HETEROGENEITY OF MONONUCLEAR PHAGOCYTES IN INTERSTITIAL LUNG DISEASES

Omslag illustratie: Immuno-enzymkleuring voor het CD4 antigeen (blauw d.m.v. alkalische fosfatase) en het CD8 antigeen (bruingeel d.m.v. peroxidase) op T lymfocyten in combinatie met een enzymkleuring voor zure fosfatase op macrofagen (rood).

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HETEROGENEITY OF MONONUCLEAR PHAGOCYTES IN INTERSTITIAL LUNG DISEASES

HETEROGENITEIT VAN MONONUCLEAIRE FAGOCYTEN

BIJ INTERSTITIËLE LONGZIEKTEN

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
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Hendrik Christiaan Hoogsteden

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Aan Gerrie
Aan mijn ouders

HETEROGENEITY OF MONONUCLEAR PHAGOCYTES IN INTERSTITIAL LUNG DISEASES

CONTENTS

Some books are to be tasted,
others to be swallowed, and
some few to be chewed and digested.

Francis Bacon

VOORWOORD (PREFACE)

Interstitiële longziekten vormen een heterogene groep aandoeningen gekenmerkt door een diffuse ontsteking van het longparenchym (bindweefsel van de long), de alveoli (longblaasjes) en in vele gevallen ook de bronchi en bronchioli (luchtwegen).

Hoewel er veel verschillende interstitiële longziekten worden onderscheiden, is er een grote overeenkomst in klinische presentatie. Dikwijls klaagt de patiënt over kortademigheid en hoesten. Het longfunctie-onderzoek toont meestal een daling van de totale longcapaciteit, de vitale longcapaciteit, de diffusiecapaciteit voor koolmonoxide en de compliantie of rekbaarheid. In rust tonen de bloedgassen veelal een te laag zuurstofgehalte en een toegenomen alveolo-arterieel verschil in zuurstofspanning. Bij inspanning daalt de arteriële zuurstofspanning. In vele gevallen komen later in het beloop van veel interstitiële longaandoeningen longfibrose, pulmonale hypertensie en rechts-decompensatie voor.

De röntgenfoto toont bij deze groep van aandoeningen niet-specifieke diffuse veranderingen. Veelal zijn er fijnvlekkige longafwijkingen te zien in een reticulair of nodulair patroon. Soms bestaat er een diffuse ontsteking van het longparenchym zonder dat dit op de röntgenfoto zichtbaar is. Dit kan het geval zijn bij patiënten met sarcoïdose, extrinsieke allergische alveolitis, sommige vasculitiden en cryptogene fibroserende alveolitis. Een alveolitis (ontsteking van de longblaasjes) gaat veelal gepaard met een ophoping van vocht en ontstekingscellen zodat op de röntgenfoto op plaatsen waar normaal lucht aanwezig is dan met vocht gevulde longblaasjes kunnen worden waargenomen. Daarnaast zijn er longaandoeningen die een ontsteking van het interstitium van de long geven zonder ontsteking van de longblaasjes. In dat geval worden fijne netvormige verdichtingen waargenomen op de röntgenfoto. Een groot aantal interstitiële longziekten uit zich echter zowel in een ontsteking van de alveoli als van het interstitium. Wanneer het ziekteproces aanleiding geeft tot een longfibrose zijn er op de röntgenfoto veelal cysteuze holten te zien die de long het aspect geven van een honingraat.

Wanneer er bij een patiënt aanwijzingen bestaan voor een interstitiële longziekte is het belangrijk om de oorzaak ervan te achterhalen. Het is noodzakelijk om uit te zoeken of de patiënt tijdens het werk bepaalde stoffen heeft geïnhaleerd, of hij bepaalde medicamenten inneemt, of er een bepaalde systeemziekte zoals reumatoïde artritis of lupus erythematosus bestaat, en of hij tijdens zijn hobby met bepaalde antigenen in contact komt (duivenmelkers!). Veel van de bovengenoemde zaken kunnen namelijk aanleiding geven tot een interstitiële longziekte. In een deel van de gevallen kan de röntgenfoto behulpzaam zijn omdat sommige interstitiële longziekten vrij kenmerkende afwijkingen geven.

Vaak is het niet mogelijk om met behulp van anamnese, lichamelijk onderzoek en longfunctie-, laboratorium- en röntgenonderzoek tot een diagnose te komen. In dat geval is meer invasieve diagnostiek noodzakelijk waarbij veelal getracht wordt om een stukje longweefsel voor microscopisch onderzoek te verkrijgen.

Bij een zogenaamde open longbiopsie wordt door de thoraxchirurg een ingreep verricht

waarbij op verschillende plaatsen weefsel uit de long wordt genomen. Er wordt zowel normaal als abnormaal uitziend longweefsel weggenomen zodat alle overgangen tussen normaal en ziek longweefsel onder het microscoop kunnen worden bestudeerd. Het voordeel van een open longbiopsie is dat een betrekkelijk grote hoeveelheid longweefsel kan worden verkregen zodat uitgebreid onderzoek kan worden verricht. Het nadeel is dat de patiënt een, weliswaar kleine, operatie onder narcose moet ondergaan.

Naast de open longbiopsie bestaat de mogelijkheid om transbronchiale biopten te nemen. Daarbij wordt een tangetje via de bronchoscoop in de long gebracht waarbij kleine biopten uit de long kunnen worden genomen. Een nadeel van de methode is dat maar betrekkelijk kleine stukjes longweefsel kunnen worden verkregen. Indien er sprake is van een diffuse interstitiële longziekte kan doorgaans met behulp van transbronchiale biopten de diagnose worden gesteld. Anders is het met een meer focale verdeling van de afwijkingen. Het is dan mogelijk dat de transbronchiale biopten net uit een stukje normaal longweefsel zijn genomen waarin de ziekte microscopisch niet is waar te nemen.

Een andere techniek is die van de bronchoalveolaire lavage. Deze techniek maakt het mogelijk om cellen en vloeistof uit de alveolaire ruimten te verzamelen. Hierbij wordt de bronchoscoop in wigpositie geplaatst in één van de kleinere bronchi. Via de bronchoscoop wordt fysiologisch zout ingespoten en onmiddellijk weer opgezogen en opgevangen. Meestal wordt ongeveer 60% van de spoelvloeistof terugverkregen; de rest wordt in de long opgenomen. Cellen en vloeistof kunnen worden gescheiden. Normaal bestaat de alveolaire celpopulatie voor ruim 90% uit macrofagen, voor 1 à 2% uit neutrofiele granulocyten en voor minder dan 10% uit lymfocyten. Bij rokers is het aantal alveolaire macrofagen in de bronchoalveolaire lavage vloeistof verhoogd. Het is aangetoond dat bij een aantal chronische en acute interstitiële longaandoeningen de celpopulaties in het interstitium en de alveolaire ruimten met elkaar overeenkomen. Hoewel analyse van ontstekingscellen in bronchoalveolaire lavage vloeistof de open longbiopsie niet kan vervangen, is in een groot aantal gevallen gebleken dat met behulp van celanalyse in bronchoalveolaire lavage vloeistof de meer invasieve diagnostiek kan worden vermeden, zodat deze techniek thans klinisch frequent wordt toegepast.

Het type ontsteking in het longinterstitium en in de longblaasjes bepaalt welke ontstekingscellen in de alveoli en dus ook in de lavage vloeistof worden gevonden. Veelal is bij ontstekingsprocessen het totale aantal cellen toegenomen, bij sommige interstitiële longziekten betreft dit de lymfocyten, bij andere weer de granulocyten. Daarnaast kan worden onderzocht of er bepaalde niet-cellulaire substanties in de lavage vloeistof aanwezig zijn, zoals vetdruppels en plantaardige bestanddelen bij aspiratie (verslikken) of asbestlichaampjes bij een longfibrose op basis van asbestinhalatie. Een alveolitis met een toename van het aantal lymfocyten wordt meestal gevonden bij sarcoïdose en extrinsieke allergische alveolitis, terwijl een toename van het percentage granulocyten vooral wordt gevonden bij fibroserende ontstekingen. Differentiële telling van de leukocyten uit de lavage vloeistof heeft in deze gevallen diagnostische betekenis. Door middel van immunologische markeranalyse kunnen subpopulaties en activatiestadia van de verschillende ontstekingscellen worden bepaald, wat een verfijning van de diagnostiek geeft. Bronchoalveolaire lavage kan anders dan open longbiopsie gedurende het ziekteproces verschillende malen worden herhaald om de invloed

van de ingestelde therapie na te gaan.

Voor een beter begrip van het ziektebeloop van interstitiële longziekten is het noodzakelijk na te gaan welke immunologische processen zich in het interstitium en de alveoli afspelen. Wanneer een bepaald antigeen in het longweefsel terecht komt, zal in eerste instantie verwerking door mononucleaire cellen plaatsvinden, waarna het antigeen aan T-lymfocyten wordt gepresenteerd en zich een immuunrespons kan ontwikkelen.

T-lymfocyten zijn niet in staat het antigeen op zich te herkennen, maar kunnen dat wel indien het antigeen samen met moleculen van het major histocompatibility complex (MHC) wordt aangeboden. In het algemeen zijn dit MHC moleculen van monocyten, macrofagen en zogenaamde 'antigeen-presenterende cellen'. Het niet-specifieke immuunsysteem (monocyten/ macrofagen en antigeen-presenterende cellen) kan dus het antigeen aanbieden aan het specifieke immuunsysteem (lymfocyten) waardoor zich een immuunrespons kan ontwikkelen. Bij zo'n immuunrespons komen allerlei stoffen vrij (cytokinen) die een stimulerende of een dempende invloed op het ontstekingsproces hebben. Dit geldt ook voor de interstitiële inflammatoire longaandoeningen. De vrijkomende cytokinen zorgen o.a. voor een verhoogde influx van mononucleaire fagocyten en granulocyten vanuit het bloed naar het interstitium en de alveoli. Naast cytokinen kunnen interstitiële en alveolaire macrofagen, maar ook granulocyten, tevens enzymen en toxische zuurstof-radicalen produceren, waardoor een beschadiging van het interstitium optreedt. Groeifactoren afkomstig van mononucleaire fagocyten kunnen bovendien fibroblasten aanzetten tot deling en productie van collageen waardoor longfibrose kan ontstaan.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven was het vergroten van het inzicht in de verandering in morfologie en immunologisch fenotype van de mononucleaire cellen die zijn betrokken bij interstitiële ontstekingen. Bij interstitiële longziekten is er een verhoogde influx van monocyten in de alveoli, die ter plaatse uitrijpen. Wij onderzochten welke veranderingen in immunologisch fenotype optreden bij de uitrijping van bloed monocyten tot alveolaire macrofagen en welke verschillen er bestaan in immunologisch fenotype van morfologisch verschillende alveolaire macrofagen. Daarnaast werd *in vitro* de invloed van diverse, ook bij interstitiële longziekten vrijkomende, cytokinen (IL-2, IL-4, GM-CSF) alsmede van een frequent gebruikte ontstekingsremmer (dexamethason) op het immunologische fenotype van mononucleaire cellen onderzocht.

CHAPTER 1

GENERAL INTRODUCTION

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1.1 STRUCTURE OF THE ALVEOLI AND INTERSTITIUM

The interstitium concerns the tissue between the basal membrane of the alveolar epithelium and the endothelium. The interstitium of the alveolar wall is in contiguity with the connective tissue surrounding the blood vessels and bronchi, and with the connective tissue network of the visceral pleura. The alveolar interstitium contains fibroblasts and extracellular matrix (1-5). The connective tissue matrix contains several types of collagen and laminin (6-9). Beside these, several other components can be found such as elastin, proteoglycans and fibronectin (8-10). In addition, mononuclear phagocytes and lymphocytes are usually present.

The integrity of the alveolar wall is essential for the normal function of the pulmonary interstitium. An influx of inflammatory cells or disruption of the basal membrane may result in interstitial lung disease.

1.2 INTERSTITIAL LUNG DISEASES

The interstitial lung diseases are a heterogeneous group of illnesses with different pathogenesis. Many of these diseases are uncommon (1-5). The etiology and pathogenesis of most of these processes remain unknown. Current medical research in interstitial lung diseases is now focused on the pathogenesis and definition of markers of disease. The performance of lung lavage in healthy normal volunteers, asymptomatics and symptomatics, offers the possibility to identify risk factors and causes for the development of interstitial lung diseases. This is useful for the prevention (for example in interstitial lung diseases in which environmental factors play a major role) and maintaining asymptomatics in a healthy condition. The interstitial lung diseases can be divided into acute or chronic inflammatory lung disorders. Frequent causes of acute interstitial lung diseases are infections with bacteria or viruses, toxic damage by inhaled fumes and gases such as chloride, and the adult respiratory distress syndrome (ARDS) (Figure 1). Chronic interstitial lung diseases may be caused by inorganic dust inhalation such as silica and talc, and organic dusts such as fungi-exogenous proteins leading to a farmer's lung or bird breeder's lung. Although the etiology is not always clear, self-perpetuating mechanisms are set in motion (11). Inflammatory cells can damage connective tissue components and distort the normal architecture of the interstitium. The specific immune system may also be involved in perpetuating chronic interstitial inflammation by the influx and activation of lymphocytes such as in collagen-vascular diseases, hypersensitivity pneumonitis, cryptogenic fibrosing alveolitis (also called idiopathic pulmonary fibrosis) and sarcoidosis (1-5).

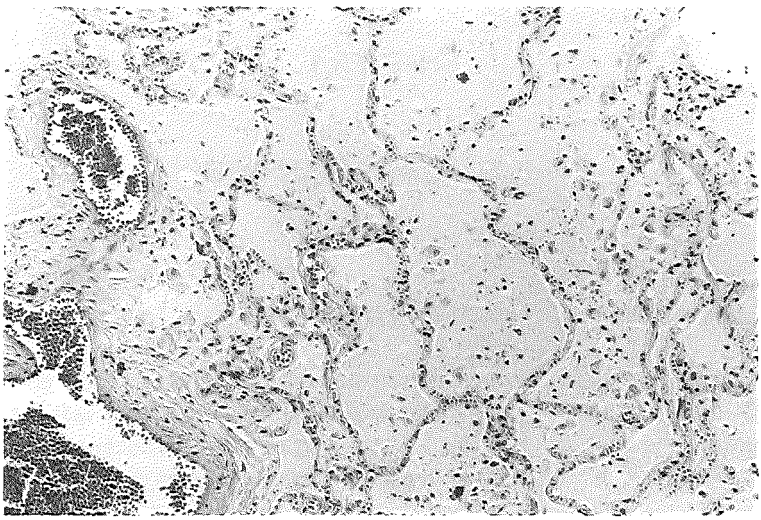

Figure 1. Acute alveolitis, early phase. The alveoli are filled with edema. Hyaline membranes are absent.

The pathological picture is complex. Often focal damage is found in combination with epithelial and connective tissue repair.

In addition, in patients with interstitial lung diseases not only a shift in the quantity but also in the types of inflammatory cells is found in the lower respiratory tract. This is caused by an increased recruitment of monocytes, neutrophils, eosinophils or lymphocytes from the circulation to the interstitium and alveoli. Analysis of cells and proteins in bronchoalveolar lavage (BAL) fluid can substitute more invasive diagnostic procedures. There are also limitations on this procedure since BAL samples only the airspaces and not the interstitium. However, inflammation of the alveolar walls and damage to vascular endothelium is frequently accompanied by changes in cell-profile and proteins in the BAL fluid (12,13) (Figure 2). Furthermore, in certain conditions the recovered cells in BAL fluid reflect changes in the lung parenchyma (14-16). In several cases these changes have prognostic value (17-19). Analysis of BAL fluid is only one of the possible investigations when a patient is submitted to the hospital. BAL fluid only generally reflects inflammatory changes that more or less parallel the severity of the disease. It is therefore necessary to develop more precise parameters for monitoring patients. Some disorders are characterized by a lymphocytic inflammatory process, others by a predominance of macrophages, neutrophils or eosinophils (Figure 3). To date it is unclear why the course of the various interstitial lung diseases is so different. Lung lavage gives the opportunity to study the morphologic, immunologic and functional differences of the cells present in the alveoli during progression of the disease. Over the past years, a lot of work has been done on the immunologic characterization of the lymphocytes in the alveoli. Because a wide range of myeloid monoclonal antibodies has become available, it is now possible to study differences in immunologic phenotype of mononuclear phagocytes and to correlate them with differences in cell function and course of the disease. This may result in clues to monitor development of disease in asymptomatics. Since the different inflammatory cells can release

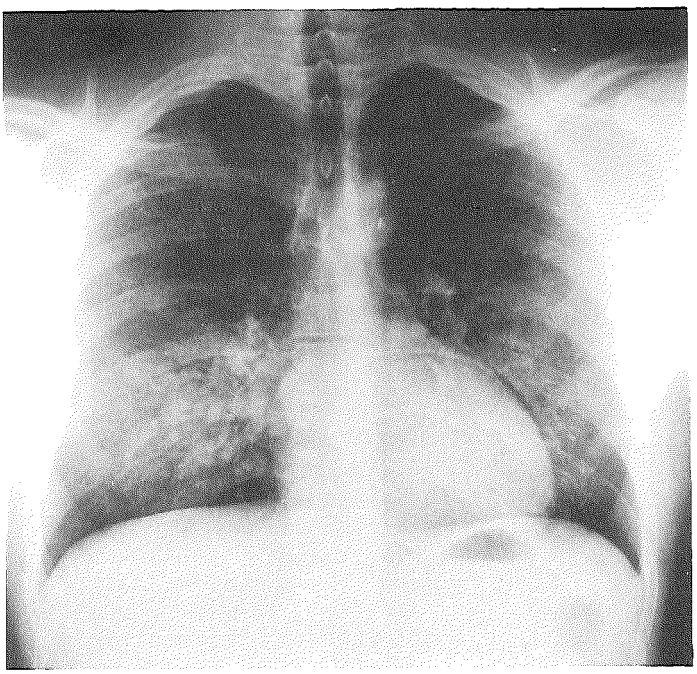

Figure 2. Chest X-ray film from a patient with idiopathic pulmonary hemosiderosis during an exacerbation. Typical findings include bilateral infiltrates predominant in the lower lung zones.

different cytokines, they can cause different types of inflammation, i.e. fibrotic or granulomatous inflammation (20,21). These cytokines activate and attract other cells to the site of inflammation (1-5). To date only a few cytokines have been investigated in interstitial lung diseases, i.e. IL-1, IL-2 and IFN-γ. These were predominantly studied in granulomatous interstitial lung diseases. It is unknown in most of the interstitial lung diseases which cytokines are released by the inflammatory cells and how to stop their release in order to prevent development of disease in asymptomatics and progression in symptomatics. Since several genes coding for cytokines have been cloned, it is now possible to obtain recombinant cytokines and to study their effects on inflammatory cells in BAL fluid. However, it is unknown yet which receptors for cytokines are present on cells in BAL fluid.

Neutrophils, eosinophils and monocytes/macrophages usually release degradative enzymes. Among these enzymes are all kinds of proteinases such as elastases, collagenases and plasminogen activators (8,10). Elastases are enzymes that degrade elastin, collagenases cleave collagen, while plasminogen activators convert plasminogen to plasmin, a proteinase that can degrade fibrin and other proteins. It is by these enzymes that inflammatory cells may damage the so-called extracellular matrix or connective tissue of the pulmonary interstitium (8,10).

Repair mechanisms may replace both damaged cells and extracellular matrix. However, excessive repair processes may distort and obliterate the architecture of the lung, producing permanent pulmonary dysfunction leading to complaints of progressive dyspnea (1-5). Peptides generated from the degradation of elastin, collagen and fibrin possess chemotactic activity for a number of inflammatory cells and fibroblasts (1-5). Furthermore, the presence of an intra-al-

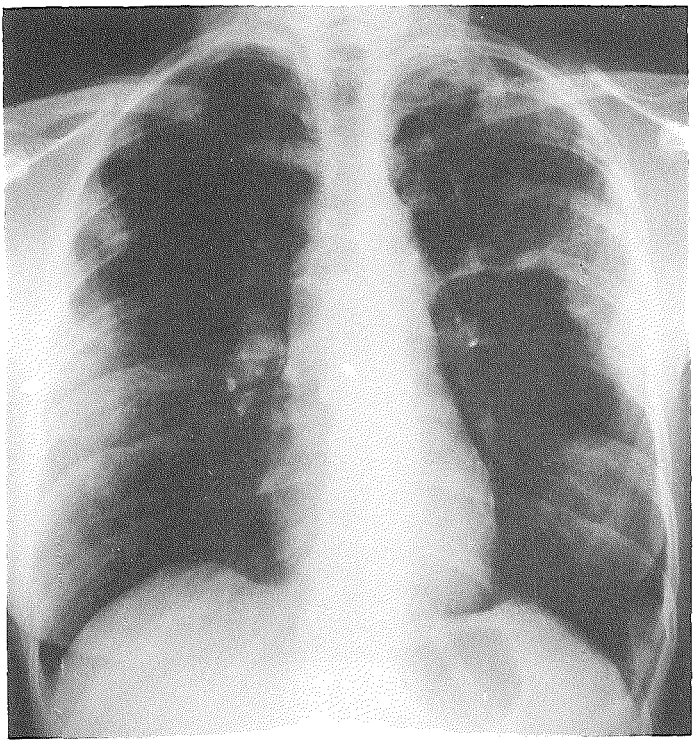

Figure 3. Chest X-ray film from a patient with eosinophilic pneumonia showing characteristic dense peripheral infiltrates.

Figure 4. Acute alveolitis, late phase. Hyaline membranes line the alveolar spaces. Inflammatory cells are present in the interstitium.

veolar exudate of fibrin and hyaline provides an alternative surface for repair and stimulates the proliferation of fibroblasts and the deposition of connective tissue at abnormal sites (Figure 4). Activated macrophages release growth factors that induce fibroblast growth and deposition of connective tissue (22-24).

From the above it is clear that interstitial lung disease is only partially a disease of the interstitium. The inflammatory process often involves the respiratory bronchioles as well as the alveolar walls and airspaces. It may heal completely or may result in the development of an excess of connective tissue with distortion of lung architecture and honeycombing, leading to a restrictive lung function.

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CHAPTER 2

INFLAMMATION AND INTERSTITIAL LUNG DISEASES

CHAPTER 2

INFLAMMATION AND INTERSTITIAL LUNG DISEASES*

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2.1 PATHOGENESIS OF GRANULOMATA

INTRODUCTION

Granulomatous inflammations are characterized by organized aggregates of mononuclear phagocytes and often plasma cells and T lymphocytes (1). Granulomata result from the presence of a persistent agent which the macrophage is unable to destroy or to which a strong immune reaction exists. On occasions the granulomata are of unknown origin such as in sarcoidosis (2). One of the basic functions of a granulomatous reaction is to protect the host against the invading antigen.

The usual response after inhalation of organic or inorganic material is acute inflammation, with increased numbers of monocytes and granulocytes. The monocytes/macrophages and granulocytes usually phagocytize, digest and remove the inciting agent. However, if the macrophage fails in this process and the invader persists within the macrophage, cytokines are continuously produced (Tables 1 and 2) which in turn attract more monocytes, lymphocytes and plasma cells (3-5). The newly-arrived monocytes have an immature morphology and resemble monocytes found in PB (6). The loose infiltrate of monocytes turns into an aggregate of mature macrophages. The monocytes become larger, develop cytoplasmic organelles and their membranes become ruffled. These changes which result in mature macrophages, are probably due to local factors. After prolonged antigenic stimulation, the activated macrophages may differentiate further into epithelioid cells. The development of alveolar macrophages into epithelioid cells has been demonstrated *in vitro* (1). Epithelioid cells are larger than macrophages and have extensive cytoplasm, filled with organelles. Epithelioid cells then differentiate into multinucleated giant cells. These cells, also called Langhans cells, have several nuclei distributed along the cell membrane leaving the central area of the cytoplasm free. The giant cell has little endoplasmic reticulum and the lysosomes appear to undergo degeneration (7). These cells together result in granulomata (Figure 1).

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TABLE 1. Relevant cytokines and their cellular source.

Cytokine[1]	Acronym	Cellular source
Interferon-γ	IFN-γ	Activated T cells, natural killer (NK) cells
Interleukin 1	IL-1 α and β	Macrophages, endothelial cells, NK cells, B cells, fibroblasts, epithelial cells
Interleukin 2	IL-2	Activated $CD4^+$ T cells
Interleukin 3	IL-3	Activated $CD4^+$ T cells, activated $CD8^+$ T cells
Interleukin 4	IL-4	Activated $CD4^+$ T cells
Interleukin 5	IL-5	Activated $CD4^+$ T cells
Interleukin 6	IL-6	Fibroblasts, T cells, monocytes/macrophages, endothelial cells, B cells, keratinocytes
Interleukin 7	IL-7	Activated $CD4^+$ T cells
Interleukin 8	IL-8	Activated $CD4^+$ T cells
Tumor necrosis factor α	TNF-α	Macrophages, T cells, thymocytes, B cells, NK cells
Tumor necrosis factor β	TNF-β	T cells

1. Cytokines are produced by cells in response to activation signals.
From: F.R. Balkwill and F. Burke, Immunol. Today 10, 299-304, 1989.

Usually granulomata are divided into immunologic and non-immunologic ones (8). The non-immunologic (foreign body) granulomata are composed of non-specific cells, i.e. monocytes/macrophages and multinucleated giant cells. The immunologic granulomata are composed of monocytes/macrophages, dendritic cells, multinucleated giant cells, T lymphocytes and plasma cells (8).

NON-IMMUNOLOGIC GRANULOMATA

In contrast to the immunologic granulomata with delayed-type hypersensitivity, the cells in non-immunologic foreign body granulomata show a relatively low turnover. Migration of monocytes from PB and proliferation of local monocytes/macrophages is usually low (1). Usually T lymphocytes are absent and lymphokines are not produced (9). Many indigestible foreign bodies are known to induce a granulomatous reaction such as talc crystals, polysaccharides, oils, fine wood particles, aluminium, quarts and silicates (9-15). The granulomata they cause are called "foreign body" granulomata and the giant cells surrounding these particles, foreign body giant cells. In foreign body granulomata lymphocytes are not

TABLE 2. Relevant cytokines and their cellular targets and effects.

Cytokine	Cellular target(s)	Effects
IFN-γ	Macrophages, T cells, B cells, NK cells	MHC class I and class II antigen induction in general. MHC class II expression on endothelial cells, fibroblasts and monocytic cells. Antimicrobial tumoricidal and antiviral activity of monocytes/macrophages. Increase of NK cell activity. Down-regulation of IL-4 induced IgE production.
IL-1 α and β	Neutrophils, macrophages, T cells, B cells, fibroblasts, endothelial cells, epidermal cells	Lymphokine release from activated T cells and fibroblasts. Growth of fibroblasts. Release of PGE_2, collagenase and acute phase proteins. Fever. Chemotactic for neutrophils, macrophages and lymphocytes. Increase of NK cell activity.
IL-2	T cells, B cells, macrophages	Growth and proliferation of B cells and T cells. Lymphokine production by T cells. Increase of NK cell activity. Increase of monocyte cytotoxicity.
IL-3	Multipotential stem cells, mast cells*	Differentiation and maturation of hemopoietic progenitor cells.
IL-4	T cells, B cells, macrophages, mast cells*	Proliferation of activated T and B cells. Increased expression of Fc receptors and HLA-DR on B cell lines. Induction of expression of CD23 (FcεR).
TNF-α	Tumor cells*, fibroblasts*, macrophages*, neutrophils*, eosinophils* and endothelium*	Stimulates release of IL-1 from macrophages* and endothelial* cells. Increase of T cell mediated cytotoxicity*, antibody dependent cellular cytotoxicity (ADCC) and NK cell activity*.
TNF-β	T cells*	Lymphocyte and NK cell mediated cytotoxicity*. Chemotactic factor for fibroblasts and monocytes, inhibition of T and B cell proliferation.
GM-CSF	Hemopoietic progenitor cells	Stimulates proliferation and differentiation of hemopoietic progenitor cells. Effects on cell morphology, motility, cytotoxic activities and phagocytosis of neutrophils, monocytes and eosinophils.

* Mouse
From: F.R. Balkwill and F. Burke, Immunol. Today 10, 299-304, 1989.

involved. Beryllium does not cause simple foreign-body granulomata, because beryllium binds to proteins resulting in hypersensitivity granulomata with T cells at the periphery (16,17). Silicates result in a special form of inflammation, because silicium acid causes death of the macrophage. This leads to an inflammatory reaction with migration of PB monocytes and neutrophils, and proliferation of fibroblasts. The damage may be quite extensive resulting in fibrosis (18). Since the foreign bodies usually persist in the lung they continue to stimulate granulomatous reactions, unless they are removed by mucociliary clearance or by the lymphatics.

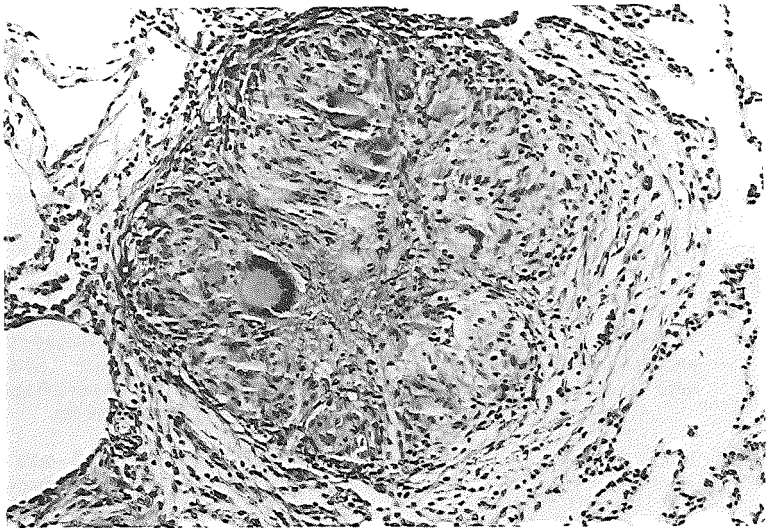

Figure 1. Miliary tuberculosis: epithelioid granulomata with many Langhans type giant cells.

IMMUNOLOGIC GRANULOMATA

Immunologic granulomata are more complex than foreign body granulomata. The granulomata grow faster and are usually larger than foreign body granulomata. The accelerated formation is apparently immunologically driven since T lymphocytes are abundant and activated. The granulomatous reaction can be transferred by T lymphocytes and can be suppressed by thymectomy or the administration of anti-lymphocyte globulin (19).

Tuberculosis

In tuberculosis, granulomata formation is often prominent in the lung (20). Intracellular parasites such as Mycobacterium tuberculosis often persist and can evoke the granulomatous response. Prolonged intracellular survival is the key to the virulence of Mycobacterium tuberculosis and is caused by failure of the bacilli-containing phagosomes to fuse with lysosomes. This fusion defect protects the bacilli from being killed by digestive enzymes. The sequence of events has been demonstrated in an animal model (21). The maturation of loose collections of monocytes into mature granulomata in infection with Mycobacterium tuberculosis requires only a few days. After that stage, the typical granulomata develop, with epithelioid, multinucleated giant cells and lymphocytes. A characteristic feature of granulomata in tuberculosis is the central necrosis which is absent in sarcoidosis (1).

Skin reactions to Mycobacterium tuberculosis indicate that cell-mediated immunologic reactions involving T lymphocytes play an important role. This is probably reflected in granulomata because the centrally placed epithelioid and multinucleated giant cells may

process antigen while dendritic cells present antigen to T lymphocytes (22-24). It has been demonstrated in humans that mycobacterial protein antigens can stimulate PB monocytes to release IL-1 (25). In addition, it was demonstrated that killed mycobacteria (Bacillus Calmette-Guérin, BCG) administered intravenously in rats caused a higher level of cytotoxicity and IL-1 release by alveolar macrophages of sensitized animals (26). Activated T lymphocytes may release cytokines that in turn can stimulate the antigen presenting capacity and bactericidal function of the centrally located epithelioid and multinucleated giant cells.

It is still questionable whether tumor necrosis factor (TNF-α) plays a role in the central necrosis seen in tuberculous granulomata. It has been demonstrated that activated macrophages are the source of TNF-α (27) and that TNF-α stimulates the release of IL-1 from macrophages (28). TNF-α has been demonstrated in the serum of BCG-infected mice after challenge with endotoxin (29,30). It has to be elucidated whether TNF-α is released in tuberculous granulomata and can potentiate the central necrosis caused by hydrolytic enzymes from epithelioid and giant cells.

The presence of delayed-type hypersensitivity can greatly augment the development of granulomata (31). It has been demonstrated in mice that antigen-specific delayed-type hypersensitivity is mediated by helper T lymphocytes (32). In granulomata induced by BCG the rate of development and number of epithelioid cells are greatest when delayed-type hypersensitivity is maximal and bacillary products are still present. This high rate is probably caused by cytokines derived from stimulated $CD4^+$ T lymphocytes (33). The onset of delayed-type hypersensitivity in tuberculosis is coincident with the development of caseous necrosis (34).

The granulomatous hypersensitivity reaction is clinically one of the most important immunologic responses.

Sarcoidosis

Sarcoidosis is a systemic disorder of unknown origin with non-caseating granulomata often most prominent in the lung (Figure 2). Bronchoalveolar lavage in patients with sarcoidosis has shown that the absolute and relative number of $CD4^+$ T lymphocytes in BAL fluid is often increased (35). At sites of disease activity, the lymphocytes recovered by BAL are representative of the interstitial lymphocyte population (36). Patients with advanced disease tend to have a greater number of granulomata and a less prominent alveolitis.

Monocyte-derived cells and lymphocytes interact through a complex series of stimulatory and inhibitory signals. The increased influx of relatively young PB monocytes and T lymphocytes may facilitate this interaction. This interaction may account for some local division of monocytes and lymphocytes in sarcoidosis (37).

Lymphocyte activation requires amongst others the release of cytokines by monocyte-derived cells while activation of monocyte-derived cells partially depends on lymphocyte derived cytokines. In order to activate T lymphocytes, it is also necessary that the monocyte-derived cell presents the "antigen" to the T lymphocyte. T lymphocytes recognize antigen in combination with a particular type of MHC molecule (38). Cytotoxic T lymphocytes recognize antigen in association with MHC class I molecules (HLA-A, -B and -C), whereas $CD4^+$ T lymphocytes

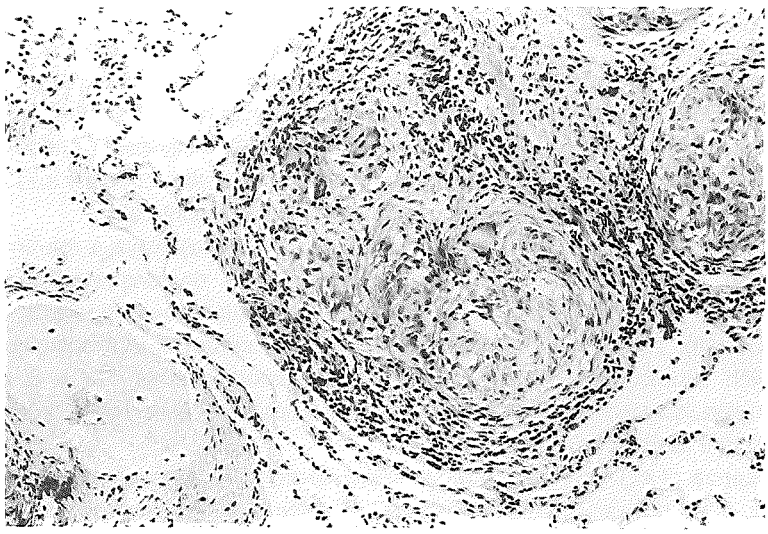

Figure 2. Sarcoidosis: epithelioid granulomata and some Langhans type giant cells (transbronchial biopsy).

recognize antigen in association with MHC class II molecules (HLA-DR, -DQ and -DP). In humans the majority of alveolar macrophages and dendritic cells are positive for HLA-DR (6,23), which is required for effective recognition of antigen by $CD4^+$ T cells. If a $CD4^+$ T lymphocyte is activated by MHC class II molecules in conjunction with antigen, IL-2 and IFN-γ are produced (2).

Activation of alveolar macrophages and PB monocytes results in the production of IL-1 (39). Alveolar macrophages have a limited capacity to produce IL-1 compared with PB monocytes (40). The expression of the IL-1-beta gene, however, appeared to be the same in alveolar macrophages and PB monocytes, suggesting that there is a difference in transcription of the IL-1-beta gene between these two cell types (41). In contrast, alveolar macrophages from sarcoidosis patients release IL-1 (42), although the expression of the IL-1-beta gene is not increased (43). After stimulation with lipopolysaccharide (LPS), alveolar macrophages of sarcoidosis patients produce more IL-1 than those of healthy volunteers (44). It seems that sarcoid alveolar macrophages are already primed *in vivo* and can produce significantly more cytokines than control macrophages.

Sarcoid macrophages in addition produce TNF-α after stimulation with LPS (45). TNF-α can stimulate the release of IL-1 from alveolar macrophages (28).

IL-1 is chemotactic for T lymphocytes and initiates T lymphocyte activation (46). Furthermore, it has been demonstrated *in vitro* that IL-1 promotes the binding of T lymphocytes to endothelial cells (47). It is suggested that if this is also the case *in vivo*, IL-1 may amplify granulomata formation in sarcoidosis patients.

