></td>
<td></td>
<td>0.82</td>
<td>4.63</td>
<td>1.22</td>
</tr>
<tr>
<td>D1D7</td>
<td>95.4</td>
<td>5.44</td>
<td>96.3</td>
<td>&lt;1</td>
<td>35.7</td>
<td>85.3</td>
<td>95.3</td>
</tr>
<tr>
<td>SE</td>
<td>3.51</td>
<td>1.12</td>
<td>0.21</td>
<td></td>
<td>0.45</td>
<td>1.24</td>
<td>3.41</td>
</tr>
</tbody>
</table>

The above values represent the mean (x) and standard error (SE) of total morphologically identifiable macrophages within each separated AM subset, that express positivity with the monoclonal antibodies RFDR1, RFD9, EBM11, UCHM1, anti-c3b & anti-Fc receptor, and antifibronectin.

* refers to the degree of intensity of the staining
# For clarity,
D1= RFD1+D7- macrophages
D7= RFD1-D7+ macrophages
D1D7= RFD1+D7+ macrophages
TABLE 5.5

PHENOTYPIC FEATURES OF MACROPHAGE SUBSETS ISOLATED FROM 10 SARCOID PATIENTS

<table>
<thead>
<tr>
<th>AM SUBSET#</th>
<th>RFDR1</th>
<th>RFD9</th>
<th>EBM11</th>
<th>UCHM1</th>
<th>ANTI-c3b</th>
<th>ANTI-Fc</th>
<th>ANTI-F.NECTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>x</td>
<td>100</td>
<td>&lt;1</td>
<td>95.5</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.52</td>
<td>&lt;1</td>
<td>95.7</td>
<td>&lt;1</td>
<td>97.5</td>
<td>62.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.91</td>
<td>0.41</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>weak*</td>
<td>1.21</td>
<td>weak*</td>
</tr>
<tr>
<td>D7</td>
<td>x</td>
<td>95.7</td>
<td>&lt;1</td>
<td>97.5</td>
<td>&lt;1</td>
<td>62.0</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>3.11</td>
<td>0.14</td>
<td>0.41</td>
<td>0.91</td>
<td>1.21</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>weak*</td>
<td>1.21</td>
<td>weak*</td>
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<tr>
<td>D1D7</td>
<td>x</td>
<td>95.6</td>
<td>45.5</td>
<td>96.2</td>
<td>&lt;1</td>
<td>36.4</td>
<td>92.2</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>2.41</td>
<td>3.12</td>
<td>0.41</td>
<td>0.17</td>
<td>1.45</td>
<td>1.45</td>
</tr>
</tbody>
</table>

The above values represent the mean (x) and standard error (SE) of total morphologically identifiable macrophages within each separated AM subset, that express positivity with the monoclonal antibodies RFDR1, RFD9, EBM11, UCHM1, anti-c3b & anti-Fc receptor, and antifibronectin.

* refers to the degree of intensity of the staining
# For clarity,
  D1=RFD1+D7- macrophages
  D7=RFD1-D7+ macrophages
  D1D7=RFD1+D7+ macrophages
**Fig. 5.2**

The correlation between the proportion of morphologically identifiable lymphocytes and RFD1+D7+ macrophages in the lavage of normal subjects (a), and patients with pulmonary sarcoidosis (b).
NORMAL

% LYMPHOCYTOSIS

RFD1+D7+ CONC X10^5 CELLS

SARCOID

% LYMPHOCYTOSIS

RFD1+D7+ CONC X10^5 CELLS
to be directly proportional to the lymphocytosis (Fig 5.2). The correlation coefficient was calculated to be 0.936 (p<0.001) in the normal group, and 0.947 (p<0.001) in the sarcoid group.

**ACID PHOSPHATASE REACTION**

In normal BAL, over 70% of RFD1+D7+ cells were acid phosphatase positive (ACP); much higher than that seen in classic 'phagocytic' RFD1-D7+ macrophages (p<0.001) (Fig.5.3). Lysozomal enzyme activity was low in RFD1+D7- cells. In active sarcoid BAL, significantly greater proportions of RFD1+D7+ cells were ACP positive (p<0.001). The ACP content was decreased in the other two subsets (Fig 5.3).

**PHAGOCYTOSIS**

Phagocytic capacity using fluorescent latex beads was observed in all three isolated AM subpopulations from both normal and sarcoid test samples. In the normal group, 64.1 ± 9.12% of RFD1-D7+ and 73.0 ± 8.11% of RFD1+D7+ AM showed a significantly higher phagocytic capacity, when compared to only 39.3 ± 4.01% of RFD1+D7- cells (p<0.001). Phagocytosis was observed to be significantly increased in RFD1+D7+ AM from sarcoid BAL (84.2 ± 1.51%) (p<0.001) (Fig 5.4); and decreased in RFD1-D7+ cells (50.4 ± 5.11%; p<0.001). There was no statistical difference in phagocytic capacity of sarcoid RFD1+D7- macrophages from normal.

Changes in cell morphology were accompanied by total inhibition of phagocytosis in all cultures containing cytochalasin B.

**HEXOSE MONOPHOSPHATE SHUNT ACTIVITY**

Overall, G-6-PD levels in unfractionated sarcoid AM, as quantitated in terms of mean relative density of "reaction product" per unit area of cells, was significantly
Fig. 5.3

The diagram represents the mean percentage of acid phosphatase positive macrophages in unfractionated BAL, and in each of the separated macrophage subpopulations, in normal and sarcoid subjects

In this figure,
RFD1=RFD1+D7- macrophages
RFD7=RFD1-D7+ macrophages
RFD1D7=RFD1+D7+ macrophages
% ACID PHOSPHATASE+ CELLS

- NORMAL
- SARCOID

BAL  RFD1  RFD7  RFD1D7
Fig. 5.4

Cytospin of RFD1+D7+ macrophages separated from sarcoid BAL, showing phagocytosis of fluorescent latex beads. Original magnification x 600
Fig. 5.5

Analysis of hexose monophosphate shunt activity in unfractionated normal (a) and sarcoid (b) lavage.

Each histogram shows the proportion of total number of readings of relative density per unit area that fell within selected limits (as shown in the key) on the bottom axis (mean ± SE).
increased (2.48±0.32), in comparison to that in unfractionated normal AM (1.07±0.28) (p<0.001). The scatter of G-6-PD levels in both groups is shown in Figs 5.5. Analysis of the isolated three AM subsets revealed a diverse spectrum of HMS activity. In both normal volunteers and patients with active sarcoidosis, the highest HMS activity was observed in the RFD1+D7- AM, with that in the normal being slightly lower (2.45±0.12) than in the sarcoid (3.60±0.23). G-6-PD levels were minimal in RFD1-D7+ cells in both groups (0.83±0.08 and 1.04±0.10 for normals and sarcoids respectively). HMS activity in RFD1+D7+ AM was 1.19 ±0.35 for the normal group, and 2.06+0.38 for sarcoid samples (Fig 5.6). In all samples the background control was 0.85±0.02.

**EFFECT OF AM SUBPOPULATIONS ON ALLOGENEIC PBM PROLIFERATION**

 Cultures were set up using aliquots (1x10^4 cells) of unfractionated macrophages and each of the separated subsets with a standard responder population of 1x10^5 allogeneic normal PBM in 7 normal volunteers (Table 5.6), and 10 sarcoid patients (Table 5.7). The stimulating factor (SI) for each AM subset in these experiments was recorded as the factor by which each of these macrophage subpopulations was able to enhance (or suppress) allogeneic PBM reactivity above (or below) that produced by unfractionated BAL cells (SI was reduced to unity by division) (Fig 5.7). The matched samples in each experiment are joined by hatched lines. The data shows that while RFD1+D7- macrophages from both normal and sarcoid groups are clearly strong stimulators of allogeneic PBM (exhibiting SI of 2 - 10), RFD1+D7+ cells from the same subjects show only weak or negative activity (SI 0.1 - 3.0). Furthermore when compared to the normal group, RFD1+D7+ AM from sarcoid patients seem to have an increased suppressive influence on allogeneic PBM responsiveness, such that this was completely abolished.
Fig. 5.6

Hexose monophosphate shunt activity in each of the three macrophage subsets separated from sarcoid patients. Each graph shows the percentage proportion of readings of relative density of reaction product per unit area for each macrophage subset that fell within selected limits on the bottom axis.

In this figure,
RFD1=RFD1+D7- macrophages
RFD7=RFD1-D7+ macrophages
RFD1D7=RFD1+D7+ macrophages
### TABLE 5.6

**ALLOGENEIC MIXED LYMPHOCYTE REACTIONS IN 7 NORMAL SUBJECTS**

<table>
<thead>
<tr>
<th>SUBJECTS</th>
<th>1</th>
<th>2</th>
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<th>4</th>
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<tbody>
<tr>
<td>BAL</td>
<td>110 ± 4.1</td>
<td>87 ± 15.9</td>
<td>196 ± 6.1</td>
<td>80 ± 28.3</td>
<td>86 ± 13.0</td>
<td>185 ± 39.9</td>
<td>120 ± 9.1</td>
</tr>
<tr>
<td>BALM</td>
<td>153 ± 34.7</td>
<td>86 ± 29.6</td>
<td>237 ± 89</td>
<td>41 ± 4.5</td>
<td>44 ± 1.2</td>
<td>75 ± 11.5</td>
<td>100 ± 1.8</td>
</tr>
<tr>
<td>PBM</td>
<td>8429 ± 2655</td>
<td>8604 ± 1451</td>
<td>1132 ± 71</td>
<td>4464 ± 555</td>
<td>1241 ± 294</td>
<td>2002 ± 450</td>
<td>4000 ± 26</td>
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<tr>
<td>PBMM</td>
<td>147 ± 13.7</td>
<td>157 ± 79.8</td>
<td>108 ± 20.6</td>
<td>30 ± 4.0</td>
<td>148 ± 5.0</td>
<td>118 ± 26.1</td>
<td>128 ± 9.1</td>
</tr>
<tr>
<td>BALM</td>
<td>4100 ± 1121</td>
<td>7958 ± 363</td>
<td>1309 ± 210</td>
<td>4735 ± 376</td>
<td>3559 ± 336</td>
<td>6293 ± 926</td>
<td>4100 ± 2.7</td>
</tr>
<tr>
<td>PBM</td>
<td>84 ± 13.2</td>
<td>87 ± 7.1</td>
<td>140 ± 44.1</td>
<td>182 ± 20</td>
<td>137 ± 58.2</td>
<td>700 ± 16.4</td>
<td>200 ± 2.0</td>
</tr>
<tr>
<td>D107</td>
<td>121 ± 19.8</td>
<td>81 ± 8.7</td>
<td>94 ± 31.5</td>
<td>96 ± 28.8</td>
<td>126 ± 3.0</td>
<td>92 ± 19.3</td>
<td>121 ± 5.7</td>
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<tr>
<td>D7</td>
<td>13118 ± 642</td>
<td>9825 ± 1124</td>
<td>198 ± 8.0</td>
<td>9357 ± 589</td>
<td>8459 ± 447</td>
<td>6898 ± 551</td>
<td>17630 ± 96</td>
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<tr>
<td>D107</td>
<td>40520 ± 3156</td>
<td>20404 ± 775</td>
<td>4181 ± 1685</td>
<td>14360 ± 1644</td>
<td>9654 ± 1362</td>
<td>12031 ± 919</td>
<td>22140 ± 12</td>
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<tr>
<td>D7</td>
<td>9 ± 3.2</td>
<td>4 ± 0.3</td>
<td>15 ± 6.7</td>
<td>5 ± 0.9</td>
<td>6 ± 1.0</td>
<td>9 ± 4.0</td>
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Each of the above values represents the mean ± SE incorporated $^3$H-Tdr of triplicate wells in counts per minute.
<table>
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<tr>
<th>Patients</th>
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<tr>
<td>BAL</td>
<td>238 ± 80.1</td>
<td>54 ± 20.2</td>
<td>106 ± 24.9</td>
<td>124 ± 12.0</td>
<td>160 ± 11.5</td>
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<td>BALM</td>
<td>257 ± 24.5</td>
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<td>58 ± 9.0</td>
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<td>60 ± 7.2</td>
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<td>PBM</td>
<td>28502 ± 4360</td>
<td>1833 ± 179</td>
<td>7079 ± 988</td>
<td>9541 ± 311</td>
<td>10200 ± 301</td>
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<tr>
<td>PBMM</td>
<td>236 ± 40.1</td>
<td>103 ± 77</td>
<td>69 ± 5.2</td>
<td>201 ± 126</td>
<td>212 ± 52</td>
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<td>BALM {</td>
<td>16798 ± 3318</td>
<td>3061 ± 278</td>
<td>5258 ± 611</td>
<td>9082 ± 1689</td>
<td>8600 ± 150</td>
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<td></td>
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<tr>
<td>D1D7</td>
<td>281 ± 38.1</td>
<td>44 ± 9.2</td>
<td>182 ± 38.6</td>
<td>150 ± 8.3</td>
<td>110 ± 11.2</td>
</tr>
<tr>
<td>D1</td>
<td>135 ± 27.1</td>
<td>40 ± 5.9</td>
<td>110 ± 23.9</td>
<td>98 ± 47.7</td>
<td>140 ± 20.4</td>
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<tr>
<td>D1D7 {</td>
<td>22016 ± 2620</td>
<td>1094 ± 214</td>
<td>4486 ± 583</td>
<td>9504 ± 1011</td>
<td>2580 ± 167</td>
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<tr>
<td>D1 {</td>
<td>35271 ± 16872</td>
<td>362 ± 513</td>
<td>10822 ± 386</td>
<td>12541 ± 1023</td>
<td>9460 ± 246</td>
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<td>PBM }</td>
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Background control

8.3 ± 1.3
8.0 ± 1.0
9.0 ± 1.0
23 ± 7.3
100 ± 0.4

Each of the above values represents the mean ± SE incorporated 3H-TdR of triplicate wells in counts per min.
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</thead>
<tbody>
<tr>
<td>BAL</td>
<td>186 ± 14.2</td>
<td>123 ± 13.6</td>
<td>91 ± 7.6</td>
<td>140 ± 4.6</td>
<td>107 ± 8.3</td>
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<tr>
<td>BALM</td>
<td>196 ± 12.0</td>
<td>79 ± 15.9</td>
<td>71 ± 17.9</td>
<td>50 ± 10.4</td>
<td>71 ± 9.4</td>
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<tr>
<td>PBM</td>
<td>1477 ± 378</td>
<td>1032 ± 264</td>
<td>1495 ± 142</td>
<td>1313 ± 145</td>
<td>1015 ± 95.7</td>
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<tr>
<td>PBMM</td>
<td>129 ± 10.1</td>
<td>113 ± 1.2</td>
<td>62 ± 10.3</td>
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<td>83 ± 10.2</td>
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<tr>
<td>BALM }</td>
<td>2465 ± 10.2</td>
<td>2748 ± 142</td>
<td>6176 ± 70.8</td>
<td>5252 ± 142</td>
<td>6555 ± 101</td>
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<td></td>
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</tr>
<tr>
<td>D7</td>
<td>138 ± 20.1</td>
<td>114 ± 18.1</td>
<td>266 ± 11.5</td>
<td>76 ± 9.4</td>
<td>61 ± 7.1</td>
</tr>
<tr>
<td>D1</td>
<td>339 ± 10.5</td>
<td>268 ± 12.3</td>
<td>419 ± 13.0</td>
<td>100 ± 16.5</td>
<td>201 ± 15</td>
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<td>2412 ± 145</td>
<td>5758 ± 76.3</td>
<td>5248 ± 610</td>
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<td>6591 ± 130</td>
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</tr>
<tr>
<td>D1 }</td>
<td>18606 ± 1850</td>
<td>18805 ± 142</td>
<td>23373 ± 78.2</td>
<td>17817 ± 229</td>
<td>16677 ± 152</td>
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<tr>
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<td></td>
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</tr>
<tr>
<td>Background control</td>
<td>9.0 ± 1.2</td>
<td>7.6 ± 1.0</td>
<td>10.1 ± 0.9</td>
<td>8.4 ± 1.4</td>
<td>7.4 ± 0.9</td>
</tr>
</tbody>
</table>

Each of the above values represents the mean ± SE incorporated $^3$H-Tdr of triplicate wells in counts per minute.
Allogeneic MLR were set up using normal PBM (1 x 10^5 per well) as the responder population with aliquots (1 x 10^4 per well) of unfractionated AM and each of the separated subsets in both normal and sarcoid groups. The volume per well of each culture was always made up to 200 ul using supplemented RPMI medium. Each value in this figure represents the mean factor by which each test sample (RFD1+D7-, RFD1-D7+, RFD1+D7+ macrophage subsets) is able to enhance (or suppress) allogeneic PBM reactivity above (or below) that produced by unfractionated BAL cells. Matched samples are joined by hatched lines. In all above cultures the SE of the mean was less than 1.5
THE EFFECT ON RFD1+D7- CELL INDUCING ACTIVITY BY RFD1+D7+ MACROPHAGES

4 x 10^3 RFD1+D7- AM from 5 healthy subjects, were admixed with 1 x 10^5 PBM; and varying concentrations of the RFD1+D7+ AM were added. In those cultures containing only RFD1+D7- AM and PBM, a 3-fold increase in allogeneic PBM reactivity was observed. This PBM proliferative response was however reduced by as much as 14%, when 2 x 10^3 RFD1+D7+ cells were added to the same cultures. This suppression of allogeneic MLR was dose-dependent: The addition of 8 x 10^3 RFD1+D7+ AM causing a 43% reduction in PBM reactivity (Fig 5.8). The admixture of RFD1+D7+ macrophages alone with PBM failed to cause any stimulation throughout all concentrations used.