In patients with active sarcoidosis usually lymphadenopathy and/or parenchymal infiltrates are present on the chest X-ray film (Figure 3) and the proportion of T lymphocytes in BAL fluid is augmented, with an increased CD4/CD8 ratio (16). These T lymphocytes release IL-2 (48,49)

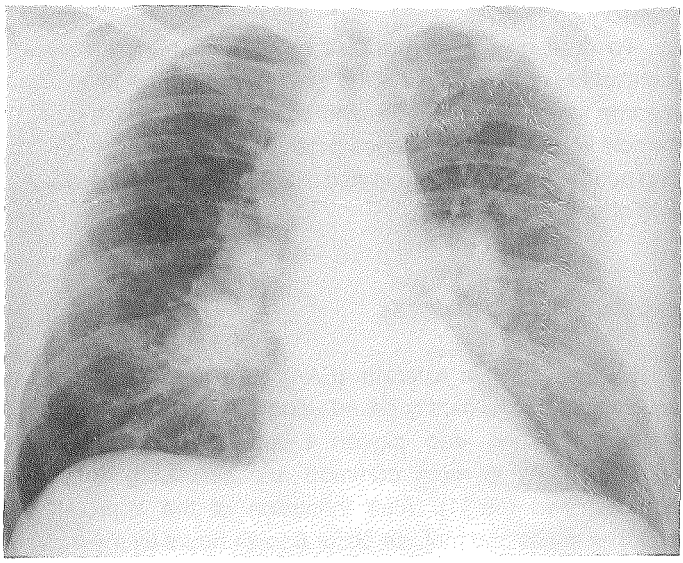

Figure 3. Chest X-ray film from a patient with sarcoidosis. Note the massive bilateral hilar and paratracheal lymphadenopathy and parenchymal infiltrate.

and other cytokines such as monocyte chemotactic factor (MCF) and macrophage migration inhibitory factor (MIF) (50). Other investigators, however, reported a decreased production of IL-2 by sarcoid T lymphocytes (44). This discrepancy may be due to the fact that transcription of the IL-2 gene occurs at sites of disease activity and is not sustained if the T cells are removed from the site of the disease activity (51).

It has been demonstrated that release of IL-2 in active pulmonary sarcoidosis is primarily from activated HLA-DR^+ $CD4^+$ T lymphocytes, (52). In addition, sarcoid alveolar macrophages express receptors for IL-2, in contrast to normal alveolar macrophages where little or no binding of IL-2 is found (53,54). These cells bind monoclonal antibody against the IL-2 receptor only after stimulation with IFN-γ. This indicates that in sarcoidosis macrophages are activated and may respond to IL-2 and other mediators elaborated by activated T lymphocytes, with the production of cytokines. An additional finding concerns elevated concentrations of soluble IL-2 receptors in serum samples and BAL fluids in active sarcoidosis (55). Immunohistochemical stainings of lymph nodes of patients with sarcoidosis confirmed the findings reported in BAL, i.e. the presence of IL-2 receptors on macrophages, epithelioid and multinucleated giant cells (54).

Other metabolites known for their immunoregulatory features are the arachidonic acid (AA) products derived from alveolar macrophages. Increase in prostaglandin (PG) synthesis occurs in macrophages in response to a variety of stimuli. The increase in PGE_2 may account for a negative feed-back regulation of macrophage and T lymphocyte activity (55,56). PGE_2 has been shown to inhibit a number of lymphocyte responses, including T lymphocyte proliferation, cytokine production and cytolysis (55,56). Thus, while stimulation of T lymphocytes causes cytokine release which stimulates PGE_2 production by macrophages, this PGE_2 can subsequently inhibit T cell cytokine formation by negative feed-back. In animal models it has been demonstrated that PGE_2 modulates pulmonary granulomata induced by Schistosoma

mansoni eggs (57).

To date only a few studies have been performed concerning the production of AA metabolites in sarcoidosis. One study reported the production of AA metabolites by sarcoid macrophages *in vitro* before and after stimulation with calcium ionophore. It was found that sarcoid alveolar macrophages produced less PGE_2, thromboxane-B_2 (TXB_2), 6-keto-PGF1α and hydroxyeicosatetraenoic acid (HETE) in comparison with control macrophages, both before and after stimulation (58).

Extrinsic allergic alveolitis

Extrinsic allergic alveolitis (EAA) can be induced by the inhalation of some antigenic substances and is mostly based upon a combination of type III and type IV allergic reaction. In farmer's lung and pigeon breeder's lung, T lymphocyte mediated delayed-type hypersensitivity is most common and circulating antibodies of the IgG type are often demonstrated (59-61). After inhalation of the appropriate antigen, symptoms of fever, breathlessness and malaise occur 6 to 24 hours after exposure. Symptoms and signs usually resolve within 48 hours. Often diffuse shadowing on the chest X-ray exists (Figure 4). Upon histologic examination lymphocytes, plasma cells and non-caseating granulomata are found. These granulomata are composed of epithelioid cells and multinucleated giant cells surrounded by a mantle of lymphocytes. Often the respiratory bronchioles are also involved in the process. In advanced cases, interstitial fibrosis may develop (62).

The BAL fluid of patients with EAA often contains an increased percentage of T lymphocytes with a decreased CD4/CD8 ratio (63,64).

Sensitized T lymphocytes interact with macrophages and release cytokines which in turn activate macrophages. Following stimulation with the appropriate antigen, T lymphocytes in BAL fluid or PB transform into lymphoblasts and produce cytokines. These T cells start to divide only in symptomatic patients (65). T lymphocyte activation is apparent from the high HLA-DR expression on high numbers of $CD8^+$ T lymphocytes in symptomatics (65). Also asymptomatic patients may show increased numbers of $CD8^+$ T lymphocytes in BAL fluid, although these are not activated (66).

Experiments in guinea pigs have shown that these animals could be sensitized with Micropolyspora faeni which is responsible for farmer's lung in humans (67-69). This makes it possible to study the dynamics of the characteristic granuloma formation in an animal model.

To date the immunopathogenesis of EAA is not completely understood. Precipitating antibodies to the antigens are found in symptomatics and asymptomatics, while in BAL fluid of both groups increased numbers of T lymphocytes with a decreased CD4/CD8 ratio can be demonstrated. Symptomatics, in contrast to asymptomatics, demonstrate T lymphocytes in BAL fluid that proliferate when stimulated with the specific antigen. In an animal model the disease can be transferred with sensitized lymphocytes, giving histopathologic changes in the lung after challenge with the appropriate antigen. Much is still unknown about the disease, but it is clear from human and animal studies that sensitized $CD8^+$ T lymphocytes are involved in the development of the clinical symptoms.

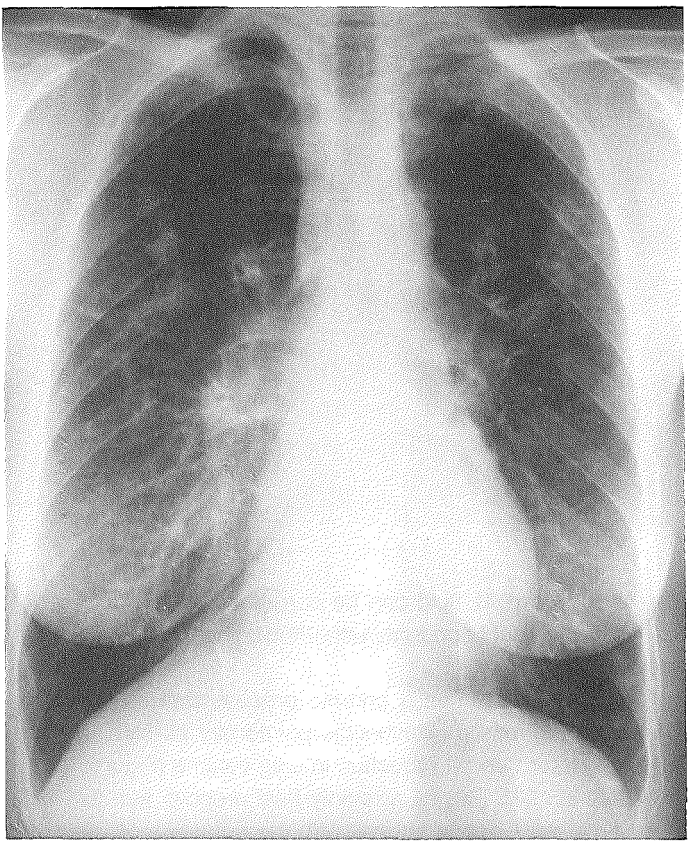

Figure 4. Chest X-ray film from a patient with chronic hypersensitivity pneumonitis. Irregular diffuse shadowing is present in both lungs.

2.2 PATHOGENESIS OF FIBROSIS

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a severe disorder that starts as an alveolitis and progresses to interstitial fibrosis (70,71). To determine the fibrosing process as idiopathic or cryptogenic, all other causes of scarring and fibrosis such as radiation, asbestosis, drug reactions, and collagen vascular diseases such as systemic lupus erythematosus and rheumatoid arthritis should be excluded.

IPF occurs most commonly in patients between 40 and 70 years old (72,73). Usually crackles are heard over the lower lung fields and they increase with progression of the disorder. Clubbing of the fingers is often present. Other symptoms which occur frequently are dyspnea, dry cough, fatigue, anorexia and weight loss (70-73). Both gallium-67 scanning and BAL are used to quantitate the extent of the alveolitis and to follow these patients (73-75).

Usually IPF patients demonstrate increased numbers of neutrophils and eosinophils in BAL fluid, the latter being associated with a reduction of the diffusion capacity of the lungs (76,77).

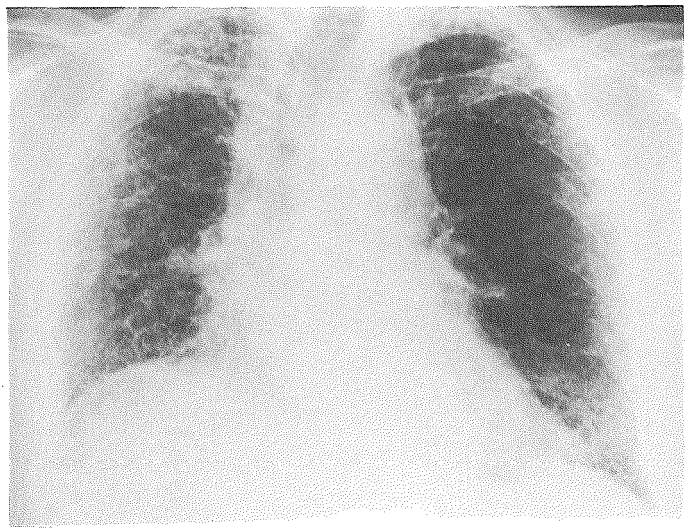

Figure 5. Chest X-ray film from a patient with idiopathic pulmonary fibrosis. Note the diffuse, coarse reticular infiltrates and small lungs.

The findings in BAL fluid can be correlated with the clinical course. BAL fluid lymphocytosis identifies a subset of patients with IPF who are likely to improve clinically in response to steroid therapy (78,79).

IPF may appear as a chronic process that remains stable for years. However, it can suddenly progress to irreversible pulmonary fibrosis. The chest X-ray film usually shows a reticular pattern and a reduction in lung volume (Figure 5). It was first described by Hamman and Rich in 1935 (80) as a rapidly fatal process with cellular thickening of the alveolar walls showing a tendency to fibrosis with the presence of large mononuclear cells, presumably of alveolar origin, within the alveolar space. In 1965 Liebow described a group of patients which he differentiated from other forms of fibrosis on histological grounds (81). He described the so-called desquamative interstitial pneumonia (DIP), in which the alveolar wall or interstitium was thickened with little fibrosis but prominent desquamation of alveolar cells into the alveolar space (Figure 6). Usual interstitial pneumonia (UIP) also shows desquamated cells but the principal finding is a chronic inflammatory cellular infiltrate in the alveolar walls with varying degrees of fibrosis. Other forms have later been described such as lymphoid interstitial pneumonia (LIP) (82) with an interstitial infiltrate consisting mainly of mature lymphocytes, a few plasma cells and histiocytes. Giant cell interstitial pneumonia (GIP) (83) describes a condition with numerous large, multinucleate giant cells in the alveolar spaces. LIP responds much better to corticosteroids than UIP. To date it is not clear whether these diseases should be considered as separate entities or rather different stages of the same fibrotic process with different reactions on corticosteroid treatment.

All other forms of lung fibrosis, such as in collagen vascular diseases may lead to a histologic picture indistinguishable from IPF. In other forms of fibrosis due to environmental substances such as asbestos or silica, one can usually find asbestos bodies or silica remnants within the fibrotic process, indicative of the etiology (84-86).

After tissue derangement with accumulation of inflammatory cells, several mediators are

released that induce the accumulation of mesenchymal cells (fibroblasts and myofibroblasts) to the site of inflammation. These cells synthesize and release connective tissue molecules such as fibronectin, collagens, elastin and proteoglycans, ultimately resulting in fibrosis.

TURNOVER OF FIBROBLASTS

The regulation of fibroblast proliferation in lung disease has been studied intensively. Fibroblasts are influenced by the extracellular matrix itself, by soluble products derived from recruited cells, and by contacts with other fibroblasts and epithelial cells (87,88). The sequence of events leading to fibrosis has been studied extensively in animal models using bleomycin, paraquat, hyperoxia, asbestos and silica (89-95). Morphologic examination of the lungs revealed that the early lesions consist of severe and irreversible endothelial and alveolar epithelial cell damage. Afterward, an inflammatory process takes place, initially dominated by polymorphonuclear leukocytes and then by mononuclear cells, but with the constant presence of granulocytes. After two to four weeks, fibroblast accumulation and proliferation are usually present (89-95). Two types of fibrosis can be demonstrated: (a) interstitial fibrosis in which the fibroblasts become activated and divide within the interstitial compartment of the alveolar wall, that is, within the space limited by the alveolar lamina; and (b) intra-alveolar fibrosis by invasion of alveolar exudates by septal connective tissue cells, migrating through gaps in the basal lamina into the alveolar exudate (92-95). Within the alveolar spaces, the exudate is broken down and replaced by fibroblasts and collagen. An important quality of fibroblasts is to contract the fibrotic mass composed of cells and matrix proteins including collagen (96). Although it has been demonstrated that fibroblasts are usually quiescent cells, *in vitro* cultures demonstrated that fibroblasts from humans with early fibrosis have a higher proliferative capacity than fibroblasts of normal lungs when stimulated *in vitro* (97).

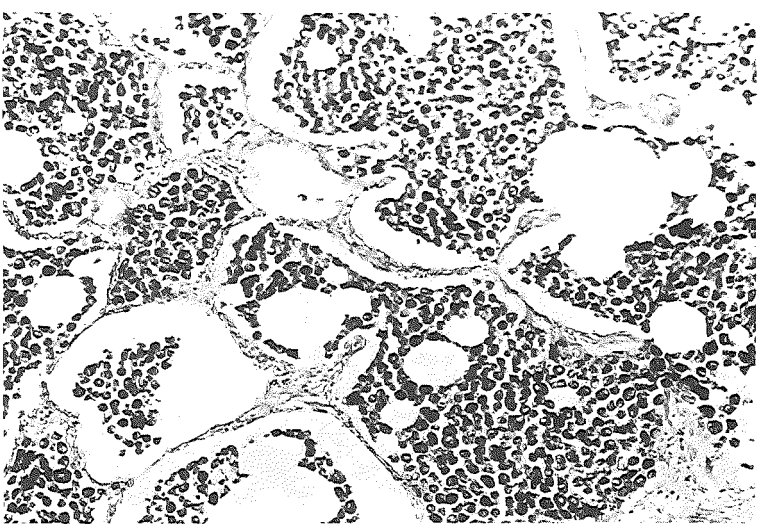

Figure 6. Desquamative interstitial pneumonia with accumulation of intra-alveolar macrophages and pneumocytes.

PRODUCTION AND DEGRADATION OF CONNECTIVE TISSUE

Fibrotic lung diseases show not only an increase in interstitial collagen but also alterations in types and location (91-93). Collagen is not a static molecule but is constantly being degraded and resynthesized. Usually the amount of collagen is constant suggesting a balance between synthesis and breakdown (98). The major cell type synthesizing collagen appears to be the fibroblast (98,99).

Collagen is the major protein found in the extracellular matrix. There is evidence for the existence of at least five polymorphic types of collagen (100). These collagens are thought to be synthesized as precursors (procollagen). During the conversion of procollagen to collagen peptide sequences are removed by a specific procollagen peptidase (101). The localization of the different types of collagen has been studied extensively (99,100). In the normal lung, type I collagen appears to be localized in the interstitium of the alveolar septa, type III appears to have a more prominent, irregular localization in the septa and perivascular, types IV and V codistribute in linear patterns on alveolar and capillary basement membranes, while type II collagen is present only in cartilaginous airways (99,100). Dramatic changes occur in types and location of collagen in fibrotic lung disease (91-93,99,100,102). There is a marked increase in type I in thickened septae. Type III is markedly reduced and seen only perivascularly. These findings are in accordance with the presence of increased procollagen III peptide in BAL fluid in patients with interstitial lung diseases (103,104). Type V is markedly increased in the interstitium and located in areas of smooth muscle proliferation, while no apparent change is noted in amount or localization of type IV collagen (100).

In addition to the increased levels of collagen, increased levels of fibronectin have also been demonstrated in fibrotic lung diseases (91-93). Fibronectin is produced by mononuclear cells and fibroblasts and contains binding sites for collagen and fibrinogen (105,106). It mediates the attachment of fibroblasts to the matrix, for example to fibrin. Due to the inflammation, compo-

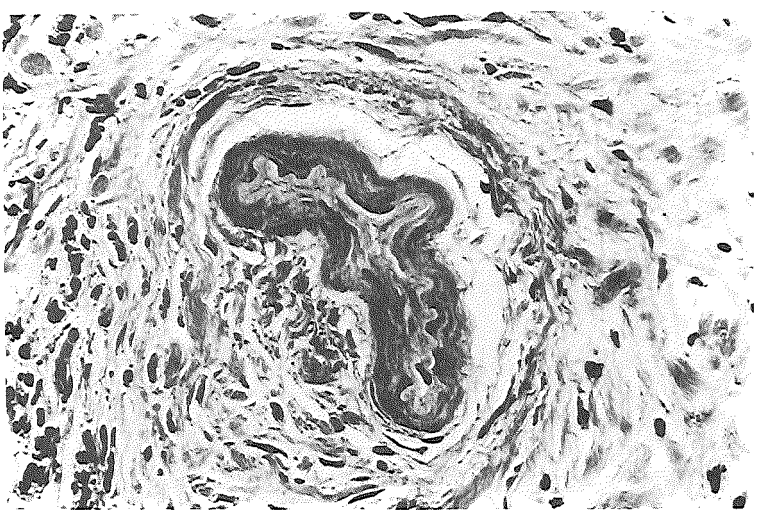

Figure 7. Compression of vein by collagen formation.

nents of plasma such as fibrin usually move into the alveolar airspaces and the increased production of fibronectin by fibroblasts facilitates their adhesion to the extravasated fibrin. Thus injury to the alveolar epithelial cells and endothelial cells with subsequent leakage of fibrin promotes the fibronectin-mediated binding of fibroblasts and facilitates pulmonary fibrosis (107).

Connective tissue is not only synthesized but also constantly broken down. Distortion of this equilibrium can further interfere with the respiratory structures (Figure 7). The resorption of connective tissue is mediated by collagenases, elastases and other proteases. (108). Mononuclear phagocytes and neutrophils are the major source of these enzymes. They are found in abundance in pulmonary interstitium and in BAL fluid of patients with fibrotic lung diseases and are for the major part responsible for the scarification in fibrotic lung diseases (74,75).

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CHAPTER 3

MONONUCLEAR PHAGOCYTES IN INTERSTITIAL LUNG DISEASES

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3.1 ORIGIN AND KINETICS OF PULMONARY MONOCYTES

Alveolar macrophages and their precursors are part of the mononuclear phagocyte system. The cells of this system include circulating blood monocytes and mononuclear phagocytes in connective tissue or fixed to the endothelial layer of the blood capillaries, in the liver known as Kuppffer cells and in the kidney known as the intraglomerular mesangial cells. Alveolar and serosal macrophages (such as peritoneal macrophages) are examples of unfixed macrophages. Brain microglia cells are cells which enter the brain around the time of birth and differentiate into fixed cells (1-4).

Dendritic cells are potent stimulator or accessory cells for the immune response. They consist of the Langerhans cell in the skin, the interdigitating cell present in the T lymphocyte area of lymph nodes and spleen, the interdigitating cells in the thymus and the veiled cells present in the afferent lymph (5). It is still questionable whether dendritic cells are derived from a distinct monocyte lineage.

The cells which comprise the mononuclear phagocyte system are widely distributed throughout the body. Proliferation and differentiation of the progenitor stem cells is under strict control of colony stimulating factors (CSF) (6).

Multi-CSF or interleukin 3 (IL-3) has the capacity to stimulate proliferation of a broad range of progenitor cells including erythroid and multi-potential progenitor cells. GM-CSF stimulates monocyte, neutrophil, eosinophil, megakaryocyte, and erythroid colony formation. G-CSF stimulates the formation of granulocytic colonies, and M-CSF (CSF-1) selectively stimulates the formation of monocytic colonies (6). More important for interstitial lung diseases is that these factors may activate monocytes (7,8) and macrophages (9-12) in terms of a respiratory burst, increased HLA-DR expression and increased phagocytosis. Activated monocytic cells in turn produce CSF and thereby regulate their own proliferation (13).

Mononuclear phagocytes

Monocytes in the circulation are heterogeneous with regard to cell density, size and morphology (14). The blood monocyte has a horseshoe-shaped nucleus and often contains faint azurophilic granules. Ultrastructurally, the monocyte possesses ruffled membranes, a well-developed Golgi complex and many intracytoplasmic lysosomes. These lysosomes contain several acid hydrolases and peroxidase which is important in intracellular killing of micro-orga-

TABLE 1. Cytokines which can modulate the phenotype and/or functional activity of mononuclear phagocytes.

Cytokine	Source	Effects
IL-1	Macrophages, others	Release of IL-1, TNF, CSF
TNF-α/cachectin	Macrophages, T lymphocytes	Release of PAF, IL-1, PGE_2; chemotaxis
IL-3	T lymphocytes	Growth of immature stages; differentiation
IL-4	$CD4^+$ lymphocytes (mouse)	Fusion, enhanced Ia expression; antigen presentation; Fcε receptor expression
M-CSF	Fibroblasts, macrophages	Growth and differentiation
GM-CSF	T lymphocytes, macrophages, endothelium	Growth, differentiation and activation
Interferon-α	Leukocytes	Antiviral activity; modulation of macrophage activation
Interferon-β	Fibroblasts	Antiviral activity; modulation of macrophage activation
Interferon-γ	T lymphocytes	Antiviral activity; macrophage activation, e.g. enhanced Ia expression; respiratory burst, down-regulation of MFR
TGF-β	Platelets, macrophages	Chemotaxis; growth factor release; macrophage deactivation

IL, interleukin; M-CSF, macrophage colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor; CSF, colony stimulating factor; PAF, platelet activating factor; PGE, prostaglandin E; MFR, mannosyl-fucosyl receptor.

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nisms (1,2). To date there are no conclusive data showing that monocytes are destined for any particular tissue when they leave the bone marrow. Migration of monocytes into the different tissues thus seems to be a random phenomenon in the absence of inflammation (5).

The origin of the alveolar macrophage has extensively been studied in humans and animals. The majority of alveolar macrophages derive from peripheral blood monocytes (15-17), while a minor part is maintained by alveolar macrophage proliferation *in situ* (18-24). Occasionally, due to inflammation, monocytes in the interstitium cross the alveolar epithelial cells and become alveolar macrophages (25). Some studies suggest that a subpopulation of alveolar macrophages may cross the alveolar epithelium to return to the interstitium and enter the lymphatics, thus providing a systemic immune response after inhalation of certain antigens (26,27).

After passing through the alveolar epithelium monocytes mature into alveolar macrophages,

probably under the influence of local factors (Table 1). Alveolar macrophages are large round or oval cells with a single bean-shaped nucleus (Figure 1). The cytoplasm contains numerous vacuoles and granules differing in size, and dust particles in varying amounts (1-3). Interstitial macrophages, on the other hand, do not have the typical inclusions of the alveolar macrophages.

The cytochemical characteristics of alveolar macrophages include a strong reaction for non-specific esterase and a positive reaction for acid phosphatase (1-3). Ultrastructurally, the cell surface of the alveolar macrophage is highly ruffled without major protrusions, the cytoplasm contains a polymorphous nucleus with a prominent nucleolus, a well-developed Golgi apparatus, mitochondria and a rough endoplasmic reticulum (4). Alveolar macrophages present on the alveolar surface can respond to chemotactic stimuli and their primary function is phagocytosis of various substances. Recently migrated monocytes are smaller and show only a few irregularly shaped lysosomal structures whereas mature macrophages display large vacuoles containing multi-lamellar granular structures. The cell surface markers of alveolar macrophages are described in Chapter 5. Briefly, the main surface markers expressed by alveolar macrophages are the mannosyl-fucosyl receptors (MFR) as well as Fc receptors, complement receptors, HLA-DR and CD11/CD18. Migration of blood monocytes and maturation to alveolar macrophages is reflected by changes in immunophenotype (28-30).

A special cell of the mononuclear-phagocyte system is the multinucleated giant cell seen in granulomatous inflammation (1,2). Sometimes these multinucleated giant cells can be demonstrated in BAL-fluid of sarcoidosis patients. It is generally believed that these cells in granulomata are fused mononuclear phagocytes (1,2,31,32). Cytokines produced by antigen or interferon stimulated lymphocytes are thought to lead to polykaryon formation of macrophages. Recently it has indeed been demonstrated that interleukin 4 (IL-4) induces the formation of multinucleated giant cells *in vitro* (32).

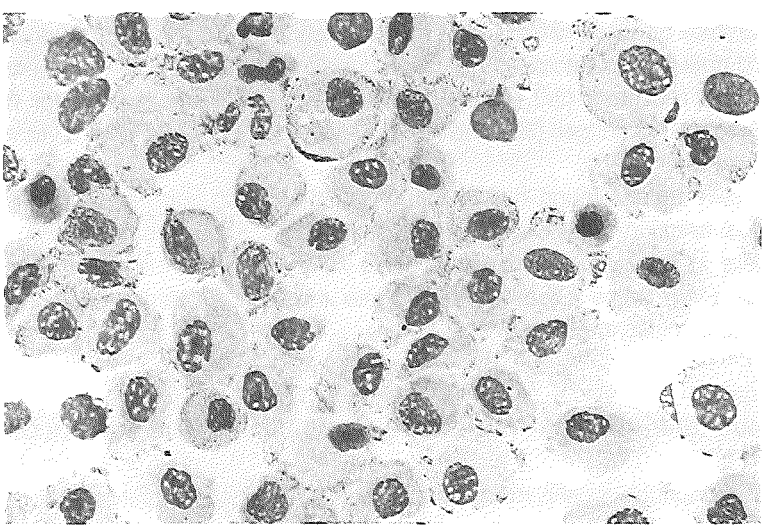

Figure 1. Alveolar macrophages in BAL fluid. Note the heterogeneity with regard to cell size.

Mononuclear cells migrated from the peripheral blood (PB) into the alveolar structures may become activated by bacterial products or T lymphocyte derived macrophage activating cytokines. Activation of alveolar macrophages may lead to secretion of harmful enzymes like collagenase and elastase which can damage the lung.

In addition, activated alveolar macrophages may alter local vascular permeability and recruit monocytes and plasma-components to the alveolar space. These newly recruited cells become activated and further enhance the ongoing inflammation.

Dendritic cells

The term dendritic cell is used to describe a group of HLA-DR$^+$ monocytic cells, that act as accessory cells in immune responses (34,35). This group comprises the above mentioned Langerhans cell in the skin, the interdigitating cell present in lymph nodes, spleen and thymus, and the veiled cells in lymph (36). They are called dendritic cells because they show many long cytoplasmic protrusions. Through active movements of these protrusions dendritic cells can form cellular clusters with T and B cells creating a microenvironment for optimal T- and B cell activation (35,36).

Langerhans cells present in the epidermis are characterized by specialized granules (the tennis racket-shaped Birbeck granules (37)). These cells are believed to carry antigens and migrate via the afferent lymphatics (where they appear as 'veiled' cells) into the paracortex of the draining lymph nodes where they interdigitate with T cells (38). These interdigitating cells present antigen to specific T lymphocytes.

Not only the morphology, but also the cytochemistry and immunophenotype of dendritic cells differ from alveolar macrophages (34). Dendritic cells contain less endocytic vesicles and lysosomes than macrophages. They do not stain for myeloperoxidase or acid phosphatase in the cytoplasm, although an eccentric nuclear acid phosphatase staining is usually present (34). They can therefore be discriminated from mononuclear phagocytes in tissue sections by the use of an acid phosphatase staining. They are abundant in HLA-DR and Fc receptors and lack mannosyl-fucosyl receptors (34). Active dendritic cells are clearly positive for RFD1 which identifies a class II MHC antigen (39), and for Ki-M4 (40,41). They show monocytic makers only in a low percentage.

Dendritic cells are also found in granulomata (42,43) and may play a role in maintaining the granulomatous reaction. Granulomata in Crohn's disease have been shown to contain epithelioid cells in the centre, surrounded by a zone of T lymphocytes, intermingled with dendritic cells (44). These observations indicate that exaggerated antigen handling and presentation takes place in granulomata.

3.2 MONONUCLEAR PHAGOCYTES IN THE DEVELOPMENT OF GRANULOMATA

The small mononuclear cells, macrophages, epithelioid and giant cells within granulomata, belong to the mononuclear phagocyte system (1,2). In granulomata of various etiology such as in sarcoidosis, tuberculosis and schistosomiasis, small monocyte-like cells evolve into

macrophages which in turn develop into epithelioid cells and giant cells (45-50). Active granulomata with a high turnover of cells are maintained by migration of newly recruited monocytic cells (45,46) and by local proliferation (51). In contrast, granulomata with a low turnover do not show intense migration and proliferation. The micro-organisms which cause these granulomata usually induce a strong delayed type hypersensitivity response in the host (52-54). The subsequent appearance of T cells within the granulomata leads to the secretion of various cytokines.

Activated macrophages within granulomata have usually been exposed to certain organic or inorganic substances and/or cytokines produced by activated T lymphocytes. Therefore mononuclear phagocytes in granulomata have the properties of inflammatory macrophages. They secrete IL-1 (55-57), TNF (58-60), IFN-γ (61) and CSF-1 (9,12). These factors promote the accumulation and activation of mononuclear phagocytes within the granulomas and in addition increase the lymphocyte population (62-67). The appearance of $CD8^+$ T lymphocytes can dampen both the extent and duration of the granulomatous reaction by suppressing $CD4^+$ T lymphocytes (68). This may be one of the explanations why EAA patients with a predominance of $CD8^+$ T lymphocytes usually do not develop granulomata early in the disease in contrast to patients with sarcoidosis in whom a predominance of $CD4^+$ T lymphocytes is often found. Other signs of monocytic activation are expression of receptors for IL-2 (59,60,70) recognized by anti-Tac monoclonal antibody, increased expression of HLA-DR antigens (71-73) and the ability to present antigen to T lymphocytes (74).

In sarcoidosis, in which an etiologic agent has not been demonstrated, alveolar macrophages present antigen more effectively than normal (75,76) and they also produce more IL-1 (55). These two factors may result in activation of T lymphocytes. It has indeed been demonstrated that this may be a local process. Local T lymphocytes express IL-2 mRNA transcripts while blood T lymphocytes generally do not. Thus the transcription of the IL-2 gene is compartmentalized (77). Expression of the IL-2 gene is rapidly turned off when the lung T cells are placed in culture. Thus activation of the IL-2 gene is not a constitutive property of T cells accumulating at sites of inflammation (77). Mononuclear phagocytes may follow similar rules, since it has been demonstrated that alveolar macrophages from sarcoid patients secrete TNF-α and IL-1 whereas monocytes from PB do not produce these cytokines (78).

TNF-α has profound effects upon other cell types. It enhances chemotaxis for leukocytes and macrophages (79,80), promotes leukostasis (80) and induces reactive oxygen metabolites (81). These properties make it an important mediator in granulomatous inflammation. The increased production of IFN-γ by lymphocytes and mononuclear phagocytes in granulomata may have considerable impact since *in vitro* studies showed that IFN-γ can activate macrophages (82-86) and can promote fusion of alveolar macrophages to form multinucleated giant cells (87), a process which also occurs *in vivo*. In addition, IFN-γ *in vitro* induces IL-2 receptors on monocytic cells and promotes the production of IL-1 (88). If this is also the case *in vivo*, IFN-γ may amplify the interactions between mononuclear phagocytes and T lymphocytes within granulomata.

The function of mononuclear phagocytes in granulomata is dependent on the stage of maturation and activation (1-4). For example, the production of TNF-α is maturation stage dependent. It has been demonstrated that mature alveolar macrophages are potent producers of TNF-α, in contrast to PB monocytes (89-91). An important feature of mononuclear

phagocytes in granulomatous inflammation is their capacity to function as accessory cells for lymphocyte proliferation. This is also differentiation stage dependent (92-94). In addition, functions of mononuclear phagocytes may be altered by inhalation of certain environmental chemicals such as silica, asbestos etc. (95). For a better understanding of the pathogenesis of granulomatous inflammations it is necessary to obtain more insight into the phenotypic and functional heterogeneity of the mononuclear phagocytes involved. To this end, the availability of monoclonal antibodies that define discrete stages of monocyte and macrophage differentiation and maturation is extremely useful (92-101).

3.3 MONONUCLEAR PHAGOCYTES IN FIBROTIC LUNG DISEASES

Mononuclear phagocytes play an important role in lung fibrosis through the release of factors that cause accumulation of mesenchymal cells such as fibroblasts, and subsequent synthesis of connective tissue (so called extra-cellular matrix) molecules such as fibronectin, collagen, elastin and proteoglycans. Alveolar macrophages can release several factors with positive growth activity for mesenchymal cells, including platelet derived growth factor (PDGF) (102-104), TNF-α and TNF-β (89,90), alveolar macrophage derived growth factor (AMDGF) (105,106), transforming growth factor α and β (TGF-α and TGF-β) (107,108) and fibronectin (109,110). These factors induce fibroblasts to migrate to sites of inflammation. Subsequently, these fibroblasts replace normal tissue by fibrotic scarring.

PDGF is a dimeric molecule consisting of two chains (A and B) that occurs as homodimer or heterodimer (111-113). It is known as a chemotactic and mitogenic factor for fibroblasts as well as neutrophils and monocytes. Normal alveolar macrophages spontaneously express the c-*sis* proto-oncogene coding for PDGF β-chain and release small amounts of PDGF. In contrast, alveolar macrophages from patients with interstitial pulmonary fibrosis release increased amounts of PDGF (102-104). In interstitial and alveolar injury monocytes enter the tissue, become macrophages and possibly secrete PDGF-like substances. PDGF deposited in the interstitium can then attract fibroblasts and smooth muscle cells. Subsequently PDGF binding to fibroblasts and smooth muscle cells induces synthesis of new extracellular connective tissue by these cells (112).

The release of TNF-α by alveolar macrophages may have an important impact in fibrotic lung diseases since it stimulates neutrophil migration across the vascular endothelium (114-116) and in addition promotes fibroblast growth (117-119).

AMDGF is produced constitutively by alveolar macrophages from patients with IPF (105,106). It stimulates proliferation of fibroblasts in conjunction with fibronectin and PDGF.

The TGF-β produced by activated alveolar macrophages (108) appears to play a role in inflammation and fibrosis. TGF-β induces monocyte chemotaxis and growth factor production (120), is chemotactic for fibroblasts (121) and stimulates the production of extracellular matrix components (fibronectin and collagen) by fibroblasts (122-125).

The release of these growth factors by activated alveolar macrophages may have important implications, not only in terms of stimulation of fibroblast growth but also in expression of adhesion molecules necessary for interaction of fibroblasts with other cells and with the extracellular matrix. It has been demonstrated that several cytokines known to be released in

interstitial lung diseases (IL-1, TNF-α, IFN-γ) induce the expression of CD54 (ICAM-1), an intercellular adhesion molecule, on fibroblasts which facilitates their interaction with other cells (126,127). Another peptide which can facilitate the adhesion of fibroblasts to extracellular matrix is fibronectin. It can be synthesized by different cell-types, including mononuclear phagocytes (108,109) and bronchial epithelial cells (128). It is chemotactic for fibroblasts and promotes the adhesion of fibroblasts to the extracellular matrix (108,109). Recently it has been demonstrated that the expression of the fibronectin gene is under control of several growth factors including TGF-β and PDGF (129). The activation of fibroblasts may result in an increased production of collagen leading to fibrosis of the extracellular matrix (130).

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CHAPTER 4

INTRODUCTION TO THE EXPERIMENTAL WORK

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Mononuclear phagocytes play a central role in interstitial lung diseases because of their ability to present antigen to lymphocytes, their capacity to synthesize cytokines and to destroy extracellular matrix. Mononuclear phagocytes arise from bone marrow precursors, differentiate into circulating monocytes and after migration (e.g. into the alveoli) further mature into macrophages which, depending on their location and state of activation, exhibit extensive morphologic, functional and phenotypic heterogeneity.

One of the aims of the study described in this thesis was to investigate the maturation of human peripheral blood monocytes (PBM) into mature alveolar macrophages (AM). In addition we wanted to investigate whether AM with a different morphology also exhibit a different immunologic phenotype and whether the characteristic immunologic phenotype of AM could be induced by culturing PBM in the presence of particular cytokines. Furthermore, we tested the hypothesis that in different interstitial lung diseases the interaction of monocytes with an inflammatory stimulus directs them into macrophages with a particular inflammatory phenotype.

Chapter 5 describes the immunologic markers of cells usually found in BAL-fluid and their recently developed nomenclature. In addition it describes which immunologic markers are expressed in the various lymphoid and myeloid differentiation stages. The relevance of immunologic marker analysis of leukocytes from BAL fluid, PB and *in vitro* stimulation assays for diagnosis and follow-up of interstitial lung diseases is also discussed in this chapter.

Chapter 6 describes the differences in immunophenotype between AM with an immature and mature morphology and between PBM and BAL macrophages. In addition, the maturation-associated differences were studied in cultured elutriator-purified monocytes.

Chapter 6, section 1 describes the immunologic phenotype of PBM and AM from BAL fluid using a broad panel of mAb. The expression of the immunologic markers on PBM was analyzed by flowcytometry using a FACScan, while the expression of the different markers on BAL macrophages was investigated by fluorescence microscopy. The data indicate clear differences in immunologic phenotype between PBM and AM. In addition it was found that AM with the morphology of PBM also exhibit an immunologic phenotype resembling PBM. Mature macrophages, on the other hand, show a diminished expression of several markers (e.g. CD14 and Monocyte-2) which are characteristic of PBM, while expression of another mononuclear phagocyte marker (e.g. RFD9) is increased.

Chapter 6, section 2 shows the expression of adhesion proteins on AM from smokers and non-smokers. While the absolute numbers of AM are 5 fold increased in smokers, the results of this study suggest that the proportion of $CD11^+/CD18^+$ AM was decreased as compared with non-smokers.

The experiments described in **Chapter 6, section 3** aimed to investigate whether highly

purified PBM cultured *in vitro* in the presence of particular cytokines, can give rise to mononuclear phagocytes with an AM phenotype. GM-CSF and IL-4 were found to be especially appropriate stimuli for inducing this phenotype as far as could be determined with our panel of monoclonal antibodies, while glucocorticoids prevented the changes in expression of the studied antigens.

Chapter 7 describes the immunologic phenotype of mononuclear phagocytes, lymphocytes and granulocytes in PB and BAL fluid in several interstitial lung disorders. These studies were undertaken in order to investigate whether or not differences in immunophenotype of mononuclear phagocytes were present. Subsequently, differences in cell samples were evaluated for their possible diagnostic value.

Chapter 7, section 1 describes the expression of monocytic surface antigens in PB and BAL fluid from patients with interstitial lung diseases. The observations in this study suggest an increased influx of blood monocytes into the alveoli in these interstitial lung disorders. Most interestingly, phenotypic differences were found between the BAL macrophage populations of the various interstitial lung diseases. The intensity of the expression of the various markers on PBM was evaluated using FACScan analysis. Using this method differences in intensity of expression of several markers was detected. It appeared that particularly CD68(Ki-M6) associated with generation of oxygen radicals during the respiratory burst tended to be elevated on monocytes in PB and macrophages in BAL fluid of IPF patients.

Chapter 7, section 2 describes the expression of cell surface adhesion glycoproteins on blood monocytes and AM in patients with interstitial lung diseases. The data presented in this chapter demonstrate that the extent of expression of these molecules on blood monocytes was different between the disease groups evaluated. AM also showed differences in the expression of these antigens, particularly in the expression of CD11b/CD18.

Chapter 7, section 3 describes the presence of an alveolitis in patients with extrapulmonary sarcoidosis. The data presented in this section demonstrate that a marked discrepancy can occur between chest X-ray abnormalities and the presence of an alveolitis as determined by immunologic marker analysis. In several cases analysis of BAL cells indicated a subclinical alveolitis. Particularly in patients with a normal chest X-ray, BAL appeared a useful tool to detect this alveolitis.

In **Chapter 7, section 4** data are presented on immunophenotyping leukocytes from the BAL fluid of patients with ocular sarcoidosis. In the majority of cases BAL analysis showed a subclinical alveolitis. Therefore, immunophenotyping of BAL leukocytes can be an important additive tool in the diagnosis of suspected ocular sarcoidosis.

The data obtained in our studies are discussed in **Chapter 8** in the perspective of literature data. Basically, PBM constitute a cell population which migrates, either randomly or selectively, into the interstitium and the alveoli. Under the influence of local factors, the monocytes mature into AM. Interaction of mononuclear phagocytes in the lungs with an inflammatory stimulus affects their immunologic phenotype.

CHAPTER 5

IMMUNOLOGIC MARKERS AND THEIR APPLICATION IN PULMONARY INFLAMMATION

CHAPTER 5

IMMUNOLOGIC MARKERS AND THEIR APPLICATION IN PULMONARY INFLAMMATION*

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INTRODUCTION

Macrophages, lymphocytes and granulocytes play an important role in local immunity and inflammatory reactions in the bronchi, bronchioli and alveoli. In inflammatory conditions there is an increased influx of cells from peripheral blood (PB) to the interstitium and alveoli. In addition to the increase in total cell-numbers, marked shifts in cell populations usually occur in the interstitium and alveoli. Analysis of cells in bronchoalveolar lavage (BAL) fluid can give insight into these shifts. Leukocytes in BAL fluid (monocytes/macrophages, lymphocytes and granulocytes) can be recognized morphologically, but the various subpopulations of monocytes/macrophages and lymphocytes in BAL fluid and PB can only be recognized by means of immunologic marker analysis.

Immunologic markers are (glyco)proteins in the cell membrane, cytoplasm or nucleus. These (glyco)proteins are called immunologic markers because their expression is detectable by various immunologic techniques, such as immunofluorescence staining, rosette-, immunogold- and immuno-enzyme techniques. Most immunologic markers can be recognized by a specific (monoclonal) antibody.

We shall discuss the international nomenclature for monoclonal antibodies (mAb) against immunologic markers. In addition we shall indicate which immunologic markers become expressed during lymphoid and myeloid differentiation. Finally, we shall discuss the clinical relevance of immunologic marker analysis of cells in BAL fluid and PB, and the general use of BAL analysis in pulmonary inflammation.

IMMUNOLOGIC MARKERS AND THE CD NOMENCLATURE

Most immunologic markers represent differentiation antigens. The majority of these are

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* *This manuscript is submitted for publication.*

expressed in more than one differentiation stage, but a combination of markers can be specific for a particular differentiation stage (1-3).

For the detection of immunologic markers predominantly mAb are used, although for some markers conventional antisera or other detection methods are available. During the last ten years an overwhelming number of mAb has been produced directed against various leukocyte antigens. To create clearness and order within the large panels of these mAb an international nomenclature has been designed during four Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; and Vienna, 1989). A large part of mAb against leukocyte antigens have been grouped into antibody clusters based on their reactivity with the same antigen (4-7). Each cluster has received its own code, the so-called CD ("cluster of differentiation" or "cluster designation") code. In this way mAb against the T cell antigens T4 and T8, for instance, have received the codes CD4 and CD8, respectively, while antibodies against the monocyte/macrophage marker gp55 received the code CD14.

In principle, CD codes are only assigned if three or more mAb from different laboratories recognize the same antigen, of which the molecular mass as well as the expression pattern has been determined. However, in a few clusters only two antibodies are present; in some other clusters antibodies of only two laboratories are available and sometimes information about the recognized immunologic marker is lacking (e.g. the molecular mass). In such cases the CD code is supplied with the letter "w" (workshop), which indicates that it concerns a preliminary clustering (4-7).

The name of the mAb which has been used to detect a specific CD molecule, can be placed in parentheses behind the CD code. In this way, the code CD3 can be extended to CD3(OKT3), CD3(Leu-4), CD3(VIT-3) or CD3(UCHT1), depending on the antibody used. Such a supplementation is important for optimal comparison of data from different laboratories, since mAb of the same cluster may slightly differ in their reaction pattern. For instance, the majority of CD3 antibodies are able to recognize CD3-ϵ chains at the cell surface of viable cells, but cytoplasmic CD3 chains (CyCD3) in fixed cytocentrifuge preparations or frozen sections can be recognized by only a few CD3 antibodies (8). Apparently not all epitopes on the CD3-ϵ chain are resistant to denaturation by acetone or ethanol fixation.

The expression patterns of several relevant immunologic markers during lymphoid and myeloid differentiation are given in Figures 1 and 2. Additional information concerning these immunologic markers (CD codes, alternative names, molecular mass) is summarized in Table 1 (see references 2 and 3 for details).

Lymphoid markers

The lymphoid system consists of a B and T cell lineage. The enzyme terminal deoxynucleotidyl transferase (TdT) is present in the nucleus of immature B and T cells, but is absent in more mature differentiation stages (9,10). Early in B cell differentiation, in the pre-B cell stage, weak cytoplasmic expression of μ immunoglobulin heavy chains (weak Cyμ) occurs (11), while more mature B cells express both immunoglobulin heavy and light chains on their cell surface membrane (SmIg) (12). Finally, the plasma cell strongly expresses cytoplasmic immunoglobulin (CyIg) heavy and light chains (13).

TABLE 1. Immunologic markers (CD codes, alternative names and molecular mass in kDa) which can be used for immunophenotyping of cells in the various stages of lymphoid and myeloid differentiation.

Immature lymphoid marker	
TdT*	terminal deoxynucleotidyl transferase (expression on nuclear membrane)
B cell markers	
CD10	common ALL antigen (gp100)
CD19	pan-B cell antigen (gp90)
CD20	B cell antigen (p35)
CD21	complement (C3d) receptor (CR2) (gp140)
CD22	pan-B cell antigen (gp135)
CD37	B cell antigen (gp40-52)
FMC7*	B cell antigen (gp105)
weak Cyμ	weak cytoplasmic expression of Ig heavy chain μ in pre-B cells
SmIg	surface membrane expression of Ig (heavy and light chains)
CyIg	cytoplasmic expression of Ig (heavy and light chains)
T cell markers	
CD1	T6 antigen (gp43, gp45, gp49)
CD2	T11 antigen; leucocyte function antigen 2 (LFA-2) (gp50)
CD3	T3 antigen (consists of at least five chains) (gp16-25)
CyCD3	cytoplasmic expression of CD3 protein chains
CD4	T4 antigen (gp60)
CD5	T1 antigen (gp67)
CD7	Tp41 antigen (gp41)
CD8	T8 antigen (gp32)
TcR-CD3	expression of the T cell receptor-CD3 complex on the cell surface
NK cell markers	
CD56	neural cell adhesion molecule (NCAM) (gp220/135)
CD57	human NK cell antigen (HNK-1) (gp110)
Pan-myeloid markers	
CD13	pan-myeloid antigen (gp150)
CD33	pan-myeloid antigen (gp67)
MPO*	myeloperoxidase (gp60/12)
Myeloid-granulocytic markers	
CD15	X hapten
CD67	granulocytic antigen (p100)
Monocyte-macrophage markers	
CD14	monocytic antigen (gp55)
CD68	macrophage antigen (gp110)
RFD9*	macrophage antigen (gp25)
Erythroid markers	
H antigen	backbone of ABO blood group proteins
GpA	glycophorin A (gp41)
Megakaryocyte-platelet markers	
CD41	GPIIb-GPIIIa complex (Glanzmann antigen) (gp145/115)
CD42	GPIX-GPIb complex (Bernard-Soulier antigen) (gp143/22/20)
CD61	GPIIIa (gp115); associated with GPIIb (see CD41)
Non-lineage-restricted markers	
CD9	p24 antigen
CD11b	complement (C3bi) receptor (CR3)
CD16	low affinity Fc receptor for IgG (FcγRIII) (gp50-65)
CD25	interleukin-2 receptor (IL-2R) (gp55)
CD34	precursor antigen (gp115)
CD38	T10 antigen (gp45)
HLA-DR	MHC class-II antigen (gp29/34)

* Non-clustered immunologic markers

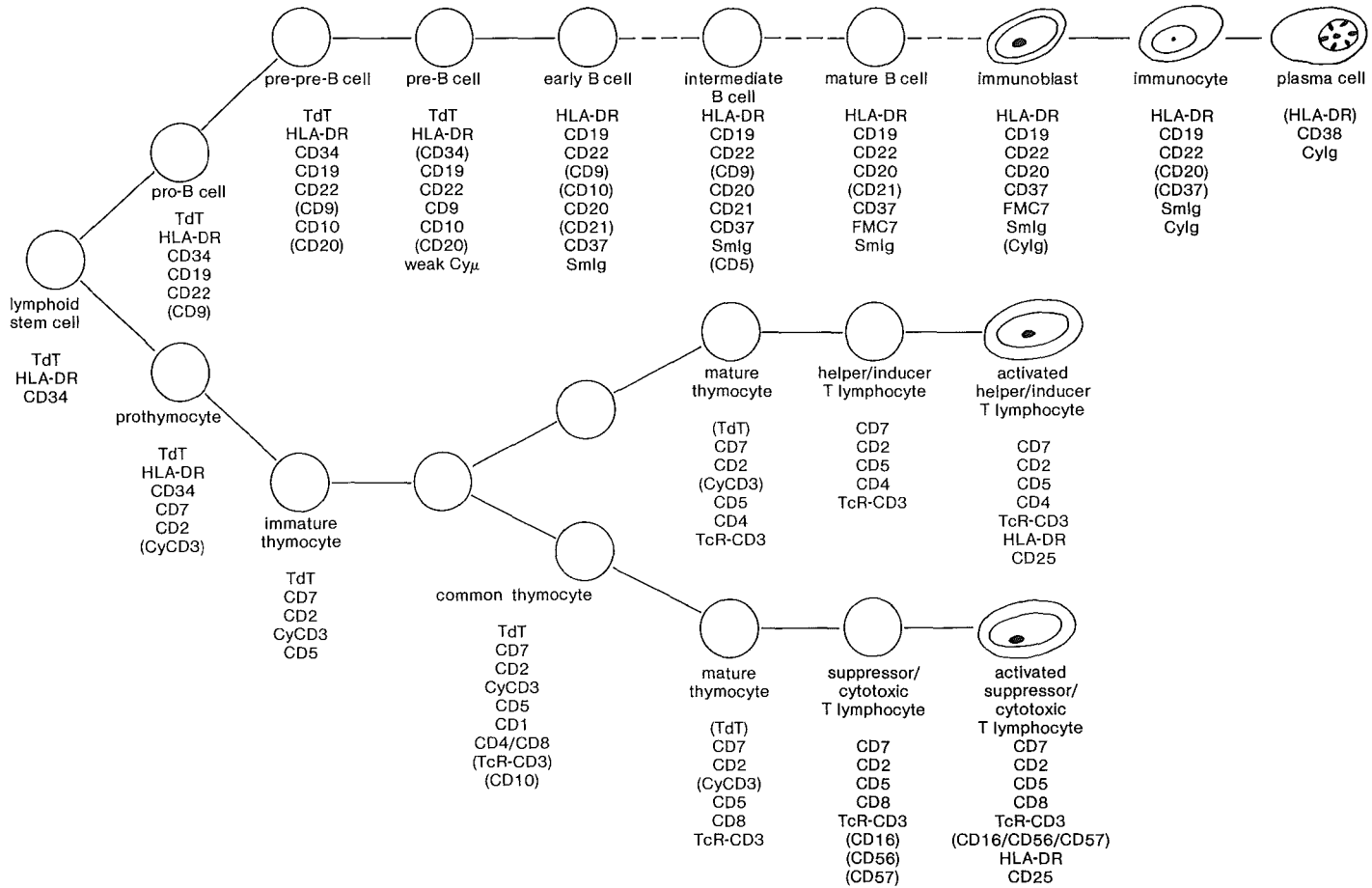

Figure 1. Hypothetical scheme of lymphoid differentiation. The expression of the various immunologic markers by cells in the different stages of lymphoid differentiation is indicated. The markers in parentheses are not always expressed (adapted from Van Dongen et al., see reference 3). Additional information concerning the immunologic markers is given in Table 1.

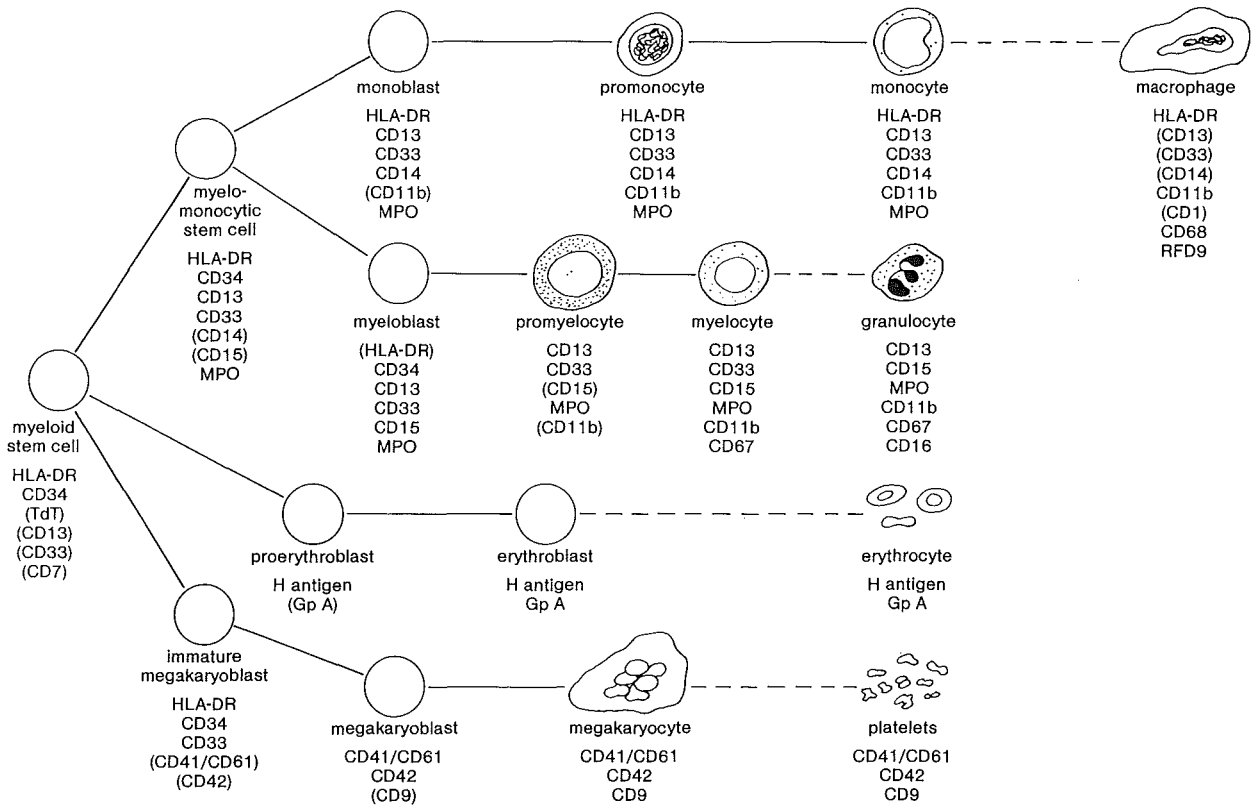

Figure 2. Hypothetical scheme of myeloid differentiation. The expression of the various immunologic markers by cells in the different stages of myeloid differentiation are indicated. The markers in parentheses are not always expressed (adapted from Van Dongen et al., see reference 3). Additional information concerning the immunologic markers is given in Table 1.

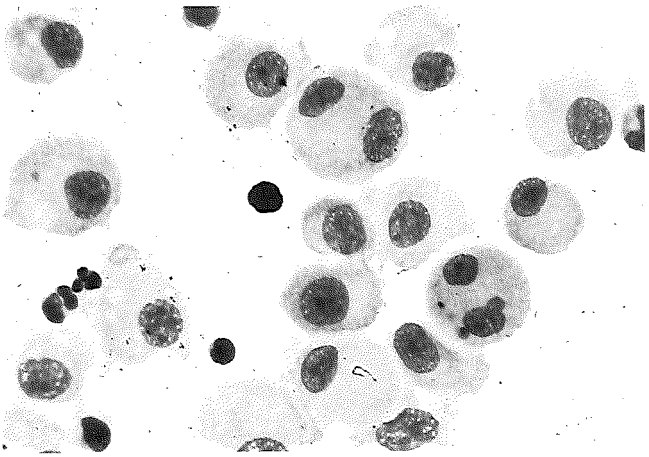

Figure 3. Normal cell differential in BAL fluid. Giemsa staining. Macrophages ± 93%, lymphocytes ± 6%, granulocytes ± 1%.

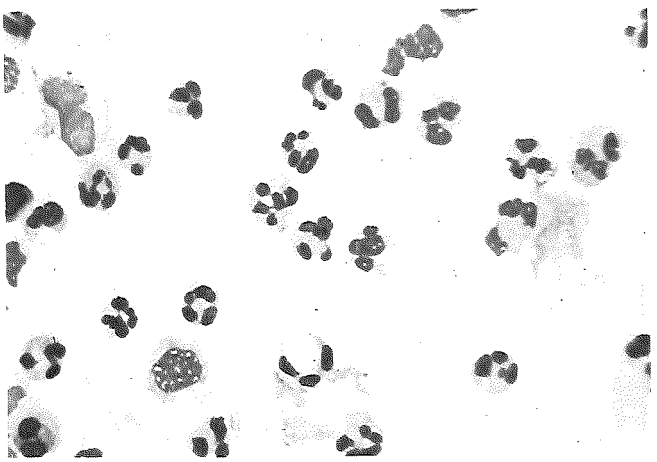

Figure 4. Cells in BAL fluid from a patient with idiopathic pulmonary fibrosis. Giemsa staining. The percentages of neutrophils and eosinophils are usually increased. A high percentage of lymphocytes may indicate a better response to therapy.

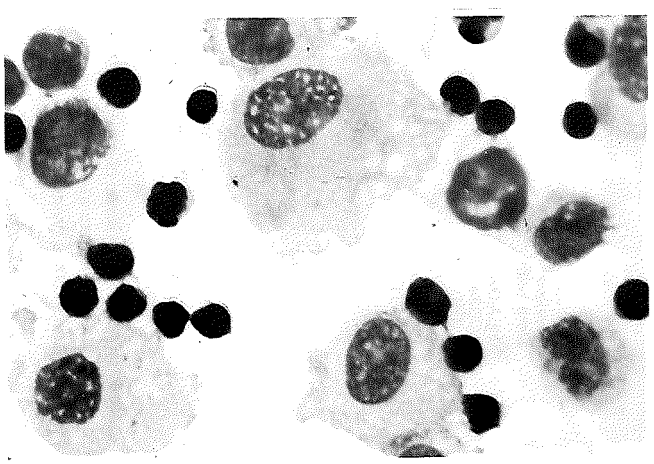

Figure 5. Cells in BAL fluid from a sarcoidosis patient. Giemsa staining. The percentage of T lymphocytes may be increased (usually in stage I and II) with an increased CD4/CD8 ratio (> 1.5, usually > 3.0).

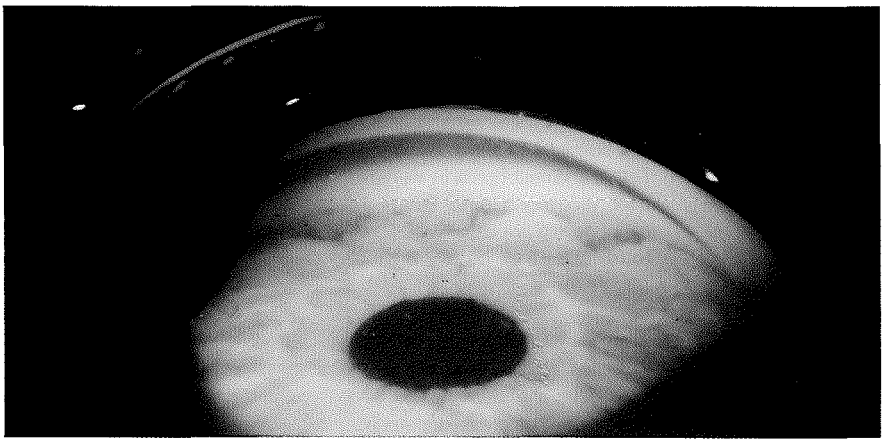

Figure 6. Granulomatous noduli in the anterior eye chamber in a patient with sarcoidosis.

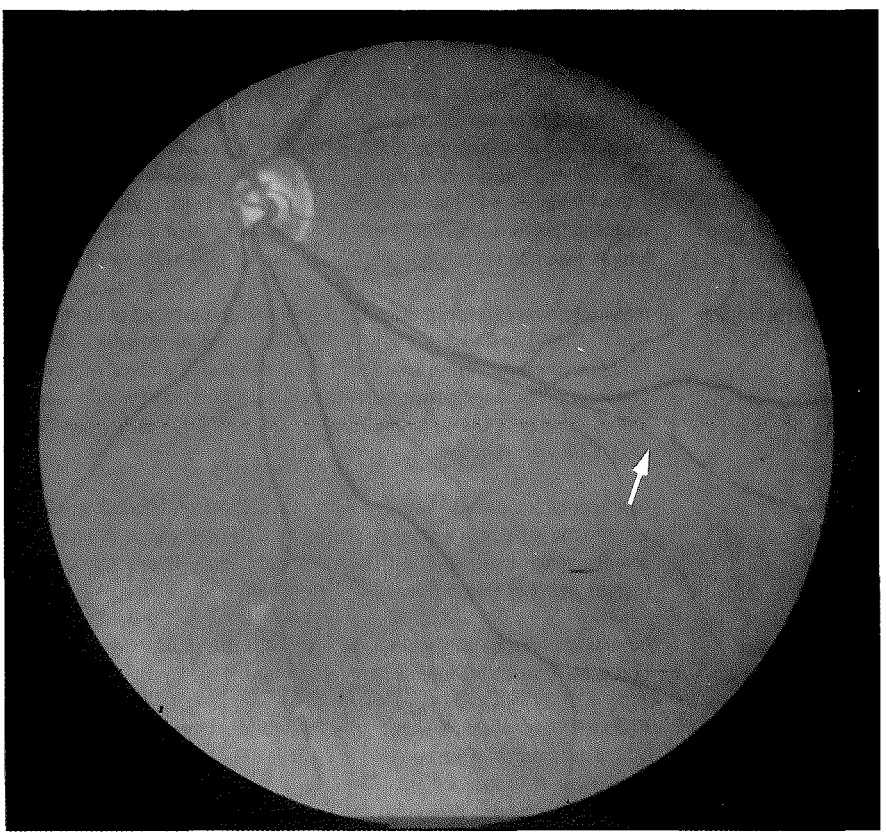

Figure 7. Vasculitis with "candle-wax" appearance in the retina in a patient with sarcoidosis.

TABLE 2. Use of bronchoalveolar lavage (BAL) analysis in pulmonary inflammation.*

Disease	Major findings in BAL fluid
Healthy individuals	The normal differential cell count shows macrophages (~93%), lymphocytes (~6%) and neutrophils (~1%) while approximately 5-10 x 10^6 cells are present after recovery (50 to 60%) of 200 ml instilled saline solution. The T lymphocytes are usually $CD4^+$ or $CD8^+$ with a CD4/CD8 ratio comparable to PB (0.8 - 3.0). Usually B lymphocytes and plasma cells are not present (Figure 3).
Idiopathic pulmonary fibrosis	Alveolitis with increased numbers of neutrophils and eosinophils (Figure 4). Activated alveolar macrophages producing growth factors for fibroblasts, fibronectin and chemotoxins (probably stimulated by immune-complexes). Patients with increased percentages of lymphocytes may show a better response to therapy.
Sarcoidosis	With alveolitis, T lymphocytes are increased ($CD3^+$,$CD5^+$), especially in stages I and II (Figure 5). Predominantly $CD4^+$ T lymphocytes are increased resulting in a high CD4/CD8 ratio (>3.0). In PB sometimes a low CD4/CD8 ratio (<0.8) is found, probably caused by compartmentalization of $CD4^+$ T lymphocytes in the lung. In 50% of patients in stage 0 or in cases of extrapulmonary sarcoidosis (Figure 6,7) the T lymphocytes and CD4/CD8 ratio are increased. In stage III often increased percentages of neutrophils and eosinophils with normal or increased percentages of T lymphocytes are found. The T lymphocytes are activated ($HLA\text{-}DR^+$). Increased numbers of $CD14^+$/$Monocyte\text{-}2^+$ macrophages are found, which are activated and secrete angiotensin converting enzyme (ACE) and interleukin 1 (IL-1). Proliferating T lymphocytes and macrophages ($Ki\text{-}67^+$, $BrdU^+$) are detectable.
Hypersensitivity pneumonitis	In symptomatics and asymptomatics increased percentages of T lymphocytes ($CD3^+$,$CD5^+$) are found. There is a tendency for an increase of $CD8^+$ T lymphocytes resulting in a low CD4/CD8 ratio (<0.8). Shortly after provocation (<4 hours) increased percentages of neutrophils and eosinophils are usually found. After 24 hours an increased percentage of T lymphocytes with a low CD4/CD8 ratio remains.
Histiocytosis X	Granulomatous disease with chronic course. Electron microscopy (EM) will identify the X-body in macrophages. Increased percentage (>4%) of $CD1^+$ alveolar macrophages (The CD1 antigen is usually found only on cortical thymocytes and Langerhans cells in the skin).
Eosinophilic pneumonia	Increased percentages (>50%) of eosinophils in BAL fluid. After treatment with corticosteroids cell differentials return to normal.
Alveolar proteinosis	With periodic acid-Schiff stain, fluid will stain and excessive surfactant globules in alveolar macrophages can be seen. These cells with large phagolysosomes have poor microbial killing. Diagnosis can be made from fluid analysis. Periodic therapeutic lavage is useful.

TABLE 2. (continued).

Disease	Major findings in BAL fluid
Gastro-oesophageal reflux with aspiration	Alveolar macrophages contain lipid loaden cytoplasmic vacuoles (Figure 8).
Congestive heart failure	Large numbers of hemosiderin loaden macrophages are present.
Idiopathic pulmonary hemosiderosis	Nearly all macrophages contain hemosiderin and exhibit a blue color in Perl's staining (Figure 9). Strong membrane expression of CD68 antigen on alveolar macrophages (Normally this CD68 antigen is found in the cytoplasm of macrophages).
Primary biliary cirrhosis	Increased percentages of T lymphocytes. $CD4^+$ T lymphocytes increased resulting in a high CD4/CD8 ratio (>3.0). These changes can also be found in patients with a normal chest X-ray. The alveolar macrophages show morphologic signs of activation.
Acquired immunodeficiency syndrome	BAL fluid often yields Pneumocystis carinii on stain and cytomegalovirus in cultures. Lymphocyte CD4/CD8 ratio is low (< 0.8). Macrophage microbial activity probably impaired. These abnormalities may be present with a normal chest X-ray. It is not useful for diagnostic purposes to determine the CD4/CD8 ratio in BAL fluid.
Occupational disease (inorganic particles such as silica, asbestos, beryllium)	Inflammation often with neutrophils. Alveolar macrophages may contain visible particles that may be seen with light-microscopy (Figure 10) or with polarized light. EM even more sensitive. Macrophages and often lymphocytes increased in number. Type II pneumocytes recovered in silica exposure.
Drug hypersensitivity	Various inflammatory cells and often antigen-responsive T lymphocytes. In amiodarone pneumonitis, EM will identify alveolar macrophages with lamellar bodies.
Asthma	No distinctive celluiar profile, although eosinophils (~5%), macrophages, neutrophils and epithelial cells are increased. In addition to mast cells, mediators may come from alveolar macrophages and possibly epithelial cells (Figure 11). Late phase mediators may attract neutrophils.
Cigarette smoker	Mainly increased cell counts 5 to 10 times (macrophages and neutrophils). The macrophages contain more pigment than those of non-smokers.
Lung cancer	Cytologic specimens often positive for malignancy. Tumor cells are usually negative for hemopoietic cell markers but part of them can show reactivity with Ki-67.

** Adapted from Dr. H.Y. Reynolds (see reference 61). Bronchoalveolar lavage, In: Textbook of respiratory medicine, Murray, J.F. and Nadel, J.A. (Eds.) W.B. Saunders Company, Philadelphia, pp. 597-611, 1988.*

Figure 8. Cells in BAL fluid from a patient with gastro-oesophageal reflux with aspiration. Oil Red O fat staining. Alveolar macrophages contain lipid laden red-stained cytoplasmic vacuoles.

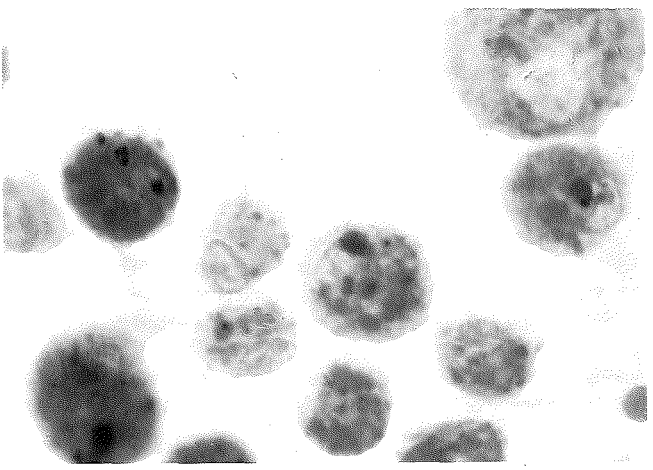

Figure 9. Cells in BAL fluid from a patient with idiopathic pulmonary hemosiderosis. Perl's staining. The hemosiderin laden macrophages exhibit a blue staining.

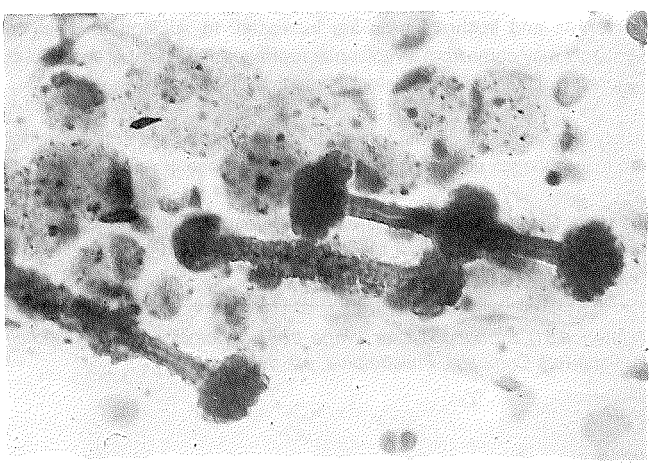

Figure 10. Asbestos bodies. The central asbestos fibres are not visible, but are surrounded by a string of protein and iron beads with bulbous expansion at both ends.

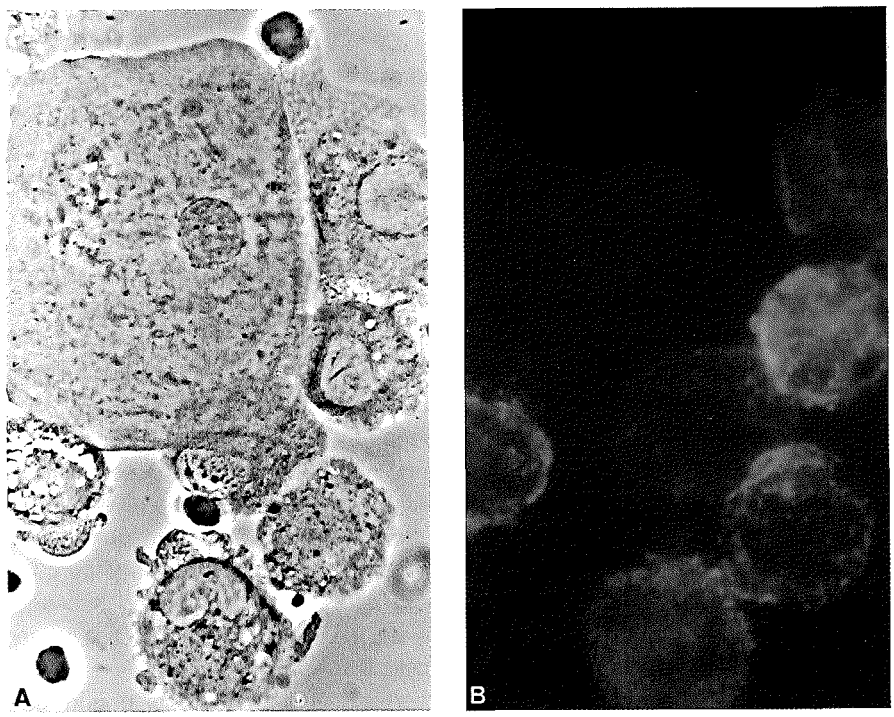

Figure 11. Immunofluorescence staining of alveolar macrophages in BAL fluid. (A) Phase contrast picture of cells present in bronchoalveolar lavage fluid. (B) Immunofluorescence staining for HLA-DR. The alveolar macrophages stain positive while the epithelial cell and the lymphocytes are negative.

Additional characterization of the various B cell differentiation stages can be performed by use of other B cell markers such as the pan-B cell markers CD19 and CD22 (14-16), the common acute lymphoblastic leukemia antigen (CALLA; CD10 antigen) and the B cell markers CD9, CD20, CD21, CD24, CD37 and FMC7 (14,15). The expression of the markers CD9 and CD24 is not restricted to the B cell lineage, but is also found in other differentiation lineages (6,7). The plasma cell is negative for the mentioned B cell markers, but does express the CD38 antigen (Figure 1) (15).

During T cell differentiation, several T cell markers appear on the cell surface membrane, resulting in the expression of many T cell antigens by functional T lymphocytes (8,10). The T cell markers CD2 and CD7 are expressed at the cell surface in virtually all stages of T cell differentiation (8,10), even the putative prothymocyte expresses these two immunologic markers (17). A valuable marker for immature T cells is the cytoplasmic expression of the CD3 antigen (CyCD3), while mature T cells express the CD3 antigen at their cell surface in close association with the T cell receptor (TcR-CD3 complex) (8,18). The expression of the various other T cell markers (such as CD1, CD4, CD5 and CD8) is indicated in Figure 1 (8,18,19).

NK cells are negative for the TcR-CD3 complex, but they do express the CD7 antigen and generally also the CD2 antigen. Most NK cells express the CD16 antigen (low affinity Fc

receptor for IgG; FcγRIII) and the CD56 antigen (neural cell adhesion molecule; NCAM) (20). A subpopulation of the NK cells also expresses the CD8 molecule and the CD57 molecule (21). The CD16, CD56 and CD57 molecules may also be expressed by a subpopulation of $CD3^+$ T lymphocytes (Figure 1). The CD16 antigen is also expressed by granulocytes (22).

Myeloid markers

The mAb which recognize surface determinants of mononuclear phagocytes made clear that the mononuclear phagocyte system is heterogeneous. Because mononuclear phagocytes probably originate from a common bone marrow precursor, the diversity in immunophenotype suggests that local factors influence the expression of immunologic markers (23). The available panels of mAb against myeloid antigens are useful to study the phenotypic heterogeneity of the cells in the various myeloid differentiation lineages (1-7).

Analogous to lymphoid differentiation also during myeloid differentiation several markers appear on the cell membrane while others disappear. Nearly all cells of the myelo-monocytic differentiation lineages (both immature and mature cells) are positive for the markers CD13 and an CD33 (3,23,24). In contrast to CD13, CD33 is not present on granulocytes (Figure 2). The CD34 antigen is present on precursors of myeloid and lymphoid cells and subsequently disappears from the more mature cells (25). In contrast, myeloperoxidase (MPO) is present on the majority of cells of the granulocytic and monocytic lineages (Figure 2) (26).