In separate co-culture experiments, varying concentrations of both cell subsets from 5 healthy volunteers (Fig 5.9a) and 5 active sarcoid patients (Fig 5.9b) were added to a fixed concentration of allogeneic PBM; the proportion of each AM subset were adjusted so as to maintain a constant total number of macrophages within each MLR. Analysing the results of these AM subsets in the normal group (Fig 5.9a) RFD1+D7+ AM alone consistently failed to stimulate allogeneic PBM. In contrast, RFD1+D7- AM alone (in concentrations of 4 x 10^3 - 1 x 10^4 per well) caused a 3 - 4 fold stimulation of PBM (over the latter's autologous reactivity). Lower concentrations of RFD1+D7- AM caused comparatively reduced reactivity, but still MLR stimulation remained significantly greater than PBM autologous reactivity (even when 1 x 10^3 RFD1+D7- cells were used). In co-culture studies using both AM subsets, it was observed that as the decreasing proportion of RFD1+D7- cells within the MLR was substituted by a corresponding proportional increase in RFD1+D7+ cells, PBM reactivity became progressively suppressed and eventually abolished at high concentrations of RFD1+D7+ macrophages.
Fig. 5.8

Down-regulation of RFD1+D7- cell inducer activity by RFD1+D7+ macrophages.

Each value on the graph represents the mean amount of incorporated radioactivity (3HT) in cpm, resulting from allogeneic MLR between a fixed number of normal PBM (1 x 10^5 per well) as the responder population, and varying proportions of RFD1+D7+ macrophages (2 x 10^3 - 8 x 10^3 cells per well): [a] alone, [b] in the presence of a constant number of RFD1+D7- cells (4 x 10^3 per well). The hatched line represents the mean amount of incorporated 3HT in cpm resulting from allogenic MLR between the above standard responder population of PBM and a fixed concentration of RFD1+D7- macrophages. In this experiment the mean autologous mixed reactivity of PBM was 2500 cpm. The volume per well of each of the above cultures was always made up to 200 ul using supplemented RPMI. Throughout all the above cultures the SE of the mean was less that 165 cpm.
ALLOGENEIC MLR cpm x 10^3

NORMAL BAL

RFD1
[4 x 10^3 CELLS/WELL]

RFD1D7
RFD1 + RFD1D7

ALLOGENEIC MLR cpm x 10^3

RFD1D7 CELLS/WELL X 10^3
Fig. 5.9

Progressive suppression of the enhancing capacity of RFD1+D7- macrophages by RFD1+D7+ cells in the normal (a) and sarcoid (b) groups.
Each value in the graphs represents the mean amount of incorporated reactivity in cpm resulting from cultures of a fixed number of allogeneic normal PBM (1 x 10^5 per well) as the responder population to: [a] varying concentrations of RFD1+D7- macrophages; [b] varying concentrations of RFD1+D7+ macrophages; [c] a fixed total number of macrophages per each well (1 x 10^4), within which the proportions of RFD1+D7- and RFD1+D7+ cells varied accordingly, so that as the number of RFD1+D7- macrophages decreased the proportion of RFD1+D7+ cells increased. The volume per well of each of the above cultures was always made up to 200 ul using supplemented RPMI. The hatched line represents the mean autologous reactivity of abnormal allogeneic PBM (1 x 10^5 cells per well). In all cultures the SE of the mean was less than 215 cpm.
Comparing this data to that obtained from equivalent experiments using AM subsets from sarcoid patients (Fig 5.9b), it appears that both sarcoid RFD1+D7- and RFD1+D7+ macrophage subpopulations are poorer stimulators of allogeneic PBM reactivity (p<0.002 and p<0.001 respectively). So that although in active sarcoid BAL RFD1+D7- cells still supported MLR better than RFD1+D7+ cells, when lower concentrations of RFD1+D7- macrophages(1x10^5) were used, the over-suppressive influence of RFD1+D7+ AM was masked by the reduced enhancing capacity of RFD1+D7- AM.

In all MLR experiments blank wells containing only culture medium but no cells consistently gave recordings of less than 20 cpm.

DISCUSSION

This study has shown that discrete macrophage subpopulations, isolated from normal and sarcoid BAL, share a similar morphology, yet exhibit phenotypic, physiological and functional characteristics implying separate capabilities. The presence of sarcoid inflammation in the lung however appears to induce certain changes in the local AM pool. In particular, a specific macrophage subset emerges in active sarcoid BAL, which reacts with both MoAb probes previously described to identify dendritic cells and 'classic' macrophages in tissue. Functional studies with these cells show for the first time that this specific subset of human AM can act to suppress the induction of T-cell responses; a function that was observed to be enhanced in sarcoid BAL.

The question of macrophage heterogeneity has assumed some importance in recent years with greater appreciation of the multiplicity of roles of these cells in the immune response (Gordon et al,1988; Johnston,1988). The advent of MoAb has enabled many workers to differentiate macrophage cell types (Hirsch & Gordon,1982; Brooks et al,1983; Hogg,1987), and in instances to link the difference in
membrane-antigen expression to functional heterogeneity (Raff et al, 1980; Sun & Lohmann-Matthes, 1982; Hancock et al, 1983; Hofman et al, 1984; Reittie et al, 1988). In addition, macrophages have been fractionated on density gradients into subpopulations which exhibit different morphological and cytochemical features (Sandron et al, 1986; Nakstad et al, 1989), as well as phagocytic (Sharma & Remington, 1981), migratory (Brannen & Chandler, 1988) and tumoricidal capacities (Evans, 1973). It has been postulated that these macrophage density subfractions represent different stages of maturation (Shellito & Kaltreider, 1984). While such studies highlight the diverse potential of macrophages in the immune system, they fail to identify and isolate discrete macrophage subpopulations as they occur 'in situ' in man.

The novel use of specific macrophage probes in conjunction with plastic plate adherence, antibody-conjugated magnetic beads and metrizamide density gradients, enabled not only the identification but also the isolation of specific, relatively homogeneous subpopulations of AM from BAL. Previous studies have reported the separation of mononuclear cell subsets with immunomagnetic particles (Lea et al, 1985, Lea et al 1986, Vartdal et al 1986), but not from BAL. Such a method has been acknowledged for its speed and preservation of cell viability. Previous workers have demonstrated that adherent AM population can be separated into AM subpopulations using a variety of density gradients (Zwilling et al, 1982; Sandron et al, 1986). It could therefore be assumed that the two adherent AM subpopulations in this current study (RFD1-D7+ and RFD1+D7+ macrophages) would have different densities. It follows therefore that if the obtained adherent AM were to be passed through an appropriate density gradient, the heavier cells, would precipitate out, leaving the lighter of the two subsets at the interface. A number of different iodinated density gradient media have been used for isopycnic cell separation (Rickwood & Birnie, 1978). However the major drawback with these substances has been their high ionic status, and high osmolarity, which tend to damage the cells banding in the dense
portions of the gradients. In addition these hypertonic solutions can give rise to changes in cell volume, which in turn affect the buoyant density of the cells to be separated (Grdina et al, 1973; Meistrich & Trostle, 1975). In contrast, metrizamide is completely non-ionic and for any given density its osmolarity is only one quarter to that of its ionic counterparts (Peters & Dahmus, 1979). Metrizamide has therefore been repeatedly reported to be non-toxic to cells.

The separation techniques used in this study have allowed a comparative cytochemical, histochemical and functional evaluation of specifically identifiable macrophage subsets obtained from normal and sarcoid BAL. A range of MoAb probes was used to analyse specific cell surface determinants on the isolated macrophage subsets. While all normal and sarcoid cell subpopulations were EBM-11 positive, none expressed positivity to UCHM1. This would imply that these AM subsets had matured and been resident for a time in the local environment from which they were obtained and not recently recruited from the circulating pool of blood monocytes: an interesting observation, as in active sarcoidosis under the influence of a chemotactic gradient, monocytes are recruited to the site of inflammation (q.v. Chapter 1). The results would thus suggest that while there is no doubt that a continual recruitment of monocytes occurs, the characteristics of AM after they settle locally do not just occur by a simple differentiation from monocytes: rather, as suggested by Riches & Hanson (1984), the maturing cell adapts to its particular environment, within which stimuli may be present that could influence expression of unique phenotypes, as well as functional capacity. In support data from Chapter 4 has shown that unfractionated human AM are deficient in their ability to promote antigen-stimulated lymphocyte proliferation; this is in contrast to in-vitro aged blood-derived macrophages (Lipscomb et al, 1986). A wide variation in C3b and Fc receptor expression, fibronectin content and lysosomal enzyme activity was observed amongst the normal and sarcoid macrophage subsets. In both groups non-adherent RFD1+D7- cells were C3b and Fc receptor negative and
poor in fibronectin content and lysosomal enzyme activity, while the adherent macrophage subsets strongly expressed C3b and Fc receptors, in addition to a high fibronectin and acid phosphatase content. Such heterogeneity in AM characteristics has been observed before and reinforces the hypothesis that AM characteristics can be influenced by local factors. Indeed Coonrod & Yoneda (1983) have observed that the lipid content in the alveolar lining can affect macrophage surface receptor expression.

Furthermore it appears that with the onset of inflammation in pulmonary sarcoidosis proportional differences are accompanied by changing characteristics within the resident AM. Many workers have previously described sarcoid-related changes in surface phenotype, specific receptor expression, phagocytosis and T-cell inductive capabilities. In particular there appears to be the emergence (in direct proportion to the lavage lymphocytosis) of the double phenotype functional suppressor AM subpopulation, with increased expression of RFD9 (which identifies epithelioid cells in tissue), and enhanced fibronectin content. Such findings raise the question of the functional significance of the presence of such cells in sarcoid BAL, and point to the possibility that these specific AM may represent those cells that have previously been observed to stimulate fibroblast recruitment and proliferation (Rennard et al, 1981). Future investigation into these specific macrophages could shed some light into the development of tissue fibrosis.

A possible controversial observation in this study was that a proportion of cells with the phenotype of dendritic cells (RFD1+D7-) in both normal and sarcoid BAL were as efficient as other macrophage types in phagocytosing inert latex beads. Such phenomena have been observed in other studies where dendritic cells have been found to be capable of phagocytosing both inert material as well as micro-organisms (Poulter & Condez, 1988). Indeed phagocytosis is now generally considered to be a major functional activity of macrophages for the uptake, localization and 'processing'
of antigens; a necessary step in cell-mediated immune reactions, without which macrophage-T-cell interaction would not occur (Unanue & Allen, 1987).

Holt et al (1988) have described the presence of non-adherent, LA positive, FcR-negative, ultra-low density mononuclear cells in rat lung parenchyma capable of APC activity in vitro, and called these cells 'putative dendritic cells'. Their function was seen to be inhibited by the presence of endogenous adherent FcR positive cells, described as 'putative macrophages'. In addition, Nicod et al (1987) described the isolation and characterisation of a potent accessory cell from human lung that was loosely adherent, FcR negative and poorly phagocytic, and which had hitherto not been obtained in BAL. One of the most exciting observations in this current study is the isolation of two discrete cell types from normal and sarcoid BAL, both possessing well-known morphological, phenotypic and physiological characteristics of the macrophage-cell lineage but exhibiting distinct functional capabilities with regard to allogeneic peripheral blood mononuclear cell reactivity. In both normal volunteers and sarcoid patients, although sarcoid-related differences in the functional capacity of these cell types was observed, RFD1+D7- macrophages strongly stimulated allogeneic MLR, while RFD1+D7+ macrophages actively suppressed it. The demarcation in function is best seen in the reconstitution experiments (Fig 5.9 a,b), where the stimulatory capacity of RFD1+D7- cells is down-regulated even by the presence of very low proportions of RFD1+D7+ macrophages. It could be argued that, as the homogeneity in each isolated macrophage subset was at best 97%, the observed functional as well as phenotypic and physiological results were possibly influenced by the contaminating cells. However, all separated cell populations were counter-checked using double-staining immunofluorescence techniques. While a minimal degree of intra-subset contamination did occur in some of the samples (<5% of cells in each subset), a proportion of morphologically identifiable macrophages (a mean of 6+3%) was found in all the isolated subpopulation samples. These cells were EBM 11+, UCHM1-, yet unreactive to the specific macrophage probes used in this study. It is
assumed that these macrophages have not yet undergone full differentiation. It can also be argued that the influence that this small proportion of cells could have on the overall results is negligible. In support, the functional reconstitution experiments showed that even at the lowest concentration of \(1 \times 10^3\), RFD1+D7+ cells still suppressed the allogeneic MLR to the same extent as the higher concentration (Fig 5.9 a,b).

It has been suggested, in previous unfractionated alveolar macrophage studies that the enhancing and suppressing effects seen with regard to T-cell reactivity could depend on the varying proportion of "stimulatory" macrophages in situ (Holt, 1979). While this may be true, it is also evident from the results in this chapter that allogeneic MLR suppression can also be a specific cell-mediated function. In both subject groups studied, throughout the varying cell concentrations, allogeneic peripheral blood mononuclear cell reactivity remained low or abolished with RFD1+D7+ macrophages, but stimulated to varying degrees with RFD1+D7- cells. This difference in functional capacity amongst AM subpopulations not only points to a possible paradox (as both 'inducer' RFD1+D7- and 'suppressor' RFD1+D7+ macrophages express HLA-DR molecules), but also the increased presence of 'suppressor' RFD1+D7+ cells in active sarcoid BAL could explain the reduced T-cell responsiveness observed in these patients in Chapter 4.

It is therefore hypothesized that under normal circumstances in man, the alveolar macrophage population is a dynamic cellular system comprising of phenotypically and functionally distinct subpopulations, the characteristics of which may change with the advent of pulmonary sarcoidosis. Indeed the emergence of particular macrophage subpopulations in response to stimuli from the local inflamed micro-environment in sarcoidosis may serve as the critical determinant of the pathogenic outcome of the disease. If this hypothesis were to be true, such findings could provide potential
criteria to stage disease activity (q.v. Chapter 1), as well as guide treatment in these patients.

**SUMMARY**

Three relatively homogeneous subpopulations of AM were isolated from the BAL of normal healthy volunteers, and active sarcoid patients. In conjunction with density separation techniques, use was made of MoAb probes, RFD1 and RFD7, specific against macrophage determinants that have previously been shown to differentiate respectively between dendritic cells and mature macrophages in normal tissue. In addition, to two AM subpopulations that respectively exhibited the phenotype of dendritic cells and mature macrophages, a third AM subset was isolated, which appeared to express both markers. All separated macrophage subsets were morphologically similar yet exhibited distinct physiological and functional features. When isolated from normal BAL those AM with the phenotype of dendritic cells (RFD1+D7-) did not adhere to glass, were strong T-cell stimulators, had weak expression of C3b and Fc receptors, poor fibronectin content and lysosomal activity; a proportion of these cells exhibited phagocytosis. This was in sharp contrast to the other two isolated AM subsets which adhered to glass, expressed C3b and Fc receptors and had high fibronectin and acid phosphatase content as well as increased phagocytic capacity. Interestingly, despite expressing HLA-DR molecules double phenotype RFD1+D7+ AM subpopulation suppressed allogeneic MLR. In particular this specific AM subpopulation was capable of down-regulating by as much as 40% the induction of T-cell responses set up by other "stimulator" macrophages. A diverse spectrum of hexose monophosphate shunt activity was observed throughout all 3 AM subpopulations, with the highest activity being recorded in the non-adherent AM.

Sarcoid-related differences in the proportion of these macrophage subsets in BAL were accompanied by differences in surface receptor expression, physiology, and function.
Isolated RFD1+D7+ and RFD1-D7+ populations showed significant increase in Fc receptor expression, and were strongly positive for fibronectin. Furthermore, an increased proportion of RFD1+D7+ cells expressed a separate antigen RFD9, and had raised acid phosphatase content. Hexose monophosphate shunt activity was raised in all sub-populations. Functional studies revealed that all sarcoid AM subsets were comparatively poor stimulators of allogeneic PBM. In particular the suppressive activity observed in normal RFD1+D7+ cells was enhanced in the equivalent sarcoid subpopulation, such that it completely abolished T-cell responsiveness.

The data presented in this study supports the concept of the presence of a dynamic heterogeneity within the AM population in the human lung. The possibility is raised that both the phenotype and function of these AM may remain flexible, yet critically balanced, and influenced by the advent of disease in the local micro-environment.
CHAPTER SIX

THE MODULATING EFFECT OF INHALED CORTICOSTEROIDS ON CELLULAR, CLINICAL, RADIOLOGICAL AND PHYSIOLOGICAL PARAMETERS IN PULMONARY SARCOIDOSIS
INTRODUCTION

Despite substantial advances in the scientific understanding of the underlying immunological features of pulmonary sarcoidosis, the lack of any clear prognostic signs in this disease leaves the question of who, and when, to treat in dispute.

One of the problems in the management of sarcoidosis appears to be made worse by the knowledge that the disease is generally benign, so that follow-up of sarcoid patients can be often sketchy and unsystematic. This has invariably led to the late discovery of lung damage in the unfortunate minority of sarcoid patients in whom the clinical course is insidious and progressive (Turner-Warwick, 1988).

Currently only those patients with Stage 2 and 3 disease (q.v. Chapter 1), with persistent abnormal lung function, are treated. When therapy is instituted, it consists of systemic corticosteroids in varying doses (20 - 60 mg prednisolone per day) and duration, producing variable clinical efficacy. Systemic corticosteroids have been shown to control the local inflammatory response in sarcoidosis by decreasing the CD4/CD8 ratio, and hence increasing CD8+ T-lymphocyte accumulation at the site of the granuloma (Ceuppens et al, 1984), suppression of local immunoglobulin production and a reduction in the lymphocytic production of IL-2 (Pinkston et al, 1987). Invariably, however, undesirable side-effects occur. For this reason systemic steroid therapy may be started too late (or curtailed too soon), with many of the patients already having developed (or proceeding to develop) pulmonary fibrosis. When this happens the lung damage is irreversible, with steroid therapy often being found to be ineffective both clinically and at the cellular level.