The CD13 antigen has been shown to represent the membrane bound enzyme aminopeptidase-N (gp150). It is likely that CD13 is involved in the transmembrane regulation of cellular processes, since this molecule consists of a transmembrane hydrophobic segment and an extracellular domain (27).

Cells of the monocytic lineage express the CD14 antigen (23,24). The human CD14 gene is located on chromosome 5 in a region encoding several growth factors and receptors, including granulocyte monocyte-colony stimulating factor (GM-CSF), interleukin (IL-)3, IL-4, IL-5, colony stimulating factor (CSF-)1, CSF-1 receptor and platelet derived growth factor (PDGF) receptor (28). It has been demonstrated that the GM-CSF and macrophage colony stimulating factor (M-CSF) induced proliferation of PB monocytes can be inhibited by CD14 antibodies (29). GM-CSF plays a role in the monocyte/macrophage differentiation of the human monoblastic U-937 cell line (30), but the precise role of the CD14 molecule in the differentiation and activation of mononuclear phagocytes is still unknown.

More immature monocytic cells are positive for the Monocyte-2 antigen (31,32). Macrophages become negative for this antigen, but may express the markers CD68 and RFD9 (33,34). The CD68 macrophage antigen is mainly expressed in the cytoplasm. In addition, mAb that discriminate between phagocytic cells (monocytes/macrophages) and immune accessory cells (dendritic cells) are now available (33-37). Dendritic cells are positive for Ki-M4 and RFD1 (36,37).

Cells of the granulocytic lineage are positive for the CD15 antigen, while more mature granulocytic cells also express CD16 and CD67 (6,7). The CD15 surface marker is involved in chemotaxis, phagocytosis and induction of a respiratory burst (38,39). The expression of the CD67 molecule is increased upon activation (7).

The H antigen, which represents the backbone of the ABO blood group proteins, and glycophorin A (GpA) can be used as markers for cells of the erythroid lineage (40,41). Cells of the megakaryocytic-platelet lineage can be recognized by use of mAb against the platelet-specific glycoproteins (GP), such as the whole GPIIb-GPIIIa complex (CD41 antigen), GPIX chain (CD42a antigen), GPIb chain (CD42b antigen) and GPIIIa chain (CD61 antigen) (Figure 2) (42,43).

Function of immunologic markers

During the past years impressive progress has been made in the analysis of the function of several immunologic markers. The LFA-1/CR3/p150,95 family (CD11/CD18 molecules) is a good example of recently obtained detailed insight into the structure and function of a family of glycoproteins expressed by lymphoid and myeloid cells. These membrane receptors play an essential role in adhesion of circulating cells to endothelium in inflamed tissues and thus affect the entry of these cells into sites of inflammation (44,45). These molecules are expressed by monocytes, granulocytes and lymphocytes, share an identical β subunit (CD18 antigen) and are distinguished by their α subunits (CD11a, CD11b and CD11c antigens) (44). MAb directed against the CD11 and CD18 glycoproteins inhibit inflammatory cell recruitment (44-47). In inflammatory conditions the expression of these molecules is upregulated (44-46,48). In addition, after stimulation endothelial cells express the intercellular adhesion molecule-1 (ICAM-1; CD54 antigen) on their surface which is a ligand for CD11a/CD18 (49). Pro-inflammatory cytokines usually found in interstitial lung disorders such as IL-1, interferon(IFN)-γ and tumor necrosis factor(TNF)-α dramatically increase the expression of the CD54 antigen on endothelial cells (50,51), thereby mediating a stronger adhesion of CD11a/CD18$^+$ cells to the endothelium.

Another important receptor present on mononuclear phagocytes is CD35, which is the receptor for the complement component C3b (6). CR1 binds immune complexes and may play a role in lung disorders which accompany autoimmune diseases, since in collagen-vascular diseases local immune complexes have been demonstrated in BAL-fluid (52,53). Antibodies against the complement receptors CR2 and CR3 are grouped into the clusters CD21 and CD11b/CD18, respectively (5-7).

A third family of receptors on mononuclear phagocytes are the Fc receptors, which are involved in phagocytosis and endocytosis. This receptor system is able to bind the Fc portion of immunoglobulins. Antibodies against the three types of Fc receptors for IgG, FcγRI, FcγRII and FcγRIII have received the codes CD64, CDw32 and CD16, respectively (5-7). The expression of these receptors by mononuclear phagocytes is probably dependent on the differentiation stage, since differences in the expression of Fc receptors exist between monocytes in PB and alveolar macrophages (22,54). Lymphokines as IFN-γ or GM-CSF are able to induce the expression of Fc receptors on the cell membrane. The expression of the low affinity Fc receptor for IgG, the CD16 antigen (FcγRIII) which is found on NK cells, mature granulocytes and macrophages is increased by IFN-γ (55) and GM-CSF (56). Another lymphokine, IL-4, is able to induce the expression of the Fc receptor for IgE (FcϵRIIb; CD23 antigen) on human monocytes (57).

The HLA-DR antigen is expressed by cells in immature hematopoietic differentiation stages,

but also by B lymphocytes, monocyte derived cells and activated T lymphocytes (Figure 2). IFN-γ induces an increased expression of HLA-DR on macrophages and many other cell types (55). The IL-2 receptor (CD25 antigen) is expressed by activated T cells and some B cells, while the CD38 antigen and the transferrin receptor (CD71 antigen) are expressed by virtually all proliferating and activated cells (58,59).

From the above it is clear that the phenotypes and functional properties of monocytes/macrophages, lymphocytes and granulocytes depend on interactions with other cells and cytokines in their local micro-environment (60). In interstitial lung diseases mononuclear phagocytes probably interact with material within the inflammation site such as damaged connective-tissue components, as well as lymphocyte and macrophage products. These interactions may drive the monocyte to mature into a macrophage with a particular inflammation-associated phenotype.

DIAGNOSTIC APPLICATION OF IMMUNOLOGIC MARKER ANALYSIS IN PULMONARY INFLAMMATION

The major part of the lymphocytes in BAL fluid are T lymphocytes positive for the markers CD3 and CD5. By means of mAb $CD4^+$ (helper/inducer) lymphocytes can be distinguished from $CD8^+$ (suppressor/cytotoxic) lymphocytes. Shifts in these T lymphocyte subpopulations have extensively been studied in interstitial lung diseases (Table 2)(61). It should be emphased that shifts in CD4/CD8 ratios are not pathognomonic and should always be interpreted in combination with clinical data. Such a combined approach, however, appears to have a high specificity (62,63). Increased absolute numbers of T lymphocytes with an increased CD4/CD8 ratio (>3.5) is highly suggestive for sarcoidosis, while a low CD4/CD8 ratio (<0.8) is highly suggestive for extrinsic allergic alveolitis (EAA). Determination of the monocyte/macrophage markers of cells in BAL fluid gives information about shifts in differentiation stages. In interstitial lung disorders the macrophages in BAL fluid have a monocytic morphology and express immunologic markers usually seen on PB monocytes, which suggests that an increased influx of PB monocytes into the alveolar compartment occurs in these disorders (31,64). In addition, in sarcoidosis the expression of CD16 molecules is upregulated (65). In disorders characterized by fibrosis, increased absolute numbers and percentages of neutrophils and eosinophils are usually found. When an appropriate diagnosis has been made, anti-inflammatory therapy is given in order to prevent functional impairment. Anti-inflammatory drugs usually result in a down-regulation of the inflammatory process. Immunologic marker analysis of cells in BAL fluid allows monitoring of the effectiveness of the therapy. The major findings of immunologic marker analysis of cells in BAL fluid from patients with pulmonary inflammation are summarized in Table 2. To underline the importance of BAL fluid analysis for diagnostic applications in pulmonary diseases we also included the relevance of conventional cytomorphology, electronmicroscopy and some functional studies in Table 2.

Immunologic marker analysis is also important for the correct interpretation of *in vitro* functional assays of PB lymphocytes and BAL cells. The investigation of the immunologic capacity of PB lymphocytes and BAL cells from patients presenting with pulmonary disorders may provide information about their immunologic competence. Lymphocyte stimulation assays

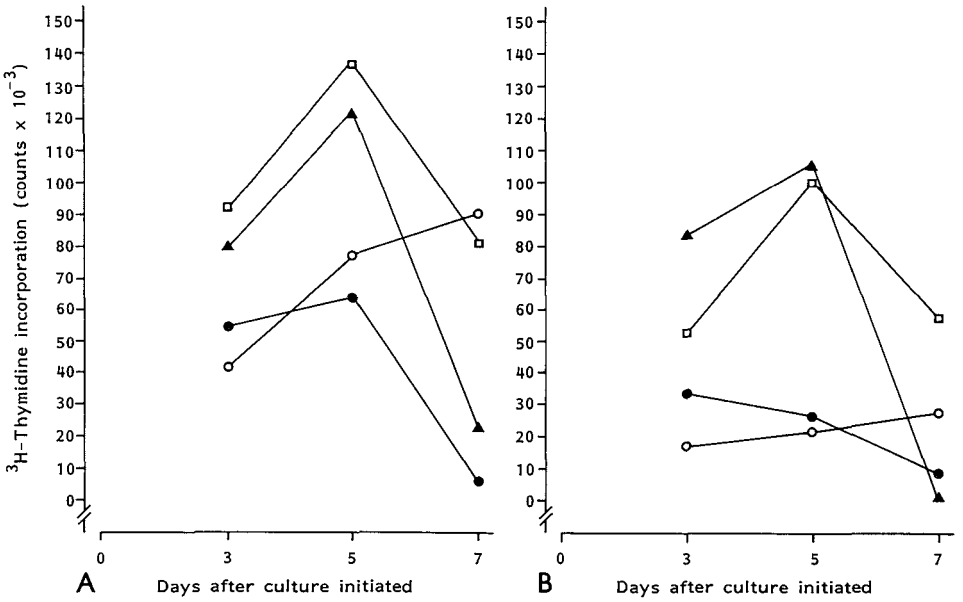

Figure 12. Kinetics of the proliferative response of PB mononuclear cells after stimulation with (**A**) PHA (Gibco Ltd., Middlesex, UK) and (**B**) ConA (Sigma Chem. Co., St. Louis, MO) in a concentration of 200 μg/ml. The cells were incubated in 96-well microtest-plates (5 x 10^5 ml^{-1}) in triplicate in 200 μl RPMI 1640 supplemented with 15% FCS and antibiotics at 37°C and 5% CO_2 in air. Lymphocyte proliferation was assayed by incubation with [^{3}H]-thymidine for the last 4 hours of culture at 3, 5 and 7 days after start of the cultures. Per well 0.5 μCi [^{3}H]-thymidine (specific activity: 5 μCi/mmol) was added. The radioactivity was measured in a liquid scintillation counter and expressed as counts per minute (cpm). Subject 1: ▲ ($CD5^+$ T lymphocytes: 52%, CD4/CD8 ratio: 1.5); Subject 2: □ ($CD5^+$ T lymphocytes: 50%, CD4/CD8 ratio: 1.9); Subject 3: ○ ($CD5^+$ T lymphocytes: 59%, CD4/CD8 ratio: 0.7); Subject 4: ● ($CD5^+$ T lymphocytes: 28%, CD4/CD8 ratio: 1.8).

with the T cell mitogens PHA and ConA are commonly applicated (66). In EAA, stimulation of PB or BAL T lymphocytes can be performed with the appropriate antigen (67). The proliferative response is usually measured with [^{3}H]-thymidine uptake. However, it is not sufficient to measure the [^{3}H]-thymidine uptake alone. Also the composition of the mononuclear cell population which is subjected to the stimulation assay, should be determined. This is illustrated in Figure 12. In this study PB T lymphocytes of four healthy individuals were subjected to stimulation with PHA and ConA and the proliferative response was measured by [^{3}H]-thymidine incorporation. It appeared that in two individuals the proliferative response after stimulation by PHA and ConA was reduced. Immunologic marker analysis of the PB cell populations showed marked differences between the individuals. In one of the two individuals with an impaired proliferative response a normal percentage of T lymphocytes with a decreased CD4/CD8 ratio was found, while the other individual had a low percentage of T lymphocytes with a normal CD4/CD8 ratio. This suggests that a reduced response is not always due to an impaired T lymphocyte function but may also be related to a shift in the composition of the cultured cell sample. Since stimulation tests are often used for clinical investigations, simultaneous

immunologic marker analysis should be performed to exclude an aberrant composition of the cultured cells as possible cause for impaired proliferative response.

CONCLUSION

Immunologic markers allow the detailed analysis of subpopulations of lymphocytes and monocytes/macrophages in PB and cells in BAL fluid. This can be used for the diagnosis and follow-up of interstitial lung disorders. In addition immunologic marker analysis should be used to determine the composition of cell samples used for *in vitro* functional assays.

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CHAPTER 6

IMMUNOLOGIC MARKERS OF BLOOD MONOCYTES AND ALVEOLAR MACROPHAGES

CHAPTER 6.1

ALVEOLAR MACROPHAGES WITH IMMATURE AND MATURE MORPHOLOGY AND PERIPHERAL BLOOD MONOCYTES EXHIBIT A DISTINCT IMMUNOPHENOTYPE*

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SUMMARY

The majority of alveolar macrophages (AM) in bronchoalveolar lavage (BAL) fluid are derived from peripheral blood monocytes (PBM). This differentiation into AM is accompanied by characteristic alterations in morphology. The immunophenotypes of BAL macrophages with an immature and mature morphology were evaluated for the expression of a broad panel of monocyte/macrophage markers and compared with PBM. The majority of PBM was positive for all markers tested except for RFD9. In contrast, mature AM were positive for RFD9, while several other markers (CD14, CD33, Monocyte-2 and CD11b) were expressed by only a small proportion of the mature AM. The immunophenotype of AM with an immature morphology differed from AM with a mature morphology, especially with respect to the markers CD14 and RFD9. Double labeling studies on BAL cells showed four subpopulations of AM with the following immunophenotypes: $CD14^+RFD9^-$, $CD14^+RFD9^+$, $CD14^-RFD9^+$ and $CD14^-RFD9^-$. The $CD14^+RFD9^-$ subset had an immature morphology resembling PBM. The $CD14^-RFD9^+$ subset, on the other hand, had a mature morphology, indicating that during AM maturation the expression of CD14 is downregulated while the expression of RFD9 is upregulated. The progressive loss of CD14 and acquisition of RFD9 were in addition found in cultured PBM. Maturation-related differences were also found in the expression pattern of the leukocyte function antigens CD11/CD18. Among the mature AM a smaller proportion of the cells expressed these antigens than among immature AM and PBM; this was the most prominent for CD11b.

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INTRODUCTION

Alveolar macrophages (AM) and their precursors are part of the mononuclear phagocyte system. Cells of this system have a common origin, comparable functions and largely similar cytomorphologic characteristics.

The origin of AM has extensively been studied in man and animals. The majority of AM are derived from PBM (5-7), while a minor part is maintained through AM proliferation *in situ* (8-13). After passing the alveolar epithelium, monocytes mature into AM, probably under the influence of local factors. Newly migrated immature AM are smaller and show only a few irregularly shaped lysosomal structures, whereas mature macrophages are larger and display large vacuoles containing multi-lamellar structures (14). This differentiation and maturation is accompanied by acquisition of acid phosphatase activity (15).

The present study aims on the comparison of the immunophenotype of PBM with that of mature and immature AM in order to increase insight into the differentiation of AM. For this purpose we performed single and double labeling studies employing a large panel of monoclonal antibodies (mAb).

MATERIALS AND METHODS

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluid and PB were obtained from 10 healthy non-smoking volunteers without chest abnormalities and with normal chest X-ray films and lung functions. All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Rotterdam.

BAL was performed after premedication with thiazinamium and local anesthesia using a lidocaine 2 percent spray. The bronchoscope was placed in wedge position in the middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL-fluid, the various laboratory analyses were carried out.

Preparation of cell suspensions for immunologic characterization

Mononuclear cells (MNC) from the PB were isolated by Ficoll density centrifugation (Ficoll Paque, density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) for 15 min at room temperature at a centrifugal force of 1,000 g. All standard washings of MNC from PB and BAL cells were performed with phosphate-buffered saline (PBS) (300 mosmol, pH 7.8) supplemented with 0.5 percent heat-inactivated bovine serum albumin (BSA) (Organon Teknika, Boxtel, The Netherlands). Washing centrifugations were performed for 5 min at 4°C at a force of 400 g.

Immunologic characterization

The BAL and PB cells were immunologically characterized by use of the following mAb: CD13(Q20) (Dr. C.E. van der Schoot, Amsterdam, The Netherlands), CD14(UCHM1) (Dr. P.C.L. Beverley, London, UK), CD33(My9) (Coulter Clone, Hialeah, FL), Monocyte-2 (Bethesda Research Laboratories, Gaithersburg, MD), U26 (Dr. R. Winchester, NY), Max 3 and Max 24 (Dr. F. Emmrich, Erlangen, FRG), RFD9 (Dr. L.W. Poulter, London, UK), CD11a(CLB-LFA 1/2) (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), CD11b(44) (Dr. N.M. Hogg, London, UK), CD11c(Leu-M5) (Becton Dickinson, San Jose, CA),

CD18(CLB-LFA 1/1) (Central Laboratory of the Red Cross Blood Transfusion Service) and anti-HLA-DR(L243) (Becton Dickinson).

Immunologic stainings of cells in bronchoalveolar lavage

For immunologic staining, 50 μl of the cell suspension (10 x 10^6 cells/ml) was incubated for 30 min at 4°C with 50 μl of the relevant, optimally-titrated antibody as described earlier by Van Dongen et al. (16). As control, cells were incubated with pooled normal mouse serum (NMS). After this incubation, the cells were washed twice and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Ig) antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands). Subsequently the cells were washed twice, and the cell pellet was mounted in glycerol/PBS (9:1), containing 1 mg of *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of the fluorochromes. Coverslips were sealed to the microscope slides with paraffin wax mixed with ceresin (BDH Chemicals).

For CD14/RFD9 double immunofluorescence staining, we used a combination of FITC-conjugated goat anti-mouse Ig antiserum and tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidine. For immunologic staining, 50 μl of the cell suspension (10 x 10^6 cells/ml) was incubated for 30 min at 4°C with 50 μl of optimally-titrated CD14(UCHM1) mAb. After this incubation, the cells were washed twice and subsequently incubated with FITC-conjugated goat anti-mouse Ig antiserum. After two washings the cells were incubated for 15 min with NMS to block free antigen-binding sites of the goat anti-mouse Ig antiserum. Thereafter the cells were washed twice and incubated with biotinylated anti-RFD9 mAb. Finally the cells were washed twice, incubated with TRITC-conjugated streptavidine, washed twice again and the cell pellet mounted in glycerol/PBS as described above.

The fluorescence was evaluated using Zeiss fluorescence microscopes, equipped with phase contrast facilities, and filtercombinations for the evaluation of FITC and TRITC (Zeiss, Oberkochen, FRG). Phase-contrast facilities were used to evaluate the morphology of the individual cells and they allowed an easy discrimination between labeled cells and contaminating fluorescent particles (16). For each immunologic marker, at least 300 cells were evaluated.

The distinction between immature and mature AM was made on morphologic criteria. Immature AM are relatively small cells ($\leq$ 20 μm) that have a small cytoplasm/nuclear ratio (< 2), a horseshoe-shaped nucleus and few lysosomal structures. Mature AM are bigger cells (> 30 μm) that have an increased cytoplasm/nuclear ratio (>2), an oval or round nucleus, elongated cellular processes called pseudopods and many lysosomal structures (Figure 1).

Isolation of monocytes by centrifugal elutriation

MNC were isolated from 500 ml of blood from normal human donors by centrifugal elutriation as described by Figdor et al. (17,18). First, MNC were separated by density centrifugation with a blood component separator. Subsequently the MNC were fractionated into lymphocytes and monocytes by centrifugal elutriation. The monocyte suspension was over 95% pure as judged by nonspecific esterase staining and contained more than 98% viable cells. Contamination with lymphocytes and granulocytes was less than 2 and 3%, respectively.

Monocyte cultures and staining

Purified monocytes were cultured in a modified Iscove's medium described by Yssel et al. (19), in which BSA is replaced by human serum albumin supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin-free (defined as less than 0.2 ng/ml of endotoxin as quantified by the Limulus amebocyte lysate assay). Monocytes (4 x 10^6/ml) were incubated at 37°C, 5% CO_2 and 100% humidity in Teflon bags (Janssen's MNL, St. Niklaas, Belgium). Monocytes cultured for 60 hours were collected from the Teflon bags. After removal of the culture supernatant the cells were washed and resuspended in PBS containing 0.5% BSA and 0.02% azide and kept on ice until they were subjected to immunologic stainings. The viability as judged by trypan

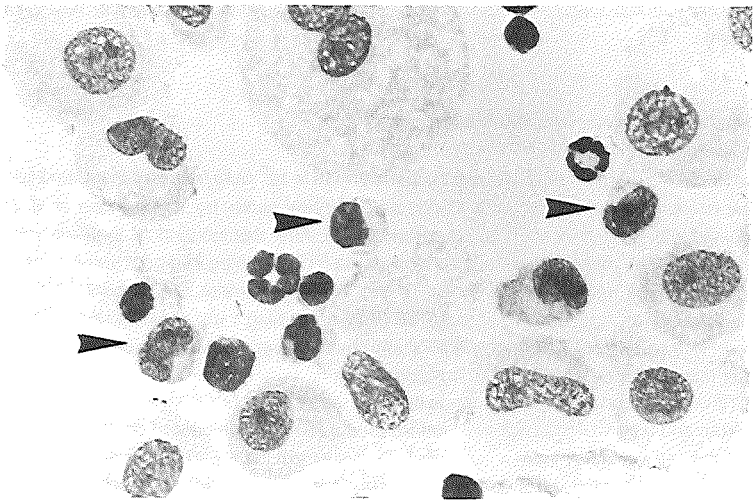

Figure 1. Light microscopy of cells present in BAL fluid. The arrows point to monocyte-like immature alveolar macrophages (AM), which are smaller as compared with mature AM.

blue exclusion exceeded 90%. The mean fluorescence intensity and the forward light scatter signal (measure for cell size) were determined by a fluorescence activated cell scanner (FACScan; Becton Dickinson). Endogenous acid phosphatase activity was visualized using the Burnstone method (20). Briefly, cytocentrifuge preparations of freshly isolated and cultured PBM were incubated at 37°C for 30 min in a mixture of naphtol AS-BI phosphate (Sigma, St. Louis, MO) as substrate and diazotized pararosanilin (Sigma) as coupling agent in acetate-barbiturate buffer (pH 5.0). After washing in PBS the slides were mounted in glycerin-gelatin (Merck, Darmstadt, FRG).

Statistical analysis

To evaluate statistically-significant differences in the expression of the various immunologic markers between PBM, immature AM and mature AM, the Wilcoxon test was used. P-values less than 0.05 were considered significant.

RESULTS

Percentage positive mononuclear cells

The immunophenotype of PBM, immature AM and mature AM is presented in Table 1. The morphology of AM is shown in Figure 1. The majority of PBM was positive for CD13, CD14, CD33, Monocyte-2, U26, Max 3 and Max 24. In addition, the majority of PBM expressed the CD11/CD18 cell surface adhesion molecules as well as HLA-DR. Only a minor part of PBM were positive for RFD9.

Marked differences in the expression of some of these markers existed between PBM, immature AM and mature AM (Table 1). The expression of CD13 was comparable between

TABLE 1. Percentages positive PBM, immature AM and mature AM in ten healthy non-smoking volunteers*.

Immunologic marker	PBM	p-value[a]	immature AM	p-value[b]	mature AM	p-value[c]
CD13(Q20)	83.9 ± 27.9	n.s.	89.6 ± 5.6	0.01	61.1 ± 23.2	0.005
CD14(UCHM1)	85.4 ± 10.2	0.05	69.8 ± 11.7	0.01	10.3 ± 11.9	0.001
CD33(My9)	84.1 ± 28.1	0.05	44.1 ± 24.9	0.01	12.7 ± 9.1	0.001
Monocyte-2	91.5 ± 3.8	0.001	77.3 ± 9.9	0.01	21.5 ± 17.2	0.001
U26	94.4 ± 3.9	n.s.	92.5 ± 7.3	0.01	79.2 ± 9.5	0.001
Max 3	96.0 ± 2.5	0.05	80.5 ± 13.4	0.01	52.1 ± 27.5	0.001
Max 24	83.9 ± 27.9	0.05	71.4 ± 27.9	0.01	46.6 ± 30.9	0.005
RFD9	0.4 ± 0.5	n.s.	5.1 ± 5.9	0.01	61.3 ± 23.9	0.001
CD11a(CLB-LFA 1/2)	96.1 ± 5.5	0.01	82.4 ± 6.5	0.05	80.3 ± 4.9	0.001
CD11b(44)	80.4 ± 28.6	0.01	63.6 ± 14.3	0.01	15.2 ± 17.5	0.005
CD11c(Leu-M5)	94.2 ± 2.5	0.001	57.6 ± 34.6	n.s.	46.4 ± 25.8	0.001
CD18(CLB-LFA 1/1)	96.0 ± 3.1	n.s.	89.5 ± 9.5	0.05	81.3 ± 17.0	n.s.
HLA-DR(L243)	90.7 ± 7.8	n.s.	89.3 ± 12.6	0.02	75.5 ± 14.7	0.05

* Means with standard deviation.
a. p-value for differences between PBM and immature AM.
b. p-value for differences between immature and mature AM.
c. p-value for differences between PBM and mature AM.
n.s. Not significant.

PBM and immature AM, but mature AM showed a decreased expression. There was also a decreased expression of CD14 on AM as compared with PBM, the most prominent on mature AM. RFD9, on the other hand, was weakly expressed by less than 1% of PBM. However, among immature AM about 5% was $RFD9^+$ and among mature AM even 61% brightly expressed RFD9. CD33, a pan-myeloid antigen present on the majority of myeloid and monocytic cells was significantly less expressed on mature AM. Also positivity for Monocyte-2 was strongly associated with PBM and immature AM. A similar pattern, but less pronounced, was found for U26, Max 3 and Max 24.

The expression of the leukocyte surface adhesion molecules CD11a, CD11b and CD11c was found to be less on immature AM than on PBM, while the expression on mature AM was even more decreased.

The expression of MHC class II molecules (HLA-DR) did not significantly differ between PBM and immature AM. Mature AM showed a slightly diminished expression of HLA-DR, although the majority appeared to be positive.

Double immunofluorescence

Double labeling of BAL cells was done in two samples. In one sample we found 12% $CD14^+RFD9^-$ AM, 17% $CD14^+RFD9^+$ AM, 41% $CD14^-RFD9^+$ AM, and 30% $CD14^-RFD9^-$ AM. In another sample we found 5% $CD14^+RFD9^-$ AM, 5% $CD14^+RFD9^+$ AM, 67% $CD14^-RFD9^+$ AM and 23% $CD14^-RFD9^-$ AM. The $CD14^+RFD9^-$ AM subset had an immature morphology resembling PBM, in contrast to the $CD14^-RFD9^+$ AM subset, which had a more mature

morphology. This suggests that during macrophage maturation expression of the CD14 antigen is downregulated in contrast to RFD9, which appears to become upregulated.

Monocyte cultures

The expression of the cell membrane antigens recognized by CD14(UCHM1) and RFD9 was determined by FACScan analysis directly after PBM isolation and after a culture period of 60 hours in three separate experiments. For freshly isolated PBM the mean fluorescence intensity of RFD9 was found to be identical to that of the negative control (NMS) (Figure 2). After 60 hours culturing, expression of the antigen recognized by RFD9 could be demonstrated, while a part of the cultured PBM population had become negative for the marker CD14 (UCHM1). The partial loss of the antigen recognized by this mAb resulted in two fluorescence peaks: one with almost the same mean fluorescence intensity as the negative control (NMS) and one with a mean fluorescence intensity identical to the value directly after isolation. (Figure 2). Cytochemical staining of the monocyte cultures for acid phosphatase showed that freshly isolated PBM contained no endogenous acid phosphatase while all 60-hours-cultured PBM had become positive.

DISCUSSION

The maturation of monocytes into macrophages is accompanied by characteristic changes in morphology, biochemistry, function and expression of surface antigens (1-4,21,22). In this study, a series of mAb, mainly directed against antigens expressed by cells of the mononuclear phagocyte lineage, were used to investigate differences in immunophenotype between PBM, immature AM and mature AM.

The majority of PBM and immature AM were positive for CD13. Mature AM, however, showed a slightly diminished expression of CD13 as compared with PBM. This may have implications for the cellular activity of the mature AM since this antigen is identical to aminopeptidase N, a membrane-bound enzyme thought to be involved in the metabolism of regulatory peptides secreted by diverse cell types (23). It is suggested that CD13 is involved in cell activation, since binding of mAb to the CD13 antigen induces a calcium influx in monocytes (24).

The CD14 antigen is expressed on human monocytes and macrophages. While its function is unknown, the CD14 gene maps to a region encoding growth factors and receptors such as interleukin 3 (IL-3), IL-4, IL-5, granulocyte/macrophage-colony stimulating factor (GM-CSF) and platelet-derived growth factor receptor (PDGFR) (25,26). The phospholipid anchoring of CD14 (27) places the molecule into a family of membrane proteins which have been implicated as signalling structures. It has been demonstrated *in vitro* that GM-CSF-induced proliferation of PBM was inhibited by CD14 antibodies (28). This suggests that the CD14 antigen is involved in cell growth. In our study marked differences in the percentages of $CD14^+$ monocytes/macrophages were found, i.e. PBM showed the highest (85.4 ± 10.2%) and mature AM the lowest (10.3 ± 11.9%) expression of this antigen. Such differences in CD14 expression by monocytes/

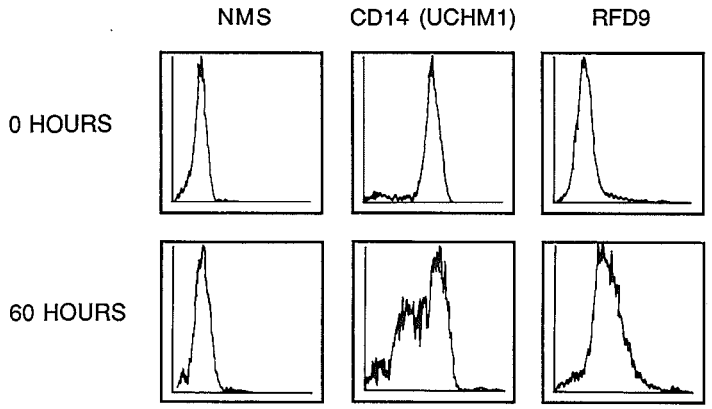

Figure 2. Histogram of immunofluorescence stainings of PBM for CD14(UCHM1) and RFD9 as analysed by the FACScan after 0 hrs and 60 hrs culture. The fluorescence intensity is shown on the X-axis and the relative number of events on the Y-axis. NMS: normal mouse serum (= negative control). Marked differences in immunofluorescence stainings were found between freshly isolated PBM and cultured PBM.

macrophages have also been reported by others, i.e. the CD14 antigen is predominantly present on circulating monocytes and could not be demonstrated on large mature tissue macrophages (29-31). However, $CD14^+$ cells do occur in the traffic areas of lymphoid tissue and then have a monocytic size and morphology (30). Hogg et al. suggested that these $CD14^+$ cells in tissues represent cells most recently derived from PB (30). This is in line with our findings since the percentage of $CD14^+$ cells among AM with an immature morphology (69.8 ± 11.7%) was significantly higher than the percentage of $CD14^+$ cells within the AM-population with a mature morphology (10.3 ± 11.9%). Furthermore we demonstrated that cultured PBM showed a diminished expression of CD14(UCHM1). The latter is in line with the findings of Hance et al. who demonstrated a loss of CD14(63D3) and CD14(MoP-9) by monocytes in culture (29).

CD33 mAb immunoprecipitate a glycoprotein with a molecular weight of 67 kilodalton (kDa). The gene encoding this gp67 was cloned and probes localized the gene to the chromosome 19 (32). The precise function of the CD33 antigen is unknown. The CD33 mAb react with multipotential myeloid progenitor cells, more mature myeloid precursors as well as PBM and AM. The CD33 antigen is also present on normal human epidermal Langerhans cells (LC) indicating a relationship between PBM and LC (33). In our study it was demonstrated that this antigen decreases in expression when PBM migrate into the alveoli. The expression further decreases when immature AM develop into mature AM.

Two other antigens present on the majority of PBM (Monocyte-2 and U26) showed a diminished expression on AM, the most prominent for Monocyte-2. Like CD14, Monocyte-2 appears to be predominantly present on circulating monocytes. The same was found for the expression of the mAb Max3 and Max24. Thus end-stage maturation *in vivo* does not necessarily lead to the expression of all monocyte/macrophage differentiation antigens.

Simultaneously with the above-mentioned loss of several surface antigens expression of other antigens is acquired, as has been reported for RFD7 and RFD9 (34-39). RFD7 is a 77 kDa

antigen present on acid phosphatase positive tissue macrophages but absent on PBM and LC (35). It has been demonstrated that cultured bone marrow cells are negative for RFD7 (39). In the presence of M-CSF, however, the bone marrow cells develop first into monocyte-like macrophages of which only a minority express RFD7 and subsequently develop into more mature macrophages (with endogenous peroxidase activity) of which the majority express RFD7 (39). Also in lung biopsies AM were positive for both RFD7 and RFD9 (35). We demonstrated that RFD9 was expressed on only a small part of the PBM (0.4 ± 0.5%). The expression of RFD9 was clearly increased in immature AM in BAL fluid (5.1 ± 5.9%), while the majority of mature AM was $RFD9^+$ (61.3 ± 23.9%). This indicates that during maturation of AM, the RFD9 antigen becomes progressively expressed. This is in line with the findings of Poulter et al. (35,36) who also showed RFD7 and RFD9 expression on mature macrophages. Recently it was demonstrated that the interstitial macrophage population in lung tissue from patients with cryptogenic fibrosing alveolitis do not express RFD9, whereas AM in the alveolar lumen were RFD9-positive (38). Apparently environmental factors may regulate the expression of particular cell surface antigens at the specific site where monocytes have migrated to.

Double immunofluorescence staining with CD14 and RFD9 showed that the small monocyte-like immature AM were $CD14^+RFD9^-$, the intermediate AM were $CD14^+RFD9^+$ or $CD14^-RFD9^-$, while the mature AM were $CD14^-RFD9^+$. These data confirm the above findings from single immunofluorescence staining for CD14 and RFD9.

When highly (elutriator) purified $CD14^+$ $RFD9^-$ PBM were cultured *in vitro*, these cells showed a progressive loss of CD14 and acquisition of RFD9. Furthermore, we found that the cells became positive for acid phosphatase and that their morphology changed into that of more mature cells. These results are in line with the above described findings on macrophage maturation *in vivo*.

Between PBM, immature AM and mature AM also marked differences were found in the expression of the CD11/CD18 leukocyte adhesion protein family. The CD11/CD18 leukocyte adhesion glycoprotein family includes three heterodimeric molecules which consist of a specific α chain (CD11a, CD11b or CD11c) and a common β chain (CD18) (40,41). They play an essential role in many adhesion-related functions, such as migration, chemotaxis and phagocytosis (40,41). This suggests an important role of these molecules in inflammatory processes. We demonstrated that nearly all PBM expressed these leukocyte function antigens. In contrast, the percentages immature and mature AM expressing the CD11/CD18 adhesion molecules were much lower, particularly the expression on mature AM was decreased. The reduced expression of these molecules may account for a less effective interaction with T cells and other cells. It has been demonstrated in mice (42-44) and man (45) that AM are inefficient accessory cells in specific T cell proliferation. Recently also Lyons et al. (46) demonstrated that in man the expression of CD18 was significantly lower on AM than on PBM, although in their study another technique (flow cytometry) and another monoclonal antibody for CD18 (MHM-23) was used. Furthermore, they demonstrated that the inability of AM to stimulate resting T lymphocytes correlated with decreased antigen-specific T lymphocyte-macrophage binding (46). It has been demonstrated in mice that circulating monocytes, after migration into tissues, give rise to cells of the mononuclear phagocyte lineage, many of which are $CD11a^-$ (47,48). Others have demonstrated a reduced expression of CD11b in tissue macrophages (49). These combined data are in line with the above described finding that mature AM do not exhibit

efficient accessory cell activities.

We could not demonstrate significant differences in HLA-DR positivity between PBM and immature AM, but the percentage of HLA-DR$^+$ cells among mature AM (75.5 ± 14.7%) was slightly lower than the percentage of HLA-DR$^+$ cells among immature AM (89.3 ± 12.6%) ($p=0.02$). These data are in line with the findings of others who also demonstrated that nearly all AM express HLA-DR (50-52), although in those studies no distinction was made between monocyte-like immature AM and mature AM. Even in patients with overt pulmonary inflammation no differences in HLA-DR expression between disease groups and controls could be demonstrated (52). Our finding that the HLA-DR expression on mature AM is slightly less than on immature AM ($p=0.02$) is in line with the findings of cultured AM in man (53) and mice (54,55). In these studies the expression of HLA-DR and Ia antigens, respectively, diminished after culturing the AM for a period of 4 days or longer.

In conclusion, in this paper we demonstrate that PBM and AM express partly different combinations monocyte/macrophage surface antigens. Furthermore, differences in immunophenotype were found between AM with an immature and a mature morphology, especially with respect to the expression of the CD14 and RFD9 antigens. This may be related to differences in cell functions of the different stages of maturation.

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CHAPTER 6.2

EXPRESSION OF THE CD11/CD18 CELL SURFACE ADHESION GLYCOPROTEIN FAMILY ON MONONUCLEAR PHAGOCYTES IN BRONCHOALVEOLAR LAVAGE FLUID AND PERIPHERAL BLOOD FROM SMOKERS AND NON-SMOKERS*

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SUMMARY

The CD11/CD18 leukocyte surface adhesion glycoprotein family consists of three different heterodimeric molecules which play an essential role in adhesion-related functions such as migration, chemotaxis and phagocytosis. This suggests an important role of these molecules in inflammatory processes. The three molecules consist of a specific α chain (CD11a, CD11b or CD11c) and share a common β chain (CD18).

The expression of the cell adhesion glycoprotein family on alveolar macrophages (AM) and peripheral blood monocytes (PBM) was studied in bronchoalveolar lavage (BAL) fluid samples and PB from 11 smokers and 10 non-smoking healthy volunteers. Smokers showed increased numbers of macrophages in their BAL-fluid as compared with non-smokers. This is probably due to an increased recruitment of blood monocytes to the alveoli, since the numbers as well as percentages of cells with a monocyte-like morphology were significantly increased in BAL fluid samples from smokers. The proportion of $CD11^+/CD18^+$ AM in the BAL fluid from smokers, however, was decreased as compared with AM from non-smokers and PBM. This suggests that tobacco smoke might play a role in the down-regulation of these leukocyte adhesion glycoproteins on AM.

INTRODUCTION

Smoking induces a chronic inflammatory process in the airways with an abundance of

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* *This manuscript is submitted for publication.*

inflammatory cells, particularly alveolar macrophages (AM). Although in bronchoalveolar lavage (BAL) fluid from smokers increased numbers of AM are found, smokers are known to be more frequently affected by respiratory tract illness than non-smokers (1-3). The abundant AM are potent producers of reactive oxygen radicals and myeloperoxidase products (4-7). Chronic exposure to oxidants may contribute to the damage of connective tissue and parenchymal cells of the lung and may therefore play an important role in the pathogenesis of emphysema (8). The recruitment of mononuclear phagocytes seems to be induced by cigarette smoke (6).

Mononuclear phagocytes are equipped with various adhesive receptors which mediate cell-cell interactions (e.g. the binding to endothelial cells) that are important for the migration into extravascular tissue. The CD11/CD18 leukocyte adhesion glycoprotein family consists of three molecules, the leukocyte function-associated antigen-1(LFA-1), the C3bi receptor (CR3, Mac-1) and the p150,95 molecule, which differ in the α subunit (CD11a, CD11b and CD11c, respectively) and share a common β subunit (CD18) (9).

Because of the increased recruitment of peripheral blood monocytes (PBM) to the alveoli in smokers, during which they have to pass the vascular endothelium to reach the alveoli, and because of the role of the CD11/CD18 cell surface molecules in adhesion to endothelium and in monocyte/macrophage activation, we investigated the expression of the CD11/CD18 cell surface adhesion molecules on AM and PBM from smokers and non-smokers.

MATERIALS AND METHODS

Subjects and bronchoalveolar lavage

BAL fluid was obtained from 11 smokers (3 males and 8 females; mean age 38.4 years; range:27-73 years) and 10 non-smokers (4 males and 6 females; mean age 37.5 years; range: 25-60 years). The subjects did not receive any medication. They had no history of chest abnormalities and had no signs of asthma or chronic obstructive pulmonary disease (COPD). They had a normal chest X-ray film and normal lung function. Approval for all lavage studies was obtained from the Medical Ethics Committee of the Erasmus University/University Hospital Rotterdam. BAL was performed with a bronchoscope placed in wedge position in the middle lobe. Four aliquots of 50 ml sterile saline were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL fluid the various laboratory analyses were carried out. All standard washings of BAL cells were performed with phosphate buffered saline (PBS) (300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated bovine serum albumin (BSA) (Organon Teknika, Boxtel, The Netherlands). Washing centrifugations were performed for 5 min at 4°C with a force of 400 g. Cell differentials were done on May-Grünwald stained cytocentrifuge preparations. At least 300 cells were counted.

A distinction was made between monocyte-like AM and more mature AM. Monocyte-like cells in BAL-fluid were relatively small cells ($\leq$ 20 μm) with a horseshoe-shaped nucleus and small cytoplasm/nuclear ratio (< 2), in contrast to macrophages that have an increased size (> 20 μm) with a round or oval nucleus and many lysosomal structures.

Immunologic characterization of BAL cells

The BAL cells were immunologically characterized by use of the following monoclonal antibodies (mAb): CD11a(CLB-LFA 1/2) and CD18(CLB-LFA 1/1) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), CD11b(44) (Dr. N.M. Hogg, London, UK) and CD11c(Leu-M5) (Becton Dickinson, San Jose, CA).

For immunologic staining, 50 μl of the cell suspension (8 x 10^6 cells per ml) was incubated for 30 min at 4°C

with 50 µl of the relevant optimally-titrated antibody. As a control, cells were incubated with pooled normal mouse serum (NMS). After this incubation the cells were washed twice and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin (Ig) antiserum (Nordic, Immunological Laboratories, Tilburg, The Netherlands). Subsequently the cells were washed twice and the cell pellet was mounted in glycerol/PBS (9:1), containing 1 mg *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of FITC. Coverslips were sealed to the slide with paraffin wax mixed with ceresin (BDH Chemicals, Poole, UK) (10). The fluorescence was evaluated using Zeiss fluorescence microscopes, equipped with phase contrast facilities. The phase contrast facilities were used to evaluate the morphology of the cells such as discrimination between small monocyte-like macrophages and lymphocytes. In this way it was possible to determine reliably which proportion of the monocyte-like cells and macrophages expressed the various immunologic markers. For each immunologic marker analysis at least 200 cells were evaluated.

Immunologic characterization of PB cells

Mononuclear cells (MNC) from PB were isolated by Ficoll density centrifugation (Ficoll Paque; density 1.077 g/cm^3, Pharmacia, Uppsala, Sweden) for 15 min at room temperature at a centrifugal force of 1,000 g. All standard washings and immunologic staining of MNC from PB were done as described above for BAL cells. The mean fluorescence intensity was measured by analysis with a fluorescence activated cell scanner (FACScan; Becton Dickinson). We have tried to evaluate BAL macrophages with the FACScan. However, AM from smokers showed such a high autofluorescence that they could not reliably be analyzed in this way.

Statistical analysis

To investigate whether there were statistically-significant differences in expression of the different immunologic markers between smokers and non-smokers, the Mann-Whitney test was used. To investigate differences in expression of the different immunologic markers within either group, the Wilcoxon test was used. P-values of 0.05 were used as the level of significance.

RESULTS

Total and differential cell counts from BAL fluid

The total number of recovered BAL cells in smokers (mean: 36.9 x 10^6) was significantly increased as compared with non-smokers (mean: 8.9 x 10^6) (Table 1). In non-smokers a tendency to increased percentages of lymphocytes was found. Due to the wide range, this difference was not statistically significant ($p = 0.08$). The absolute numbers of lymphocytes in the recovered BAL fluid did also not significantly differ between smokers and non-smokers. Although the percentages of macrophages were not different between the groups, the absolute number of macrophages was significantly increased in the smokers group. In addition, the percentages and the absolute numbers of cells with a monocyte-like morphology (resembling monocytes in PB, i.e. small dense cells without prominent vacuoles) were significantly increased in smokers (mean: 2.3%; mean 0.8 x 10^4/ml recovered BAL fluid) as compared with non-smokers (mean: 1.0%; mean 0.07 x 10^4/ml recovered BAL fluid).

TABLE 1. Total and differential cell counts from BAL fluid*.

Recovered BAL fluid	Study groups: Non-smokers n=10		Smokers n=11	
Recovered volume in ml	122.0 ± 23.9		113.1 ± 36.0	
Total cell recovery x 10^6	8.9 ± 5.2		36.9[a] ± 15.8	
Differential cell count	%	x 10^4/ml	%	x 10^4/ml
- Lymphocytes	5.9 ± 7.7	0.4 ± 0.4	1.3 ± 1.5	0.4 ± 0.4
- Granulocytes	1.9 ± 2.9	0.1 ± 0.1	1.1 ± 1.6	0.5 ± 0.3
- Macrophages	93.1 ± 7.7	6.7 ± 3.1	95.2 ± 3.7	30.6[b] ± 11.3
- Monocyte-like cells	1.0 ± 0.1	0.07 ± 0.03	2.3[c] ± 1.4	0.8[d] ± 0.7

* Data given are means ± 1 SD (May-Grünwald Giemsa staining). The absolute number is given as cells x 10^4/ml recovered BAL fluid. Significantly different from non-smokers: a ($p < 0.001$), b ($p < 0.001$), c ($p = 0.04$), d ($p < 0.001$).

Expression of cell surface adhesion proteins by BAL macrophages and PB monocytes

The expression of the cell surface adhesion proteins CD11a, CD11b, CD11c and CD18 on AM and PBM is presented in Table 2. The absolute numbers of macrophages expressing the leukocyte function antigens CD11a, CD11c and CD18 were increased in smokers as compared with non-smokers. The numbers of positive macrophages (x 10^4 per ml BAL fluid) recovered from smokers and non-smokers were for CD11a: 23.8 ± 3.6 vs. 6.4 ± 0.9 ($p = 0.0001$); for CD11b 5.6 ± 1.9 vs. 2.2 ± 0.7 ($p = 0.02$); for CD11c: 16.0 ± 3.5 vs. 4.6 ± 1.1 ($p = 0.0008$); and for CD18: 24.1 ± 3.4 vs 6.2 ± 0.9 ($p = 0.0001$), respectively.

Nearly all PBM appeared to express the adhesion proteins analyzed. No statistically significant differences could be demonstrated between smokers and non-smokers. The fluorescence intensity of the CD11/CD18 stainings on PBM as measured by the FACScan did not show differences between smokers and non-smokers either (Table 2).

DISCUSSION

Chronic pulmonary inflammation is a consistent feature of smoking. It has been demonstrated that macrophages accumulate in the alveoli and respiratory bronchioli of smokers (7). Smoker's lungs also have alveolar septal hypercellularity which is directly related to alveolar wall destruction (11). The strong increase in absolute numbers of AM in BAL fluid was also found in our study. In addition we have found higher numbers and percentages of cells with a monocyte-like morphology in BAL fluid of smokers, probably caused by an increased influx of PBM into the alveoli. It has been suggested that AM take up tobacco smoke products and

TABLE 2. Expression of cell surface adhesion proteins on PBM and AM from BAL fluid*.

	non-smokers n=10				smokers n=11			
marker	AM in BAL		PBM		AM in BAL		PBM	
	%	x 10^4/ml	%	FI	%	x 10^4/ml	%	FI
CD11a	94.7 ± 4.1	6.4 ± 0.9	98.5 ± 0.7	347 ± 36	74.8[a] ± 17.5	23.8[b] ± 3.6	96.1 ± 5.5	324 ± 57
CD11b	30.3 ± 21.7	2.2 ± 0.7	87.6 ± 10.6	218 ± 46	14.9 ± 13.3	5.6[c] ± 1.9	80.4 ± 28.6	186 ± 50
CD11c	63.5 ± 21.1	4.6 ± 1.1	91.7 ± 6.8	218 ± 45	47.8 ± 20.6	16.0[d] ± 3.5	94.2 ± 2.5	193 ± 48
CD18	91.6 ± 7.1	6.2 ± 0.9	98.1 ± 0.9	337 ± 36	76.2[d] ± 18.7	24.1[e] ± 3.4	96.0 ± 3.1	315 ± 38

* Data given are means ± 1 SD. The absolute numbers are given as cells x 10^4/ml recovered BAL fluid. Significantly different from non-smokers: a ($p=0.004$), b ($p=0.001$), c ($p=0.02$), d ($p=0.008$), e ($p=0.001$).
Fluorescence intensity (FI) data were calculated by the formula: FI = mean fluorescence of cells labeled with reactive mAb minus mean fluorescence intensity of cells labeled with NMS (linear scale). No significant differences were found between percentages of positive monocytes or FI between smokers and non-smokers.

as a reaction become activated and release chemotactic factors (12). These factors could be the cause of an increased influx of PBM. During migration of mononuclear phagocytes from the vascular compartment to extravascular tissues, such as the lung, adhesive interactions with other cell types are of great importance.

We have analysed the expression of the leukocyte surface adhesion molecules CD11/CD18 on AM and PBM from smokers and non-smokers. These adhesion molecules are three structurally related glycoprotein complexes that play a central role in the above mentioned cellular interactions. The α subunit of the CD11/CD18 complex appears to control the adhesive specificity of the glycoprotein. The CD11b antigen functions as a C3bi complement receptor while CD11a and CD11c antigens are involved in cell-cell adhesion (13-15). The CD11a(LFA-1) antigen cooperates with CD54(ICAM-1) in T cell-mediated killing and helper cell responses, and in antibody-dependent cell-mediated killing (16-18). The induction of CD11a expression on mononuclear phagocytes is tightly regulated. It has been demonstrated that macrophage-activating factor (MAF), interferon-γ (IFN-γ) and endotoxin induce CD11a expression on macrophages *in vitro* (19). The CD11/CD18 cell surface adhesion molecules belong to a larger family of molecules called integrins, which include a receptor for fibrinogen and receptors for extracellular matrix components such as collagen, fibronectin and laminin. The expression of these receptors has also been demonstrated on monocytes/macrophages (20-22). The increased numbers of alveolar macrophages in smokers may become attached to the extracellular matrix which they may breakdown by the release of proteases (12).

In a study of Tollerud et al. (23) also increased numbers of macrophages were found in BAL fluid of smokers as compared with non-smokers, while no differences in the numbers of circulating monocytes could be demonstrated. The present study extends these data to the leukocyte function antigens. Percentages PBM expressing leukocyte function antigens did not differ between smokers and non-smokers. Also the degree of expression of the various chains of the CD11/CD18 complex as measured by flowcytometry was similar. This indicates that the increased influx of PBM into the alveoli of smokers is neither related to increased

percentages of circulating monocytes positive for the leukocyte function antigens, nor to an increased expression of leukocyte function antigens. One of the explanations for an increased influx of monocytes into smoker's alveoli might therefore be an increased expression of the ligand of CD11a/CD18 i.e. CD54(ICAM-1) on endothelial cells within the lung, induced by the inflammatory process. The expression of CD54 on endothelial cells is necessary for effective cellular interactions mediated through the CD11a/CD18 - CD54 adhesion step (24). Antibodies directed against CD54(ICAM-1) could prevent the migration of cells to the interstitium and alveoli (25). It has in addition been demonstrated that products from activated macrophages such as interleukin 1 (IL-1) increase the expression of CD54 on endothelial cells (24), which is in line with finding that IL-1 enhances leukocyte-vessel wall adhesion at sites of inflammation (26).

Smokers AM produce significantly more superoxide anions (26) and these have been demonstrated to induce production of IL-1 (28). The IL-1 production by AM is indeed increased in smokers (29), while the absolute numbers of AM are five-fold increased (Table 2). Activated AM of smokers releasing these pro-inflammatory mediators might increase the expression of CD54 on pulmonary vascular endothelial cells and thereby facilitate the migration of PBM to the alveoli. Adhesive processes of enhanced numbers of $CD11a^+/CD18^+$ AM may again trigger their IL-1 production, because stimulation of the adhesion proteins CD11a/CD18 and CD11b/CD18 has been demonstrated to result in IL-1 production in human monocytes (30). This cascade of events is probably enhanced by continuous smoking.

We found that the proportion of PBM expressing the CD11/CD18 leukocyte function antigens was higher than the proportion of BAL macrophages expressing these markers. This indicates that during migration of PBM to the alveoli the expression of cell surface adhesion proteins decreases. These findings are in line with the findings of other investigators who demonstrated that circulating monocytes, after migration into tissues, give rise to cells of the mononuclear phagocyte series, many of which are $CD11^-/CD18^-$ (15,31-35). Apparently the down-regulation of CD11/CD18 expression on macrophages is related to maturation. Although increased numbers of AM were found in the smokers group, the proportion of $CD11^+/CD18^+$ AM was decreased as compared to non-smokers. This suggests that in smokers a larger part of the AM have reached their final maturation stage and/or their maximum phagocytosis capacity. On the other hand, it might be that components in tobacco smoke directly down-regulate the expression of CD11/CD18 leukocyte function antigens on AM.

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CHAPTER 6.3

INDUCTION OF A MACROPHAGE-LIKE IMMUNOPHENOTYPE ON PERIPHERAL BLOOD MONOCYTES BY INTERLEUKIN 4 IS INHIBITED BY GLUCOCORTICOIDS*

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SUMMARY

Elutriator purified human peripheral blood monocytes (PBM) were cultured *in vitro* under non-adherent conditions in the presence of interleukin 2 (IL-2), IL-4, granulocyte-monocyte-colony stimulating factor (GM-CSF), or dexamethasone. The immunophenotype of these purified and subsequently cultured PBM was compared with that of alveolar macrophages (AM) using a panel of monocyte/macrophage monoclonal antibodies (mAb). The majority of PBM was positive for all markers tested except RFD9. In contrast, almost all AM were positive for RFD9, while a smaller percentage was positive for the CD14 antigen. The expression of the CD11a and CD13 antigens did not significantly differ between PBM and AM.

During culturing, PBM became progressively positive for acid phosphatase, RFD9 and CD13. Part of the PBM lost the antigen recognized by CD14, while the expression of CD11a remained stable. These changes were increased by IL-4 and GM-CSF, partly inhibited by dexamethasone, but not influenced by IL-2. This suggests that during culturing PBM approach the phenotype of AM except for the CD13 antigen which is, in contrast to cultured PBM, not increasingly expressed by AM.

These findings suggest that certain cytokines stimulate macrophage maturation and that glucocorticoids amongst others may modulate the cytokine-induced maturation and function of monocytes/macrophages in inflammatory processes.

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* *This manuscript is submitted for publication.*

INTRODUCTION

Peripheral blood monocytes (PBM) belong to the mononuclear phagocyte system, which is widely distributed throughout the body (1,2). Alveolar macrophages (AM) are mainly derived from PBM, but also local replication of AM has been demonstrated (3,4). The morphologic and immunologic phenotype of AM differ from that of PBM (5,6). It is believed that during their migration from blood to alveoli PBM undergo a maturation process including a change of their phenotype. The precise nature of the stimuli leading to these changes is still unknown.

It has been shown that lung tissue is a major source of GM-CSF, and it has been proposed that this cytokine in addition to others controls macrophage heterogeneity (7-9). Local production of cytokines such as interleukin 2 (IL-2) and IL-4 during inflammatory reactions in the lung may influence the immunophenotype of AM, while such inflammatory processes can be modulated by glucocorticoids. In the present study we investigated the effects of human recombinant IL-2 (rIL-2), rIL-4, GM-CSF and dexamethasone on the immunophenotype of cultured PBM and compared their immunophenotype with that of AM as representatives of mature mononuclear phagocytes.

MATERIALS AND METHODS

Isolation of peripheral blood monocytes.

Human PBM were isolated from 500 ml of blood from healthy volunteers as described earlier (10). Briefly, mononuclear cells were separated by density centrifugation with a blood component separator. Next, the mononuclear cells were fractionated into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was over 95% pure and contained more than 98% viable cells as judged by trypan blue exclusion. The monocyte preparation contained less than 2% lymphocytes and less than 3% granulocytes.

Isolation of alveolar macrophages.

Bronchoalveolar lavage (BAL) was performed in 10 healthy, non-smoking volunteers after premedication with thiazinamium and local anesthesia using a lidocaine 2 percent spray. All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Rotterdam. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL-fluid, the various laboratory analyses were carried out.

Detection of surface membrane determinants.

For immunofluorescence stainings of PBM and AM the following monoclonal antibodies (mAb) were used: CD11a (CLB-LFA 1/2; Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), CD13 (Q20; Dr. E.M. van der Schoot, Amsterdam, The Netherlands), CD14 (UCHM1; Dr. P.C.L. Beverly, London, UK), and RFD9 (Dr. L.W. Poulter, London, UK) were used as control antibodies. Fifty μl of the PBM cell suspension (2×10^6 cells/ml) was incubated for 30 min at 4°C with 50 μl of one of the optimally-titrated mAb. Incubation was performed in 96-well microtiter plates. After three washings PBM were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti mouse-immunoglobulin antiserum (Nordic Immunologic Laboratories, Tilburg, The Netherlands) for 30 min at 4°C. After another three washings the cell pellets were

resuspended for analysis of the fluorescence intensity (FI) by means of a FACScan (Becton Dickinson). The FI is expressed as the difference between the mean channel value of PBM labeled with a reactive mAb and the mean channel value of PBM labeled with the isotype matched control mAb.

For immunologic staining of AM, 50 μl of the cell suspension (8 x 10^6/ml) was incubated for 30 min at 4°C with 50 μl of the relevant, optimally titrated mAb. After this incubation, the cells were washed twice and subsequently incubated with an FITC-conjugated goat anti-mouse-immunoglobulin antiserum for 30 min at 4°C. After two washings the cell pellet was mounted in glycerol/PBS (9:1), containing *p*-phenylenediamine (1 mg/ml) (BDH Chemicals, Poole, UK) to prevent fading of FITC. Coverslips were sealed to the slide with paraffin wax mixed with ceresin (BDH Chemicals). Fluorescence was evaluated using Zeiss fluorescence microscopes, equipped with phase contrast facilities. For each mAb at least 200 cells were evaluated.

All standard washings were performed with phosphate-buffered saline (PBS) solution (PBS; 300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated bovine serum albumin (BSA) (Organon Teknika, Boxtel, The Netherlands).

Monocyte cultures.

PBM were cultured in a modified Iscove's medium as described previously (11), in which BSA is replaced by human serum albumin. The medium was supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin-free (defined as less than 0.2 ng/ml of endotoxin as quantified by the Limulus amebocyte lysate assay). PBM (4 x 10^6/ml) were cultured at 37°C, 5% CO_2 and 100% humidity in Teflon bags (Janssen's MNL, St-Niklaas, Belgium) for 60 hours. For detection of surface membrane determinants PBM were harvested from the Teflon bags. Culture supernatant was removed after centrifugation, after which the PBM were washed twice.

IL-2, IL-4 and GM-CSF.

rIL-2 was kindly provided by Eurocetus (Amsterdam, The Netherlands). rIL-4 was a generous gift of Dr. K. Arai (DNAX Research Institute, Palo Alto, CA). Human rGM-CSF was used as a supernatant (1%) of transfected COS-7 cells.

Dexamethasone.

Dexamethasone micronisatum used in these experiments was obtained from Duchefa BV (Haarlem, The Netherlands). A stock solution of 2 x 10^{-3}M dexamethasone in ethanol was prepared. Concentration of ethanol during culture was less than 0.1%.

Staining for acid phosphatase.

Endogenous acid phosphatase activity was visualized using the Burnstone method (12). Briefly, cytocentrifuge preparations of freshly isolated and cultured PBM were incubated at 37°C for 30 min in a mixture of naphthol AS-BI phosphate (Sigma, St. Louis, MO) as substrate and diazotized pararosaniline (Sigma) as coupling agent in acetate-barbiturate buffer (pH 5.0). After washing in PBS the slides were mounted in glycerin-gelatin (Merck, Darmstadt, Germany).

Statistical analysis.

To evaluate statistically-significant differences in the expression of the various immunologic markers between PBM and AM, the Wilcoxon test was used. P-values less than 0.05 were considered significant.

RESULTS

Immunophenotype of freshly isolated PBM and AM.

The most prominent differences in immunophenotype between PBM and AM concerned the expression of RFD9 and the CD14 antigen (Table 1). RFD9 was virtually absent on PBM, while 85±13% of the AM expressed this membrane antigen. In contrast, most of the PBM were positive for CD14, while only 23±15% of the AM were positive. The results with the other mAb are listed in Table 1.

Effects of IL-2, IL-4, GM-CSF and dexamethasone on the immunophenotype of cultured PBM.

PBM were cultured for 60 hours in the presence of either IL-2 (1000 U/ml), IL-4 (100 U/ml), GM-CSF (1%) or dexamethasone (5×10^{-7} M), or without one of these supplements.

After culturing for 60 hours without supplements, CD13 showed a slightly increased expression (Figure 1 and Table 2). The expression of RFD9 greatly increased. Interestingly, the single fluorescence peak of CD14 in freshly isolated PBM transformed into two peaks after 60 hours of culture, representing a positive and a negative subpopulation (Figure 1). This was interpreted as the emergence of a CD14 negative monocyte subpopulation from the initial CD14 positive population. CD14 positive and CD14 negative monocytes were scattered throughout the relatively homogeneous monocyte population as defined by the forward and sideward light scatter signals (data not shown).

Culturing with IL-2 did not result in additional shifts in the expression patterns of the antigens recognized by CD11a, CD13, CD14 and RFD9, in contrast to GM-CSF which resulted in a decreased CD14 and an increased RFD9 expression. (Figure 1, Table 2).

When IL-4 was added to the culture medium, the changes in expression of CD14 and RFD9 were more pronounced. IL-4-induced an FI for RFD9 of 262 (control: 203). Only a single CD14 negative population, with FI of 49, was detectable. The IL-4 induced down-regulation of the determinant recognized by CD14 was accompanied by an up-regulation of the determinant re-

TABLE 1. Percentages of positive PBM and AM directly after isolation*.

	PBM	AM	p-value
CD11a (CLB-LFA 1/2)	98.5 ± 0.7	94.7 ± 4.1	n.s.
CD13 (Q20)	78.3 ± 29.1	68.4 ± 16.6	n.s.
CD14 (UCHM1)	87.4 ± 9.5	23.0 ± 15.2	0.005
RFD9	0.3 ± 0.6	84.9 ± 5.8	0.001

* Mean with standard deviation.
PBM, peripheral blood monocyte as measured in the monocyte-gate of the FACScan.
AM, alveolar macrophages as counted on the fluorescence microscope equipped with phase-contrast facilities.
n.s., not significant.

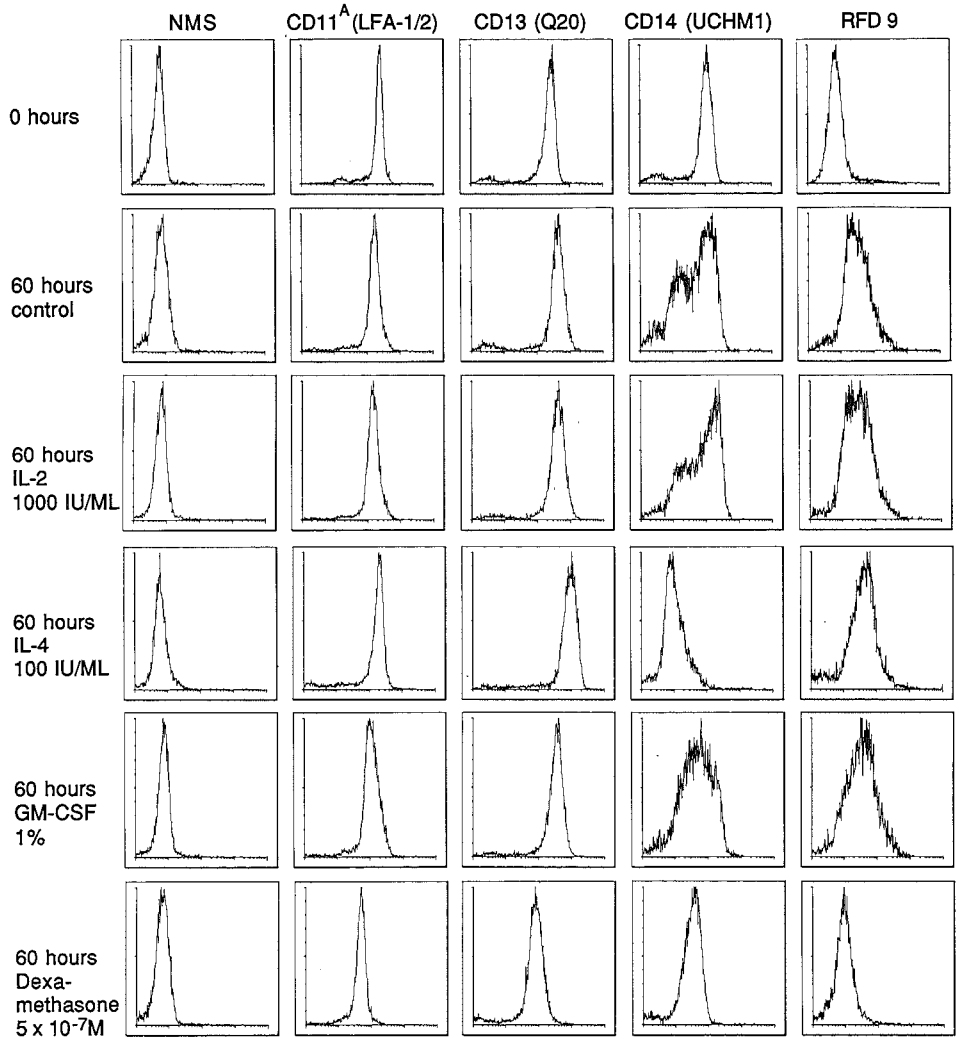

Figure 1. The expression of various cell membrane antigens on freshly isolated PBM (0 hours) and on PBM cultured for 60 hours as analysed by FACScan. The log fluorescence intensity is shown on the X-axis. The relative number of events on the Y-axis. PBM were cultured in the presence of either IL-2 (1000 U/ml), IL-4 (100 U/ml), GM-CSF (1%) or dexamethasone (5×10^{-7} M) or without any of these supplements. NMS: normal mouse serum (= negative control).

cognized by CD13. The expression of the CD11a antigen was not affected (Figure 1).

After culture in the presence of dexamethasone (5×10^{-7} M) also a single monocyte population was observed with respect to the positivity for CD14. However, the FI of this population was relatively low as compared with the CD14 positive population of the control incubation. The expression of CD13 and CD11a antigen diminished under the influence of dexamethasone, while the increase of RFD9 was almost totally prevented.

Acid phosphatase (an enzyme-cytochemical marker for monocyte-derived macrophages)

TABLE 2. Effects of *in vitro* culturing of PBM and the presence of cytokines or dexamethasone in the culture medium on the immunophenotype in one representative experiment*.

	CD11a (CLB-LFA 1/2)	CD13 (Q20)	CD14 (UCHM1)	RFD9
Freshly isolated PBM	394	408	315	17
Cultured PBM[a]				
- Control	350	453	57/307	203
- IL-2 (1000 U/ml)	345	458	75/341	211
- IL-4 (100 U/ml)	376	553	49	262
- GM-CSF (1%)	317	432	260	222
- Dexamethasone (5×10^{-7} M)	233	295	194	46

* Data are expressed as fluorescence intensity (FI) calculated by subtracting the mean channel number of cells labeled with NMS from the mean channel number of cells labeled with the relevant monoclonal antibody. A representative experiment out of five is shown.
a. PBM were cultured in Teflon bags for 60 hours.

appeared to be negative in PBM, weakly positive in cultured PBM and strongly positive in AM (Figure 2).

DISCUSSION

This study describes differences in immunophenotype between purified and subsequently cultured PBM and AM using a variety of monocyte/macrophage mAb. Most freshly isolated PBM were positive for CD11a, CD13 and CD14, in contrast to the surface antigen recognized by RFD9. The majority of AM was positive for CD11a and RFD9. Of the AM 68% was positive for CD13, while only 23% was positive for CD14. The most marked differences therefore concerned the extent of expression of CD14 and RFD9, while the expression of the CD13 antigen was not significantly different. The observed differences in immunophenotype between PBM and AM were reason for us to study the immunophenotype of PBM during *in vitro* culture and the possible influences of cytokines and glucocorticoids.

One of the CD11/CD18 leukocyte adhesion molecules, CD11a, was used as a reference membrane glycoprotein, because it has been shown earlier that the expression of CD11a on PBM was not affected by *in vitro* culture, whether or not in the presence of IL-4 (9). Here we confirm that nearly all freshly isolated PBM and AM express CD11a. During culture the CD11a expression remained stable, although IL-4 slightly upregulated CD11a in some experiments. Dexamethasone progressively diminished the expression of CD11a. This decrease could be an important mechanism of glucocorticoids to modulate cell-cell interactions during inflammatory processes.

CD13 recognizes the enzyme aminopeptidase N (13). This enzyme has been postulated to function in the final stages of the cleavage of N-terminal aminoacids from peptides and in the inactivation of regulatory peptides. It was found that 78% of the PBM strongly express this marker. On AM this figure was slightly lower (68 ± 17%), but not significantly different from

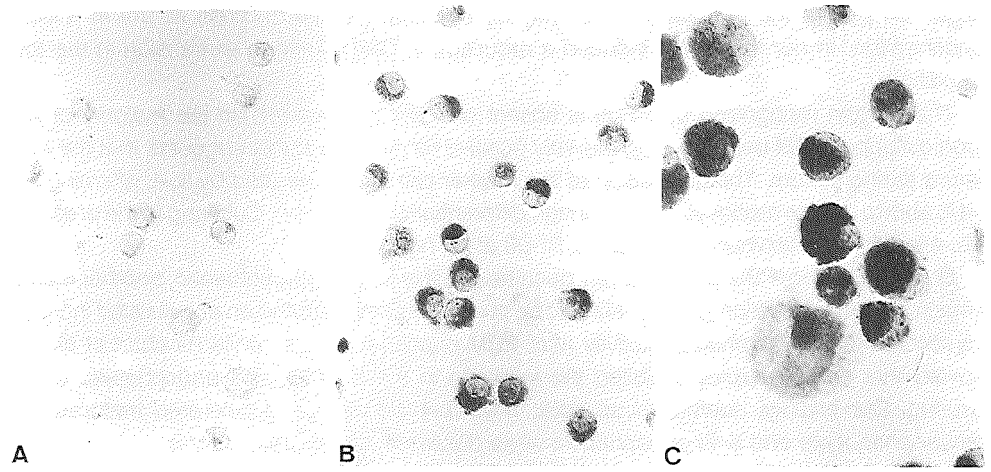

Figure 2. Acid phosphatase staining: **A.** Freshly isolated PBM. **B.** PBM cultured for 60 hours. **C.** AM. The acid phosphatase staining is negative in PBM, weakly positive in cultured PBM and strongly positive in AM.

PBM. During *in vitro* culture of PBM the expression of CD13 slightly increased, which could be further enhanced by IL-4, while IL-2 and GM-CSF had no such additional effect on the expression of CD13. The difference in expression of CD13 between AM and cultured PBM may be caused by a different microenvironment of AM in which IL-4 is absent. Another possibility is that AM themselves produce cytokines which selectively downregulate particular cell surface molecules. Dexamethasone reduced the culture-induced increase in expression of CD13. It has been suggested that aminopeptidase N is involved in cell activation since binding of mAb to the CD13 antigen induces a calcium influx in monocytes (14). This may explain why dexamethasone can have a beneficial effect in pulmonary disorders associated with monocyte/macrophage activation.

The CD14 antigen represents a 55 kD glycoprotein. The function of this protein is still unknown, but since it maps to chromosome 5 within a region containing genes encoding growth factors (IL-3, GM-CSF, M-CSF) and receptors (c-*fms*), it may function as a receptor involved in myeloid differentiation (15). Alternatively, the protein may be involved in phagocytosis (16). The protein is anchored to the plasma membrane via a phosphatidyl-inositol (PI) linkage and is absent from stem cells and early myeloid progenitor cells. During *in vitro* culture of PBM the expression pattern of CD14 changed markedly resulting in the appearance of both a $CD14^-$ and a $CD14^+$ subpopulation. This was apparent already after 18 hours of culturing and still detectable at 60 hours. The emergence of a $CD14^+$ and $CD14^-$ subpopulation may be linked to functionally distinct monocyte subsets. Gidlund et al. (17) demonstrated that the $CD14(Leu\text{-}M3)^+$ subset has potent phagocytic capacity, in contrast to the $CD14^-$ subset which had a marked antigen presenting capacity. Najar et al. (18) and Andreesen et al. (19) found that particular factors in serum influenced the morphologic and immunologic phenotype during human monocyte/macrophage maturation. Andreesen et al. (19) showed that the expression of CD14 on PBM was downregulated during culture in the absence of serum. We cultured the PBM in the presence of serum but nevertheless found downregulation of the CD14

antigen on part of the population. This may be because we used human serum at a lower concentration. In our study IL-4 induced a reduction in CD14 antigen expression in the total population.

The antigen recognized by RFD9 is absent on PBM but present on the majority of AM. Upon culturing, PBM become progressively positive for RFD9 and this suggests that the PBM mature during culture. The acquisition of this marker can be accelerated by IL-4, affirming that IL-4 is able to induce monocyte-macrophage differentiation *in vitro* (9). Culturing in the presence of IL-4 induces a maximum positivity for RFD9 at 60 hours.

During culturing, PBM progressively became positive for acid phosphatase, another marker of macrophage maturation (20,21). Abramson et al. (22) demonstrated an accelerated increase in lysosomal acid phosphatase activity after PBM exposure to IL-4, while Rinehart et al. (23) showed that corticosteroids inhibited the increase in the level of acid phosphatase during culturing. Our findings confirm these data. Thus IL-4 induces an accelerated maturation in immunophenotype and acid phosphatase while dexamethasone prevents this.

Similar maturational changes by GM-CSF on U-937 cells and PBM have been described by Geissler et al. (24). They demonstrated that GM-CSF resulted in increased acetate esterase activity, acquisition of macrophage morphology and CD11b expression. Eischen et al. (25) demonstrated that GM-CSF allows the full differentiation of PBM into macrophages and increases the phagocytic and cytotoxic properties of cultured PBM. The finding in our study that IL-2 in contrast to IL-4 and GM-CSF had neither effect on expression of the antigens recognized by CD13, CD14 and RFD9 on PBM nor on acid phosphatase activity might be caused by the absence of functional IL-2 receptors on PBM. These receptors only become expressed after activation of PBM and AM (26).

In conclusion, the findings presented here support the notion that maturation of PBM to more mature mononuclear phagocytes, as achieved by *in vitro* culture and evaluated by the expression of specific cell membrane determinants, is accelerated by IL-4 and GM-CSF, while this process can be inhibited by dexamethasone.

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CHAPTER 7

MONONUCLEAR PHAGOCYTES IN INTERSTITIAL LUNG DISEASES

CHAPTER 7.1

DIFFERENCES IN EXPRESSION OF MONOCYTE/MACROPHAGE SURFACE ANTIGENS IN PERIPHERAL BLOOD AND BRONCHOALVEOLAR LAVAGE CELLS IN INTERSTITIAL LUNG DISEASES*

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SUMMARY

In this paper the surface antigens of monocytic cells in bronchoalveolar lavage (BAL) fluid were analysed in 10 patients with sarcoidosis, 8 patients with idiopathic pulmonary fibrosis (IPF), 9 patients with extrinsic allergic alveolitis (EAA), and 10 healthy volunteers, and compared with the surface antigens of peripheral blood monocytes (PBM) of the same individuals.