Against this background, there is clearly a need for a therapeutic regimen that is safe enough to commence early in disease, that can be targeted to the lung, and that can be shown (as with systemic steroids) to modulate the underlying immunological
dysfunction seen in this disease.

Inhaled corticosteroids became available 15 years ago for the treatment of patients with bronchial asthma. Early attempts to use inhaled steroids for treatment of pulmonary sarcoidosis were unsuccessful (Williams, 1981), probably because the daily dose was too low, and the disease too advanced to be treatable with inhaled corticosteroids. With the development of new glucocorticosteroids for inhalation with an improved ratio between anti-inflammatory effects and systemic potency, and with a different lung pharmacokinetic profile, the idea of using inhaled steroids in pulmonary sarcoidosis was reviewed. In 1982, the new steroid budesonide became available and its pharmacological profile indicated that it could be useful in these patients (Brattsand et al, 1982). Budesonide is a non-halogenated glucocortico-steroid, which is structurally related to 16x-hydroxy prednisolone. The exact mechanism of action remains obscure. Both in vitro and in vivo experiments have shown that budesonide is not metabolized locally in the lungs; it is thought that the drug is absorbed intact, and then undergoes extensive first pass metabolism in the liver, with rapid inactivation by biotransformation into metabolites of minimal activity (Clissold & Heel, 1984)

Preliminary studies (Selroos, 1986a) using inhaled budesonide in a dose of 1600 ugm per day, have been shown to produce clinical benefit with minimal side-effects in active pulmonary sarcoidosis. As yet, it has not been documented whether such therapy is effective in modulating local immune reactivity, nor has it been established that the inhaled mode of administration is effective in actually depositing the drug at the site of the alveolitis.

Studies on both inhaled and systemic steroids have so far largely focused on their effect on lavage lymphocytes. However, both the T-cell activation and the granuloma
formation occurring in sarcoidosis are mechanisms controlled by macrophage-like cells (Hunninghake et al, 1984). In Chapters 4 and 5, it was demonstrated that phenotypic and functional aberrations within this macrophage population exist in active sarcoidosis. Such alterations have been shown by others to reflect changes in clinical status (Ainslie et al, 1989). One might therefore hypothesize that the persistence of granulomata and fibrosis seen in pulmonary sarcoidosis are features determined as much by alveolar macrophages as by T-lymphocytes. Thus, in order for steroids to be properly evaluated as a means of treating pulmonary sarcoidosis their immuno-regulatory effect on the macrophage population in the lung must also be ascertained.

A study was therefore designed to compare the effects of inhaled and systemic steroids on the phenotype and functional capacity of alveolar macrophages obtained by BAL from a homogeneous group of previously untreated active sarcoid patients. The observed local cellular events were correlated with clinical, radiological and physiological parameters.

PATIENTS AND METHODS

Subject groups

The study population consisted of 30 patients, with at least a 12 month history of biopsy-proven sarcoidosis: 20 males and 10 females mean±S.E. age 38.4±5.68 years; 1 smoker (1 pack per day). None of the patients had received any treatment prior to the study. All 30 patients had unequivocal bilateral parenchymal shadowing (grade III chest x-ray), in addition to impaired pulmonary function. There was no objective evidence of extrathoracic sarcoidosis in any of the patients. Sixteen of the 30 sarcoid patients had a raised SACE (mean ±S.E. 84.2±3.75 mmol/min/ml); the ESR was within normal limits in all recruited patients. A control population of 10 healthy volunteers, all non-smokers, 9 males and 1 female, mean ±S.E. age 22.0±2.68 years, were recruited in accordance with the selection criteria set out in
Chapter 2.

Study design

Prior to commencement, all subject groups had a full clinical evaluation with documentation of any presenting symptoms. All 30 sarcoid patients reported symptoms of cough, dyspnoea on exertion and intermittent chest pains. Six of the patients also complained of limited exercise tolerance (mean 6 minute walk on level: 200 m). The patients were sequentially randomised (without discrimination) into one of three groups (Fig 6.1). Formal written consent was obtained from each subject in the study after full details of the investigation had been explained. The study period was for 18 months: chest x-rays, pulmonary function and BAL were performed on each subject before the study, and repeated at the 4-month and 18-month time points. Immunological analysis and functional capacity studies were carried out on all BAL samples. All chest-x-rays were read by two independent radiologists. In addition, all normal volunteers and sarcoid patients were seen at monthly intervals by the author; when a full physical examination was carried out, and details of progress and/or side-effects taken and compliance ensured. In the case of the inhaled regimens, the aerosol canisters were weighed prior to use and checked monthly. Using the standard 400-dose metered inhalation aerosols, each canister is expected to last for about one month.

Ten of the patients with sarcoidosis received budesonide (Pulmicort, Astra Pharmaceuticals) in a dose of 800 ugm twice daily. Each dose was given as 4 single puffs (200 ugm each) delivered from a pressurised metered dose inhaler via a 750 cm$^3$ spacer device with a one-way inhalation valve (Nebuhaler, Astra Pharmaceuticals). Two deep inhalations followed by 10 second breath-holding were taken after each puff. Another 10 sarcoid patients and 10 healthy volunteers received a placebo equivalent in number of puffs, mode of administration and study duration. The dose of the inhaled aerosol remained unchanged for the 18-month duration. This
30 Symptomatic patients  
Biopsy proven sarcoidosis  

10 Healthy volunteers  

INITIAL ASSESSMENT  
- chest x-ray (CXR)  
- lung function (PFTs)  
- BAL  

10 Sarcoid patients  
18 months oral prednisolone  
30mg/day reducing to 10mg alternate days  

20 Sarcoid patients  
18 months double blind study  
10 patients placebo equiv.  
10 patients inhaled budesonide  
800 ug bd  
Both delivered in 4 single puffs via nebulizer  

Drug distribution assessment teflon particles labelled with technetium-99M  

Reassessment at 4 and 18 months  
- CXR, PFTs, BAL.  

Figure 6.1. A schematic diagram of the study design
part of the study, during which recruited patients took inhaled steroid or placebo equivalent, was single blind. Another 10 sarcoid patients received systemic corticosteroids in the form of oral enteric-coated prednisolone, starting at a dose of 20 mg a day for 1 month and reducing to a maintenance dose over the subsequent months as outlined in Fig 6.2.

**Aerosol deposition analysis**

This part of the investigation was carried out with the help of Dr S Newman in the Department of Thoracic Medicine, RFH. Pressurised aerosol deposition was assessed prior to commencing treatment in the 10 sarcoid patients who received budesonide therapy, by incorporating Teflon particles labelled with technetium 99 m into placebo pressurised canisters (Newman et al, 1981). The particles had a mass median aerodynamic diameter of 3 \( \mu \text{m} \), which is similar to that of budesonide particles produced by the Nebuhaler. The percentage of the dose deposited in the lung and oropharynx was assessed by gamma camera, and the 24-hour whole lung retention of the insoluble Teflon particles (measured by 5 cm diameter collimated scintillation probes) was taken as a measure of alveolar deposition.

**Bronchoalveolar lavage**

BAL was performed and all samples processed using the standard procedure described in Chapter 3. Prior to each lavage, bronchial washings from the same segment of the right middle lobe (using 2 x ten-ml aliquots each of 0.9% normal saline) were performed, and the aspirates sent for microbiological culture (in order to exclude current infection and opportunistic growth).
**BUDESONIDE**

INHALED VIA NEBUHALER AT A DOSE OF 800 UGM TWICE A DAY (7.00 am, 8.00 pm CONSISTENTLY FOR 18 MONTHS.

**PREDNISOLONE**

TAKEN ORALLY IN ENTERIC-COATED FORM, AT 7.00-8.00 am, AS FOLLOWS:

- 30mg daily for 1 month
- reduced to
- 25 mg daily for 1 month
- 20 mg daily for 1 month
- 15 mg daily for 1 month
- 12.5 mg daily for 1 month
- 10 mg daily for 1 month
- 10/5mg* for 1 month
- 10/0mg* for the rest of the study

* alternate days

**Fig. 6.2 DRUG REGIMENS USED IN THIS STUDY**
**Preparation of Peripheral Blood Mononuclear Cells**

All subjects had 20 ml of peripheral blood taken by venepuncture at the time of BAL. These were processed in the same way as the method used in Chapter 4.

**Immunocytological analysis**

Cytospins were prepared from the processed lavage fluid, and the cells analysed for single and double antigen expression using the standard techniques described in Chapter 3. In this study the MoAb probes used were RFD1 and RFD7 (q.v. Table 4.2)

**Autologous Mixed Lymphocyte Reactions (AMLR)**

The BAL and PBM suspensions used in AMLR were adjusted to a concentration of 1 x 10^6 cells/ml. For control tests, separate aliquots of these suspensions were initially incubated in the presence of mitomycin C (25 ul per ml of cell suspension) for 45 minutes at 37 °C in 5% humidified CO\textsubscript{2} to block cell division. Following this, the treated cell samples were washed three times in RPMI 1640.

AMLRs were then set up for each subject at each time-point in the study, in triplicate, in microtitre wells using the following cell populations: BAL alone (1 x 10^6 cells per well); control wells of mitomycin treated BAL and PBM (1 x 10^6 per well) respectively. These cultures were then incubated at 37 °C in 5% humidified CO\textsubscript{2} for 4 days. After this, each well was pulsed with 2 uCi tritiated thymidine (\textsuperscript{3}HT) (Amersham, 5Ci-mmol\textsuperscript{-1}), and further incubated for 18 hours. The cultures were then harvested using a semi-automatic cell harvester (Titertek-flow, Laborat, Inc, McLean Va). The amount of incorporated radioactivity was measured in a liquid scintillation counter, and expressed as average counts per min (cpm) of triplicate cultures. These results were recorded as Stimulation Index (SI) which was defined as the factor by which cpm \textsuperscript{3}HT of cells is increased over the cpm \textsuperscript{3}HT of same cells
treated with mitomycin. Blank wells, containing medium only, were set up as background controls.

**STATISTICAL ANALYSIS**

Quantitative data were expressed as mean ± SE; wherever relevant, this was analysed as outlined in Chapter 2.

**RESULTS**

Aerosol deposition pattern

In the 10 sarcoid patients who received budesonide therapy, 21.1 ± 2.01% of the dose was deposited in the lungs, of which 9.64 ± 2.11% appeared in the alveoli, and 11.6 ± 1.82% on the conducting airways (tracheobronchial zone). The remainder of the dose was either deposited in the oropharynx (11.2 ± 1.80%) or retained in the Nebuhaler and actuator (67.6 ± 2.23%)

Microbiology Results

All bronchial washings set up for culture, including anaerobes, fungi and acid-fast bacilli, were negative in all study groups.

Clinical effects

At 4 months, all 10 sarcoid patients receiving inhaled budesonide reported an improvement in their symptoms, in particular a reduction of their cough. While symptomatic relief, with a decrease in dyspnoea and cough, was also obtained in 9 out of 10 patients receiving systemic corticosteroids, one patient on prednisolone complained of persistent wheezing.

At 18 months 9 of the 10 patients on inhaled budesonide remained symptomatically well; however 1 patient relapsed, and reported a marked reduction in exercise
tolerance with an increase in cough and dyspnoea at rest. Of the 10 patients on oral prednisolone, 6 patients described further improvement of their symptoms, 3 patients showed no further significant change (including the patient with wheezing at 4 months), while 1 patient complained of increased dry cough and exertional dyspnoea. No significant change in symptoms was reported by the placebo group.

Throughout the study all normal volunteers remained well, with no change in clinical status.

**Radiographic Effects**

Three of the 10 patients on inhaled budesonide had significant resolution in their radiographic shadowing at 4 months, which remained unaltered at 18 months. In another 3 patients in this group, there was no change in radiographic appearance over the 18 months. Four patients receiving inhaled steroid had increased parenchymal shadowing by 18 months.

In contrast, 4 of the 10 patients on systemic steroids had complete resolution of their radiographic appearance by 4 months, and this persisted up to 18 months. In 5 other patients in this group, significant clearing of the X-ray shadowing was only seen at 18 months. In 1 patient receiving systemic steroids, a progressive increase in interstitial infiltrates was observed over the study period.

No radiological changes were observed in the healthy volunteer group. Only 1 patient in the inhaled placebo group had complete radiographic resolution at 4 months, which remained the same at 18 months. There was no change in 6 patients in this group, while 3 patients showed an increase in their parenchymal shadowing.

**Pulmonary Function Analysis**

The results are summarized in Fig.6.3 and Fig.6.4.
Fig. 6.3

Comparative analysis of the lung function parameters used during the study period

PEFR = peak expiratory flow rate

FEV₁ = forced expiratory volume in 1 second

FVC = forced vital capacity
Comparative analysis of lung function parameters continued.

TLC = total lung capacity
TLCO = diffusion capacity (transfer factor)
KCO = transfer coefficient
Non-specific changes in lung function were seen in the inhaled steroid group throughout the study; the most significant being in the TLCO and KCO at 4 months (p< 0.001) and at 18 months (p<0.005). Much more obvious was the improvement in pulmonary function in the patients receiving systemic steroids. In this group, the mean±SE FEV₁ and FVC rose from 77±7.6% and 81.3±6.7% predicted respectively, to 88.4±5.5% and 93.2±5.0% after 4 months of oral prednisolone (p<0.001, in both). A similar increase in PEFR was also noted. In addition, the TLC and TLCO both increased from 84.9±4.8% and 79.1±7.9% respectively, to 93.9±4.0% and 84.8±6.6% at 4 months (p<0.001, in both). This improvement in lung function was sustained above baseline at 18 months.

A significant improvement in FVC and TLCO (p<0.002 and p< 0.001 respectively) was seen in the inhaled placebo group only at 18 month. There were no significant changes in lung function in the normal group throughout the study.

Adverse Effects
Throughout the study none of the patients receiving inhaled budesonide or placebo equivalent reported any side-effects. This also applied to the healthy volunteer group.

Nine of the 10 patients on systemic steroids developed acne on their face, chest and back. All patients in the systemic steroid group reported an increase in body weight (in excess of half a stone) in the first 4 months. Their mean weight was still above that prior to the study at 18 months. Ankle oedema was observed in 1 patient at 18 months. However none of these patients developed any problem with blood pressure, blood glucose or glycosuria. One patient on oral prednisolone complained of epigastric discomfort (there had not been a prior history of dyspepsia or peptic ulceration).
**BAL Differential Cell Count**

All 30 patients with sarcoidosis had a greater percentage lymphocyte count in their BAL (38.3 ± 1.81%) pre-treatment, than the normal population (8.82 ± 3.41%) (p<0.001). At 4 months, there was a decrease in BAL lymphocytosis in those patients receiving budesonide from 42.3 ± 6.8% to 31.6 ± 5.24% (p<0.001), which reduced further to 22.3 ± 1.90% at 18 months. A similar, but more marked change, was seen in the patients on systemic steroids (from 36.9 ± 7.81% to 19.7 ± 2.10% at 4 months (p<0.001), and 18.9 ± 12.0% at 18 months. No such change was seen in the other two groups (Fig 6.5c). Similar changes were observed in total cell count, and absolute number of macrophages (Fig 6.5a,b).

**BAL immunocytological analysis**

At the start of the study all sarcoid patients had a higher proportion of macrophages expressing RFD1+D7- and RFD1+D7+ than the normal volunteers (Figs 6.6 a,c ).

After 4 months treatment with inhaled budesonide, there was a reduction in the proportion of all macrophage subsets, and in particular a dramatic decrease in the proportion of macrophages expressing RFD1+D7+ (21.4 ± 3.14 % to 8.54 ± 2.01%) (p<0.001), which was still low at 18 months (11.9 ± 1.72%) (p<0.001)(Fig 6.6c).

Similar reductions in the proportion of RFD1+D7- and RFD1+D7+ macrophages were noted in patients receiving systemic steroids; however, the striking observation was the marked rise in macrophages expressing RFD1-D7+ (from 43.2 ± 0.17% to 76.9 ± 1.91 at 4 months (p<0.001), and 89.0 ± 4.22% at 18 months (Fig 6.6b).

Although changes in macrophage subset proportions were seen in the lavage of the inhaled placebo groups (sarcoid and normal), these tended to be inconsistent.
Fig. 6.5

The mean and standard error of (a) total cell count, (b) absolute number of macrophages, and (c) percentage lymphocytosis in the BAL during the study period, is given for each group investigated.
Fig. 6.6

The mean and standard error percentage proportion of each of the 3 macrophage subsets in BAL during the study period is given for each group investigated.
Autologous mixed lymphocyte reactions

The AMLR experiments in this study were performed on unmanipulated BAL populations. Although it is accepted that this caused varied proportions of macrophages and lymphocytes to be present in different samples, this approach represents the only way in which the in situ capacity of these cells can be ascertained rather than the potential capacity produced by concentrations adjusted in vitro (q.v. Discussion below).

In normal subjects the incorporation of $^3$HT in PBM cells showed an eight-fold increase over mitomycin-treated cultures (SI 7.82 ± 0.80%) (Fig 6.7b). This was markedly higher when compared to the SI of their lavage (0.75 ± 0.24) (Fig 6.7a). This PBM reactivity in normals was suppressed by admixture with autologous BAL cells (SI 1.13 ± 0.36) (Fig 6.7c). There was no marked change in any of these cell reactions after inhaled placebo.

All sarcoid patients exhibited a reduced reactivity in peripheral blood compared to normal (p<0.001)(Fig 6.7b). When PBM was admixed with autologous BAL prior to any treatment, further reduction in PBM AMLR was seen (Fig 6.7c). After 4 months of treatment with inhaled budesonide, there was a striking increase in PBM reactivity from SI (1.61 ± 0.14 to 6.6 ± 1.71 ) (p<0.001), as well as BAL reactivity (from SI 0.82 ± 1.01 to 2.90 ± 0.92) (p<0.001) (Figs 6.7a,b). In addition, following budesonide, alveolar macrophages were much better stimulators of autologous PBM than were equivalent populations tested before treatment (from SI 0.12 ± 0.51 to 3.16 ±1.13) (p<0.001). These changes were sustained after 18 months of inhaled steroid.