The absolute numbers of alveolar macrophages (AM) were increased in all disease groups as were the numbers of small monocyte-like cells, indicating an increased influx of PBM into the alveoli, which was the most prominent in EAA patients.

In all groups investigated, the percentages PBM positive for the monoclonal antibodies (mAb) CD13, CD14, CD33, U26 and Max3 were higher than the percentages of BAL macrophages positive for these markers, while the Max24 marker was equally expressed. In all groups the percentages of AM positive for RFD9 and CD68 were higher than the percentages positive PBM. The absolute numbers of $CD13^+$ macrophages were increased in IPF and EAA patients, probably due to the increased influx of monocytic cells.

The three mAb in the CD68 cluster, i.e. Ki-M6, Ki-M7 and Y2/131, demonstrated marked differences in expression on PBM as well as on AM. This is probably because CD68(Ki-M6) recognizes a different epitope than CD68(Ki-M7) and CD68(Y2/131). The latter two become increasingly expressed by AM and this is paralleled by an increased CD68(Ki-M6) expression. The expression of CD68, which is associated with the generation of oxygen radicals during the respiratory burst and increased chemiluminescence, tended to be elevated on PBM and AM of IPF patients, although with a broad range.

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INTRODUCTION

Recruitment of monocytes from the circulation into inflamed tissue plays an important role in macrophage accumulation (1). Macrophages play a central role in interstitial inflammatory lung disorders and it has been demonstrated that they are significantly increased in bronchoalveolar lavage (BAL) fluid of patients with sarcoidosis, idiopathic pulmonary fibrosis (IPF) and extrinsic allergic alveolitis (EAA) (2-4).

Monocytes are generated in the bone marrow, enter the circulation and migrate to different tissues in response to environmental stimuli (1,5,6). In situations with overt pulmonary inflammation, macrophages are also able to replicate (7,8). Differentiation of myelomonocytic cells from pluripotent stem cells to mature monocytes and macrophages and activation during inflammation is accompanied by a variety of changes in morphology, biochemistry, function and expression of cell surface antigens (9-12). Monoclonal antibodies (mAb) are useful to detect these differences in expression of monocytic surface antigens (13,14). The function of these surface antigens can be studied with mAb by using them as blocking antibodies (15-17). Different stages of the monocyte life cycle are characterized by different combinations of surface antigens, which are related to the functions of the respective differentiation stages (18-20).

This study was aimed to investigate differences in the expression of monocytic cell surface antigens on PBM and alveolar macrophages (AM) from BAL fluid of patients with different interstitial disorders, i.e. sarcoidosis, IPF and EAA.

MATERIALS AND METHODS

Subjects and bronchoalveolar lavage

BAL fluid was obtained from 10 patients with sarcoidosis, 8 patients with IPF, 9 patients with EAA and 10 healthy volunteers. In case of sarcoidosis and IPF, the diagnosis was based on the clinical history, physical examination, radiologic findings as well as on positive findings on histologic examination of biopsy specimens, e.g. non-caseating granulomas in sarcoidosis and extensive fibrosis in patients with IPF. In case of EAA, all patients had a history of exposure to organic dusts followed by exposure-related respiratory symptoms. Furthermore, EAA patients had specific precipitating antibodies to antigens of the particular dusts. The EAA patients had an antigen exposure four days or less prior to the lavage. The patients did not receive corticosteroids or other medication and did not smoke. The control group consisted of 10 healthy, non-smoking volunteers without chest abnormalities and with normal chest X-ray films and lung functions. Approval for all lavage studies was obtained from the Medical Ethics Committee of the Erasmus University/University Hospital Rotterdam.

BAL was performed with a bronchoscope placed in wedge position in the middle lobe and four aliquots of 50 ml sterile saline were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL fluid the various laboratory analyses were carried out. All standard washings of BAL cells were performed with phosphate-buffered saline (PBS) (300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated bovine serum albumin (BSA) (Organon Teknika, Boxtel, The Netherlands). Washing centrifugations were performed for 5 min at 4°C with a force of 400 g.

Immunologic characterization of BAL cells

The BAL cells were immunologically characterized by use of the following mAb: CD3(Leu-4), CD4(Leu-3a),

CD8(Leu-2a) (Becton Dickinson, San Jose, CA), CD13(Q20) (Dr. C.E. van der Schoot, Amsterdam, The Netherlands), CD14(My3) (Coulter Clone, Hialeah, FL), U26 (Dr. R. Winchester, NY), CD68(Y2/131) (Dr. D.Y. Mason, Oxford, UK), Max3 and Max24 (Dr. F. Emmrich, Erlangen, FRG), RFD9 (Dr. L. Poulter, London, UK), CD68(Ki-M6), CD68(Ki-M7) and Ki-M8 (Behring, Marburg, FRG).

For immunologic staining, 50 μl of the cell suspensions (8×10^6 cells per ml) was incubated for 30 min at 4°C with 50 μl of the relevant, optimally-titrated antibody. As a control, cells were incubated with normal mouse serum (NMS). After this incubation the cells were washed twice and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands). Subsequently the cells were washed twice and the cell pellet was mounted in glycerol/PBS (9:1), containing 1 mg *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of FITC. Coverslips were sealed to the slide with paraffin wax mixed with ceresin (BDH Chemicals). The fluorescence was evaluated using Zeiss fluorescence microscopes equipped with phase contrast facilities. The phase contrast facilities were used to evaluate the morphology of the cells (e.g. recognition of lymphocytes, small monocyte-like macrophages and mature macrophages) and for discrimination between labeled cells and contaminating fluorescent particles. In this way it was possible to determine which proportion of the monocytes and macrophages expressed the various immunologic markers. For each immunologic marker at least 300 cells were evaluated.

We have tried to evaluate BAL macrophages with a FACScan (Becton Dickinson). However, macrophages show a very high autofluorescence. Although it is no problem to evaluate BAL lymphocytes on the FACScan, it is difficult to evaluate BAL macrophages on the FACScan. Usually the fluorescence intensity of the background is much higher than the specific signal. Therefore we have chosen to evaluate BAL cells by fluorescence microscopes, equipped with phase contrast facilities.

Immunologic characterization of PB cells

Mononuclear cells (MNC) from PB were isolated by Ficoll density centrifugation (Ficoll Paque, density: 1.077 g/cm^3, Pharmacia, Uppsala, Sweden) for 15 min at room temperature at a centrifugal force of 1,000 g. All standard washings and immunologic stainings of MNC from PB were done as described above for BAL cells. The fluorescence intensity and the forward light scatter signal (cell size) were measured by FACScan analysis. The fluorescence data are expressed as fluorescence intensity (FI) according to the formula FI = mean fluorescence of cells labeled with a reactive antibody minus mean fluorescence of cells labeled with NMS.

Statistical analysis

Comparisons of cells from BAL fluid and PB within patients of the various groups (i.e., sarcoidosis, IPF and EAA) were done with Wilcoxon's test. Parameters of groups of patients with sarcoidosis, IPF or EAA were compared with the healthy volunteers using the Mann-Whitney test. Comparisons between sarcoidosis, IPF and EAA were done with the Kruskal-Wallis test, supplemented by pairwise application of the Mann-Whitney test if the former test indicated statistically significant differences. Five percent was considered the limit of statistical significance.

RESULTS

Total and differential cell counts from BAL fluid

The cell counts in the various disease groups and healthy volunteers are summarized in Table 1. The total number of recovered BAL cells was increased in the disease groups as compared with the healthy volunteers. The percentages of lymphocytes were increased in the sarcoidosis and EAA groups, while the percentages of granulocytes were increased in the IPF

TABLE 1. Total and differential cell counts in BAL fluid from patients with interstitial lung diseases and healthy volunteers*.

Recovered BAL fluid	Sarcoidosis n=10	IPF n=8	EAA n=9	Controls n=10
Recovered volume in ml	134.0 (27.2)	101.4 (30.8)	122.2 (24.2)	122.0 (23.9)
Total cell recovery x 10^6	21.4[b] (14.5)	19.8[c] (10.2)	43.6[d] (29.4)	8.9 (5.2)
Differential cell count %				
- Macrophages	62.3[d] (16.9)	62.3[g] (18.6)	52.3[e] (20.7)	93.1 (7.7)
- Granulocytes	3.4 (3.8)	25.5[e,i,j] (12.6)	9.9[b,k] (5.8)	1.9 (2.9)
- Lymphocytes	35.7[e,f] (17.1)	6.1 (8.2)	37.2[g,h] (17.9)	5.9 (7.7)
- Monocyte-like cells	1.8[n] (0.5)	6.5[o] (8.4)	5.5[p] (8.1)	1.0 (0.1)
CD4/CD8[a] ratio	4.6[m] (3.5)	n.d.[l]	0.7 (0.2)	n.d.[l]

* Mean with standard deviation in parentheses. IPF, Idiopathic pulmonary fibrosis; EAA, Extrinsic allergic alveolitis.

a. CD4/CD8 ratio in the lymphocyte population as determined by immunologic markers.
b. Different from healthy volunteers ($p < 0.002$).
c. Different from healthy volunteers ($p < 0.009$).
d. Different from healthy volunteers ($p < 0.0002$).
e. Different from healthy volunteers ($p < 0.0001$).
f. Different from IPF ($p < 0.0005$).
g. Different from healthy volunteers ($p < 0.0003$).
h. Different from IPF ($p < 0.001$).
i. Different from sarcoidosis ($p < 0.002$).
j. Different from EAA ($p < 0.008$).
k. Different from sarcoidosis ($p < 0.01$).
l. n.d.: CD4/CD8 ratio not determined if < 10% T lymphocytes in BAL fluid.
m. Different from EAA ($p < 0.001$).
n. Different from healthy volunteers ($p < 0.01$).
o. Different from healthy volunteers ($p < 0.006$).
p. Different from healthy volunteers ($p < 0.03$).

and EAA groups. The CD4/CD8 ratio in the lymphocyte population was increased in sarcoidosis and decreased in EAA. In all three patients groups the percentages of macrophages appeared significantly lower than in the healthy volunteers, while there were no statistically significant differences between the disease groups. However, because of the increased total cell recovery, the absolute number of AM was increased in all disease groups (Table 1).

Phenotype of AM and PBM

The immunologic characterization of the AM is presented in Table 2. For most of the monocytic markers used (CD13, CD14, U26, CD33, CD68, Max3, Max24 and Ki-M8) the proportion of positive AM in the three patients groups did not differ from the healthy volunteers. The absolute numbers of $CD13^+$ macrophages were significantly increased in IPF and EAA as compared with the healthy volunteers due to the increased absolute numbers of AM in these disorders. In EAA, the absolute numbers of AM positive for the markers CD14, Max3, Max24 and RFD9 were also significantly increased. In IPF the percentages of $Max24^+$ and $RFD9^+$ AM were slightly lower than in the healthy volunteers, 83.0 ± 11.8% versus 93.0 ± 5.9% ($p<0.04$) for Max24, and 81.6 ± 10.6% versus 84.9 ± 23.3% ($p<0.03$) for RFD9 (Table 2). In contrast, the percentage and the absolute number of $CD68(Ki\text{-}M6)^+$ macrophages appeared to be increased

TABLE 2. Immunologic characterization of BAL macrophages from patients with interstitial lung diseases and healthy volunteers*.

Monoclonal antibody	Sarcoidosis n=10		IPF n=8		EAA n=9		Control n=10	
	%	x 10^4/ml	%	x 10^4/ml	%	x 10^4/ml	%	x 10^4/ml
CD13(Q20)	51.1 (27.5)	5.5 (4.7)	79.2 (14.5)	10.9[a] (6.6)	76.8 (16.2)	11.9[b] (8.2)	68.4 (16.6)	5.2 (1.3)
CD14(My3)	49.3 (25.7)	5.2 (4.0)	28.8 (14.1)	3.8 (2.6)	55.7 (21.0)	8.7[c] (5.7)	49.3 (23.3)	3.5 (0.9)
CD33(My9)	48.0 (29.9)	4.1 (2.6)	32.6 (33.5)	5.8 (6.0)	45.1 (27.8)	5.8 (4.6)	42.9 (34.4)	3.1 (1.2)
CD68(Ki-M6)	4.0 (7.6)	0.4 (0.8)	9.9[b] (7.1)	1.5[b] (1.2)	7.9 (6.6)	0.9 (1.0)	2.2 (2.6)	0.2 (0.2)
CD68(Ki-M7)	48.0 (24.4)	5.0 (3.3)	68.7 (28.5)	13.4 (11.8)	49.6 (28.3)	8.4 (7.1)	17.6 (18.1)	4.8 (5.6)
CD68(Y2/131)	33.6 (28.5)	3.3 (2.5)	62.5 (32.0)	12.6 (12.2)	44.1 (35.8)	7.9 (6.6)	45.0 (19.6)	4.5 (1.7)
U26	86.2 (15.9)	8.1 (3.9)	85.4 (10.8)	14.5 (10.8)	84.7 (9.7)	13.4 (6.7)	91.7 (7.6)	7.0 (1.4)
Max3	73.7 (17.4)	6.8 (3.0)	76.8 (20.9)	14.3 (11.3)	77.9 (19.1)	12.1[e] (6.8)	81.6 (17.0)	6.4 (1.5)
Max24	84.6 (15.8)	8.1 (3.7)	83.0 (11.8)	12.5 (10.4)	87.9 (9.4)	14.2[e] (7.4)	93.0 (5.9)	7.2 (1.6)
Ki-M8	85.4 (8.3)	8.3 (3.8)	86.1 (10.0)	14.5 (10.6)	83.2 (12.4)	13.6 (7.2)	82.7 (8.6)	7.6 (2.1)
RFD9	86.8 (16.3)	7.7 (3.3)	81.6[a](10.6)	11.5[c] (8.3)	78.8[c] (8.0)	13.3[d] (7.8)	84.9 (23.3)	5.8 (0.9)

* Mean with standard deviation in parentheses. The absolute numbers are given as positive macrophages x 10^4/ml recovered BAL fluid. IPF, Idiopathic pulmonary fibrosis; EAA, Extrinsic allergic alveolitis.

a. Different from healthy volunteers ($p < 0.03$).
b. Different from healthy volunteers ($p < 0.02$).
c. Different from healthy volunteers ($p < 0.01$).
d. Different from healthy volunteers ($p < 0.004$).
e. Different from healthy volunteers ($p < 0.04$).

in IPF (9.9 ± 7.1%) as compared with the healthy volunteers (2.2 ± 2.6%) ($p<0.02$) (Table 2).

The immunologic characterization of PBM is given in Table 3. One of the most striking differences between AM and PBM are the higher percentages of PBM positive for the majority of the mAb used, i.e. CD13, CD14, CD33, U26, Max3 and Ki-M8. Statistical analysis indicated that this difference was significant in the disease groups as well as the healthy volunteers. The percentages of PBM positive for two of the mAb used, RFD9 and CD68, appeared to be lower than the percentages of AM positive for these markers. The percentage of $RFD9^+$ PBM was less than 2% and the percentage of $CD68(Ki\text{-}M6)^+$ PBM was less than 3% for all groups together. In BAL fluid the percentage of $RFD9^+$ macrophages was 78.1 ± 18.3% and the percentage of $CD68(Ki\text{-}M6)^+$ macrophages was 6.0 ± 3.0% for all groups together. The percentage of $CD68(Ki\text{-}M6)^+$ PBM was significantly increased in IPF patients as compared with healthy volunteers and the other disease groups ($p<0.02$) (Table 3).

The extent of expression (FI) of markers on PBM is also presented in Table 3. For all mar-

TABLE 3. Immunologic markers and fluorescence intensity (FI) of PBM from patients with interstitial lung diseases and healthy volunteers*.

Monoclonal antibody	Sarcoidosis n=10		IPF n=8		EAA n=9		Control n=10	
	%	FI	%	FI	%	FI	%	FI
CD13(Q20)	74.8 (32.4)	145 (109)	94.5 (1.9)	189 (34)	82.2 (31.1)	190 (130)	78.3 (29.1)	218 (77)
CD14(My3)	89.5 (6.7)	367 (48)	92.5 (3.6)	339[a,b] (41)	87.8 (6.2)	408 (56)	89.0 (10.1)	385 (34)
CD33(My9)	90.3 (5.1)	185 (56)	82.9 (33.5)	139[c,d,e] (33)	91.0 (5.9)	202 (41)	89.3 (8.7)	218 (51)
CD68(Ki-M6)	0.3 (0.5)	-26[f,l] (26)	14.1[g,h] (33.7)	40[k] (50)	0.0 (0.0)	-13 (48)	0.2 (0.3)	14 (27)
CD68(Ki-M7)	21.5 (23.0)	89 (89)	36.5 (28.7)	84 (35)	37.6 (26.5)	86 (61)	52.4 (29.5)	103 (42)
CD68(Y2/131)	23.5 (15.0)	35 (38)	23.9 (2.1)	37 (28)	23.8 (19.7)	62 (65)	27.4 (16.8)	83 (44)
U26	92.8 (7.3)	201 (51)	95.3 (2.5)	206[f] (32)	92.9 (7.9)	246 (41)	93.7 (6.5)	251 (35)
Max3	94.0 (2.4)	162[d] (29)	96.0 (1.4)	158[f,i] (26)	94.0 (1.4)	205 (21)	96.6 (1.8)	190 (27)
Max24	91.6 (4.9)	141 (16)	57.8 (47.4)	127[j,k] (64)	87.4 (6.7)	163 (31)	92.0 (4.2)	175 (23)
Ki-M8	76.5 (20.0)	168 (49)	62.7 (38.7)	187 (80)	73.7 (33.8)	183 (44)	79.4 (6.4)	187 (24)
RFD9	0.4 (0.7)	10 (46)	1.1 (1.2)	-11 (23)	0.6 (0.7)	-6 (41)	0.3 (0.6)	6 (33)

* Mean with standard deviation in parentheses. Fluorescence data are expressed as fluorescence intensity (FI) calculated by the formula: FI = fluorescence of cells labeled with reactive mAb minus fluorescence of cells labeled with normal mouse serum (NMS).

a. Different from sarcoidosis ($p < 0.03$).
b. Different from EAA ($p < 0.008$).
c. Different from sarcoidosis ($p < 0.04$).
d. Different from EAA ($p < 0.02$).
e. Different from healthy volunteers ($p < 0.0009$).
f. Different from healthy volunteers ($p < 0.04$).
g. Different from healthy volunteers ($p < 0.02$).
h. Different from EAA ($p < 0.01$).
i. Different from EAA ($p < 0.002$).
j. Different from healthy volunteers ($p < 0.01$).
k. Different from EAA ($p < 0.04$).
l. Different from IPF ($p < 0.05$).

kers evaluated no differences were found in FI in the sarcoidosis and EAA groups as compared with the healthy volunteers. In IPF the mean FI for several markers was lower than in healthy volunteers. On the other hand, the expression of CD68(Ki-M6) was significantly increased in patients with IPF (Table 3). No differences in expression of RFD9 were found between the three disease groups and healthy volunteers.

DISCUSSION

We have analysed the expression of a panel of monocytic antigens on AM and PBM in sarcoidosis, IPF and EAA by means of mAb. Some of the markers studied have a known

function, while the activity of other markers is still unknown. It is generally believed that the changes in functional activity of monocytes/macrophages parallels the phenotypic maturation (19). It has for example been demonstrated that the capacity of PBM to stimulate a mixed leukocyte reaction or to phagocytose bacteria parallels the expression of class II MHC molecules and CD11b/CD18 (C3bi complement receptor), respectively (20).

CD13 antibodies recognize a membrane-bound glycoprotein, aminopeptidase N, involved in the metabolism of regulatory peptides (21). It is present on monocytes/macrophages and granulocytes (21-23). CD13 antibodies have been demonstrated to induce a rapid rise in $[Ca^{2+}]$ associated with a respiratory burst in monocytes (24). In sarcoidosis and IPF the absolute numbers of $CD13^+$ AM were increased as compared with the healthy volunteers. Triggering of this receptor may lead to a respiratory burst in AM, a phenomenon known to be present in patients with interstitial lung disease (25).

The CD14 marker recognizes a differentiation antigen on the surface of PBM and AM. The CD14 antigen is coded by a gene on chromosome 5 in a region containing a number of growth factor and receptor genes (26). It may therefore represent a receptor for myeloid differentiation. We have found that this antigen decreases in expression during maturation of PBM *in vitro* (Chapter 6.3). In the present study we found that the percentages CD14 positive AM were lower than PBM. This has also been demonstrated by others (27-31). We demonstrated that the FI for CD14 on PBM was significantly lower in IPF patients. The lower expression of CD14 may be related to more mature or activated monocytes in PB of IPF patients since several other markers also showed a lower expression.

Although it was found recently that the CD33 antigen is a glycoprotein present on the majority of myeloid and monocytic cells, to date the function of CD33 is still unknown (32). CD33 antibodies bind to perivascular macrophages and Langerhans cells in the skin and within the hemopoietic compartment to stem cells capable of producing mixed colonies (32). Treatment of human monocytic cell lines with a variety of growth and differentiation promoting cytokines, e.g. interferon-γ (IFN-γ) and interleukin 1 (IL-1), did not alter the expression of CD33 protein on the surface membrane (32). In our study we found a decreased expression on AM in all groups as compared to PBM indicating that AM are end-stage cells in monocyte/macrophage differentiation. Furthermore, our study confirms data that CD33 expression in not influenced by cytokines since no differences could be demonstrated between the healthy volunteers and the disease groups with a prominent alveolitis, most likely associated with the production of cytokines (i.e. IFN-γ and IL-1).

Two other monocyte-differentiation antigens, Max3 and Max24, appeared to be present on the majority of PBM and AM. Earlier reports demonstrated that Max3 and Max24 are present on mature monocytic cells in PB and BAL fluid (33,34). Max3 and Max24 antigens are both present on epithelioid and giant cells in lymph nodes affected by tuberculosis and sarcoidosis and on AM. It has been demonstrated that the Max24 antigen is a phosphatidyl-inositol linked molecule and it may be involved in membrane processes (25). Antibodies against Max3 and Max24, however, do not influence the respiratory burst and chemotaxis (37). The broad reactivity of these mAb was also demonstrated in our study; the majority of PBM and AM appeared to be positive. For these antigens no differences in percentages of positive cells were found between the groups studied. The mAb U26 also reacts with more mature myelomonocytic cells (38) and this reaction pattern was also found in our study.

RFD9 was nearly absent from PBM, but positive on AM indicating that during maturation and migration this antigen becomes progressively expressed (39). The finding of a significantly lower percentage RFD9$^+$ AM in IPF and EAA may therefore be due to a higher influx of monocytic cells from the PB.

The three mAb CD68(Ki-M6), CD68(Ki-M7) and CD68(Y2/131) are pan-macrophage antibodies (40-43). The markers recognized by these mAb are restricted to cells of the monocyte/macrophage system, and these antigens are mainly confined to lysosome and phagosome structures. For CD68(Ki-M6) and CD68(Ki-M7) a stepwise increase in cytoplasmic expression has been described during differentiation of monocytes into macrophages, *in vitro* as well as *in vivo* (40,41). The CD68 antigen is functionally related to the generation of oxygen radicals during the respiratory burst in phagocytosing cells, a feature not shared by dendritic cells (41). Upregulation of CD68(Ki-M6) might furthermore be influenced by conditions with antigen stimulation since CD68$^+$ monocytic cells are absent in the fetal and postnatal intestine but abundantly present in the mature intestine (44). The low frequency of CD68(EBM11) was also described for fetal AM (45), while in our study CD68$^+$ AM were present. In our study we did not perform immunoperoxidase stainings on cytocentrifuge preparations (in which cytoplasmic expression of antigens can easily be detected), but instead incubated cells in suspension in order to analyse differences in cell surface expression between the different CD68 mAb. We demonstrated that these markers are not strictly cytoplasmic. Surface expression was also found by Cordell et al. (42). Our findings are also in line with the findings of Parwaresch et al. (40) and Kreipe et al. (41), that the expression of CD68 is increased in more mature cells. We found in all groups the percentage of CD68$^+$ AM to be higher than the percentage of CD68$^+$ PBM. Although the mAb used in our study belong to the same CD cluster, in PB as well as in BAL marked differences in percentages of positive cells were found: the percentages of CD68(Y2/131)$^+$ and CD68(Ki-M7)$^+$ monocytic cells was higher than the percentages of CD68(Ki-M6)$^+$ monocytic cells. The increased expression of the markers CD68(Ki-M7) and CD68(Y2/131) on AM was paralleled by CD68(Ki-M6). These differences in CD68 expression can be explained by the experiments of Cordell et al. (42). They investigated the expression of CD68 by transfecting two cDNA clones, selected for their ability to react with anti-macrophage antibodies of the CD68 cluster, in WOP cells. Immunoprecipitation of surface-labeled cells revealed that transfection with the longer clone caused the expression of a 110,000 kDa molecule whereas the shorter clone coded for a 70,000 kDa molecule which was not precipitated by CD68(Ki-M6). The epitope recognized by antibody CD68(Ki-M6) appeared to be dependent on expression of the sequence absent from the shorter clone. This may explain the difference found in our study. The tendency to increased numbers of CD68(Ki-M6)$^+$ AM in IPF may be of functional significance since CD68(Ki-M6) is mainly confined to the lysosome and phagosome structures of macrophages which have been demonstrated to become increasingly expressed during activation. CD68 is also involved in the generation of oxygen radicals during the respiratory burst and is related to an increase in monocyte chemiluminescence (40). Further studies are necessary to determine whether this marker has prognostic significance.

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CHAPTER 7.2

EXPRESSION OF THE CD11/CD18 CELL SURFACE ADHESION GLYCOPROTEIN FAMILY AND MHC CLASS II ANTIGEN ON BLOOD MONOCYTES AND ALVEOLAR MACROPHAGES IN INTERSTITIAL LUNG DISEASES*

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SUMMARY

The expression of molecules of the CD11/CD18 cell surface adhesion glycoprotein family and HLA-DR antigen was studied on peripheral blood monocytes (PBM) and alveolar macrophages (AM) in bronchoalveolar lavage (BAL) fluid from patients with sarcoidosis, idiopathic pulmonary fibrosis (IPF) and extrinsic allergic alveolitis (EAA). Patients with these interstitial lung diseases showed increased numbers of macrophages in BAL fluid. This is probably caused by an increased influx of PBM to the alveoli since the numbers of cells with a monocytic morphology were also significantly increased in BAL samples from patients with interstitial lung disease, most prominently in IPF and EAA.

The increased influx of PBM into the alveoli in patients with interstitial lung diseases was not reflected by an increased expression of the CD11/CD18 leukocyte function antigens on PBM.

In healthy volunteers as well as in sarcoidosis, IPF and EAA the percentages of AM positive for CD11b (the C3bi complement receptor) and CD11c were lower than among PBM. This indicates that the expression of these cell surface adhesion molecules is down-regulated during maturation and migration of PBM to the alveoli. The absolute numbers of AM positive for CD11b were increased in BAL fluid of IPF and EAA patients as compared to healthy volunteers. EAA patients also showed increased absolute numbers of AM positive for CD11a and CD11c. This differentially increased expression of these leukocyte function antigens on AM suggests the influence of locally-produced cytokines.

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* *This manuscript is submitted for publication.*

INTRODUCTION

Interstitial lung disorders such as sarcoidosis, idiopathic pulmonary fibrosis (IPF) and extrinsic allergic alveolitis (EAA) are usually characterized by increased cell numbers in the alveoli and in the interstitium (1-3). This has been shown to be due to local division (2-6) and an increased influx of leukocytes (7-10). The cells interact with each other and with components within the inflammatory site such as damaged connective tissue and cytokines derived from mononuclear cells and consequently become activated (1-3). This activation may lead to the secretion of harmful enzymes like collagenase and elastase which can damage the lung. In addition, local vascular permeability is altered and more cells and plasma-components will be recruited to the alveolar space. These newly recruited cells become activated and further enhance the ongoing inflammation (1-3).

It is believed that the adhesion between cells is essential for migration from the vascular compartment to extravascular tissues and for leukocyte mediated cytotoxicity, phagocytosis, chemotaxis and lymphocyte proliferation (11). This adhesiveness is, at least partially, mediated by a family of adhesion molecules, consisting of three different α subunits, CD11a(LFA-1), CD11b(Mac-1) and CD11c(p150,95), and a common β subunit (CD18) (11,12). CD11a/CD18 predominates on lymphocytes, CD11b/CD18 on granulocytes and CD11c/CD18 on macrophages, while monocytes express all three molecules (11,12). CD11a/CD18 can bind to its ligand CD54(ICAM-1) present on vascular endothelium and other cells. The importance of this family of molecules is illustrated by the observations that they become upregulated during inflammatory reactions (13-16) and that monoclonal antibodies against CD11/CD18 can prevent attachment of cells to vascular endothelium (17-19) and activation of granulocytes (20).

Other molecules important for effective interactions (e.g., antigen presentation) are the MHC class II antigens, which is especially involved in monocyte/T lymphocyte interactions (21-23). These molecules become also upregulated during inflammatory reactions.

Monocytic cells play an important role in interstitial lung diseases and they are usually activated under these conditions (24,25). In the present paper we studied the expression of the CD11/CD18 leukocyte function antigen family and HLA-DR on PBM and BAL macrophages in sarcoidosis, IPF and EAA.

MATERIALS AND METHODS

Subjects and bronchoalveolar lavage

BAL fluid was obtained from 10 sarcoidosis patients, 8 IPF patients, 9 EAA patients and 10 healthy volunteers. In case of sarcoidosis and IPF, the diagnosis was based on clinical history, physical examination, radiological findings as well as on positive findings on histological examination of biopsy specimens, e.g. non-caseating granulomata in sarcoidosis and extensive fibrosis in patients with IPF. In case of EAA, all patients had a history of exposure to organic dusts followed by exposure-related respiratory symptoms. Furthermore, EAA patients had specific precipitating antibodies to antigens of the particular dusts. The EAA patients had an antigen exposure four days or less prior to the lavage. The patients did not receive corticosteroids or other medication and did not smoke. The control group consisted of 10 healthy non-smoking volunteers without chest abnormalities and with normal chest X-ray films and lung functions. Approval for all lavage studies was obtained from the Medical Ethics

Committee of the Erasmus University/University Hospital Rotterdam.

BAL was performed with a bronchoscope placed in wedge position in the middle lobe and four aliquots of 50 ml sterile saline were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL fluid the various laboratory analyses were carried out. All standard washings of BAL cells were performed with phosphate buffered saline (PBS) (Organon Teknika, Boxtel, The Netherlands). Washing centrifugations were performed for 5 min at 4°C with a force of 400 g.

Immunologic characterization of BAL macrophages

The BAL macrophages were immunologically characterized by use of the following monoclonal antibodies: CD11a(CLB-LFA 1/2) and CD18(CLB-LFA 1/1) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), CD11b(44) (Dr. N.M. Hogg, London, UK), CD11c(Leu-M5) (Becton Dickinson, San Jose, CA), and anti-HLA-DR(L243) (Becton Dickinson).

For immunologic staining, 50 μl of the cell suspension (8×10^6 cells per ml) was incubated for 30 min at 4°C with 50 μl of the relevant optimally-titrated antibody. As a control, cells were incubated with normal mouse serum (NMS). After this incubation the cells were washed twice and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands). Subsequently the cells were washed twice and the cell pellet was mounted in glycerol/PBS (9:1), containing 1 mg *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of FITC. Coverslips were sealed to the slide with paraffin wax mixed with ceresin (BDH Chemicals). The fluorescence was evaluated using Zeiss fluorescence microscopes, equipped with phase contrast facilities. The phase contrast facilities were used to evaluate the morphology of the cells (e.g. recognition of lymphocytes, granulocytes and macrophages) and for discrimination between labeled cells and contaminating fluorescent particles. In this way it was possible to determine which proportion of the monocytes and macrophages expressed the various immunologic markers. For each immunologic marker at least 300 cells were evaluated.

We have tried to evaluate BAL macrophages with fluorescence-activated flowcytometry (FACScan). However, macrophages show a high autofluorescence. Although it is no problem to evaluate BAL lymphocytes by FACScan, it was difficult to evaluate BAL macrophages by FACScan. Usually the intensity of the background fluorescence was much higher than the specific signal. Therefore we have used fluorescence microscopes equipped with phase contrast facilities for evaluating BAL cells.

Immunologic characterization of PBM

Mononuclear cells (MNC) from PB were isolated by Ficoll density centrifugation (Ficoll Paque; density, 1.077 g/cm^3, Pharmacia) for 15 min at room temperature at a centrifugal force of 1,000 g. All standard washings and immunologic stainings of MNC from PB were done as described above for BAL macrophages. The fluorescence and the forward light scatter (cell size) were measured by FACScan. The fluorescence data are expressed as fluorescence intensity (FI) according to the formula FI = fluorescence intensity of cells labeled with a FITC-conjugated reactive antibody minus fluorescence of cells labeled with NMS.

Statistical analysis

Comparisons of data from BAL fluid and PB within the various groups of patients (i.e., sarcoidosis, IPF and EAA) were done with Wilcoxon test. Parameters of groups of patients with sarcoidosis, IPF or EAA were compared with the healthy volunteers using the Mann-Whitney test. Comparisons between sarcoidosis, IPF and EAA were done with the Kruskal-Wallis test, supplemented by pairwise application of the Mann-Whitney test if the former test indicated statistically significant differences. Five percent was considered the limit of statistical significance.

RESULTS

Total and differential cell counts from BAL fluid

The BAL cell counts in the various disease groups and healthy volunteers are summarized in Table 1. The total number of recovered BAL cells was markedly increased in the disease groups as compared with the healthy volunteers. The percentages of lymphocytes were increased in the sarcoidosis and EAA groups, while the percentages of granulocytes were increased in the IPF and EAA groups. In all three patients groups the percentages of macrophages appeared significantly lower in the disease groups while there were no statistically significant differences between the disease groups (Table 1). However, the absolute numbers of AM were increased in all disease groups (Table 1). Furthermore, in all three patient groups an increased incidence was observed of macrophages with a monocytic morphology, resembling PBM. These monocyte-like macrophages were smaller and denser than most AM and only had a few granules without prominent vacuoles.

Immunologic characterization of BAL macrophages and PBM

The expression of cell surface adhesion proteins and MHC class II antigens on BAL macrophages and PBM is presented in Tables 2 and 3, respectively. Almost all macrophages in BAL fluid were positive for CD11a. There were no statistically significant differences in the percentages of $CD11a^+$ AM between the three disease groups and the healthy volunteers (Table 2). In PB there were no differences either in the percentages of $CD11a^+$ monocytes. The absolute number of $CD11a^+$ AM was increased in EAA as compared to the healthy volunteers. The percentages of AM positive for CD11b were significantly lower than the percentages of PBM expressing this marker ($p<0.007$, Wilcoxon's test). There were no differences in the percentages of $CD11b^+$ macrophages in sarcoidosis and EAA patients as compared with the healthy volunteers. In contrast, the percentage $CD11b^+$ AM in IPF patients was much higher as compared to the healthy volunteers (60.5 ± 17.8% vs 30.3 ± 11.7%, $p<0.004$).

Differences in the expression of CD11c between PBM and BAL macrophages were also seen in the disease groups and healthy volunteers. In BAL the percentage of $CD11c^+$ AM was lower than the percentage $CD11c^+$ PBM ($p<0.007$). No differences in the percentages of $CD11c^+$ BAL macrophages were seen between the disease groups and healthy volunteers. The absolute numbers of $CD11c^+$ AM, however, were markedly increased in IPF and EAA.

Nearly all PBM (>95%) were positive for CD18. AM expressing this marker were slightly less frequent (90%) ($p<0.01$). No differences in relative frequency were found between the disease groups and the healthy volunteers, while the absolute number of AM expressing this marker was increased in EAA.

The expression of MHC class II antigens appeared to be the same on PBM and AM, while no differences between the disease groups and healthy volunteers were found both in PB and BAL.

TABLE 1. Total and differential cell counts in BAL fluid from patients with interstitial lung diseases and healthy volunteers*.

Study group	Total cell recovery x 10^6	Differential Cell Count[a]							
		Lymphocytes		Granulocytes		AM		Monocyte-like AM	
		%	x 10^4/ml	%	x 10^4/ml	%	x 10^4/ml	%	x10^4/ml
S (n=10)	21.4[b] (14.5)	35.7[e,f] (17.1)	6.3[e,g] (5.1)	3.4 (3.8)	0.7 (1.2)	62.3[d] (16.9)	9.3 (5.0)	1.8[l] (0.5)	0.2 (0.2)
IPF (n=8)	19.8[c] (10.2)	6.1 (8.2)	1.0 (1.2)	25.5[e,i,j] (12.6)	5.0[d,h] (3.2)	62.3[g] (18.6)	13.6[l] (11.6)	6.5[m] (8.4)	1.3[p] (1.3)
EAA (n=9)	43.6[d] (29.4)	37.2[g,h] (17.9)	15.4[e,h] (16.4)	9.9[b,k] (5.8)	3.7[i] (3.8)	52.3[e] (20.7)	14.9[n] (8.8)	5.5[n] (8.1)	1.3[p,r] (1.6)
HV (n=10)	8.9 (5.2)	5.9 (7.7)	0.4 (0.4)	1.9 (2.9)	0.1 (0.1)	93.1 (7.7)	6.7 (3.1)	1.0 (0.1)	0.07 (0.03)

* S, sarcoidosis; IPF, idiopathic pulmonary fibrosis; EAA, extrinsic allergic alveolitis; HV, healthy volunteer; AM, alveolar macrophage.

a. The absolute numbers are given as cells x 10^4 per ml recovered BAL fluid. Figures represent the mean with the standard deviation in parentheses.
b. Different from healthy volunteers ($p < 0.002$).
c. Different from healthy volunteers ($p < 0.009$).
d. Different from healthy volunteers ($p < 0.0002$).
e. Different from healthy volunteers ($p < 0.0001$).
f. Different from IPF ($p < 0.0005$).
g. Different from healthy volunteers ($p < 0.0003$).
h. Different from IPF ($p < 0.001$).
i. Different from sarcoidosis ($p < 0.002$).
j. Different from EAA ($p < 0.008$).
k. Different from sarcoidosis ($p < 0.01$).
l. Different from healthy volunteers ($p < 0.01$).
m. Different from healthy volunteers ($p < 0.006$).
n. Different from healthy volunteers ($p < 0.03$).
o. Different from IPF ($p < 0.007$).
p. Different from healthy volunteers ($p < 0.001$).
q. Different from IPF ($p < 0.05$).
r. Different from sarcoidosis ($p < 0.01$).

TABLE 2. Expression of the cell surface adhesion proteins and HLA-DR on BAL macrophages from patients with interstitial lung diseases and healthy volunteers*.

mAb	Sarcoidosis n=10		IPF n=8		EAA n=9		Control n=10	
	%	x 10^4/ml	%	x 10^4/ml	%	x 10^4/ml	%	x 10^4/ml
CD11a	91.7 (6.7)	8.6 (3.9)	89.1 (9.7)	13.6 (11.2)	82.2(12.9)	13.4[e] (7.4)	94.7 (4.1)	6.4 (0.9)
CD11b	30.1 (21.1)	2.5 (2.6)	60.5[a,b](17.8)	8.2[d,e](5.9)	27.2(24.7)	3.9[f] (4.9)	30.3(11.7)	2.2 (0.7)
CD11c	61.5 (13.3)	5.7 (3.0)	75.3 (9.8)	11.7 (10.1)	72.4(14.0)	11.1[g,h](4.5)	63.5(11.1)	4.6 (1.1)
CD18	92.5 (9.1)	8.5 (4.0)	85.3 (11.9)	12.9 (11.3)	83.6(12.9)	13.4[i] (7.0)	91.6 (7.2)	6.2 (0.9)
HLA-DR	88.3 (13.3)	8.0 (3.8)	90.9 (7.7)	13.0 (8.4)	77.8(20.6)	12.3 (6.7)	89.0(10.8)	6.2 (1.1)

* Mean with standard deviation in parentheses. The absolute numbers are given as cell x 10^4/ml recovered BAL fluid.

a. Different from healthy volunteers ($p < 0.004$).
b. Different from sarcoidosis and EAA ($p < 0.008$).
c. Different from healthy volunteers ($p < 0.02$).
d. Different from healthy volunteers ($p < 0.001$).
e. Different from sarcoidosis ($p < 0.02$).
f. Different from healthy volunteers ($p < 0.05$).
g. Different from healthy volunteers ($p < 0.002$).
h. Different from sarcoidosis ($p < 0.006$).
i. Different from healthy volunteers ($p < 0.008$).

Extent of expression of immunologic markers on PBM

Besides the percentages of PBM positive for the different immunologic markers, also the intensity of the fluorescence was measured with the FACScan (Table 3). We used NMS as a negative control. The data are expressed as FI (see Materials and Methods). In patients with sarcoidosis and EAA no differences were found in FI of the CD11/CD18 complex and MHC class II antigens as compared with the healthy volunteers. In contrast, marked differences in expression of these antigens were found in IPF. The FI of CD11a on PBM was lower in IPF patients than in healthy volunteers ($p<0.0009$), and sarcoidosis ($p<0.02$) and EAA patients ($p<0.008$). No difference in FI of CD11b was found between IPF and the other groups. The FI of CD11c, however, also appeared to be lower in IPF ($p<0.002$). The FI of CD18 was also lower in IPF than in the other groups, while no differences in expression of HLA-DR were found (Table 3).

DISCUSSION

In this study we investigated the differential cell count in BAL fluid, the expression of the CD11/CD18 leukocyte function antigen family and MHC class II antigen expression on PBM and BAL macrophages in healthy volunteers and patients with interstitial lung diseases.

It was found that the total cell count in BAL fluid was markedly increased in all disease groups, with substantial differences in cell profile between the groups, i.e. an increase in the percentages of lymphocytes in sarcoidosis and EAA and an increase in the percentages of granulocytes and macrophages in IPF and EAA. In addition we found an increase in monocyte-like cells suggesting an increased influx of PBM to the alveoli. These findings confirm earlier

TABLE 3. Expression of the fluorescence intensity (FI) of the cell surface adhesion proteins CD11/CD18 and HLA-DR on PBM from patients with interstitial lung diseases and healthy volunteers*.

mAb	Sarcoidosis n=10		IPF n=8		EAA n=9		Control n=10	
	%	FI	%	FI	%	IF	%	IF
CD11a	95.9 (1.3)	319 (41)	98.3 (0.7)	280[a,b,c] (36)	97.2 (1.9)	350 (46)	98.5 (0.7)	347 (36)
CD11b	88.3 (6.7)	193 (38)	91.5 (4.9)	174 (30)	91.0 (5.0)	172 (60)	87.6 (10.6)	218 (46)
CD11c	92.1 (3.8)	188 (56)	95.9 (2.0)	139[d] (40)	93.7 (3.8)	194 (68)	91.7 (6.8)	218 (45)
CD18	90.9 (18.4)	311 (43)	97.4 (1.1)	246[a,c,e] (57)	96.8 (1.6)	318 (47)	98.1 (0.9)	337 (36)
HLA-DR	88.8 (10.9)	165 (41)	97.1 (1.1)	150 (66)	91.6 (6.1)	165 (54)	93.6 (7.2)	181 (56)

* Mean percentages with standard deviation in parentheses. The fluorescence intensity (FI) is calculated as described in the Materials and Methods-section.

a. Different from sarcoidosis ($p < 0.02$).
b. Different from healthy volunteers ($p < 0.0009$).
c. Different from EAA ($p < 0.008$).
d. Different from healthy volunteers ($p < 0.002$).
e. Different from healthy volunteers ($p < 0.0003$).

described findings in patients with interstitial lung disease (2,3,26-29).

The increased influx of PBM into the alveoli in patients with interstitial lung disorders was not associated with differences in the percentages CD11/CD18$^+$ PBM between the disease groups and the healthy volunteers. High frequency of CD11/CD18 expression (≥ 90%) on PBM have also been demonstrated by Hance et al. for CD11b(OKM1) (28) and by Marwitz et al. for CD11a(CLB-LFA1/2), CD11b(OKM1) and CD11c(Leu-M5) (30). While in patients with IPF the highest number of monocyte-like cells was found in BAL fluid, the density of CD11/CD18 (especially CD11b and CD11c) on PBM was slightly lower than in the other groups. It is unclear whether or not this is related to the disease process in IPF.

Although the expression of CD11/CD18 was not increased on PBM in the disease groups, the affinity for their natural ligand may well be increased by inflammatory mediators, thus accounting for the increased influx of PBM into the alveoli. It has for example been demonstrated that leukocytes in the absence of an inflammatory stimulus do not show increased adhesion (39-41), while formyl-methionyl-leucyl-phenylalanine (FMLP) and phorbol myristate acetate (PMA) stimulated leukocytes did. This was not caused by newly recruited receptors but by changes in preexisting surface molecules (39). It has also been demonstrated that the CD18 common β chain was not phosphorylated in resting cells but became strongly phosphorylated in cells stimulated with PMA *in vitro* (42). Thus the ability of the CD11/CD18 family to mediate adhesive reactions is under strict physiological control. Another cause for the increased influx of mononuclear cells into the alveoli in interstitial lung diseases may be the increased expression of the ligand CD54 (ICAM-1) by vascular endothelium (11,12,43,44). This may be due to inflammatory mediators such as interferon-γ (IFN-γ), IL-1 and tumor necrosis factor (TNF) (45-52).

A prominent finding in the disease groups and healthy volunteers is that the percentages of CD11b$^+$ and CD11c$^+$ AM in BAL fluid were lower than the percentage of positive PBM, while no differences in percentages of CD11a$^+$ and CD18$^+$ monocytes/macrophages could be

demonstrated between PB and BAL fluid. It is also striking that the percentage and absolute number of $CD11b^+$ AM were significantly increased in IPF and EAA patients as compared to the healthy volunteers. The CD11b molecule (C3bi receptor) binds immune complexes and may therefore play an important role in these interstitial disorders. In some auto-immune disorders such as in collagen-vascular diseases, local immune complexes have been demonstrated in BAL fluid (53,54) and the increased numbers of $CD11b^+$ AM may bind these complexes and become activated. CD11b also binds fibrinogen (11) which is usually present in increased amounts in BAL fluid of IPF patients. Stimulation of this receptor has been demonstrated to induce IL-1 release (55), which has chemotactic activity (46).

PBM remain $HLA\text{-}DR^+$ after migration to the alveoli. Our finding of a very high percentage of $HLA\text{-}DR^+$ macrophages in lavage fluid confirms results from other centers in healthy volunteers (56,57) and patients with interstitial lung diseases (58). Poulter et al. (59) using scanning and integrating microdensitometry found that the mean density of HLA-DR antigen on BAL macrophages of sarcoidosis patients was increased. This is probably due to locally-released cytokines (e.g., IFN-γ) derived from activated T lymphocytes since we could not demonstrate an increase of HLA-DR expression on PBM in our study.

In summary, we demonstrated increased numbers of monocyte-like AM in BAL fluid of patients with interstitial lung diseases suggesting an increased influx of PBM. The increased number of monocyte-like AM in BAL fluid in the interstitial disorders studied was not associated with a generally increased expression of leukocyte function antigens on PBM. The percentages of $CD11b^+$ and $CD11c^+$, but not $CD11a^+$ AM were lower than the proportion of PBM positive for these markers in all disease groups as well as in healthy volunteers. However, among AM from IPF and EAA patients the absolute numbers of cells positive for the complement C3bi receptor (CD11b) were significantly increased. This is probably due to local factors since the expression of CD11/CD18 and HLA-DR on PBM was not different between the groups. It is suggested that other factors such as increased expression of CD54 on pulmonary vascular endothelium or modified CD11/CD18 molecules in interstitial lung disorders might be responsible for the increased influx of monocytic cells into lung. Currently we are investigating the expression of CD54 on vascular endothelium in histologic specimens of patients with interstitial lung disease.

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CHAPTER 7.3

BRONCHOALVEOLAR LAVAGE IN EXTRAPULMONARY SARCOIDOSIS*

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SUMMARY

Twenty-one patients presenting with extrapulmonary sarcoidosis, 20 patients with pulmonary sarcoidosis and 12 healthy volunteers were investigated. They were evaluated for roentgenographic findings as well as for immunologic marker expression of cells in bronchoalveolar lavage (BAL) fluid. The patients presenting with extrapulmonary sarcoidosis could be divided into two groups: 9 of 21 (43 per cent) presented with a stage I or stage II chest X-ray film, while 12 of 21 (57 per cent) had no chest X-ray film abnormalities (stage 0).

In all three groups of sarcoidosis patients, a significant increase of $CD3^+$ T lymphocytes in the BAL fluid was found as compared to the healthy volunteers. However, the percentages of T lymphocytes in BAL fluid of patients with extrapulmonary sarcoid lesions and a normal (stage 0) chest X-ray film were significantly lower as compared to patients with extrapulmonary sarcoidosis and an abnormal (stage I, II) chest X-ray film, while the latter patient group did not differ from the patients with pulmonary sarcoidosis. This suggests that in patients with extrapulmonary sarcoidosis a gradual progression of the T cell alveolitis may occur. Furthermore these data indicate that a marked discrepancy between chest X-ray film abnormalities and the presence of an alveolitis as determined by immunologic marker analysis exists in more than 50 per cent of the patients with extrapulmonary sarcoidosis.

INTRODUCTION

Pulmonary involvement is the most common and perhaps most important manifestation of sarcoidosis (1,2). Sarcoidosis is, however, a multisystem disorder with granulomatous inflammatory changes which can develop in almost every organ. Acute granulomatous uveitis

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may be the initial manifestation of sarcoidosis. Also, involvement of the laryngeal mucosa has been encountered. Even sarcoid of the cervix and uterus has been reported, although this is unusual. Besides symptomatic extrapulmonary lesions, asymptomatic "punched out" lesions in bone are reported (3).

In patients presenting with extrapulmonary sarcoid lesions, interstitial pulmonary changes, with or without hilar adenopathy, may be present (1,2). There may be a normal chest X-ray film, but conclusions from roentgenographic examination may underestimate the alveolitis already present.

The objective of this study was to assess the alveolitis in patients with an extrapulmonary sarcoid lesion as the first symptom.

MATERIALS AND METHODS

Patients and healthy volunteers

Bronchoalveolar lavage (BAL) fluid was obtained from 21 patients presenting with extrapulmonary sarcoidosis (7 men, 14 women; mean age: 44 ± 22.8 years), 20 patients presenting with pulmonary sarcoidosis (11 men, 9 women; mean age: 39 ± 14.3 years) and 12 healthy volunteers (9 men, 3 women; mean age 37 ± 16.0 years). The group presenting with extrapulmonary sarcoid lesions was separated in two groups, consisting of a group of 12 patients with a normal (stage 0) chest X-ray film and a group of 9 patients with an abnormal (stage I, II) chest X-ray film.

In 12 patients with extrapulmonary sarcoidosis and a stage 0 chest X-ray film, the diagnosis was based on the presence of noncaseating granulomata in biopsy specimens. From these 12 patients, one patient had a positive cervical lymph node biopsy, four patients had a positive liver needle biopsy, one patient had a positive epiglottis biopsy, one patient had a positive lachrymal gland biopsy, one patient had a positive larynx biopsy and one patient had a positive skin biopsy, while in three patients the diagnosis was based upon a positive Kveim test. In the other nine patients presenting with extrapulmonary sarcoidosis, but who upon further examination also appeared to have a positive chest X-ray film, one patient had a positive eyelid biopsy, three patients had positive transbronchial biopsies, one patient had positive endometrium biopsies, one patient had a positive skin biopsy, two had a positive liver needle biopsy and one patient had a positive Kveim test, presenting noncaseating granulomata.

In the 20 patients presenting with pulmonary sarcoidosis, the diagnosis was also based on the presence of noncaseating granulomata in biopsy specimens. From these 20 patients, two had a positive cervical lymph node, 16 had positive transbronchial biopsies, while in two patients a positive Kveim test was found. The patients did not receive corticosteroids, nor other medication. Those patients who smoked were excluded from the study.

The control group consisted of 12 healthy nonsmoking volunteers without chest abnormalities and with a normal chest X-ray film and lung function. Ethical approval was obtained for all the lavage studies.

Bronchoalveolar lavage

The BAL was performed after premedication with thiazinamium and local anesthesia with lidocaine, 2% spray. The bronchoscope was placed in wedge position and 4 aliquots of 50 ml sterile saline were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL fluid, the immunologic marker analysis was performed.

Cell samples

Cytocentrifuge preparations of BAL cells were stained with May-Grünwald-Giemsa for cell differentiation. At

least 500 cells were counted. The BAL cells and mononuclear cells from peripheral blood (PB) were analyzed for the expression of the T cell marker CD3(Leu-4), the T helper marker CD4(Leu-3) and T cytotoxic/suppressor marker CD8(Leu-2), the myeloid marker CD15(VIM-D5) and the monocytic marker CD14(My4) (4). A fluorescein conjugated goat anti-mouse-immunoglobulin antiserum was used as a second step reagent. The fluorescence was evaluated using fluorescence microscopes (Zeiss), equipped with phase contrast facilities. For each immunologic marker at least 300 cells were evaluated. Morphologic information of the labeled cells was obtained by use of phase contrast.

Statistical analysis

To investigate whether there were statistically significant differences between the four groups studied, the Kruskal-Wallis test was used for each parameter. In case of significance of this test the three disease groups (extrapulmonary sarcoidosis, extrapulmonary + pulmonary sarcoidosis, and pulmonary sarcoidosis) were each compared to the group of healthy volunteers by the Mann-Whitney test, while the Kruskal-Wallis test was used again to evaluate any differences between the three groups, followed by pairwise comparisons if indicated. The p-values less than 0.05 were considered significant.

RESULTS

The immunologic analysis of the BAL and PB cells in the 21 patients with extrapulmonary sarcoid lesions as first symptom are summarized in Table 1. Also the location of the sarcoid lesions, the recovered BAL volume, total BAL cell count and the results of the immunologic marker analysis are given for each individual patient.

The majority of the patients 16 of 21 (76%) had eye symptoms, while five of 21 (24%) had other manifestations of sarcoidosis such as bone cysts and hypercalcemiae (one patient), endometrium sarcoid lesions (one patient), erythema nodosum (one patient), epiglottitis (one patient) and laryngopharyngitis (one patient). According to the chest X-ray film examination, 12 of 21 patients (57%) presented with a normal chest X-ray film (stage 0), while 6 of 21 patients (29%) presented with a stage I chest X-ray film and 3 of 21 patients (14%) with a stage II chest X-ray film. None of the patients presented with a stage III chest X-ray film. In patients presenting with extrapulmonary symptoms, 6 of 21 patients (29%) had less than 15% $CD3^+$ lymphocytes in BAL fluid, while 15 of 21 patients (71%) had more than 15% $CD3^+$ lymphocytes in BAL fluid (Table 1). The data about the T lymphocyte populations in the PB of the patients with extrapulmonary sarcoidosis are within the normal range (Table 1).

The mean values of the immunologic marker analysis of BAL cells of the different groups are given in Table 2. The patient group presenting with extrapulmonary sarcoidosis was divided into two groups: one group with a normal chest X-ray film (stage 0) and a second group with an abnormal chest X-ray film with pulmonary involvement (stage I, II). The two groups were compared with a patient group with pulmonary sarcoidosis (stage I, II) and a group of healthy volunteers. The mean percentages of $CD3^+$ lymphocytes in BAL were significantly increased in the three patient groups as compared to the healthy volunteers. Although the percentages $CD3^+$ lymphocytes in BAL of patients presenting with extrapulmonary lesions and a normal (stage 0) chest X-ray film were significantly increased as compared to the healthy volunteers, they were significantly lower as compared to patients with extrapulmonary symptoms and an abnormal (stage I, II) chest X-ray film, 16.3 ± 16.3% and 30.4 ± 13.4%, respectively ($p < 0.003$).

TABLE 1. Clinical data and immunologic marker analysis of cells in BAL and PB in patients presenting with extrapulmonary sarcoidosis.

	Patient Diagnosis	Sex	Age (yr)	Chest[a] X-ray findings	BAL (recovered volume, ml)	Total cell recovery (x 10^6)	Immunologic marker analysis (% positive cells)									
							BAL						PB[c]			
							CD3 (Leu-4)	CD4 (Leu-3)	CD8 (Leu-2)	CD4/CD8 ratio	CD14 (My4)	CD15 (VIM-D5)	CD3 (Leu-4)	CD4 (Leu-3)	CD8 (Leu-2)	CD4/CD8 ratio
1	Uveitis, anterior	M	50	0	100	23.6	5	2	2	n.d.[b]	92	3	44	40	13	3.2
2	Perivasculitis, eye	F	55	0	150	10.3	56	45	4	11.2	49	3	34	25	10	2.5
3	Panuveitis	F	67	0	170	10.6	16	14	3	4.6	81	2	59	45	11	4.1
4	Perivasculitis, eye	M	56	0	140	24.7	18	10	6	1.6	82	2	48	21	19	1.1
5	Bone cysts, hypercalcemiae	F	54	0	160	24.2	40	34	11	3.0	51	4	28	15	21	0.7
6	Eyelid inflammation	M	37	II	90	14.2	21	24	5	4.8	69	2	59	30	25	1.2
7	Uveitis, perivasculitis, eye	F	36	II	140	17.2	21	15	6	2.5	81	0	57	23	26	0.9
8	Perivasculitis, eye	F	59	0	85	19.8	2	2	1	n.d.	96	2	56	44	17	2.5
9	Endometritis	F	39	I	100	8.4	35	30	10	3.0	39	21	71	38	23	1.7
10	Epiglottitis	F	36	0	100	11.0	16	13	2	6.5	83	2	31	27	18	1.5
11	Chorioretinitis	F	29	0	120	4.3	16	5	10	0.5	81	5	62	48	18	2.6
12	Panuveitis	M	67	I	150	8.0	17	15	2	7.5	80	3	43	42	17	2.5
13	Uveitis anterior	M	49	0	100	28.0	2	1	1	n.d.	95	3	59	37	20	1.9
14	Panuveitis	F	49	I	130	10.0	38	48	16	3.0	36	0	56	55	13	4.2
15	Panuveitis	F	34	II	150	23.8	16	15	2	7.5	82	1	59	39	11	3.5
16	Lachrymal glanditis	F	43	0	160	4.4	10	7	4	1.6	86	3	54	43	20	2.2
17	Erythema nodosum	F	27	I	150	20.7	49	40	13	3.2	41	6	34	25	10	2.5
18	Panuveitis	F	65	I	80	4.2	51	37	12	3.0	48	3	68	30	33	0.9
19	Perivasculitis, eye	F	32	0	120	20.2	4	3	1	n.d.	94	2	49	28	30	0.9
20	Laryngopharyngitis	F	30	0	160	31.6	10	7	2	3.5	89	2	72	32	22	1.5
21	Uveitis, anterior	M	26	I	120	20.6	26	26	4	6.9	63	7	47	39	12	3.3

a. Stage 0 : No chest X-ray abnormalities (12 patients)
Stage I : Hilar lymphadenopathy (6 patients)
Stage II : Hilar lymphadenopathy and pulmonary infiltration (3 patients)
Stage III : Pulmonary infiltration without hilar lymphadenopathy (0 patients).
b. n.d. : CD4/CD8 ratio not determined if < 10% lymphocytes in BAL.
c. Percentages of positive cells after Ficoll density centrifugation.

TABLE 2. Immunologic marker analysis of cells in BAL in patients with extrapulmonary sarcoidosis, pulmonary sarcoidosis and healthy volunteers.

Diagnosis	n	Sex	Age	BAL (recovered volume, ml)	Total cell recovery (x 10^6)	BAL (% positive cells) CD3 (Leu-4)	CD4 (Leu-3)	CD8 (Leu-2)	CD4/CD8 ratio	CD14 (My4)	CD15 (VIM-D5)
Extrapulmonary sarcoidosis	3	M[a]	46.7[b]	130.0	17.7	16.3[e]	11.9[e]	3.9[c]	4.1	81.6[e]	2.8
normal chest X-ray (0)	9	F	(12.5)	(30.0)	(9.3)	(16.3)	(13.8)	(3.4)	(3.4)	(15.7)	(1.0)
n=12											
Extrapulmonary sarcoidosis +	4	M	42.2	120.3	14.1	30.4[f]	27.8[f]	7.8[d]	4.6	59.9	4.8
pulmonary involvement, chest X-ray (I, II)	5	F	(15.1)	(27.0)	(6.8)	(13.4)	(12.0)	(5.1)	(2.1)	(19.2)	(6.6)
n=9											
Pulmonary sarcoidosis	11	M	39.0	140.2	24.8	29.4[f]	27.9[f]	5.4[e]	7.8	27.8[d]	3.3
chest X-ray (I, II)	9	F	(14.3)	(21.7)	(9.5)	(19.2)	(19.8)	(3.6)	(7.1)	(14.1)	(2.4)
n=20											
Healthy volunteers	9	M	37.3	130.0	14.4	3.1	1.4	2.0	n.d.[g]	48.0	2.5
normal chest X-ray (0)	3	F	(16.0)	(27.0)	(4.5)	(2.6)	(1.5)	(2.8)		(21.6)	(2.3)
n=12											

a. M = Male, F = Female.
b. Mean, with standard deviation between parentheses.
c. Significantly different from healthy volunteers ($p < 0.05$).
d. Significantly different from healthy volunteers ($p < 0.01$).
e. Significantly different from healthy volunteers ($p < 0.001$).
f. Significantly different from healthy volunteers ($p < 0.0001$).
g. n.d.: CD4/CD8 ratio not determined, since 11 of 12 healthy volunteers had < 10% lymphocytes in BAL.

No significant difference could be demonstrated between the patients presenting with extrapulmonary sarcoidosis plus pulmonary involvement and the patients presenting with pulmonary sarcoidosis. In BAL of all three sarcoidosis patient groups, the percentages of $CD4^+$ lymphocytes were significantly increased as compared to the healthy volunteers, while the percentages of $CD8^+$ lymphocytes were less increased, resulting in a high CD4/CD8 ratio.

No significant differences in the percentages of $CD4^+$ lymphocytes in BAL could be demonstrated between the patients with extrapulmonary lesions plus pulmonary involvement (chest X-ray film stage I or II) and the patients with pulmonary sarcoidosis alone (chest X-ray film stage I or II). However, the percentages of $CD4^+$ lymphocytes in BAL of patients with extrapulmonary symptoms and an abnormal (stage I or II) chest X-ray film were significantly increased as compared to the patients with extrapulmonary sarcoid symptoms and a normal (stage 0) chest X-ray film. The latter group, however, had significantly increased percentages of $CD4^+$ lymphocytes in BAL fluid as compared with the healthy volunteers ($p < 0.001$) (Table 2).

DISCUSSION

Sarcoidosis patients may present with extrapulmonary lesions due to the multisystem character of the disease (1,2). The majority of patients with extrapulmonary sarcoid lesions also have intrathoracic involvement (1,2).

In this study, it is demonstrated that 57% of the patients who presented with extrapulmonary sarcoid lesions had no roentgenographic abnormalities on the chest X-ray film, while the remaining 43% of the patients had an abnormal chest X-ray film. Since immunologic analysis of BAL cells is useful in the assessment of the intensity of the alveolitis (5-7), we analyzed cells in BAL fluid of patients with extrapulmonary sarcoidosis. In this study we demonstrated that a marked discrepancy can be present between chest X-ray film examination and determination of the alveolitis by immunologic marker analysis of cells in BAL fluid. Our data clearly indicate that patients with extrapulmonary sarcoidosis but a normal chest roentgenogram have a significant increase of $CD3^+$ T lymphocytes in the BAL fluid as compared to normal individuals. It appeared that 50% of these patients had even 15% or more T lymphocytes. Increased percentages of lymphocytes in BAL fluid of patients with extrapulmonary sarcoidosis were also reported by Wallaert et al, indicating the presence of a lymphocyte alveolitis in these patients (8). An interesting finding in our study was that the percentages of T lymphocytes in BAL fluid of patients with extrapulmonary sarcoid lesions and a normal (stage 0) chest X-ray film were significantly lower ($p < 0.003$) as compared with patients with an extrapulmonary sarcoidosis plus an abnormal (stage I, II) chest X-ray film, while the latter patient group did not differ from patients with pulmonary sarcoidosis (Table 2). This suggests that in patients with extrapulmonary sarcoidosis a gradual progression of the T cell alveolitis may occur. Our data show that this T lymphocyte alveolitis predominantly consists of $CD4^+$ lymphocytes and to lesser extent of $CD8^+$ lymphocytes resulting in a high CD4/CD8 ratio. The mean CD4/CD8 ratios in BAL fluid of the patient groups were not significantly different.

Several reports indicate that an alveolitis precedes the formation of granulomata and that granulomata formation precedes the irreversible lung fibrosis with impairment of lung function

(1-3). Patients with extrapulmonary sarcoidosis without chest X-ray film abnormalities may not yet have developed such granulomata, although the results of this study show that a $CD3^+$ T lymphocyte alveolitis is present already. In these patients, a gradual progression of the T cell alveolitis and development of granulomata may occur, since more than 40% of the patients presenting with extrapulmonary sarcoid lesions appeared to have developed chest X-ray film abnormalities. Therefore, we suggest that not only patients with extrapulmonary sarcoidosis and chest X-ray film abnormalities, but also those without chest roentgenogram abnormalities require long-term follow-up by a pulmonary physician until the disease has remained stable for years.

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CHAPTER 7.4

DIAGNOSTIC VALUE OF BRONCHOALVEOLAR LAVAGE IN OCULAR SARCOIDOSIS*

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SUMMARY

Bronchoalveolar lavage (BAL) is an investigation which has already proven its value in the diagnosis and follow-up of patients with pulmonary sarcoidosis. It also appears to be a valuable contribution to the diagnosis of patients presenting with ocular sarcoidosis.

We evaluated the findings in BAL in 16 cases with suspected ocular sarcoidosis (14 cases of uveitis, one with eyelid-swelling and one with an inflammatory process of the lacrimal gland). BAL was positive in 11 cases i.e. showed a lymphocytosis with predominantly $CD4^+$ T lymphocytes. There was one patient with ocular signs very suspect for sarcoidosis (a perivasculitis with candle wax infiltrates) with a normal percentage T lymphocytes (2%) in BAL. In two cases BAL was positive and showed a subclinical alveolitis, whereas no changes were seen on the chest X-ray film and in angiotensin converting enzyme (ACE) level.

INTRODUCTION

Sarcoidosis is a systemic disorder characterized by non-caseating granulomata. The granulomata are formed by collections of inflammatory cells derived from the mononuclear phagocyte system e.g. macrophages, epithelioid cells, multinuclear giant cells and lymphocytes. Current concepts of the pathogenesis of sarcoidosis suggest that a disturbance in T lymphocyte activity leads to the formation of epithelioid cell granulomas, which can either disappear or change to fibrotic tissue (1-3). In the lung a mononuclear cell alveolitis, comprised of activated T lymphocytes and activated macrophages, precedes and modulates the formation of granulomas (1).

Although only biopsy confirms the diagnosis of sarcoidosis, other methods of investigation can make the diagnosis highly probable (4,5). Bronchoalveolar lavage (BAL) is a technique in

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which alveolar cells are assembled and immunologically characterized. A lymphocytosis in BAL fluid of 10% or more with an elevated CD4/CD8 T lymphocyte ratio is highly suggestive for an alveolitis caused by sarcoidosis (6,7).

BAL makes early detection of the alveolitis in sarcoidosis possible (1,3,8). It has already proven its value in the contribution to the diagnosis and follow up of patients with pulmonary sarcoidosis (3,8,9). The importance of BAL as a diagnostic tool is stressed by the fact that a subclinical alveolitis can be detected when the patient has no complaints and has no abnormalities on the chest X-ray film. This has major implications on the follow-up of the patients.

Ocular sarcoidosis is found in 11-32% of the patients with sarcoidosis (9-13) and in 7% it is the first sign of the disease (14). It occurs in 4% of the patients with uveitis (8). In patients with ocular sarcoidosis James found in 74% of the cases pulmonary involvement (15). As we earlier described, we perform a chest X-ray film and a routine uveitis screening blood assay including determination of the ACE serum level in most of our cases with uveitis (16). If chest X-ray film changes or an elevated ACE level are found or if we have a strong suspicion of sarcoidosis on clinical findings (granulomatous uveitis, perivasculitis with candle wax drippings), the patients are referred to the pulmonary medicine department. The pulmonologist will add pulmonary function tests and BAL to the investigations.

In this article we evaluate the diagnostic value of BAL in patients with suspected ocular sarcoidosis.

PATIENTS AND METHODS

Patients

From January 1983 until September 1986 BAL was performed in 16 cases with suspected ocular sarcoidosis. Six men and 10 women were included. The mean age was 45 years (varying from 18 to 68 years). In 14 cases an uveitis was found. In 8 cases a panuveitis, in 3 an anterior uveitis and in 3 a posterior uveitis. One patient had swollen eyelids and one patient had an inflammatory process of the lacrimal gland. In all cases chest X-ray films and ACE level determinations were performed previously.

Bronchoalveolar lavage

BAL was performed by the pulmonologist after premedication with thiazinamium and local anesthesia with lidocain 2% spray. The bronchoscope was placed in the middle lung lobe and 4 aliquots of 50 ml sterile saline were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection of the BAL fluid the various laboratory analyses were performed. After centrifugation the cells were immunologically characterized by monoclonal antibodies against T lymphocytes.

RESULTS

The ocular findings and the characteristics of BAL cells of our patients are shown in Table 1. BAL showed a lymphocytosis of more than 10% lymphocytes in 11 cases. In these cases an elevated CD4/CD8 T lymphocyte ratio was found consistent with the alveolitis of patients

TABLE 1. General characteristics of patients with ocular sarcoidosis.

Patients no.:	Age (yrs)	Sex f/m	ACE[a]	Chest X-ray positive + negative –	BAL[b] T lympho-cytes	CD4/CD8[c] ratio	Ocular signs
1.	68	f	29.7	–	2%	n.d.[e]	perivasculitis, candle wax signs
2.	68	f	18.5	–	15%	5.6	panuveitis, papiledema OS[f]
3.	66	m	18.7	+	50%	10.2	panuveitis, perivasculitis (snowballs)
4.	64	f	20.6	+	45%	3.0	panuveitis, perivasculitis
5.	59	f	20.1	+	60%	3.0	panuveitis, CME[d] and papiledema OD[g]
6.	54	f	15.5	–	52%	18.0	perivasculitis
7.	49	m	13.2	–	3%	n.d.	uveitis anterior
8.	49	m	9.2	–	2%	n.d.	uveitis anterior
9.	43	f	20.0	+	20%	1.6	inflammatory process lacrimal glands
10.	37	m	19.1	+	25%	5.4	superior eyelid swelling ODS[h]
11.	35	f	30.5	+	20%	2.7	panuveitis, perivasculitis
12.	32	f	19.1	–	2%	n.d.	panuveitis, perivasculitis
13.	27	m	19.5	–	5%	n.d.	chorioretinitis, preretinal membranes
14.	26	m	29.4	+	54%	3.0	panuveitis granuloma iris
15.	25	f	19.7	+	24%	6.9	uveitis, anterior
16.	28	f	18.7	+	15%	10.0	panuveitis (snowballs)

a. ACE = Angiotensin converting enzyme. Normal value for our laboratory 7-20 U/L.
b. BAL = Bronchoalveolar lavage. Lymphocytosis > 10% suggestive for alveolitis.
c. CD4/CD8 = T Helper/T Suppressor ratio. Elevated CD4/CD8 ratio consists with an alveolitis of patients with sarcoidosis.
d. CME = cystoid macular edema.
e. n.d. = CD4/CD8 ratio not determined if < 10% lymphocytes in BAL.
f. OS = Oculus sinister
g. OD = Oculus dexter
h. ODS = Oculus dexter + sinister

with active sarcoidosis. In 9 of these cases chest X-ray film abnormalities were found. In these 9 patients only 3 showed an elevated ACE level. In 1 case a positive Kveim test was found and 3 other cases showed non-caseating granulomas on biopsy.

In 2 patients (patients 2 and 6, Table 1) without chest X-ray film abnormalities and with normal ACE levels an alveolitis could be demonstrated by a markedly increased percentage of T lymphocytes in BAL fluid. BAL showed normal percentages of T lymphocytes (5-10%) in 5 patients. One of these patients (patient 1, Table 1) had no chest X-ray film abnormalities, but showed an elevated ACE level and ocular findings highly suspect for sarcoidosis. The other 4 patients without chest X-ray film abnormalities and with a normal ACE level had severe ocular symptoms, possibly sarcoidosis, requiring further investigation. This was the reason why BAL was performed.

Lung-function tests appeared to be normal in all cases. In 2 patients with chest X-ray film abnormalities and an elevated percentage T lymphocytes in BAL, ACE levels were normal at the time of diagnosis, but became elevated during follow up.

DISCUSSION

In the literature several articles can be found on diagnostic methods in patients with ocular

sarcoidosis (13,15,17).

Although biopsy is the only accepted confirmation of the diagnosis it is not always possible to obtain biopsy material. If no visible lesions are available to biopsy, the best approach is a transbronchial lung biopsy. The diagnosis is then reached in about 75% of the patients (18). When the biopsy specimens are not diagnostic, a more invasive approach is needed: mediastinoscopy (95% positivity) or open lung biopsy (100% positivity).

In ophthalmology lacrimal gland biopsies are positive in about 20% of the cases (17). Blind conjunctival biopsies performed on patients with suspected sarcoidosis give positive results in about 10% of the cases (13,19). However, conjunctival biopsies when taken from suspicious conjunctival follicles can confirm the diagnosis in 40% of the cases (13). We did not notice conjunctival follicles in our patients and no blind conjunctival biopsies were taken.

At present a combination of several diagnostic methods, other than biopsy, may add to make the diagnosis of sarcoidosis highly probable (5). James developed a 13 point scheme (starting with the ocular examination and ending with advise on therapeutics) in the diagnostic management of ocular sarcoidosis (15). Weinreb and Tessler also extensively described all the known tests and they suggest classifying patients on the basis of the results in "sarcoid-suspect", "sarcoid-presumed" or "sarcoid-highly-probable" (5).

When it is difficult to obtain biopsy material, BAL may be useful in the diagnosis of sarcoidosis. A lymphocytosis in BAL fluid of 10% or more T lymphocytes is highly suggestive for an alveolitis (6,7). Such an alveolitis can be demonstrated in different diseases such as: sarcoidosis, extrinsic allergic alveolitis, histocytosis-X and in collagen diseases like scleroderma, systemic lupus erythematosus (SLE) and rheumatoid arthritis (20). An increased percentage of T lymphocytes in BAL fluid with an elevated CD4/CD8 T lymphocyte ratio is highly suggestive for an alveolitis caused by sarcoidosis and is not found in the other above mentioned diseases.

In 1985 Boscher et al. published an article on the value of BAL in the diagnosis of isolated uveitis (21). In a group of 19 patients BAL and biopsy were positive in 6 cases. They found no correlation between these results and the ACE level determination.

In our 16 cases BAL was positive in 11 cases, so that these cases are "sarcoid-highly-probable". No false positive cases were demonstrated but there was one false negative case. These findings are in accordance with Boscher et al. (21). In 2 cases BAL showed elevated percentages of T lymphocytes, although chest X-ray films and ACE levels were normal. This indicates that these patients had an alveolitis and should therefore remain under control to detect progression of pulmonary involvement. Like Boscher et al. we did not find a correlation between BAL T lymphocytes and ACE level (21).

In conclusion BAL is an important additive investigation in the diagnosis of suspected ocular sarcoidosis, also when chest X-ray film and ACE level are normal.