No similar changes were seen in the sarcoid placebo group except for an increase in PBM stimulation [from SI 3.02 ± 0.45 to 5.06 ± 0.10 at 4 months (p<0.001), and
Fig. 6.7

The autologous mixed lymphocyte reactivity expressed as mean stimulation index is given for various cell combinations tested during the study period.

Cells tested were unfractionated bronchoalveolar lavage cells (BAL); peripheral blood mononuclear cells (PBM); and a mixture of the two (BAL/PBM).

Results on each of the four study groups are compared:
- normal volunteers receiving inhaled placebo (Normal);
- sarcoid patients receiving systemic steroids (S. steroid);
- sarcoid patients receiving inhaled steroid (I. steroid); and
- sarcoid patients receiving inhaled placebo (I. placebo)
In the patients receiving systemic corticosteroids the most striking observation was the marked increase in PBM reactivity, as early as 4 months (Fig 6.7b). At the same time, a lesser increase in BAL cell reactivity was seen, suggesting that even in this group the stimulating capacity of AM was enhanced (Fig 6.7a,c).

**DISCUSSION**

This study shows that in the treatment of pulmonary sarcoidosis, inhaled steroids such as budesonide, administered via a 750 cm² spacer device in a relatively small dose of 800 µmg twice daily, achieves approximately 10% alveolar deposition and effective symptomatic relief with no adverse effects. Furthermore, in just 4 months, the inhaled therapy was shown capable of modulating the tested features of the aberrant immunological reactions existent in the lung in this disease (although changes in CXR and lung function were not marked). Inhaled steroids not only produced a significant decrease in lavage lymphocytosis but concurrently produced a change in the phenotype and functional characteristics of the alveolar macrophage population.

In this study following budesonide treatment, there was a striking increase in PBM and BAL AMLR reactivity; in addition, the AM from these treated patients were better stimulators of PBM in the AMLR. Such observations might be explained by the change in phenotype witnessed within AM population subsequent to therapy. Specifically, there was a marked reduction in the proportion of RFD1+ macrophages also expressing RFD7. As the cell populations in each lavage were not manipulated, it is not possible from results presented here to identify the specific subset potentially responsible for these functional changes. However, investigation of the lavage populations as they appear *in situ*, makes these observations more relevant to the situation *in vivo*.
Interestingly, systemic steroids were also shown to have an effect on the local AM population in the lung. In this regard, although a reduction in double phenotype AM occurred, this was also coupled by a dramatic switch in phenotype to RFD7. As these phenotypically distinct AM subsets have been shown to express different functions (q.v. Chapter 5), the different effect of both inhaled and systemic steroids on these cell types might be taken to imply that, such therapies are directly affecting macrophage-lymphocyte interactions in the lung. Whether this modulation is occurring as a result of a direct action of steroid on the local macrophage population, or an indirect action via effects on lymphocytes or mediators present, remains to be elucidated.

However, it could be argued that these observations were due not to the inhaled and systemic steroid treatment but to the variable clinical course of sarcoidosis, and to the fact that the phenotype and presumably functional capacity of the macrophage population is known to alter during the course of the disease (Ainslie et al, 1989). In an attempt to limit such variables, a group of sarcoid patients who seemed to be in the same stage of their disease (in so far as clinical status, pulmonary function and radiological grading were concerned) were recruited. In addition in all the patients, a diagnosis of sarcoidosis had been made over a year prior to the current study. The results presented are also justified by the fact that, the dramatic changes witnessed in certain macrophage subsets with inhaled budesonide and oral prednisolone were not observed in the sarcoid and normal placebo groups. It may appear from Fig 6.6 that the difference in pre-treatment percentage of RFD1+D7+ macrophages between the patient and placebo groups might have biased the result after treatment. This possibility is discounted by the normal group having an even smaller proportion of RFD1+D7+ macrophages before treatment, which did not significantly change after the study period. The same can be said for the RFD1-D7+ macrophages (Fig 6.6).
Both in the inhaled and systemic steroid groups, the clinical and immunological changes were not accompanied by any striking improvement in chest X-ray appearances, or lung function. However, the rate of clinical improvement and radiological clearing were much more noticeable in the systemic steroid group. This is not surprising, perhaps, in view of the comparatively larger doses of systemic steroid given. Yet even with systemic corticosteroids, it is now recognised that the relief of clinical symptoms may not always be paralleled by objective improvement (Scadding & Mitchell, 1985). Indeed in a recent study on the effect of systemic corticosteroid therapy on the breathing pattern in interstitial lung disease Renzi & Renzi (1988) showed no significant change in the volume and time components of the respiratory cycle or the ventilatory drive parameters. On the other hand, it is also true that any changes at the cellular level would be expected to precede gross changes in pulmonary function and radiological appearances.

In an 18 month study on the effect of inhaled budesonide in pulmonary sarcoidosis, Selroos et al (1986) demonstrated a significant clinical improvement in symptoms and pulmonary function. They also observed a decrease in BAL lymphocytosis, as well as normalisation of the increased T-cell CD4/CD8 ratio usually seen in active disease. Yet no significant changes were observed in radiological appearances. Erkkila et al (1988) found that inhaled budesonide could also influence the biochemical profiles in patients with early sarcoidosis in the same way as systemic steroids. In particular, budesonide had a significant effect on SACE, serum beta2-microglobulins and BAL concentration of hyaluronan (described as a marker of fibroblast activation).

Systemic corticosteroids in varying doses (20 - 60 mg prednisolone a day) have been shown to induce a shift in the distribution of T-cell subsets towards a lower CD4/CD8 ratio; a change observed by Ceuppens et al (1984) to be independent of the persistent high lymphocytosis in lavage. Pinkston et al (1987) showed that systemic steroids also suppressed the exaggerated release of IL-2 by lung T-lymphocytes, with
consequent reduction in lung T-cell proliferation. The study in this chapter has extended these observations, and clearly showed that such therapeutic regimes can also influence the sarcoid-related aberrations within the lung's macrophage population.

**SUMMARY**

The effect of inhaled corticosteroids, on the phenotype and functional capacity of macrophages obtained by BAL from patients with pulmonary sarcoidosis, was studied and compared to that of systemic corticosteroids. The results in both groups were correlated with clinical status and therapeutic efficacy. Drug distribution studies showed that 10% of the inhaled drug was deposited in the alveolar region. Clinical symptomatic improvement was noted in both patient groups treated with inhaled and systemic steroids, although the latter produced a much more rapid improvement both subjectively and objectively. However oral corticosteroids (but not inhaled) caused systemic side-effects. Changes at the cellular level, on both the lymphoid and non-lymphoid cell populations were observed with both inhaled and systemic steroids. Both produced a decrease in BAL lymphocytosis (more marked with oral prednisolone); inhaled steroids produced a concomittant change in the phenotype and functional characteristics of the local AM population; in particular, a decreased proportion of the RFD1+D7+ macrophages. While similar changes were seen in the systemic steroid group with regard to RFD1+D7- and RFD1+D7+ AM, there was in addition a marked increase in macrophages expressing RFD7 only. No similar changes were observed in the placebo groups. The results suggest that inhaled steroids can modulate the aberrant immunological reactions existent in the lung in pulmonary sarcoidosis. As such they have a place in the early treatment of pulmonary sarcoidosis, in an effort to abort the potential persistence of granulomata in the lung parenchyma and subsequent development of fibrosis. The author postulates that this effect may occur through an action on the local AM population.
CHAPTER SEVEN

AN ANALYSIS OF LUNG LAVAGE CELLS OBTAINED FROM PATIENTS WITH PRIMARY BILIARY CIRRHOSIS: A COMPARISON IS MADE TO THOSE FROM PATIENTS WITH ACTIVE PULMONARY SARCOIDOSIS
INTRODUCTION

Sarcoidosis shares many overlapping features (clinical, histological and immunological) with other chronic multisystem granulomatous disorders, in which the clinical manifestations may also involve the lung. One such disease is primary biliary cirrhosis (PBC) (q.v. Chapter 1). PBC is a chronic progressive liver disease, of unknown aetiology, and characterized by a granulomatous, destructive inflammation of intrahepatic bile ducts with eventual portal fibrosis and cirrhosis (Scheuer, 1967). The rate at which the disease progresses varies considerably, producing a wide clinical spectrum (Christensen et al, 1980). Raised serum bilirubin concentrations reflect a poor prognosis (Shapiro et al, 1979), while patients with focal granulomatous lesions in their livers have a better prognosis (Scheuer, 1973). Just as has been described in sarcoidosis, factors determining the progression of PBC are ill-understood. A number of abnormalities of the immune system have been described in PBC (James S P et al, 1983; Kaplan, 1987); whether these arise as a consequence of the liver disease or whether they are implicated in either the initiation or progression of the disease process remains unclear.

The true incidence of pulmonary complications in patients with PBC is unknown; however, the possible involvement of the lung during the course of PBC is acknowledged (Turner-Warwick, 1968; Stanley et al, 1973; Sherlock & Scheuer, 1973). Observations range from reports of associated fibrosing alveolitis (Turner-Warwick, 1968) to the presence of parenchymal granulomata mimicking pulmonary sarcoidosis (Stanley et al, 1973). Radiological parenchymal shadowing and lung function defects (such as reduced diffusion capacity and airways obstruction) have also been cited (Golding et al, 1973). The significance of such associations remains controversial, with some authors suggesting that the radiological lung shadowing and defective pulmonary function in PBC are secondary to pulmonary vascular disorders rather than coincidental interstitial disease (Stanley & Woodgate, 1972). Recently Wallaert et al (1986a) observed the presence of an
alveolitis in the lungs of PBC patients who had normal chest X-rays and lung function. This subclinical alveolitis was characterized by an increased number of T-helper lymphocytes in the BAL of these patients. This implies that the alveolitis may be the first manifestation of interstitial lung disease in PBC, occurring even before the pulmonary disorder becomes clinically overt. A similar situation has been described in other non-pulmonary disorders, such as rheumatoid arthritis and Crohn's disease (Fulmer, 1982; Wallaert et al, 1985; Smiejan et al, 1986; Voisin et al, 1988). Furthermore, reports have appeared that describe a subclinical T-lymphocytic infiltrate in the BAL of patients with extrathoracic manifestations of sarcoidosis, but who have normal chest X-rays (Wallaert et al, 1982; Wallaert et al, 1986b). Such observations raise the possibility of an initial, identical, immunological response in these disorders, the clinical expression of which may be partly influenced by stimuli in the environment of the patient. Indeed in both sarcoidosis and PBC the causative stimulus remains unknown and the clinical progression highly variable; in addition, in a group of patients, the presenting features are common to both diseases such that no clear-cut distinction can be made.

BAL offers the possibility of assessing the emergence of the subclinical alveolitis in patients with PBC, and allows monoclonal antibodies (MoAb) to be used as probes to define the state of activation and immunocompetence of the cell subsets involved in the inflammatory process. Such studies would not only help to reveal the underlying immunological reactions occurring in the lungs of patients with PBC, but would also permit a detailed evaluation of the relationship of these findings to those documented from patients with sarcoidosis. The results may provide clues to the aetiology of these two disorders, and could benefit the management of those PBC patients in whom the alveolitis progresses and becomes symptomatic: in particular as there are, as yet, no clear guidelines for the treatment of inflammatory lung disease in PBC.
MATERIALS AND METHODS

Subjects

The study population consisted of 10 patients with biopsy-proven PBC (diagnosis >1 year): 8 females, 2 males; mean age ± S.E. 46.7 ± 5.34 years, 2 smokers (one pack/day). All were asymptomatic as regards their chest, with normal chest radiographs and lung function parameters. None of the patients were receiving any immunosuppressive therapy at the time of the study, and none complained of dry eyes and/or mouth, or arthralgia. All PBC patients had been screened by serological tests for other immunological disorders and found to be negative (Table 7.1). Prognostic staging of PBC was assessed by serum bilirubin levels and histological analysis of liver biopsies. Fifteen patients with biopsy proven pulmonary sarcoidosis were recruited; all non-smokers, 12 males, 3 females; mean age ± S.E. 38.9 ± 2.41 years. All had abnormal chest radiographs ranging from bilateral hilar lymphadenopathy (BHL) (5 patients) to BHL with interstitial shadowing (4) to interstitial shadowing alone (6). None were receiving any form of treatment at the time of the investigation. A population of 10 healthy controls: non-smokers; 9 males, 1 female mean age ± S.E. 22.4 ± 1.21 years. All had normal chest x-ray and pulmonary function.

Bronchoalveolar Lavage

BAL was performed, and all samples processed with the standard procedure described in Chapter 3. After counting the cells, the BAL concentration was adjusted in each case to 3 × 10^5 cells per ml, and cytospins prepared from each sample.

Immunocytological analysis

To discriminate particular macrophage subsets involved in the inflammatory process, the macrophage markers RFD1, RFD7 and RFD9 were used. The cells from each recruited subject, were analysed for single or double antigen expression using the
### Table 7.1

Details of PBC patients in this study

<table>
<thead>
<tr>
<th>TEST</th>
<th>Mean±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR</td>
<td>30.5±1.56</td>
</tr>
<tr>
<td>SACE mmol/min/ml</td>
<td>41.0±1.5</td>
</tr>
<tr>
<td>Bilirubin umol/l</td>
<td>34.5±10.3</td>
</tr>
<tr>
<td>Asp. Transaminase IU/l</td>
<td>40.1±6.43</td>
</tr>
<tr>
<td>Alk. Phosphatase IU/l</td>
<td>514±40.1</td>
</tr>
<tr>
<td>Gamma GT IU/l</td>
<td>90±11.4</td>
</tr>
<tr>
<td>Total Protein g/l</td>
<td>94±4.13</td>
</tr>
<tr>
<td>Albumin g/l</td>
<td>30±6.45</td>
</tr>
<tr>
<td>Corrected Calcium mmol/l</td>
<td>2.39±0.41</td>
</tr>
<tr>
<td>IgG g/l</td>
<td>14.7±1.34</td>
</tr>
<tr>
<td>IgA g/l</td>
<td>1.6±0.43</td>
</tr>
<tr>
<td>IgM g/l</td>
<td>3.4±1.23</td>
</tr>
<tr>
<td>Rheumatoid factor</td>
<td>Titre&lt;1:80</td>
</tr>
<tr>
<td>Antinuclear factor</td>
<td>Titre&lt;1:40</td>
</tr>
<tr>
<td>Antimitochondrial antibodies</td>
<td>Titre&gt;1:160</td>
</tr>
<tr>
<td>Smooth muscle antibodies</td>
<td>Titre&lt;1:40</td>
</tr>
<tr>
<td>Thyroid antibodies</td>
<td>Titre&lt;1:40</td>
</tr>
<tr>
<td>Parietal cell antibodies</td>
<td>Titre&lt;1:40</td>
</tr>
</tbody>
</table>
standard techniques described in Chapter 3. The panel of MoAb probes used in this study are outlined in Table 7.2. T-lymphocyte subpopulations were identified using MoAbs OKT4 and RFT8. The state of activation of such lymphocytes was analysed with RFDR1, anti-tac and RFT2 (the latter acting only as an activation marker at a dilution of 1 in 5). In addition, MoAbs UCHL1 and SN130 were used as probes to investigate the immunological competence of the cell subsets involved in the alveolitis.

**STATISTICAL ANALYSIS**
Quantitative data were expressed as the mean ± S. E.; wherever relevant, data were analysed as described in Chapter 2.

**RESULTS**

*Bronchoscopic Findings*
No evidence of any bronchial infection or inflammation was found in any subject. There was no significant difference in percentage return of lavage fluid in any of the study groups (72.0 ± 11.0% to 73.0 ± 7.42% of the instilled volume).

*Differential Cell Counts*
Overall, the group of patients with PBC had a significantly raised lymphocyte proportion in their BAL when compared to normal (p < 0.01) (Fig. 7.1). Within the PBC patient group, it appeared that 6 of the 10 patients had markedly raised proportions of lymphocytes in their lavage (mean ± SE 27.6 ± 4.32% of total count) compared to normal (5.0 ± 3.41%). While the other 4 PBC patients had the same lymphocyte proportions (4.80 ± 2.44%) as the normal volunteers. All patients with pulmonary sarcoidosis had a marked lymphocytosis (29.2 ± 4.17%). The 6 PBC patients with lymphocytosis had a raised lavage total cell count (14.3 ± 0.67 x 10^6 cells), as did the sarcoid group (16.6 ± 0.3 x 10^6 cells), in contrast to the other 4 PBC patients and normal volunteers (5.23 ± 0.14 x 10^6 and 7.11 ± 0.25 x 10^6)
TABLE 7.2 Monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>MOL WT ANTIGEN</th>
<th>SOURCE</th>
<th>SPECIFICITY IN NORMAL TISSUE</th>
<th>REFERENCE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFDR1</td>
<td>28/33 kD</td>
<td>RFHSM</td>
<td>Framework epitope on HLA-DR molecule</td>
<td>Janossy (1986)</td>
</tr>
<tr>
<td>RFD1</td>
<td>28/33 kD</td>
<td>RFHSM</td>
<td>Interdigitating cells small proportion B cells</td>
<td>Poulter (1986)</td>
</tr>
<tr>
<td>RFD7</td>
<td>77 kD</td>
<td>RFHSM</td>
<td>Mature macrophages</td>
<td>Poulter (1986)</td>
</tr>
<tr>
<td>RFD9</td>
<td>-</td>
<td>RFHSM</td>
<td>Epithelioid cells; Tingible body MO</td>
<td>Munro (1986)</td>
</tr>
<tr>
<td>OKT4</td>
<td>62 kD</td>
<td>ORTHO</td>
<td>Helper-inducer T-cells</td>
<td>Bach (1986)</td>
</tr>
<tr>
<td>RFT8</td>
<td>30-32 kD</td>
<td>RFHSM</td>
<td>Suppressor-Cytotoxic T-cells</td>
<td>Janossy (1985)</td>
</tr>
<tr>
<td>RFT2</td>
<td>40 kD</td>
<td>RFHSM</td>
<td>Pan-T; preferentially expressed on blast cells</td>
<td>Poulter (1985)</td>
</tr>
<tr>
<td>ANTI-TAC</td>
<td>53 kD</td>
<td>DAKO</td>
<td>IL-2 receptors</td>
<td>Uchiyama (1981)</td>
</tr>
<tr>
<td>SN130</td>
<td>200-200 kD</td>
<td>RFHSM</td>
<td>Recognised distinct isoforms of the leucocyte common antigen</td>
<td>Munro (1988)</td>
</tr>
<tr>
<td>UCHL1</td>
<td>180 kD</td>
<td>P Beverley (Middlesex Hosp)</td>
<td></td>
<td>Smith (1986)</td>
</tr>
</tbody>
</table>

* Only first author names are given; full reference is given in appropriate section.
Fig. 7.1

This represents the percentage of morphologically identifiable lymphocytes in the BAL of each subject investigated. In comparison to the normal volunteers, lymphocyte proportions in BAL were significantly higher in both PBC patients (p<0.01) and sarcoid patients (p<0.001).
Percentage lymphocytosis in BAL

SARCOID  NORMAL  PBC  study group

<table>
<thead>
<tr>
<th>Percentage lymphocytosis</th>
<th>BAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>
respectively). The BAL macrophage percentage proportions were thus altered accordingly, being lower than normal in those 6 PBC patients with lymphocytes, and normal in the 4 PBC patients without lymphocytosis. The viability of the retrieved lavage cells from all 3 study groups was > 95%.

**Immunocytological Analysis**

In all subjects, over 83% of lymphocytes in BAL were T-cells: of which more than 82% expressed UCHL1 (Tables 7.3 and 7.4). There was no significant difference between any of the groups. A marked increase in CD4/CD8 T-cell ratio was seen in the group of PBC patients with lymphocytosis (4.13 : 1) and sarcoid patients (5.60 : 1); compared to normal volunteers (1.80 : 1) and PBC patients without lymphocytosis (1.60 : 1) (Table 7.3). A small proportion of T-lymphocytes expressed markers of activation, being positive to MoAbs RFDR1, anti-tac and RFT2. This was evident in those PBC patients exhibiting lymphocytosis in their BAL (7.51 ± 2.13%, 5.88 ± 1.75%, 2.21 ± 0.19% respectively; as well as in the patients with sarcoidosis (10.0 ± 0.51%, 5.01 ± 0.94%, 3.10 ± 0.31% respectively). No T-cell activation was detected in the other two groups (Table 7.4).

Within the non-lymphoid cell population, an increase in proportion of RFD1+D7- cells was seen in PBC patients with lymphocytosis (31.2 ±1.91%) and in sarcoid patients (46.3 ± 5.15%); in contrast to the normal subjects (13.6 ± 2.11%) and the PBC group without lymphocytosis (6.52± 1.10%). Interestingly in the former two groups, there was an increased emergence of RFD1+ cells that also expressed RFD7. No significant difference was seen in the proportion of RFD9+ cells in any of the groups. In those PBC patients without lymphocytosis, a greater proportion of macrophages was RFD1-D7+ (Table 7.5).
TABLE 7.3

The Percentage Proportion and Distribution of T-Lymphocytes in the BAL of PBC, sarcoid and normal subjects

<table>
<thead>
<tr>
<th></th>
<th>% T-cells</th>
<th>CD4:CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC with lymphocytosis (N = 6)</td>
<td>95.8± 3.51</td>
<td>4.13:1*</td>
</tr>
<tr>
<td>PBC without lymphocytosis (N = 4)</td>
<td>83.7±10.8</td>
<td>1.60:1</td>
</tr>
<tr>
<td>Normal volunteers (N = 10)</td>
<td>89.2±7.32</td>
<td>1.80:1</td>
</tr>
<tr>
<td>Sarcoid patients (N = 15)</td>
<td>97.0±1.24</td>
<td>5.60:1*</td>
</tr>
</tbody>
</table>

* P = < 0.001 compared to normal

The proportion of B-cells was consistently <1% in each group.
### TABLE 7.4 Phenotypic Characteristics of lavage T-Lymphocytes in each group

<table>
<thead>
<tr>
<th></th>
<th>SN130+</th>
<th>UCHL1+</th>
<th>HLA-DR1+</th>
<th>RFT2+</th>
<th>ANTI-TAC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC with lymphocytosis (N = 6)</td>
<td>0.60±0.42</td>
<td>97.3±0.14</td>
<td>7.54±2.32*</td>
<td>5.88±1.75*</td>
<td>2.21±0.19**</td>
</tr>
<tr>
<td>PBC without lymphocytosis (N = 4)</td>
<td>0.25±0.31</td>
<td>64.5±22.2</td>
<td>0.25±0.33</td>
<td>1.01±0.72</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Normal volunteers (N = 10)</td>
<td>1.10±0.52</td>
<td>82.0±3.42</td>
<td>2.01±0.84</td>
<td>1.22±0.31</td>
<td>1.04±0.11</td>
</tr>
<tr>
<td>Sarcoid patients (N=15)</td>
<td>0.91±0.23</td>
<td>96.0±1.01</td>
<td>10.0±0.51*</td>
<td>5.01±0.94*</td>
<td>3.10±0.32**</td>
</tr>
</tbody>
</table>

All figures above represent percentage total number of lymphocytes (mean ± SE)

* p = <0.001 compared to normal, in each case

** p = <0.01 compared to normal
TABLE 7.5
Distribution of Macrophage Subsets in BAL of PBC Patients Compared to Normal and Sarcoid Subjects

<table>
<thead>
<tr>
<th></th>
<th>RFD1</th>
<th>RFD7</th>
<th>RFD9</th>
<th>RFD1D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBC with</td>
<td>31.2±1.91*</td>
<td>38.1±5.71</td>
<td>51.7±1.61</td>
<td>18.5 ± 7.21*</td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>(N = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBC without</td>
<td>6.52±1.10</td>
<td>93.0±1.64**</td>
<td>50.7±2.10</td>
<td>3.42 ± 1.24</td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>(N = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>13.6±2.11</td>
<td>20.2±1.51</td>
<td>48.2±1.64</td>
<td>6.41 ± 2.10</td>
</tr>
<tr>
<td>volunteers</td>
<td>(N = 10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcoid</td>
<td>46.3±5.15*</td>
<td>30.4±6.42</td>
<td>45.0±1.10</td>
<td>26.5 ± 6.54</td>
</tr>
<tr>
<td>(N = 15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All figures above represent percentage of total number of morphologically identifiable macrophages (mean ± SE)

* \( p = <0.001 \) compared to normal

** \( p = <0.0001 \) compared to normal
TABLE 7.6

Correlation of BAL lymphocytosis with histological and biochemical staging of PBC

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Liver Biopsy Stage</th>
<th>Serum Bilirubin (umol/L)</th>
<th>% Lavage Lymphocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>52</td>
<td>1</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>60</td>
<td>2</td>
<td>12</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>56</td>
<td>2</td>
<td>44</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>67</td>
<td>2</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>65</td>
<td>2</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>42</td>
<td>4</td>
<td>17</td>
<td>35</td>
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<tr>
<td>7</td>
<td>M</td>
<td>53</td>
<td>4</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>57</td>
<td>4</td>
<td>93</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>54</td>
<td>4</td>
<td>86</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>71</td>
<td>3/4</td>
<td>24</td>
<td>10</td>
</tr>
</tbody>
</table>
**Relationship of BAL Data to Biological and Histological Staging**

The normal range for serum bilirubin level in this study was 5 - 17 umol/L. By this criteria, 6 of the patients with PBC had hyperbilirubinaemia, of whom only 3 showed a lymphocytosis in their lavage. All the remaining 4 PBC patients with normal serum bilirubin levels had BAL lymphocytosis (Table 7.6).

Only 1 of the PBC patients had early disease on liver biopsy staging (Stage 1); this patient had increased lymphocyte numbers in lavage. Four patients showed moderately advanced PBC (Stage 2), 3 of whom had lymphocytosis. The remaining 5 PBC patients had late stage disease in their liver biopsies; only 2 of these patients had lymphocytosis (Table 7.6)

**DISCUSSION**

This study has shown that a proportion of patients with PBC can develop a subclinical inflammation in their lower respiratory tract. The alveolitis was characterized by an increased number of activated T-helper lymphocytes, with a concomitant increase in the proportion of macrophage-like cells that expressed positivity to RFD1. A great majority of these cells also expressed RFD7. In contrast, the proportion of both lymphoid and non-lymphoid cell subsets in the PBC patients without evidence of a lavage lymphocytosis reflected those of normal subjects.

The lavage results obtained from the PBC patients with a lymphocytosis in their BAL suggest that, with the insidious onset of a lymphocytic infiltration into the lung, a switch from one cell phenotype to another occurs within the macrophage population, leading to the emergence of selective macrophage subsets. This is a situation that has also been observed to occur in pulmonary sarcoidosis, and has been related to potential changes in macrophage function (q.v.Chapters 4 and 5). Indeed this study suggests that in a number of PBC patients, the changes within their lung cell populations reflect the
There is in fact considerable clinical and histological overlap in both PBC and sarcoidosis. Pulmonary function abnormalities are common in patients with PBC, and are often similar to those found in sarcoidosis (Stanley et al., 1973). In the early stages of PBC a high frequency of liver granulomas are found. Hepatic granulomas are also common in sarcoidosis, and moreover intrahepatic cholestasis has also been reported. Immunologically, both show evidence of skin anergy, delayed-type hypersensitivity reactions, and increased HLA-DR expression on effector mononuclear cells. (Colussi, 1986; Kaplan, 1987). Indeed, there have been reports of the possible co-existence of both sarcoidosis and PBC in the same patient (Fagin et al., 1983).

In the majority of investigations into PBC associated lung involvement, the focus has usually centred on radiological and pulmonary function assessment in patients who tended to be symptomatic. While such studies confirm that PBC patients can indeed develop latent pulmonary involvement during the course of their liver disease, the data from the current investigation on asymptomatic PBC patients reinforces the immunological similarities with sarcoidosis.

The findings in this chapter agree with those of Wallaert et al. (1986a), who also found no relationship between the lung lymphocytosis and duration or stage of the PBC. Accepting that the patient group in the current study was small, and that no direct correlation was observed between BAL lymphocytosis and stage of PBC, the results appear to hint that the incidence of the observed subclinical alveolitis might be greater in those PBC patients who only had histological evidence of focal granulomatous lesions in their liver. This hypothesis would not be contradictory, as previous studies on patients in different stages of their pulmonary sarcoidosis have shown that the degree of lymphocytosis in BAL tends to decrease in the presence of advanced fibrotic disease (Keogh & Crystal, 1982). By the same token, the absence of an alveolitis in
the lavage of advanced PBC patients does not discount the possible presence of disease in their lung tissue.

As a control group of non-PBC liver disease patients was not included in this study, it could be argued that the observed alveolitis in some of the PBC patients is non-specific and secondary to liver damage. Previous authors, however, have shown that the lavage of patients with non-PBC liver disorders such as alcoholic cirrhosis reflects that of normal volunteers (Wallaert et al, 1986a). The alveolitis observed in PBC is also unlike that found in the lavage of patients with pulmonary viral infection, in whom there is an increase in the proportion of CD8+ T-lymphocytes as well as B-cells, with no change in the non-lymphoid population (Milburn et al, 1989).

The results may be interpreted as a reflection of systemic changes, that is as simply reflecting peripheral blood ratios. Although this study did not investigate peripheral blood lymphocyte subsets, previous authors have demonstrated that BAL lymphocyte findings in PBC patients do not bear any correlation to comparative peripheral blood markers (Wallaert et al, 1986a). Even if the former were the case, the lymphocytosis observed in the lungs of a major proportion of the PBC patients at best suggests a subclinical alveolitis, and at worst a nascent pneumonitis.

Rodriguez-Roisin et al (1981) concluded that the observed lung disease in PBC patients was probably due to associated Sjogren's syndrome; yet none of the PBC patients selected for this study appeared to have any associated clinical disease (as excluded by pre-study investigations). As no lung biopsies were performed in the current investigation (patients were asymptomatic with clear CXR), it may seem that the current data only reflects the existence of an inflammation in the lower respiratory tract, and does not necessarily represent interstitial lung involvement. However as described in Chapter 1, it is now accepted that the initial lesion in
granulomatous lung disorders is a mononuclear cell alveolitis, that may proceed to granuloma formation within the lung parenchyma.

The insidious presence of aberrations within the lymphoid and non-lymphoid cell populations in the BAL of a proportion of patients with PBC raises the possibility of an emergence of granuloma-producing mechanisms in the lungs of these patients similar to those active in their liver. Furthermore the similarity to the alveolitis seen in patients with sarcoidosis supports the hypothesis that these two disorders may share a similar initial immunological response to an as yet unknown stimulus, with the resultant clinical expression being partly influenced not only by the internal genetic make-up of the individual but also by the external environment.

SUMMARY
BAL was performed on a group of patients with PBC, who were clinically asymptomatic with normal CXR and lung function, in order to investigate the basis of previously reported early lung involvement in this disease. Both lymphoid and non-lymphoid cell populations in the lavage were analysed using immunocytological methods to determine their proportions and phenotypic features in order to gain information as to possible immune mechanisms active in the lung of these patients. Six of the 10 patients in our study showed evidence of a lymphocytic alveolitis. The results were compared with control groups of normal volunteers and patients with active pulmonary sarcoidosis. The 6 PBC patients with lymphocytosis had a raised CD4/CD8 T-cell ratio, similar to the sarcoid patients. A proportion of these T-lymphocytes expressed markers of activation (HLA-DR1+, anti-tac+, RFT2+). This increased T-cell activation was similar to that in the sarcoid patients. This was not seen in the PBC patients without lymphocytosis, and the normal volunteers. In addition, those PBC patients with a lymphocytosis, and in the sarcoid patients, there was a significant increase in the proportion of macrophages that expressed RFD1.
Much of this increase was due to cells that also expressed RFD7. No such changes were seen in either the normal group or the PBC patients without lymphocytosis. The latter patients seemed to have a relatively greater proportion of mature phagocytes (RFD1-D7+). The author postulates that these observations may indicate the emergence in the lung of a granuloma producing mechanism similar to that occurring in the liver in PBC. By comparison, the alveolitis found in PBC is consistent with that observed in interstitial granulomatous lung disorders such as sarcoidosis.
CHAPTER EIGHT

GENERAL DISCUSSION
ALVEOLAR MACROPHAGES: THEIR CRITICAL ROLE IN THE IMMUNE RESPONSE OF THE NORMAL LUNG

The induction and expression of T-cell dependent immunity within the human lung is governed by the same overall regulatory mechanisms that apply to the immune system in general. However, certain unique features are present in the lung: in particular the association of the large vascular bed, and the overlying epithelium with the external environment. Such a relationship, of necessity, would dictate that local T-cell activation in response to incoming antigen must be tightly controlled. This is therefore likely to involve the effective operation of highly selective control mechanisms, which would restrict the induction and expression of potentially tissue-damaging immune responses in the lung.

In light of this, it is known that the vast proportion of antigenic material entering the respiratory tract is sequestered by a series of overlapping filtration mechanisms, including the mucociliary escalatory system. This is supplemented by local secretory antibody which protects the upper airways, and a mobile AM population which patrols the alveolar surface.

In comparison to other macrophages, these AM possess a unique position in tissue response to external stimuli. They reside in a well-oxygenated air-tissue interface, with direct continuous exposure to inhaled microbial pathogens and environmental pollutants. By virtue of this, it would therefore be appropriate for AM to acquire a physiological and functional specialization, that is consequent on their immediate milieu. Indeed, AM have been shown to undergo special metabolic adaptation (Oren et al, 1963), and to possess non-specific phagocytic and microbicidal activities. Furthermore marked differences in antibody-binding capacity between AM, and macrophages from other sites, have also been shown in vitro and in vivo (Lasser, 1983). The ability to acquire novel properties through the acquisition of appropriate
surface receptors for lymphokines, as well as the possession of a wide armamentarium of biologically active substances, empowers AM to exhibit an immunoregulatory function on both cell-mediated and humoral immune responses, when challenged by a variety of specific stimuli.

This apparent pleuripotential nature of AM begs the question as to whether all these different functions in vivo are performed by one cell type, or whether there exist functionally distinct subpopulations within the macrophage-cell lineage in the normal human lung. The answer to this intriguing question may perhaps be reconciled by the data presented in this thesis. By using monoclonal antibody probes directed against specific macrophage determinants, it was possible to identify three phenotypically distinct subpopulations within the normal AM pool. Even in health, it appears that such AM subsets exist in varying proportions to one another (q.v. Chapter 4). While two of the identified AM subsets respectively showed the phenotype of dendritic cells and classic macrophages, a third AM subset was observed to express both these phenotypes. When isolated, differences in the phenotype of these three AM subsets were seen to be related to quantitative differences in surface receptor expression (e.g. C3b and Fc receptors), and lysosomal enzyme content. In addition, a diverse spectrum of phagocytic capacity, and hexose monophosphate shunt activity was observed amongst the isolated AM subpopulations (q.v. Chapter 5). Superimposed on this, the data in Chapter 5 suggests that the presence of phenotypically distinct macrophage subsets, identified by specific macrophage probes, really reflect functionally distinct subpopulations, with peculiar immunoregulatory capabilities, in vivo.

In particular, the in vitro MLR experiments suggest that within the normal lung, there may exist both 'stimulator'* macrophages with T-cell enhancing properties, as

* The words 'stimulator' and 'inducer' will be used interchangeably in this discussion.
well as 'suppressor' macrophages capable of down-regulating the induction of T-cell responses set up by other cells. In support of this hypothesis, many workers (Daniele et al, 1977; Kaltrieder, 1982; Yeager et al, 1982) have independently commented on the apparent varying accessory cell function of AM. Indeed, the initial studies of Daniele et al (1977) suggested that human AM enhanced T-cell responses, while Yeager et al (1982) indicated that human AM suppressed mitogen-induced lymphocyte proliferation.

All these observations raise the possibility that, within the normal lung milieu, there exists an intricate inter-relationship between 'stimulator' and 'suppressor' macrophage cell types capable of influencing the overall function of the AM pool, with consequent effect on T-cell responsiveness. The events portrayed in the experiment shown in Fig. 5.9a (Chapter 5, p.159) are an excellent *in vitro* example of the cell-to-cell interaction potentially occurring *in vivo* in the human lung. In particular, it demonstrates that shifts in the proportions of these macrophage cell types, and thus their ratio to each other, could tip the balance in overall T-cell responsiveness. In accordance with such observations, Holt et al (1985), using animal experiments, elegantly showed that the *in vitro* antigen-presenting capacity of non-adherent Fc receptor negative mononuclear cells, could be inhibited by the presence of endogenous adherent Fc receptor positive macrophages.

It is thus exciting to speculate that such a balance of enhancing and suppressive forces, exerted by a dynamic heterogeneous AM pool on the local T-lymphocyte population, provides the basis of an effective immunologically-mediated host defence in the lung. In support, data in Chapters 5 and 7 show that a shift in this critical balance occurs with the advent of lung inflammation, promoting the emergence of AM subpopulations with distinctive features. As such therefore, the concept arises that within the normal
lung in man, there must also exist a constantly changing balance between heterogeneous AM capable of various roles: the activation of particular roles being constantly influenced by stimuli in the immediate milieu. Such a set-up would then induce appropriate functional specialization at the cellular level. This would give rise to functionally distinct AM subpopulations, each with the potential to directly influence the action of other immunocompetent cells in the local micro-environment. But what specific roles would these AM subsets be expected to play in the day-to-day defence mechanisms of the human lung?

In vitro MLR studies in this thesis conclusively showed that those macrophages, with the phenotype of RFD1+D7-, were capable of transmitting immunogenic signals to allogeneic T-lymphocytes, thereby enhancing their proliferation. As such, these RFD1+D7- cells appear to be similar in function to the putative dendritic cells, previously described by Poulter et al (1986). It could be speculated that these T-cell enhancing macrophages originate from peripheral blood monocytes that have migrated to the lung, where following differentiation and maturation, they retain their monocyte antigen-presenting capacity but lose the monocyte-related surface phenotype. Indeed, the majority of RFD1+D7- macrophages were UCHM1-. In support, Nicod et al (1987) described the presence, within the AM pool, of non-adherent, low density, Fc receptor-poor macrophages capable of both antigen-presenting activity and soluble factor production. Furthermore, the presence of such 'inducer' RFD1+D7-macrophages within the lung is justified by the fact that the induction and expression of the immune response to inhaled antigen within the lung, requires the interaction between T-helper cells and antigen-bearing accessory cells within a micro-environment conducive to T-cell activation (Unanue 1981).

The outcome of such an interaction would however be ineffectual without the presence
of effector-cell macrophages, whose prime function would be to sustain the initiated immune reaction. There is thus a need for cells that would act as scavengers of the lower respiratory tract, removing foreign material entering the lungs by phagocytosis. Such cells would have to possess phagocytosis-related surface receptors such as C3b and Fc receptors, and have a high lyzosomal enzyme content. These are the characteristic features of classic macrophages, and as such are portrayed by the RFD1-D7+ AM isolated from BAL in Chapter 5. These macrophages account for the majority of cells in normal lavage. In fact under normal circumstances, a significant proportion of antigen deposited within the terminal air spaces is phagocytosed by these resident AM (Hocking & Golde, 1979).

It would now seem that, with the existence of 'stimulator' (RFD1+D7-), and 'effector' (RFD1-D7+) AM, host defence within the lung would be complete. If so, then what is the significance of those macrophages that appear to have the phenotype of both dendritic cells and classic macrophages - so-called RFD1+D7+ cells? The presence of such RFD1+D7+ macrophages in normal BAL, albeit in small numbers (Chapter 4), and their increased proportion with the onset of disease (Chapters 4, 5 and 7), would suggest that their presence in the lung is a purposeful one. In vitro experiments have shown conclusively that these macrophages are capable of suppressing the T-cell responses set up by 'inducer' RFD1+D7- cells. If this apparent in vitro 'suppressor' macrophage influence on 'inducer' macrophage action is to be reflected in vivo, then it could be conceived that the role of these 'suppressor' macrophages in the normal lung, is to down-regulate the potentially harmful cascade of immunological events initiated by a macrophage-lymphocyte interaction. In this way, even under steady-state conditions in the lung, such 'suppressor' macrophage activity would still be needed to dampen any 'overstimulating' effects of antigen-presenting cells, thereby influencing overall T-cell responsiveness. Such a 'suppressor' mechanism would presumably itself be also under tight critical control:
as such this may be partly dictated by the inciting stimulus, and partly by events in the immediate environment. In support of the existence of such a mechanism in vivo is the recently-reported presence of 'suppressor' macrophages in human colonic mucosa, where such cells have been speculated to down-regulate the local immune responses arising as a result of a continuous insult of stimuli (Allison et al, 1988). Furthermore it appears that the proportion of these 'suppressor' macrophages increases in disease states, in which chronic perturbances of the immune system occur. This has been independently observed in both animals and humans (Eggers & Wunderlich, 1975; Waldmann et al, 1976; Ainslie et al, 1989).

But how could a particular AM subpopulation exert a 'suppressor' influence on T-cell responsiveness?

Previous studies have suggested that the 'suppressor' function of macrophages may be due to a decreased ability to take up and process antigen, and then to present it to lymphocytes in association with Class II MHC determinants (Ferro et al, 1987a). The question therefore becomes even more intriguing, as the majority of 'suppressor' RFD1+D7+ macrophages in the current studies, expressed HLA-DR (one of the MHC Class II molecules said to be critical to antigen presentation; q.v. Chapter 1). This raises the possibility that the presence of HLA-DR is not the sole determinant of the antigen-presenting capacity of a cell. In support, Unanue (1984) suggested that although the minimum requirement for an antigen-presenting cell is the expression of HLA-DR, a given HLA-DR bearing cell may not necessarily present all or any antigen, or indeed be capable of stimulating all T-cells. In fact, differences in functional DR-antigen expression, with shielding of the reactive epitopes in the DR-complex by N-linked carbohydrate moieties, have been postulated as explanations for macrophage 'suppressor' activity (Ferro et al, 1987b). Other possibilities could include: (i) active suppression of the lymphocyte response either directly or through the secretion
of certain products (Pryjma et al, 1989); (ii) the reduced secretion of soluble 'stimulator' factors, such as IL-1 and IL-6 (Weivers et al, 1984); (iii) the lack of other surface antigens distinct from (yet additive to DR), in stimulating T-lymphocyte responses e.g. HLA-DQ (Bach, 1984; Ettensohn et al, 1989). In addition, some reports have stressed that membrane-bound accessory molecules, such as LFA-1 and LFA-3*, are crucial for antigen-presenting cell-T-cell interactions (Krensky et al, 1983). Such molecules could well be absent or 'hidden' in the population of 'suppressor' RFD1+D7+ macrophages.

Whatever the mechanism of T-cell down-regulation, it remains to be seen whether excessive regulation by such macrophages may be causal in the aetiology of disease, or whether such regulation is a secondary response to the primary disorder. It does however seem likely that within the healthy human lung, there exists a sophisticated intrinsic macrophage-mediated regulation of local T-cell activation that maintains immunological homeostasis under steady-state conditions. The balance between 'stimulator' and 'suppressor' macrophages is therefore critically controlled in health. In the event of an upset, this could lead either to inappropriate enhancement, or suppression, of immune reactivity resulting in hypersensitivity lung injury, or reduced resistance to infection, respectively.

It is proposed that the above phenomena are only possible if they reflect a continuum of functional changes induced by AM maturation, in response to exogeneous stimuli arising in the local micro-environment.

* described as members of a supergene family of adhesion molecules, collectively termed as "integrins"
BAL: ITS VALUE IN ASSESSING THE CELLULAR EVENTS IN PULMONARY SARCOIDOSIS

The preceding discussion has highlighted the concept of steady-state conditions within the healthy lung, as being critically maintained by an intricate local cell-to-cell interaction, in close association with different stimuli in the immediate milieu. Data presented in this thesis offers cogent evidence that, within the lungs of sarcoid patients studied, a situation has developed wherein aberrations in macrophage-lymphocyte interaction occur as a result of inflammatory changes arising in situ. In keeping with the current concepts of sarcoidosis, it follows that these cellular aberrations are destined to play a central and critical role in the pathogenic outcome of the epithelioid granulomatous inflammation in sarcoid patients. Indeed, current studies have shown that such cellular events can occur insidiously, even before any subjective or objective evidence comes to surface (q.v. Chapter 7). Others have observed that these changes at the cellular level can reflect alterations in disease activity (Ainslie et al, 1989). At the same time the results in Chapter 6 suggest that, while changes in cell phenotype and function in the sarcoid lung can be effectively modulated by therapeutic regimes, the return to normal (as seen in situ) may long precede any objective improvement.

It would therefore follow that the ideal parameter for assessing disease activity in pulmonary sarcoidosis, would be one that could directly capture the presence of disease in situ, while simultaneously reflecting the events involved in the maintenance and outcome of the granulomatous response in the individual sarcoid patient.

In this regard it must be appreciated that the inflammatory cellular hyperactivity in sarcoidosis is localised to the lung; and is not reflected in the peripheral blood (q.v. Chapter 1). Therefore while routine clinical parameters, such as blood tests, may be
useful in the diagnosis of sarcoidosis, they are generally not helpful in reflecting and evaluating the character and intensity of the underlying effector cell mechanisms.

Similarly, although in clinical practice, widespread use is made of the chest radiograph and lung function parameters, comparison of such tests with open lung biopsies have shown that, overall, they bear little relationship to the status of the ongoing in situ alveolitis (q.v. Chapter 1). This would appear to be somewhat surprising, as it would be expected that physiological indices of functional impairment (such as reduced lung volumes, reduced diffusion capacity, and arterial hypoxaemia) would be sensitive enough to assess the integrity of the alveolar structures. However repeated independent studies (Carrington & Gaensler 1978; Keogh & Crystal 1980) have suggested that such lung function parameters may not be specific enough for the extent of the early parenchymal involvement. The same is true for the chest radiograph (Epler et al 1978).

In support, clinical experience shows that physiology tests and chest x-rays are not always reliable in monitoring clinical progress in sarcoid patients, whether they are treated or not. Radiographic shadows that appear non-fibrotic, and that are known to be of short duration, may show diminution (or clearing) either spontaneously or with therapy. On the other hand, longer-standing fibrotic changes may show little objective alteration to treatment, despite the initial subjective improvement claimed by some patients (Scadding & Mitchell, 1985).

Early pulmonary function abnormalities may progress slowly with time, and spontaneous improvement to normal values is unusual (Scadding & Mitchell, 1985). In addition such functional impairment may only be partially reversed by systemic therapy, despite complete improvement of symptoms and clearing of radiographic
shadows. In support, Smellie et al (1961) found that while ventilatory capacity may improve, severely impaired gas-exchange indices were little changed. In contrast, other reports (McClement et al, 1953; Stone et al 1953) have shown a deterioration in lung function following steroid treatment, at the same time that serial biopsies on these patients showed conversion of granulomata to fibrosis. This suggests that although clinical and radiographic criteria may respond dramatically to treatment, severe abnormalities of function may persist, reflecting the insidious transformation of visible granulomatous disease to less apparent early pulmonary fibrosis.

In general, although in practice worsening radiographic shadows and/or pulmonary function are taken as an indication of increasing disease severity and therefore the introduction of treatment, it remains unclear as to what role the maintenance of a clear chest X-ray and/or maximum pulmonary function has to the eventual outcome of the disease. These comments lead to the speculation that the traditional use of clinical, radiological and physiological criteria to stage sarcoid patients is neither specific for, nor sensitive to, the cellular aberrations occurring in the lungs of these patients. Rather, such criteria at best can only indicate the overall state of the sarcoid individual, and at the cellular level, only evaluate the extent of the overall derangement to the alveolar structures (as caused by the intense granuloma formation and fibrosis) (Keogh & Crystal, 1980; Crystal et al, 1981; Keogh et al, 1981). These investigations therefore tend to portray what has occurred before, and not the events occurring at the time of evaluation.

As from previous discussion, it can be assumed that cellular components in the lung are the critical determinants of parenchymal injury in pulmonary sarcoidosis, it would appear that the clinician needs criteria that can be used repetitively to: (a) evaluate the intensity of the inflammatory cellular response as it occurs in situ; and (b) distinguish this from the extent of derangement to the alveolar structures, that
has resulted from previous cellular events. In this context, when compared to the other parameters, BAL would appear to be able to offer a dynamic and kinetic impression of what is present, or developing, on the gas exchange surface where increasing insidious inflammatory changes could potentially lead to the loss of alveolar-capillary units, with irreversible physiological dysfunction.

Accordingly, the studies presented in this thesis bear testimony to the concept that BAL is the most rational and sensitive approach available:

1. To obtain appropriate samples of cells directly from the lower respiratory tract for analysis
2. To detect early pulmonary inflammation before the advent of any structural derangement and physiological dysfunction.
3. To monitor the natural outcome of the initial cellular changes
4. To assess the influence of the immediate micro-environment on disease progression, and
5. To evaluate the modulation of such events with therapeutic regimes.

Yet set against such a logical approach, continues a worldwide controversy regarding the precise role of BAL in the day-to-day management of pulmonary sarcoidosis. Many would argue that despite its good points, in comparison to other objective criteria available, BAL remains an invasive procedure: its potential adverse effects being directly related to the technique used. Data from Chapter 3 shows that arterial hypoxaemia during lavage is inevitable, but that the degree of O₂ desaturation can be minimized by instilling smaller volumes of fluid. Albertini et al (1974) have also suggested the importance of avoiding prolonged procedure times. At the same time, the current studies suggest that manoeuvres such as pre-warming of the instilled fluid, and maintaining a negative suction pressure below 80mmHg, would reduce the incidence of symptoms e.g. chest pain, cough, wheezing. But above all, it would appear
that even if such events were to occur during and/or after the lavage, they are completely reversible, and are not detrimental to the patients (provided that they have an $\text{FEV}_1 > 1\text{L}$, and a $\text{Pa}_2 > 10\text{KPa}$ at rest; Rossi,1986).

In the light of these suggestions, BAL would appear to be a relatively safe procedure. Yet for the use of BAL to be meaningful and fruitful, the investigator must also appreciate certain basic concepts regarding sarcoidosis. Indeed, by the very nature of the disease, cellular events within the lung may be constantly and insidiously changing, even within the same individual. In addition, shifts within the immunocompetent cell population in the lung could (and indeed do) parallel disease progression (Ainslie et al, 1989). There is therefore a place for the judicious repeated use of BAL in a given patient. This may enable a cellular profile to emerge that could suggest a pattern for the evolving pathogenic inflammatory mechanisms within the given individual. This might be helpful not only to stage disease activity and predict eventual outcome, but might also prove invaluable in making therapeutic decisions.

It is also important to recognize that sarcoidosis is subject to a diverse spectrum of clinical expression and outcome, which varies not only within the same individual, but also within the patient population as a whole. Such variability may not only result from individual differences in genetic make-up, but perhaps also from the diversity of local stimuli influencing the specific behaviour of the immunocompetent cells at the inflammatory site so that, although clinical and radiological parameters may not be specifically helpful in monitoring disease progression, their adjunct use prior to any comparative study seems essential. In this way, patients with sarcoidosis can be chosen to form 'homogeneous' groups, at least in terms of clinical disease expression: appropriate comparative analysis can then be made. In support, Ward et al (1989) recently reported that the mode of disease presentation, and in some patients the time
since onset of symptoms, may in part explain why different centres (Hollinger et al 1985; Buchalter et al 1986; Turner-Warwick et al 1986) have reported conflicting results regarding the value of BAL in predicting the outcome in sarcoidosis. With this in mind, particular attention was paid to strict selection criteria in all studies in this thesis, so that homogeneous groups of patients could be compared and assessed.

As used in Chapters 4 - 7, BAL presents a snap-shot view of the in situ cell-to-cell interactions in the normal lung, and their behaviour in response to the local inflammatory changes in the sarcoid lung. The information obtained, not only sheds light on the intriguing immunopathogenic mechanisms operative in sarcoidosis, but could also potentially lead to unravelling the initial inciting stimulus or stimuli in this disease.

**CHANGES WITHIN AM SUBPOPULATIONS: CRUCIAL TO THE FORMATION AND OUTCOME OF THE SARCOID GRANULOMA**

It follows from the previous discussion that all the ingredients necessary to mount an active cellular immune response in the early stages of sarcoidosis, are present in the lung. Analysis of the cells obtained from the lower respiratory tract of sarcoid patients by BAL, suggests that the granulomatous inflammation is heralded by a local mononuclear cell infiltrate, comprising of macrophages and T-lymphocytes. Furthermore, this initial accumulation of cells may occur insidiously, in the absence of any subjective or objective evidence (q.v. Chapter 7).

The studies in Chapter 4 clearly demonstrate that, in a clinically homogeneous group of patients suffering from active pulmonary sarcoidosis, changes within the lavage lymphocyte populations are accompanied by aberrations within the local AM pool. Not only may the total number of AM in sarcoid BAL increase, but consistent alterations in the phenotype and function of these cells are also present. These results support the
concept of a macrophage-lymphocyte interaction at the site of granulomatous inflammation in sarcoidosis. Yet if this is so, then a paradox exists in the data presented! For like normal AM, unfractionated AM from active sarcoid patients were observed to abolish autologous T-lymphocyte reactivity. Such a result would appear to be contradictory in the light of previous reports of sarcoid AM showing increased DR expression (Basham et al, 1983; Campbell et al, 1986a), and enhanced antigen presenting capacity (Toews et al, 1986). But is it?

Current experiments have suggested that within the actively changing AM population, there exists not only (a) those macrophages capable of presenting antigen to, and stimulating, T-lymphocytes (thereby initiating the granulomatous response), but also (b) other phenotypically and functionally distinct 'effector' and 'suppressor' macrophage-like cells, potentially capable of modulating the granuloma and its resolution or progression to fibrosis. Indeed, it was speculated in the previous discussion that the critical balance between these AM subpopulations in the lung is constantly influenced by events in the immediate milieu. In support, current data has demonstrated that sarcoid BAL consistently contained a high proportion of macrophages with the phenotype of dendritic cells (RFD1+D7-). Much of this increase was however due to the raised numbers of AM expressing the double phenotype of both dendritic cells and classic macrophages, the putative 'suppressor' RFD1+D7+ macrophages (q.v. Chapter 4). In addition, with the advent of sarcoid inflammation in the micro-environment, such shifts in the proportion of these AM subpopulations were accompanied by not only an increase in hexose monophosphate shunt activity in all three subsets (as would be expected in the presence of inflammation), but also by sarcoid-related differences in surface antigen expression, physiology and function (qv. Chapter 5). The result is the emergence of AM subpopulations with distinctive features in the BAL of active sarcoid patients. Such developments not only explain why unfractionated AM from active sarcoid BAL may fail
to support T-cell reactivity, but they also highlight the possible influence imparted by local environmental stimuli on the resident cell population. But what purpose do these cellular shifts in the lower respiratory tract of sarcoid patients have in the initiation, maintenance, and outcome of the immune response in sarcoidosis? This intriguing question must be answered before the effects of exogenous stimuli on these local cellular components can be appreciated.

If one could conceive the speculated roles played by the isolated AM subpopulations in the normal host defence (discussion above) as a model of the immune response, then it could be postulated that in a susceptible patient, under an appropriate as yet unidentified stimulus, antigen-presenting macrophages (RFD1+D7- equivalent) take up antigen, process it and present it in a recognizable immunogenic form to CD4+ T-lymphocytes. This would be done in the context of MHC Class II antigens, in conjunction with IL-1 and other activating signals (q.v. Chapter 1; Fig. 1.3). In support of this hypothesis, two independent studies have shown that AM from active sarcoid patients have enhanced capacity to present recall antigen to T-cells in vitro (Venet et al, 1985; Toews et al, 1986). This macrophage-lymphocyte interaction sparks off the development of a localised cellular immune response, that is responsible for the initial alveolitis described by others in early sarcoidosis (Crystal et al, 1984; Thomas & Hunninghake, 1987).

It is as yet unknown whether such 'inducer' macrophages activate sarcoid-specific CD4+ T-cells. This suggestion becomes more exciting as the rise in number of local CD4+ T-cells, and the increase in CD4-helper : CD8-suppressor T-cell ratio appears to be compartmentalised and absent in the peripheral blood (Semenzato et al, 1982). Do specific CD4+ T-cells migrate from the blood to the site of the presumed immunological insult under the control of a chemotactic factor, or does local proliferation of helper T-cells at the site of granuloma formation occur after
migration of a sub-population of activated lymphocytes? One clue as to what might happen, lies in the events observed in the alveolitis of patients with chronic beryllium disease (Saltini et al, 1989). In this disorder, a substance beryllium acts as a Class II-restricted antigen, which stimulates local proliferation and accumulation in the lung of beryllium-specific CD4+ T-cells. It would seem that such observations support the view of lymphocyte migration from the peripheral blood to the lung under the influence of chemotactic factors; once in the lung, proliferation of CD4+ T-cells is maintained by local activation of beryllium-specific T-cell clones. As chronic beryllium disease appears to be similar to sarcoidosis (with the notable exception that the inciting stimulus in the latter is unknown), could it be that the same mechanism of selective CD4+ T-cell activation by 'inducer' macrophages occurs in sarcoidosis?

Whatever the mode of T-cell activation, it is clear that consequent on this reaction, biologically active mediators are released by the T-cells into the local milieu. One such substance is IFN-Y, increased levels of which have been shown in active sarcoid lavage (Robinson et al, 1985). IFN-Y is a potent activator of macrophages: indeed it has been observed in vitro to increase HLA-DR expression on sarcoid AM (Basham et al, 1983). It is interesting to note that the intensity of RFDR1 staining on RFD1+D7-macrophages was markedly increased in sarcoid lavage (q.v. Table 5.5).

In addition it appears that upon T-cell activation, a monocyte-macrophage activation to the site of inflammation occurs, by the creation of a gradient of monocyte chemotactic activity between the blood and the lung. This is achieved through the secretion of MCF and MIF (Hunninghake et al 1980a; Wahl et al 1975). In this way not only do the local resident AM expand, but circulating monocytes are also attracted to the site of the alveolitis, where they presumably undergo differentiation and maturation into appropriate AM subtypes. This would account for the observed local total increase of the AM pool, and the emergence of specific macrophage cell types. In this way it could
be conceived that an increased proportion of the recruited monocytes in sarcoidosis would retain their antigen presenting activity as they mature into AM, in an effort to sustain local T-lymphocyte activation and combat the inciting agent. It may however be argued from the data shown in Chapter 4 that, in contrast to normal blood monocytes, sarcoid peripheral blood mononuclear cell reactivity is comparatively reduced. In keeping with this, current experiments demonstrate that the enhancing effect of isolated 'inducer' RFD1+D7- macrophages from sarcoid patients, is diminished when compared to equivalent macrophages from the normal.

At the same time under the local inflammatory conditions, one could speculate that within the resident *classic* macrophage population, a proportion of cells could be induced to develop new phenotypic and physiological characteristics, enabling them to take up novel functions. In support, current studies show an increase in the proportion of macrophages with the double phenotype of RFD1+D7+ in sarcoid BAL. Such cells share a number of features with *classic* macrophages (equivalent RFD1-D7+ cells): adherence to glass, acid-phosphatase positive, and phagocytosis-associated C3b and Fc receptors. Furthermore *in vitro* experiments have observed that IFN-Y can increase the proportion of RFD1+ cells developing, while suppressing D7-antigen expression (Poulter et al, 1987). It is therefore possible that within the sarcoid micro-environment, one of the roles of the secreted IFN-Y is to induce the emergence of D1-antigen on macrophages that already express the D7-phenotype. By suppressing the further development of the D7-phenotype, INF-Y can induce a switch in the resident AM population towards an increasing proportion of 'suppressor' RFD1+D7+ macrophages.

If such events were reflected *in vivo*, they would explain the shifts observed towards particular AM subpopulations in the BAL of active sarcoid patients. Yet another interesting observation in the current studies was that a proportion of RFD1+D7+
macrophages in sarcoid patients showed the increased expression of a separate antigen D9 (which identifies epithelioid cells). As epithelioid cells are important structural units of the sarcoid granuloma, this observation provokes the intriguing question of what function these cells have. Apart from non-specific ACE production (Silvestein et al, 1979), the physiological role of the epithelioid cell is still unknown (Epstein & Fukuyama 1989). Could it be that yet again under local stimuli, effete RFD1+D7+ macrophages change their phenotype (and perhaps their function), and undergo morphological differentiation into epithelioid cells? In support, IFN-Y has been observed in vitro to promote epithelioid cell, and giant cell formation when added to cultured macrophages (Weinberg et al 1984)

But what is the in situ effect of the emerging 'suppressor' RFD1+D7+ macrophages on the outcome of the epithelioid cell granuloma?

It is recognized that sarcoid granulomata are usually benign, and that they usually resolve spontaneously. This would imply that the immune response set up has effectively removed the inciting agent, or has markedly decreased the antigen load (without leaving any secondary sequelae). For this to happen in the face of an increasing spiral of cell activation, it would not be wrong to envisage that controlling influences must be in operation.

Current studies have shown that the increase in 'suppressor' RFD1+D7+ macrophages in BAL, is directly proportional to the lymphocytosis present (q.v. Chapter 5). In addition, the 'suppressor' function of these cells seems to be enhanced in sarcoid patients, when compared to normal equivalents. It is thus tempting to postulate that the marked rise in 'suppressor' macrophages in active sarcoidosis is aimed at controlling T-lymphocyte responsiveness from potentially escalating, and resulting in disruption of the local integral alveolar-capillary units. In support, Ainslie et al (1989) noticed reduced levels of CD7 (a marker of blast formation in T-cells), and HLA-DR
expression on CD4+ T-lymphocytes, at the same time as proportions of 'suppressor' RFD1+D7+ macrophages were increasing in sarcoid BAL. It would follow that such macrophages could arise as part of a secondary response to stimuli in the immediate milieu, in order to contain the events arising from the initial macrophage-T-cell interaction. Could this be why the enhancing effect of 'inducer' RFD1+D7- macrophages in sarcoid patients is diminished when compared to equivalent macrophages in the normal?

The presence of in situ mechanisms that control the initial sarcoid response has been postulated by other workers. Data from stable sarcoid patients has shown an increase in CD8+ suppressor T-lymphocytes in BAL (Hunninghake & Crystal 1981a). A similar switch in immunoregulatory T-cells is also seen in animal models of granulomatous lung disorders (Chensue et al 1980; Chensue et al, 1981). While CD4+ T-cells are seen to be prominent during the process of granuloma formation, later during a time of granuloma size regression, suppressor T-cells predominate. It is postulated that such a switch in T-cell phenotype may be secondary to the development of a particular genotype of DQ on the surface of macrophages (Matsushita et al,1987; Sasazuki & Matsushita,1987). In the light of this, could one conceive an inter-relationship between CD8+ T-lymphocytes and RFD1+D7+ 'suppressor' macrophages, aimed at modulating the active cellular events? Indeed, macrophages from active sarcoid patients have been observed to release increased amount of PGE2, TNF and IFN-Y (Wolter et al, 1983; Foley et al, 1988; Robinson et al, 1985). There is formal evidence showing that all these cytokines possess inhibitory activity, with direct down-regulation of T-cell responsiveness (Pryjma et al, 1989). PGE2 can also induce the production of CD8+ suppressor T-lymphocytes (Elmasry et al, 1987), as well as inhibit the expression of MHC Class II molecule (Steeg et al, 1982). In addition, the potential in vitro effect of IFN-Y, in inducing a switch in local AM phenotype towards an increased proportion of 'suppressor' macrophages, has already been discussed.
From the preceding discussion, a situation is conceived whereby, upon the advent of sarcoid inflammation, events are set in motion that could ensure a continuum of functional differentiation at the cellular level, aimed at restoring a new equilibrium between 'stimulator' and 'suppressor' cells, in the presence of biologically active mediators. This would hopefully contain the inciting stimulus, or stimuli, and resolve the granulomatous reaction. Support for a similar situation in vivo can be obtained from observations of the development of the Kveim-induced granuloma.

Kveim-induced granulomata in positive responses resemble their spontaneous counterparts in almost all aspects of light and electron microscopic structures (James & Thompson, 1955; Mitchell et al, 1977), as well as in their cellular composition and phenotype (Mishra et al, 1983). It has been shown (Munro et al, 1986a; Munro, 1986b) that in the first few days following injection, the first sign of a cellular reaction is an accumulation of macrophages with high lyzosomal hydrolase activity surrounding the dermal deposition of the injected suspension. If the response proceeds to well-formed epithelioid granulomas, at about the 10th day, an influx of macrophages (showing signs of activation) occurs at the site. This is immediately followed by the appearance of lymphocytes, predominantly of the CD4+ phenotype. At this stage electron microscopic studies have clearly shown an intimate association between the lymphocytes and macrophages. These findings correlate well with what has been described in the sarcoid alveolitis as studied by BAL, and with the 'putative' early lesions obtained by transbronchial biopsies in Stage 1 sarcoid patients (Semenzato et al, 1986). Progressive events in the Kveim response show morphological and phenotypic changes taking place within the macrophage population, as they differentiate into epithelioid cells to giant cell formation. Finally at 4 weeks, within the central core of the mature sarcoid granuloma are found tightly packed RFD1+D7- macrophages and epithelioid cells interspersed with CD4+ T-lymphocytes. This is surrounded by a loosely arranged mantle of CD8+ and CD4+ T-cells, and
macrophages. Interestingly, there is a lack of DNA synthesis in the centre of the granuloma; however, proliferating macrophages and lymphocytes are found in the mantle zone. Evidence for this is obtained by the localisation of the increased expression to Ki67 (a monoclonal antibody that binds to nuclear antigens, expressed by cells in G1, G2, M and S proliferative cycle) to this zone (Chilosi et al, 1988). It is easy to assume therefore that within the sarcoid granuloma, the mantle zone represents the site of the cell proliferation, and probably the source of lymphokines and interferons. In addition it seems that the CD8+ suppressor lymphocytes are restricted to the outer zone, suggesting that T-helper cells are selectively attracted to the centre of the granuloma by antigen-presenting macrophages. Such findings are highly suggestive that the direction of spread of cells occurs from the outside to the inside of the granuloma. More important, they place the controlling 'suppressor' mechanisms at the periphery of the granuloma. This stimulates the in vivo picture of 'suppressor' cells being attracted to the periphery of the granuloma (as a secondary response), in order to contain the initial inflammatory cellular activity.

In the light of the previous discussion, it would be intriguing to postulate that such 'suppressor' mechanisms would also include the in situ differentiation of macrophages within the mantle zone, into 'suppressor' macrophages under the influence of IFN-Y secreted in this area by the proliferating cells. In support of such a progressive accumulation of 'suppressor' cells, is the observed progressive decline in the frequency of positive Kveim response in the same sarcoid patient, in whom the disease is still apparently active (as defined by a high lavage lymphocyte count) (Munro & Mitchell, 1987). This suggests that any specific sensitivity to the Kveim material previously present has been actively down-regulated by 'suppressor' mechanisms evolving in situ.

The effectiveness of such 'suppressor' mechanisms in containing the local cellular
hyperactivity in the sarcoid lung, and preventing a generalized immune reaction to the inciting stimulus, is further supported by the observed depressed peripheral blood mononuclear cell responsiveness (when compared to normal; q.v. Chapter 4). This peripheral anergy has been attributed to a number of factors: defective IL-2 production (Lyons et al, 1988); increased presence of peripheral blood natural killer cells (Campbell & Tolson, 1986); decreased production of IL-1 by monocytes (Hudspith et al, 1984), increased release of prostaglandins by monocytes (Goodwin et al, 1979), and a shift in the balance of T-helper: suppressor cell ratio in the blood towards increased CD8+ T-cells (consequent on a sequestration of CD4+ T-cells to sites of disease activity). It is however, the observations of Goodwin et al (1979) and Muragushi et al (1982) that are of interest in the light of previous discussion. They describe the presence of adherent cells of the monocyte-macrophage lineage in sarcoid peripheral blood. They also independently show that such cells were capable of depressing the function of T-cells. In this respect such cells would therefore be analogous to the 'suppressor' RFD1+D7+ macrophages identified in the BAL in the current studies. The possibility arises that simultaneous with the emergence of 'suppressor' macrophages in the lung, these cells are also present in sarcoid peripheral blood. This would explain the observed decrease in sarcoid T-lymphocyte reactivity, and the return towards normal following steroid treatment (when the proportion of RFD1+D7+ cells was greatly reduced; q.v. Chapter 6). In support, Citron & Scadding (1957) observed that local hydrocortisone injection reversed the cutaneous anergy to tuberculoprotein and 'recall' antigens. The mechanism by which 'suppressor' macrophages would be recruited to the peripheral blood is unclear. It may well be that a situation is reflected in the peripheral blood similar to that in the lung, in which a switch in monocyte phenotype and function (under an appropriate influence) results in a preferential increase of a particular monocyte subpopulation that is associated with suppression of T-cell responsiveness.

With such a sophisticated, inbuilt, macrophage-mediated regulation of local T-cell
responsiveness, it would seem that the cellular events in the sarcoid lung are following the typical pattern of a normal immune defence to an extrinsic agent. This would therefore be expected to lead to a localised self-limiting immune reaction. Indeed this is the case in the majority of patients with pulmonary sarcoidosis. What therefore goes wrong with the controlling mechanisms in the small proportion of sarcoid patients, in whom a more progressive disease develops, and results in cellular and matrix disorganization of the alveolar structures with consequent embarrassment to pulmonary function?

If one assumes that the development of fibrosis occurs as a result of an extension of the granulomatous process, then it follows that the 'suppressor' mechanisms outlined above have failed to contain the immune response. Instead, cellular hyper-responsiveness with exaggerated release of mediators occurs, and results in local tissue damage. On the other hand it could also be that 'over-enthusiastic' down-regulation of the immune response by CD8+ T-lymphocytes and 'suppressor' macrophages, have resulted in a deficient immune attack on the inciting stimulus. In support, Gallagher et al (1988) observed that whilst there was no correlation with clinical parameters, the accessory function of AM correlated significantly with the T-helper : T-suppressor cell ratio in BAL fluid. The immunological competence of AM was seen to be progressively reduced in the presence of an increasing CD8+ T-cell population.

These observations would tend to suggest that, in situations where there is increased presence of 'suppressor' macrophages and CD8+ T-cells, the immune response would be dampened and inadequate. This is however not entirely true. Results obtained from studies of BAL from patients with extrinsic allergic alveolitis, show consistent and significantly greater increases in RFD1+D7+ 'suppressor' macrophages in comparison to active sarcoid patients (Johnson et al 1989); such an increase was
concomitant with an accumulation of CD8+ T-lymphocytes. Yet in these patients, it is recognised that the granulomata present in the lung parenchyma are much less prominent than those seen in sarcoid patients (Rosen et al, 1978).

It therefore seems likely that in those sarcoid patients progressing to fibrosis, the immune response has become over-exaggerated and thus overrides the local intrinsic 'suppressor' mechanism. Indeed, it could be said that in such patients a situation has arisen whereby factors (internal and/or external) to the local milieu, have upset the \textit{in situ} delicately balanced equilibrium, resulting in progression of the granulomatous response towards fibrosis. These over-riding factors would range from the nature of the inciting stimulus, to the genetic background and immune status of the susceptible sarcoid individual. In support, it is known that both the presence and quantity of antigen deposited in the lung, determines disease activity in many granulomatous lung disorders such as tuberculosis, pulmonary fungal infections and hypersensitivity pneumonitis (Dannenberg 1974). There is no reason to think that this is different in sarcoidosis (albeit the stimulus is as yet unidentified). It follows therefore that if the stimulus persists, the cell-to-cell interaction would escalate, and as a result an overproduction of biologically active substances could be released into the immediate micro-environment. Under such conditions, further changes at the cellular level could occur that would give rise to the emergence of specific immunocompetent cells, capable of promoting the development of fibrosis. Data in Chapter 5 show that not only were the majority of RFD1+D7+ 'suppressor' macrophages positive for fibronectin, but that in sarcoidosis the intensity of this staining on the individual RFD1+D7+ macrophages was markedly increased. Could therefore a sustained heightened cellular immune reaction result consequent on persistent antigen stimulation, and in a last attempt to combat this, the RFD1+D7+ 'suppressor' macrophages release fibronectin (and possibly other similar substances) into the immediate milieu? After all the controlled release of such substances would appear to be required for the normal
repair process! In fact, in advanced stages of the disease, patients have been shown to have increased levels of fibronectin and AM-derived growth factor, in addition to enhanced expression of membrane fibronectin receptors on macrophages (Rennard et al, 1981; Bitterman et al, 1983b; O'Connor et al, 1988). As it has been observed that the proportion of RFD1+D7+ 'suppressor' macrophages increases with progressive radiographic changes (Ainslie et al, 1989), it remains to be seen whether an exaggerated presence of this subset is present at site of injury in Stage IV sarcoid patients. In fact it is only in the advanced radiographic (and physiologically debilitating) stages of the disease, that one sees a shift (from the usual) in effector cell constituents: BAL lymphocytosis is replaced by increased numbers of neutrophils (Roth et al, 1981) and mast cells (Flint et al, 1986; Bjermer et al, 1987). In these patients, the BAL also shows an increased amount of tissue factor, Factor VII (Chapman et al, 1985) and collagenase (O'Connor et al, 1988) as well as exaggerated production of high reactive oxygen metabolites (Aerts et al, 1986).

Another interesting possibility is that the immune mechanisms operative within the alveoli in the later stages of the illness, may be different from those in Stage I disease. This would imply that in the group of patients in whom the disease is persistent and progressive, the nature of the stimulus could be different. In support of this view, it has been shown on animal models that repeated inhalation of muramyl dipeptide can produce a chronic progressive granulomatous pneumonitis leading to irreversible fibrosis. This substance was found capable of suppressing the lung's immune responses to inhaled antigen (Richerson et al, 1982).

The nature of the immune response, and hence the evolution of the granulomatous process towards fibrosis may also depend on the genetic status of the individual. In Chapter 1, it was seen that particular genotypes (eg HLA-B13) are susceptible to more chronic forms of sarcoidosis (Neville et al, 1980). Other studies have shown that some patients have an inherent susceptibility to accumulate fibrin in the alveolar
spaces (Chapman et al, 1986). Could therefore HLA-B13 patients possess intrinsic abnormalities in their alveolar fibrin turnover pathways, and present an aberrant immune response? The patient's competence of his or her immune system could also dictate the quality of the cellular response. However, this thesis has shown that contrary to previous beliefs (q.v. Chapter 1), patients with sarcoidosis are capable of mounting an effective cellular immune response (albeit localised to the sites of inflammation). Indeed, even following long term targetted steroid therapy to the lungs, patients with active sarcoidosis did not appear to develop the clinical and opportunistic sequelae of significant immune suppression, as do immunodeficient patients (q.v. Chapter 6).

**MODULATION OF AM ABERRATIONS IN SARCOIDOSIS WITH THERAPY**

The main problem with treating sarcoid patients adequately in practice is the lack of a specific causal factor that can be eliminated by a therapeutic agent. On the other hand, the discussion so far has emphasized that with the help of BAL, it is now recognized (a) that the localised reaction in pulmonary sarcoidosis is made up of an intricate aberrant cell-to-cell interaction, involving both lymphoid and non-lymphoid subpopulations; (b) that changes within the AM subpopulations are occurring continuously; and (c) that these are under the sustained direct influence of cytokines secreted into the immediate micro-environment.

In the absence of a definite inciting stimulus, therapeutic regimes should be targetted to modulate the above immunological phenomena. Data from Chapter 6 show that such cellular events can be reversed by inhaled, as well as systemic, corticosteroids. Not only was there a decrease in the number of lymphocytes in lavage, but following treatment, simultaneous changes occurred within the local AM pool. Indeed the sarcoid-related differences in proportion, as well as phenotype and functional capacity
observed in AM, were reversed towards normal. Both inhaled and systemic corticosteroids managed to suppress the emergence of particular AM subsets (thought to be critically involved in the pathogenesis of sarcoidosis). Such immune modulation was accompanied by clinical improvement (q.v. Chapter 6). In support of these results, Hance et al (1985) have also shown that glucocorticosteroid therapy in sarcoid patients can alter AM phenotype towards normal with clinical efficacy.

Whether such changes in sarcoid AM profile occur via a direct action of the steroid on the macrophage (which does possess functional glucocorticoid steroid receptors; Werb et al, 1978), or indirectly via effects on the lymphocyte, is unknown. Interestingly the effect of both therapeutic regimes in the current studies has been to decrease substantially the proportion of RFD1+D7+ 'suppressor' macrophages in the lavage, at the same time that a reduction in lymphocytosis occurs. It is therefore possible that the observed changes at the cellular level reflect a situation of steroid-induced suppression of IFN-Y secretion by T-cells into the immediate milieu (Robinson et al, 1985), as well as a possible reduced expression of IFN-Y mRNA by macrophages (Arya et al 1984). As the expression of the D1-epitope has been observed to be promoted by IFN-Y (Poulter et al, 1987), a lack of this mediator might explain the reduction in proportion of RFD1+D7+cells following steroid therapy, as well as the switch in phenotype towards increased D7 seen in the systemic corticosteroid group. Indeed, hydrocortisone has been shown to suppress the production of INF-Y and other cytokines by human peripheral blood mononuclear cells (Cesario et al, 1986; Guyre et al, 1988). In addition, steroids have also been reported to be capable of suppressing the IL-2 gene, at the level of transcription of IL-2 mRNA, and thereby reducing T-lymphocyte proliferation in vivo. In this respect it is interesting to note that both inhaled and systemic corticosteroids altered the aberrant functional capacity of peripheral blood mononuclear cells (PBM), usually present in sarcoidosis. A significant increase in PBM responsiveness was observed concomitant with a
suppression of lavage RFD1+D7+ macrophages. Could this be due to the same mechanisms as postulated in the lung?

It would appear that, as suppression of the macrophage-lymphocyte reactions are brought under control by targeted or systemic therapy, the need for in situ suppressor mechanisms is removed. Such modulation of the macrophage population by corticosteroid regimes may indeed be more effective in controlling the disease process than an effect solely on the lymphocyte population. In fact, if a progression from alveolitis to granuloma formation to fibrosis (with consequent structural derangement leading to physiological dysfunction) is at the critical control of the AM population, then it is this cell population that should be made vulnerable to therapeutic attack. This hypothesis would then explain the mystery behind the ineffectiveness of cyclosporin in sarcoid patients. This immunosuppressive agent has been shown to be unable to modulate the functions of cells of the monocyte-macrophage lineage (Semenzato, 1988).

For treatment to be effective, one must be able to modulate the course of events while they are still in a pliable state, and before fibrosis sets in. In order to do this effectively, one has two choices:-

(i) in the absence of appropriate predictive criteria, to assume all sarcoid patients may progress to fibrosis and therefore to treat all patients at presentation (irrespective of their radiographic staging and physiological function). This will obviously involve the treatment of patients who may be asymptomatic at that time, and who indeed may get better spontaneously, regardless of the treatment. However such an action may prove difficult in practice, in view of the invariable side-effects of conventional systemic corticosteroids, and also because the appropriate duration of the therapy is unknown; or

(ii) to monitor the patient closely using predictive criteria that directly monitor the events at the site of disease. This thesis has presented compelling evidence that
analysis of both macrophage and lymphocyte subpopulations (and particularly the proportional relationship to one another) in the lavage of sarcoid patients, may be an invaluable tool in predicting the course of disease. This may also prove helpful in monitoring the optimal dose of steroid therapy required to suppress adequately the inflammatory events, thereby limiting adverse effects.

The results obtained with inhaled corticosteroid treatment in this thesis are encouraging. Not only do such targeted regimes allow the appropriate drug to be delivered against the in situ offending cellular components, but they can effectively alter their aberrations (which are partly responsible for the granulomatous inflammation) without any systemic upset. In fact the dose of inhaled budesonide used in this study (1600ugm per day) has been shown to be equivalent to 5.0mg per day of oral prednisolone, in terms of ability to suppress plasma cortisol level (Toogood et al,1983). In support, other studies have looked at the combined use of inhaled budesonide and systemic steroids (intravenous methylprednisolone or oral prednisolone) in treating pulmonary sarcoidosis (stages II and III) (Selroos,1987; Selroos,1988). Such combination regimes were shown to result in clinical improvement within the first 3 months of therapy; in addition they allowed lower doses of the systemic steroid to be used, thereby reducing the frequency of adverse effects.

Despite the above guidelines, clinical experience demonstrates that, whereas a proportion of patients with pulmonary sarcoidosis would demonstrate a beneficial response to steroids, others do not. Even in the group of patients who may show an initial response to corticosteroids, the disease in frequently progressive (Scadding & Mitchell, 1985). Studies have shown that the lack of response to steroid therapy is not due to a dysfunction of the glucocorticoid steroid receptor on the cells, but rather due to the fact that the steroids are unable to suppress the inflammatory process that
modulates development of fibrosis (Lacronique et al, 1984), in particular the increased release of fibronectin and AMDGF. Such studies have shown that, once AM have released both these mediators into the immediate milieu, patients are at high risk of deteriorating physiologically (Bitterman et al, 1983b). At this stage, even large doses of steroids will fail to halt the progressive downward decline of these patients. It may well be that when this occurs, therapeutic regimes should consist of drugs such as colchicine, which have been said to inhibit these growth-factor releasing substances, and thus arrest further development of the fibrosis (Rennard et al, 1984).

Even accepting that therapeutic decisions may be made more rational by using criteria that offer information of in situ cellular events, the question still remains of when to introduce therapy. Does one treat the presence of lymphocytic alveolitis, or wait to see if this resolves spontaneously? In this instance, help may be forthcoming from studies on subclinical alveolitis.

SUBCLINICAL ALVEOLITIS: ITS RELEVANCE IN CLINICAL PRACTICE

In this thesis, subclinical alveolitis signifies the presence of an active mononuclear cell infiltrate in the lungs of patients, who are otherwise free of clinical pulmonary symptoms and have normal chest X-rays, with or without normal lung function.

Current studies have demonstrated that in some patients with PBC, who were asymptomatic of any respiratory symptoms, and who had normal chest radiographs and physiological indices, there was clear evidence of an accumulation of T-cells (predominantly of the CD4+ phenotype). These cells not only expressed markers of activation, but they were also associated with changes within the AM population. From the preceding discussion, it follows that these aberrations reflect an in situ active macrophage-lymphocyte interaction (q.v. Chapter 7). Furthermore, the data presented suggests that despite distinct disease expressions, the nature of the
inflammatory response in the lung may be identical. Indeed other studies support the view that the macrophage-lymphocyte interaction described above is not pathognomonic to sarcoidosis, but can be present in the lungs of patients with other chronic granulomatous disorders (e.g., PBC, Crohn's, extrinsic allergic alveolitis) (Solal-Celigny et al., 1982; Voisin et al., 1988). A similar inflammatory infiltrate has also been shown in a high proportion of patients with extrathoracic sarcoidosis but no overt evidence of lung disease (Wallaert et al., 1982).

The results in Chapter 7 also raise the possibility that the presence of such a cellular reaction need not necessarily lead to a clinically significant pulmonary dysfunction. How true is this in clinical practice?

If one assumes that alveolitis precedes granuloma formation, which could potentially disrupt alveolar-capillary units, then the presence of a subclinical alveolitis in these patients may reflect the first step of an inflammatory pulmonary condition that could deteriorate clinically. If this were the case, then it might be reasonable to advocate that BAL should be carried out in all patients who have evidence of granulomatous disease. By the same token, the presence of an in situ alveolitis would necessitate the start of treatment, in order to prevent progression of the alveolitis, and thus alveolar-capillary unit derangement.

In practice, obvious pulmonary involvement has only been rarely described in granulomatous inflammation, other than sarcoidosis: e.g., PBC and Crohn's disease. In a recent follow-up study of 82 untreated patients with extrathoracic granulomatosis (Voisin et al., 1988), it was found that the presence of subclinical pulmonary cell infiltrates did not lead to any significant deterioration in lung function over the 1 year study period. Similarly, Cormier et al. (1986) demonstrated that none of their asymptomatic dairy farmers and pigeon breeders, who had been found to have a significant lymphocytic alveolitis in BAL, developed clinical or radiological features of a hypersensitivity pneumonitis over a 4-year period. Even in the presence of a
subclinical alveolitis in patients with predominant extrathoracic sarcoidosis, the lungs in these patients are rarely a cause for concern: indeed, treatment is often initiated and directed to control other organ involvement (eg CNS, cardiac, liver).

The above observations lead to speculation that the presence of a subclinical alveolitis in the lungs may just reflect a local expression of a systemic granulomatous disorder. Its presence in the lower respiratory tract, as detected by BAL, seems to be benign, and does not appear to lead to a clinically significant interstitial pulmonary process per se.

In this context therefore, treatment would not be appropriate, unless otherwise directed. While this guideline would presumably hold true for the majority of patients with evidence of subclinical alveolitis, it has also been observed that the incidence and magnitude of physiological dysfunction may differ not only with type of disease, but also in individual patients. In the study by Voisin et al (1988), those patients with Crohn's disease who had evidence of a subclinical alveolitis were noted to have more severe impairment of diffusion capacity and pulmonary perfusion scanning at presentation, than did patients with extrathoracic sarcoidosis or PBC. This suggests that the 'role' of the alveolitis may be quite different in clinically overlapping granulomatous disorders: could this be consequent on the antigenicity of the inciting stimulus? It would be logical therefore, that while accepting the previous guideline, close pulmonary follow-up is undertaken in those circumstances where the presence of an interstitial cell infiltrate is causing significant physiological dysfunction.
CONCLUSIONS AND FUTURE PROSPECTS
The work in this thesis has illustrated the pleuripotential nature of the AM response to its environment. As such the AM population is seen to play a critical role in the pathogenesis of pulmonary sarcoidosis.

This disease can now be visualised as an activated cellular immune response, localized to the lung possibly the site of antigen challenge. The identity of such a stimulus remains unknown. The immune reaction involves an intricate web of antigen-presenting macrophages, CD4+ helper and CD8+ suppressor T-cells, and 'suppressor' macrophages. The delicate balance between these cell types is critical to the resolution of the sarcoid granulomatous lesion, or its progression to irreversible tissue damage. Such a balance is influenced by stimuli in the immediate milieu, which may not only dictate the cellular products to be secreted, but may also be conducive to the emergence of particular cell types, capable of propagating the reaction. These cellular aberrations in the lungs of sarcoid patients can be modulated by systemic, as well as inhaled, corticosteroid therapy.

It follows that further evaluation of these cell phenotypes is essential, in order to provide reasonable criteria for staging disease activity, as well as guiding therapy in patients with active pulmonary sarcoidosis.

The expression of unique cell phenotype indicates subtle changes in the induction and expression of certain gene products consequent on an external stimulus. With the availability of molecular biological techniques, coupled with recombinant DNA technology, the intrinsic profile of the AM subpopulations can now be better delineated. These methods would be able to determine the genes that encode for surface proteins, and to define which genes are turned on (under particular circumstances) that promote the emergence of particular macrophage types. Such information would help
the understanding of the macrophage's adaptability to its environment, and its interaction with other cells - and hence the sequence of events in the immune response occurring in sarcoidosis. Could this lead to the nature of the inciting stimulus?

Analysis of the appropriate macrophage cell profiles (obtained by BAL) in sarcoid patients provides an \textit{in vivo} model for studying the effects of various drug regimens at the cellular level. It is as yet unclear how corticosteroids modulate the local cellular aberrations in sarcoidosis. Use of molecular biological techniques may provide the answer to the chemical nature of the macrophage glucocorticoid steroid receptor, and the influence thereon from one cell type to other with the onset of inflammation. In this context, then, the efficacy of the targeted therapy at the cellular level could be directed at particular cell subpopulations (perhaps with the help of liposomes), in order to control the evolution of an exaggerated immune response and prevent clinical deterioration.

Further \textit{in vitro} evaluation needs to be undertaken in order to delineate the appropriate dose and frequency of administration of inhaled therapy, and to increase its clinical efficacy in sarcoid patients. The need for such information is obvious: patients would be able to be treated early, so that the exaggerated cellular interactions are aborted before progression ensues, without fear of local or systemic adverse affects.

"THIS IS NOT THE END.
IT IS NOT EVEN THE BEGINNING OF THE END.
BUT IT IS, PERHAPS, THE END OF THE BEGINNING."

\textit{W S Churchill, 1942}
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