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CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8

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8.1 MATURATION OF MONOCYTES

Mononuclear phagocytes are derived from the common committed progenitor cell for the granulocyte and monocyte-macrophage pathways, the granulocyte-monocyte colony-forming unit (CFU-GM) (1,2). The developmental sequence of mononuclear phagocytes is as follows: CFU-GM → monoblast → promonocyte → monocyte → tissue macrophage (3). In Chapter 5, the expression of the relevant markers for each differentiation stage is indicated. Some markers are not always present but are acquired during differentiation while other markers show a diminished expression during differentiation. This diversity of the human monocyte/macrophage system as detected by monoclonal antibodies has also been demonstrated by Hogg (4) and Radzun et al. (5). A group of glycoprotein hormones termed colony stimulating factors (CSF) induce differentiation of the CFU-GM into a monoblast and subsequently into the promonocyte and circulating monocyte (2). In addition to the growth - and differentiation - promoting activities of CSF (such as IL-3, GM-CSF and M-CSF) on hematopoietic progenitor cells, CSF have been demonstrated to influence mature monocytes and macrophages. They can stimulate the expression of cell surface markers such as Fc receptors (6) and HLA-DR (7) on monocytic cells. In Chapter 6, section 3 we demonstrate that granulocyte/monocyte-colony stimulating factor (GM-CSF) and IL-4 *in vitro* stimulate peripheral blood monocytes (PBM) to express a mature macrophage phenotype. *In vivo*, PBM also undergo a process of maturation to macrophages while migrating into tissues (3) (Figure 1). Others have demonstrated other effects of various CSF such as increased phagocytosis (8) and antimicrobial activity (8,9). These findings suggest that CSF do not only influence hematopoietic progenitor cells but also influence mature monocytes and macrophages.

PBM are able to mature into two different cell types: (a) antigen-presenting "dendritic" cells that express high levels of HLA-DR antigen and do not stain for myeloperoxidase or acid phosphatase in the cytoplasm; and (b) phagocytic macrophages strongly positive for acid phosphatase (10). Ultrastructurally, dendritic cells typically contain relatively few cytoplasmic organelles associated with phagocytic function, while macrophages are rich in lysosomes and endocytic vacuoles (11-13). Dendritic cells are highly active cells with respect to accessory functions including antigen presentation and secretion of mediators such as IL-1 for initiating the T cell and T-dependent B cell activation, whereas cells generally classified as macrophages (including alveolar macrophages (AM)) have a predominant scavenger function and therefore contain higher levels of lysosomal enzymes. To date there are no conclusive data that monocytes are destined for any particular function or tissue when they leave the bone marrow.

Although dendritic cells and scavenger macrophages differ in many respects, they also

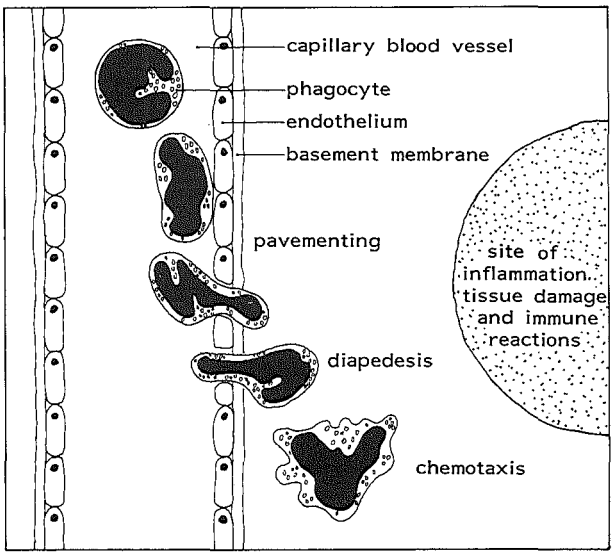

Figure 1. Monocyte migration out of a blood vessel. Monocytes adhere to the vascular endothelium (pavementing). They insert pseudopodia between the endothelial cells and dissolve the basement membrane (diapedesis). They then pass out of the blood vessel and move to the site of inflammation.

share a number of cell surface antigens and functional characteristics. Both dendritic cells and monocytes can stimulate autologous and allogeneic T cells, be it that the capacity of dendritic cells is much larger than of monocytes (14). Attempts have been made to discriminate these two cell types by employing mAb. It has been claimed that Ki-M4 (15) and RFD1 (16) discriminate dendritic cells from monocytes/macrophages. By using these mAb in combination with morphology, distinct monocyte/macrophage subsets have been demonstrated in human fetal tissue (17-19). The surface antigens CD68(Ki-M6) and Ki-M8 were found on scavenger macrophages and became progressively expressed during fetal development and particularly postnatally.

Isolation of dendritic cells from lymphatic tissue and subsequent double staining with Ki-M4 and other mAb (20) showed that dendritic cells were positive for CD71 (transferrin receptor), CD54(ICAM-1), HLA-DR and CD11b and, to a lesser extent, CD14(Leu-M3). Najar et al. (21) have demonstrated that monocyte/macrophage markers of PBM such as Fc receptors and nonspecific esterase show an inverse correlation with the expression of accessory activity, while morphologically the appearance of veils positively correlated with accessory function. In this particular study it seemed that factors in serum influence morphologic and immunologic phenotype and the accessory properties of mononuclear cells.

It has been demonstrated by Peters et al. (22) and Kabel et al. (23) that dendritic cells can develop from precursors in the monocytic pool. Kabel et al. (23) demonstrated that metrizamide has an enhancing effect on this differentiation. Like Radzun et al. (5) he demonstrated that PBM and dendritic cells share several cell surface antigens such as CD14. The findings in Chapter 6, section 1 support the view that AM are also derived from PBM. Thus our experiments together with those of Kabel et al. (23) support the view that phagocytic macrophages and dendritic cells can develop from PBM but do not exclude the possibility of distinct subpopulations among PBM that give rise to the two cell lines.

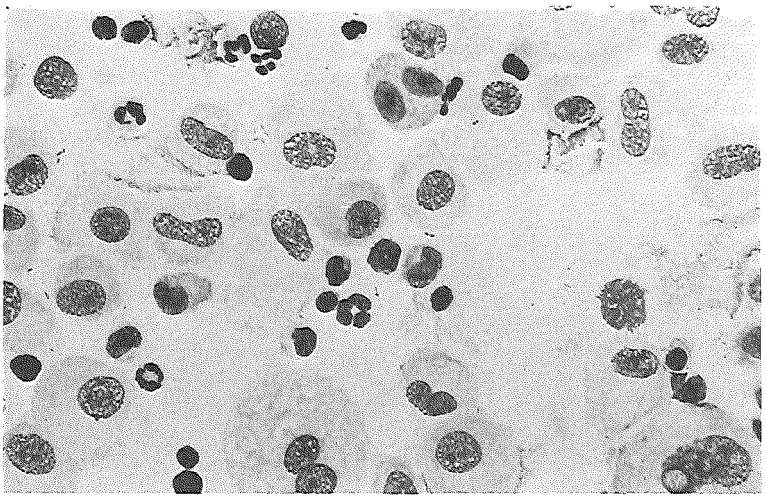

Figure 2. Cellular component of BAL fluid from a non-smoking healthy volunteer. Note the presence of AM with a different morphologic phenotype.

We describe in Chapter 6, sections 1 and 2 that immunologic markers present on PBM are also present on AM. However, AM with an immature morphology resembling PBM, i.e. small cells with only a few irregularly shaped lysosomal structures, had an immunologic phenotype also resembling PBM, whereas mature AM with large vacuoles containing multi-lamellar structures exhibited a phenotype different from PBM and from immature AM (Figure 2). This suggests that AM derive from PBM and that migration and differentiation of PBM is associated with the appearance of changes in immunophenotype.

The monocytic origin of AM has been demonstrated by Blussé van Oud Alblas et al. (24) and Adamson et al. (25). In pulmonary inflammation AM undergoing mitosis have been found by Bitterman et al. (26) indicating that apart from migration of PBM to the alveoli, macrophage replication may also play a role in the expansion of the AM population. Other findings confirming the monocytic origin of AM are from Radzun et al. (27) showing that monocytes acquire acid phosphatase activity after stimulation with lymphokine-conditioned media and acquire the morphologic appearance of AM. During maturation PBM also acquire peroxidase activity (28,29). In inflammatory situations increased numbers of AM without peroxidase activity in the endoplasmic reticulum were observed. These cells appear to reflect the monocyte influx into the lung.

Given the continuous migration of PBM into the lungs and the characteristic differences in immunologic phenotype observed between PBM and AM, i.e. the decreased expression of CD14 and the increased expression of RFD9 on AM, we investigated in Chapter 6.3 the expression of monocyte/macrophage antigens CD11a, CD13, CD14, and RFD9 and the expression of acid-phosphatase on elutriator purified PBM during *in vitro* culture. In addition the effect of recombinant cytokines (IL-2, IL-4 and GM-CSF) on the immunophenotype of the cultured cells was studied. It was found that cultured PBM acquired the morphology (Figure 3)

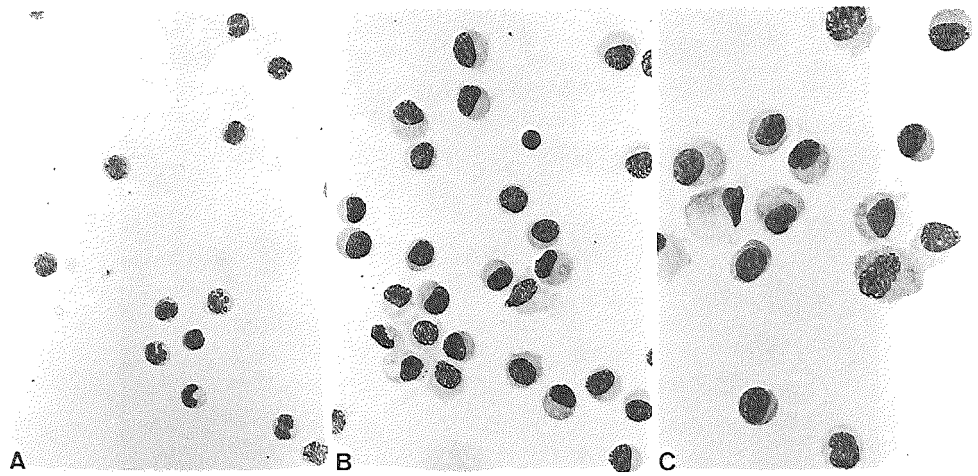

Figure 3. May-Grünwald Giemsa staining of **(A)** freshly isolated PBM; **(B)** PBM cultured for 60 hours; and **(C)** AM present in BAL fluid. An increased cytoplasm/nucleus ratio is present in cultured PBM, while AM show the greatest ratio.

and immunophenotype of mature macrophages and became positive for acid phosphatase. The latter represents a clearcut difference between mature macrophages such as AM and PBM (30). IL-2 had no effect on the expression of the different myeloid markers studied. This is probably caused by the absence of IL-2 receptors on unstimulated PBM (31). Certain cytokines can induce the receptor for one or more other cytokines that are required for stimulation. It is suggested that cytokines in serum can influence maturation associated monocyte cell surface antigen expression in a dose dependent way (32). We found that IL-4 and GM-CSF enhanced the acquisition of RFD9 and loss of CD14 on PBM, while dexamethasone inhibited these changes. The data of other markers confirmed the findings of Te Velde et al. (33), who showed increased expression of HLA-DR, CD11b(44) and CD11c(Leu-M5) and of Stuart et al. (34), who showed induction of class I and class II (HLA-DR) antigen expression after treatment of PBM with IL-4. This may explain the increased antigen presenting ability of these cells after treatment with IL-4 as has been demonstrated by Zlotnik et al. (35).

In conclusion, the experiments of Chapters 6.1 and 6.3 illustrate the importance of the local microenvironment in determining the properties of monocytes/macrophages. In addition our findings in Chapter 6.3 suggest that the anti-inflammatory influence of dexamethasone (often used in interstitial lung diseases) is amongst others mediated by inhibition or suppression of membrane receptor expression.

8.2 MONONUCLEAR CELLS IN INTERSTITIAL LUNG DISEASE

Mononuclear cells play an important role in interstitial lung diseases such as sarcoidosis, extrinsic allergic alveolitis (EAA) and idiopathic pulmonary fibrosis (IPF). In Chapter 7.1 and 7.2

increased absolute numbers of mononuclear cells in BAL fluid of patients with interstitial lung diseases are reported. These findings confirm those described by Turner-Warwick (36), Snider (37) and Hance et al. (38). The maturation associated changes in immunophenotype of mononuclear phagocytes described in Chapters 5, 6.1-6.3 were also observed in patients with interstitial lung diseases. For most of the markers studied, the percentages PBM expressing these markers were much higher than the percentages positive AM. In contrast AM were more frequently positive for some other markers, i.e. RFD9 and CD68. For most of the myeloid markers used, in the PB of the three patient groups studied no differences in percentages positive monocytes were found, in contrast to AM. The percentages of $CD68^+$ PBM and AM, however, were significantly increased in IPF patients.

CD68 has been reported to represent an intracytoplasmatically localized antigen (39-44). In these studies immunoperoxidase staining techniques on cytospin preparations were used. In contrast, using the FACScan for analyses of PBM we found membrane expression of this antigen, be it in a small proportion of the cells. Because CD68 expression increases during differentiation of PBM into macrophages, it may be confined to a function which increasingly develops during monocyte/macrophage maturation. It has been demonstrated that CD68 is mainly confined to the lysosome and phagosome structures of macrophages (39) which have been demonstrated to become increasingly expressed during activation. The observation that CD68 is functionally related to the generation of oxygen radicals during the respiratory burst in phagocytosing cells may indicate that the PBM of IPF patients are activated. In a study of Strausz et al. (42) it was demonstrated that the bulk of the production of reactive oxygen intermediates in cells from BAL fluid of IPF patients was derived from AM.

The marked differences in AM phenotype in different interstitial lung diseases are probably due to local factors, depending on location and type of inflammation. The lower expression of CD14 by AM as compared to PBM described in Chapters 6.1 and 6.2 has also been demonstrated by others (43-45). That local factors can influence the expression of cell surface antigens has been demonstrated by Firestein et al. (46) who observed that IFN-γ induces a decrease in expression of CD14 and Monocyte-2 on freshly isolated PBM. The immunophenotype of mononuclear phagocytes is also influenced by the cellular microenvironment. Noble et al. (47) showed that $RFD9^+$ macrophages were entirely absent from the interstitium in contrast to the alveoli where $RFD9^+$ macrophages were abundantly present. The discrete compartmentalization of phenotypically different macrophages within the lung suggests that in each microenvironment macrophages may contribute in a different way to lung pathology. Munro et al. (48) demonstrated that epithelioid cells and giant cells in granulomata were positive for RFD9 while macrophages in the surrounding mantle were negative. The function of myeloid antigens is now becoming progressively unraveled and in the near future it will become clear which function the recognized molecules perform. This will elucidate the function of mononuclear phagocytes at distinct anatomical sites and in different inflammatory reactions. For example, Look et al. (49) found that the human myeloid cell membrane glycoprotein CD13 is identical to aminopeptidase-N and its function is signal transduction. Goyert et al. (50) demonstrated that the CD14 monocyte differentiation antigen maps to a region of chromosome 5 encoding growth factors and receptors. These products are not all made simultaneously but at different stages of the mononuclear phagocyte life cycle, indicating that AM perform different functions at each stage of their maturation.

Mononuclear phagocytes are equipped with adhesive receptors which account for cell-cell interactions that are important for migration into extravascular tissue and interaction with antigens (51). Adherence to vascular endothelial cells, which precedes monocyte migration into extravascular tissue is dependent on interactions between the monocyte receptors CD11/CD18 and the CD54 ligand on endothelial cells (51). The expression of these receptors is described in Chapters 6.2 and 7.2. It was found that the expression of molecules of the CD11/CD18 cell surface adhesion protein family was higher on PBM than AM. This may indicate that mature macrophages have a diminished capacity as immune accessory cells which has indeed been demonstrated by Toews et al. (52). We could not demonstrate an increased expression of these antigens on PBM in smokers or in interstitial lung disease, despite increased numbers of mononuclear phagocytes in BAL-fluid from the individuals studied. The increased migration may be stimulated by an increased expression of CD54 by the endothelial cells at the sites of inflammation or by phosphorylation of the CD11/CD18 complex which increases cellular adhesion (53). Increased expression of CD11/CD18 and CD54 has been demonstrated to depend on stimulation by locally derived cytokines (54,55). In our study increased numbers of $CD11b^+$ and $CD11c^+$ AM were found in IPF patients, suggesting the local production of such inflammatory cytokines in IPF.

8.3 CLINICAL APPLICATIONS OF BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage (BAL) was initially used as a therapeutic measure in order to remove secretions in patients with alveolar proteinosis and cystic fibrosis. The introduction of the flexible bronchoscope made it possible to perform subsegmental lavage using small volumes of lavage fluid. Under local anesthesia this is a safe procedure to isolate cells from the lungs for subsequent studies of changes that occur during pulmonary inflammation.

BAL is currently widely applied to study different lung diseases (56-59). The lingula and middle lobe are the sites most commonly lavaged, because fluid can be recovered from these locations most easily when the patient is in a supine position. In Chapter 5 we demonstrate which immunologic markers can be used for immunophenotyping of cells in BAL fluid. The cells usually present in BAL fluid are T cells (5-10%), monocytes/macrophages (85-90%) and neutrophils, eosinophils and basophils (1-2%), while mature B cells are scarce. This cell distribution has also been found by others (56-59). In non-smoking adults, cell yields equal 8-10 x 10^6 cells in the recovered BAL fluid. The cells can be visualized by light-microscopy using Giemsa staining in order to make a proper cell differential.

In patients with overt pulmonary inflammation, marked differences in cell yield and cell differential occur. In smokers and in patients with interstitial lung disease, the cell yield in BAL fluid is up to five times larger (Chapters 6.2 and 7.1-7.3) as compared to healthy non-smoking volunteers. This contrast between the lavage cells from smokers and non-smokers and the other groups emphasizes the need to study both the total cell number and the cell differential. It has been suggested (60,61) that proteinases derived from excessively increased numbers of activated cells in smokers may damage the extracellular matrix resulting in alteration of lung structure and function.

The changes in cell number and differential in patients with interstitial lung disorders is

described in Chapters 5 and 7. Lymphocytes are usually increased in BAL fluid of patients with sarcoidosis and hypersensitivity pneumonitis. In sarcoidosis immunologic marker analysis often reveals an increase in $CD4^+$ lymphocytes resulting in a high CD4/CD8 ratio (>3.0). In contrast, in hypersensitivity pneumonitis, BAL done shortly after exposure shows an increase in neutrophils, while after a few days an increase of $CD8^+$ T lymphocytes occurs resulting in a low CD4/CD8 ratio (<0.8). These observations have also been done by Crystal et al. (62,63). This indicates that lung lavage can be of great value as a screening test in order to select other confirming tests in the diagnosis of chronic interstitial lung disease. In patients with IPF the alveolitis is usually characterized by increased percentages of neutrophils and eosinophils (Chapter 7.1). One must be careful in the interpretation of differential cell counts from BAL fluid since advanced stages of sarcoidosis and hypersensitivity pneumonitis may be associated with fibrosis with increased numbers of neutrophils in BAL fluid, as has been demonstrated by Turner-Warwick et al. (58). Other components in BAL fluid must also be taken into account, for example asbestos bodies in combination with a high percentage of neutrophils suggests the presence of asbestosis instead of IPF (57,58).

Special stains may be helpful in the diagnosis of certain interstitial disorders such as Perl's stain for ferric iron to identify iron-laden AM in the diagnosis of pulmonary hemosiderosis (60) or a fat staining to identify lipid laden vacuoles in macrophages in gastro-oesophageal reflux (64,65).

BAL is also increasingly used for the diagnosis of infectious diseases such as in Pneumocystis carinii pneumonia, pneumonitis due to cytomegalovirus infection and others in immune compromised patients as was reported by the Task Group on BAL of the European Society of Pneumonology (66).

In Chapters 7.3 and 7.4 we demonstrate that BAL permits early detection of alveolitis in patients with extrapulmonary sarcoidosis. It was found that the percentages of T lymphocytes in BAL fluid of patients with extrapulmonary sarcoid lesions and a normal chest X-ray film were increased but lower than in patients with extrapulmonary sarcoidosis and an abnormal chest X-ray film. This suggests that in patients with extrapulmonary sarcoidosis a gradual progression of the T cell alveolitis may occur. Subclinical alveolitis in patients with normal chest X-ray films has also been demonstrated by Wallaert et al. in primary biliary cirrhosis (67), Crohn's disease (68) and in collagen-vascular diseases (69). Our data and those of Wallaert et al. (67-69) indicate that an alveolitis does not necessarily lead to a clinically significant process although a marked discrepancy between chest X-ray film abnormalities and the presence of an alveolitis as determined by immunologic marker analysis exists. BAL may have prognostic value since Wallaert et al. (69) demonstrated in patients with collagen vascular diseases that the presence of neutrophils in BAL fluid was associated with a progressive deterioration.

In conclusion, BAL provides the opportunity to sample cells present in the inflamed lung. This is of value in the initial diagnosis, especially if attention is paid to the differential inflammatory cell count and immunologic marker analysis. Thereby more invasive diagnostic procedures can often be prevented. If necessary, additional information on the BAL cells can be obtained by special staining methods. Its value in following activity of disease is still unproven. Further studies are therefore necessary to investigate whether other aspects of cells in BAL fluid such as activation markers or components of the supernatant, alone or in combination with the already studied markers, have prognostic significance.

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SUMMARY

Interstitial lung diseases are a heterogeneous group of illnesses with different pathogeneses. In interstitial lung diseases there often is an increased influx of cells from the peripheral blood (PB) to the interstitium and alveoli. Besides the increase in total cell numbers, often marked shifts in the cell populations occur.

This thesis describes the immunophenotype of the cells involved in three types of interstitial lung diseases, namely sarcoidosis, idiopathic pulmonary fibrosis (IPF) and extrinsic allergic alveolitis (EAA). Emphasis is put on monocytic cells and macrophages. The cell surface markers of monocytes and macrophages as well as the immunophenotype of cultured purified monocytes have been studied. In addition surface antigens of cells from PB and bronchoalveolar lavage (BAL) fluid have been studied in the forementioned interstitial lung diseases.

In **Chapter 1** the structure of the alveoli and interstitium is described. Interstitial lung diseases are only partially diseases of the interstitium. The inflammatory process often involves the respiratory bronchioles as well as the alveolar walls and airspaces. The inflammation may heal completely or may result in the development of an excess of connective tissue with the distortion of lung architecture and honeycombing, leading to a restrictive lung function.

In **Chapter 2** the pathogeneses of granulomata and fibrosis are described. Granulomatous inflammations are characterized by organized aggregates of mononuclear phagocytes, dendritic cells, plasma cells and T lymphocytes. It is described that granulomatous reactions occur in several interstitial lung disorders, e.g. tuberculosis, sarcoidosis and EAA. Fibrosis may appear as a chronic process that remains stable for years, but it can also suddenly progress to an irreversible pulmonary fibrosis. To identify the fibrosing process as idiopathic or cryptogenic, all other causes of fibrosis and scarring such as radiation, asbestosis, drug reactions, and collagen vascular diseases such as systemic lupus erythematosus and rheumatoid arthritis should be excluded.

In **Chapter 3** the role of monocytes in granulomata and fibrosis is described. Monocytes in granulomata may become activated by bacterial products or T lymphocyte derived cytokines. These activated monocytes in turn produce mediators that among others attract other monocytes. The newly-arrived monocytes develop into epithelioid cells and multinucleated giant cells. These cells together result in granulomata.

Monocytes play also a role in lung fibrosis through the release of factors that cause accumulation of mesenchymal cells such as fibroblasts, and subsequent synthesis of connective tissue (so called extracellular matrix) molecules such as fibronectin, collagen, elastin and proteoglycans.

Chapter 4 is an introduction to the experimental work that is described in Chapters 5, 6 and 7.

In **Chapter 5** the immunologic markers and their recently developed nomenclature are described. Immunologic markers are proteins, sometimes with one or more carbohydrate

groups, located in the cell membrane, cytoplasm or nucleus. These (glyco)proteins are called immunologic markers because they can be visualized by different immunologic techniques. The immunologic markers are recognized by a specific, usually monoclonal, antibody. Many of them become increasingly expressed during lymphoid and myeloid differentiation. Other markers become upregulated during activation by certain cytokines such as IFN-γ and disappear again after deactivation. Immunologic marker analysis of cells in BAL fluid from patients with interstitial lung diseases gives information about shifts in differentiation stages and activation of the inflammatory cells in the BAL fluid. This information is also relevant to the interpretation of *in vitro* stimulation tests of cells from BAL fluid and PB, since an impaired response might be due to an aberrant cell composition.

In **Chapter 6.1** it is investigated whether alveolar macrophages (AM) with an immature morphology resembling blood monocytes (PBM), i.e. small cells which only show a few irregularly shaped lysosomal structures, have an immunologic phenotype different from mature AM, which display large vacuoles containing multi-lamellar structures. This was done with a broad panel of monoclonal antibodies (mAb). The majority of PBM was positive for these markers in contrast to AM. The immunophenotype of AM with an immature morphology was different from mature AM. Most markers studied appeared to be increased on immature AM. The most prominent differences were found for the markers CD14 and RFD9. The marker CD14 is expressed on the great majority of immature AM, in contrast to the marker RFD9. Based on these findings double immunofluorescence staining was performed. Double labeling of BAL cells showed AM with the following immunophenotype: $CD14^+RFD9^-$, $CD14^+RFD9^+$, $CD14^-RFD9^+$ and $CD14^-RFD9^-$. The $CD14^+RFD9^-$ subset had an immature morphology resembling PBM, in contrast to $CD14^-RFD9^+$ macrophages, indicating that during macrophage maturation expression of CD14 is downregulated, while RFD9 is upregulated.

The expression of another set of molecules, i.e. the cell surface adhesion glycoproteins CD11/CD18 on PBM and AM is described in **Chapter 6.2**. This family of glycoproteins consists of three molecules, the leukocyte function associated antigen-1 (LFA-1), the C3bi receptor (CR3; Mac-1) and p150,95. These are heterodimeric antigens with different α subunits (CD11a, CD11b or CD11c, respectively) and a common β subunit (CD18). In non-smokers the percentages PBM expressing cell surface adhesion glycoproteins were higher than the percentages BAL macrophages expressing these markers. This indicates that during migration of PBM into the alveoli the cell surface adhesion glycoproteins decrease in expression. In the smokers group, the absolute number of AM was five-fold increased, but the proportion of receptor positive cells (CD11a, CD11b, CD11c and CD18) in smokers was decreased, indicating either a down-regulation of these cell surface adhesion glycoproteins on smokers AM or shedding of their CD11/CD18 molecules from smokers AM. Although increased absolute numbers of AM were found in BAL fluid from smokers, no differences could be demonstrated in the percentages PBM expressing these markers, nor in fluorescence intensity as measured by flow immunocytometry (FACScan). This indicates that the increased influx of PBM into the alveoli in smokers is not reflected by an increased expression of the above mentioned cell surface adhesion glycoproteins on PBM in smokers.

The modulation of the immunophenotype of elutriator purified monocytes under culture conditions is described in **Chapter 6.3**. It is demonstrated that the expression of CD11a did not change during culturing and that a slight increase of CD13 expression occurred, while the

expression pattern of CD14 changed markedly resulting in the appearance of both a $CD14^-$ and $CD14^+$ subpopulation. However, the RFD9 expression clearly increased upon culturing. GM-CSF and IL-4 enhanced these processes, whereas dexamethasone prevented the changes in expression of the above mentioned markers. During culturing of mononuclear phagocytes the forward and sideward light scatter changed, indicating that the granularity and the cell volume increase during maturation. After culturing, PBM resembled AM not only in terms of morphology and immunophenotype, but also with regard to positivity for endogenous acid phosphatase.

The immunophenotype of PBM and AM in interstitial lung diseases is described in **Chapter 7.1**. This was done using a broad panel of myeloid mAb. The expression of the various markers on PBM was evaluated using FACScan. In all groups the percentages PBM positive for CD13, CD14, CD33 and Max3 were higher than the percentages BAL macrophages positive for these markers. The mAb Max24 was equally expressed on PBM and AM, while the percentages of AM positive for RFD9, CD68(Ki-M6) CD68(Ki-M7) and CD68(Y2/131) were higher than the percentages positive PBM. Thus maturation is associated with a diminished expression of certain markers, while others such as RFD9 and CD68 become upregulated.

The CD68(Ki-M6) marker is associated with the generation of oxygen radicals during the respiratory burst and increased chemiluminescence. Due to the increased cell count in the BAL fluid of EAA patients, the absolute numbers of positive monocytes/macrophages in BAL fluid were significantly increased for most of the markers studied. In IPF patients, the percentages of $CD68(Ki\text{-}M6)^+$ monocytes/macrophages were elevated in both PB and BAL fluid. In sarcoidosis, however, no differences in percentages $CD68^+$ cells could be demonstrated as compared to the healthy volunteers.

The expression of the cell surface adhesion glycoprotein family CD11/CD18 on PBM and AM from patients with interstitial lung diseases is described in **Chapter 7.2**. In PB no differences in the percentages positive monocytes could be demonstrated between the three disease groups and healthy volunteers. The percentage and the absolute number of $CD11b^+$ AM, however, were significantly increased in IPF patients. Similarly, in IPF and EAA patients the absolute number of $CD11c^+$ AM was increased. These results suggest that the increased influx of PBM into the alveoli of patients with interstitial lung diseases is not reflected by a generally increased expression of CD11/CD18 cell surface adhesion glycoproteins, neither among PBM, nor among AM. The increased expression of these cell surface adhesion glycoproteins on AM in some interstitial lung diseases suggests activation by locally produced cytokines in these conditions.

In **Chapter 7.3** the alveolitis of patients with extrapulmonary sarcoidosis is described. The patients with extrapulmonary sarcoidosis could be divided in those with an abnormal chest X-ray film (stage I or stage II) and those with a normal (stage 0) chest X-ray film, and were compared with sarcoidosis patients with an abnormal chest X-ray film alone. In all three groups of sarcoidosis patients a significant increase in T lymphocytes in the BAL fluid was found. However, the percentages of T lymphocytes in BAL fluid from patients with extrapulmonary sarcoid lesions and a normal (stage 0) chest X-ray film were significantly lower than in patients with extra-pulmonary sarcoidosis and an abnormal (stages I or II) chest X-ray film, while the latter patient group did not differ from the patients with pulmonary sarcoidosis. This suggests that in patients with extrapulmonary sarcoidosis a gradual progression of the T cell alveolitis

may occur. Furthermore, these data indicate that in patients with extrapulmonary sarcoidosis a marked discrepancy may exist between chest X-ray film abnormalities and the presence of an alveolitis as determined by immunologic marker analysis of cells from BAL fluid.

In **Chapter 7.4** the diagnostic value of BAL in ocular sarcoidosis is described. BAL was positive in 11 out of 16 cases. In two cases BAL showed elevated percentages of T lymphocytes, although the chest X-ray film and ACE level were normal. This indicates that these patients have a subclinical alveolitis and should therefore remain under control. Together these data suggest that analysis of cells in BAL fluid is an important additive tool in the diagnosis of suspected ocular sarcoidosis.

In conclusion, the studies in this thesis show that the available panel of monocytic mAb can be used to define the several stages of monocyte/macrophage maturation. The local microenvironment influences the immunophenotype of the monocyte/macrophage cells. Most importantly, cell-differentials, special stainings and immunologic markers of BAL leukocytes can be used as an additional diagnostic tool in interstitial lung diseases. Thereby the use of more invasive diagnostic procedures can often be prevented.

SAMENVATTING

Interstitiële longziekten vormen een heterogene groep aandoeningen met verschillende oorzaken en een verschillend beloop. Er is bij interstitiële longziekten vaak een toegenomen migratie van cellen uit het bloed (PB) naar het interstitium en de alveoli. Naast een toename van het totale celaantal treedt er dikwijls ook een verschuiving op in de samenstelling van de alveolaire celpopulatie.

In dit proefschrift worden de immunofenotypen beschreven van cellen die een rol spelen bij drie typen interstitiële longziekten, namelijk sarcoïdose, idiopathische pulmonale fibrose (IPF) en extrinsieke allergische alveolitis (EAA). De nadruk wordt gelegd op monocyten en macrofagen. Er werd onderzoek gedaan naar zowel de immunologische markers van monocyten en macrofagen als naar het immunofenotype van gezuiverde en vervolgens gekweekte monocyten. Daarnaast werd gekeken naar monocytaire markers op cellen in het PB en de bronchoalveolaire lavage (BAL) vloeistof van patiënten met interstitiële longziekten.

In **hoofdstuk 1** wordt de struktuur van de alveoli en het interstitium beschreven. Interstitiële longziekten zijn slechts ten dele een ziekte van het interstitium. Het ontstekingsproces betreft dikwijls tevens de respiratoire bronchioli en de alveoli. Het proces kan volledig genezen, maar kan ook aanleiding geven tot de vorming van een excessieve hoeveelheid bindweefsel, met een verstoring van de architectuur van de longen, een honingraattekening op de thoraxfoto en een restrictieve longfunktie.

In **hoofdstuk 2** wordt de pathogenese van granulomata en fibrose beschreven. Granulomateuze ontstekingen worden gevormd door georganiseerde aggregaten van mononucleaire fagocyten, dendritische cellen, plasmacellen en T-lymfocyten. Granulomateuze ontstekingsreakties treden op bij verschillende interstitiële longziekten zoals tuberculose, sarcoïdose en EAA. Longfibrose kan chronisch verlopen en jaren stabiel blijven, maar kan ook plotseling toenemen tot een irreversibele fibrose. Om een fibrotisch proces idiopatisch te noemen moeten alle andere oorzaken van fibrose zoals bestraling, asbestose, geneesmiddelen reakties, en bindweefselziekten zoals lupus erythematosus en reumatoïde artritis worden uitgesloten.

In **hoofdstuk 3** wordt de rol van monocyten in granuloomvorming en fibrose beschreven. Monocyten in granulomen kunnen worden geaktiveerd door bacteriële produkten of door van T-lymfocyten afkomstige cytokinen. De geaktiveerde monocyten produceren op hun beurt weer mediatoren die onder andere monocyten aantrekken. Deze nieuw-gearriveerde monocyten ontwikkelen zich tot epithelioid cellen en meerkernige reuscellen. Deze cellen worden betrokken bij de granuloomvorming ter plaatse. Monocyten spelen eveneens een belangrijke rol bij fibrose doordat zij stoffen produceren die mesenchymale cellen (zoals fibroblasten) aantrekken, en de produktie van grondstoffen voor bindweefsel (z.g. extracellulaire matrix) induceren zoals fibronectine, collageen, elastine en glycoproteïnen.

In **hoofdstuk 4** worden de experimenten geïntroduceerd die beschreven worden in de hoofdstukken 5, 6 en 7.

In **hoofdstuk 5** worden de immunologische markers en hun recent ontwikkelde nomenclatuur beschreven. Immunologische markers zijn eiwitten, al dan niet voorzien van suikergroepen, die in de celmembraan, in het cytoplasma of in de celkern voorkomen. Deze eiwitten worden immunologische markers genoemd, omdat hun aanwezigheid kan worden aangetoond met immunologische technieken. Deze immunologische markers worden meestal herkend door een specifieke monoklonale antistof (mAb). De expressie van veel van deze markers neemt gedurende de lymfoïde en myeloïde differentiatie toe. Andere markers komen tot expressie na activatie van de cel door bepaalde cytokinen zoals IFN-γ, om weer te verdwijnen wanneer de cel in de rustfase terugkeert. Onderzoek van immunologische markers van cellen in de BAL vloeistof van patiënten met een interstitiële longziekte geeft informatie over verschuivingen in differentiatiestadia en activatie ten gevolge van deze ziekte. Onderzoek van immunologische markers is ook van belang voor de interpretatie van *in vitro* stimulatietesten van cellen uit BAL vloeistof en PB, omdat de hoogte van de respons direkt samenhangt met de samenstelling van het onderzochte celmonster.

In **hoofdstuk 6.1** wordt onderzocht of alveolaire macrofagen (AM) die er "onrijp" uitzien en die lijken op monocyten in het bloed (PBM), d.w.z. kleine cellen met slechts enkele lysosomale strukturen met onregelmatige vorm, een immunologisch fenotype hebben dat verschilt van rijpe macrofagen. De laatste bevatten grote vacuolen met multilamellaire structuren. Dit onderzoek werd verricht met een uitgebreid panel mAb. De meeste PBM brachten de onderzochte markers tot expressie. Dit in tegenstelling tot de AM. Het immunologische fenotype van minder "rijpe" AM verschilde van dat van rijpe AM. De meeste onderzochte immunologische markers werden sterker tot expressie gebracht door onrijpe AM dan door rijpere AM. De meest opvallende verschillen in expressie werden gevonden voor de markers CD14 en RFD9. De marker CD14 komt tot expressie op de meerderheid van de onrijpe AM, in tegenstelling tot de marker RFD9, die juist op de rijpere AM meer tot expressie komt. Op basis van deze bevindingen werd een dubbelkleuring toegepast. Dubbelkleuring van cellen in de BAL vloeistof toonde vier subpopulaties AM aan met het volgende immunologische fenotype: $CD14^+RFD9^-$, $CD14^+RFD9^+$, $CD14^-RFD9^+$ en $CD14^-RFD9^-$. De $CD14^+RFD9^-$ AM subset had de kenmerken van onrijpe AM en toonde gelijkenis met PBM, in tegenstelling tot de $CD14^-RFD9^+$ AM subset, die de kenmerken van rijpe macrofagen had. Deze gegevens wijzen erop dat gedurende de uitrijping van macrofagen de expressie van CD14 omlaag gaat, terwijl die van RFD9 juist toeneemt.

In **hoofdstuk 6.2** wordt de expressie van een ander panel markers op PBM en AM beschreven, de zogenaamde cel adhesie eiwitten CD11/CD18. Deze groep membraaneiwitten bestaat uit drie antigenen, te weten: het Leukocyten Funktie Antigeen-1 (LFA-1), de C3bi receptor (CR3; Mac-1) en p150,95. Dit zijn antigenen bestaande uit twee eiwitketens, samengesteld uit verschillende α-ketens (respectievelijk CD11a, CD11b en CD11c) en een gezamenlijke β keten (CD18). PBM brachten de cel adhesie eiwitten in een hoger percentage tot expressie dan AM. Dit duidt erop dat gedurende de migratie van PBM naar de alveoli een vermindering van expressie van cel adhesie eiwitten optreedt. Bij de groep rokers was het absolute aantal AM een faktor vijf verhoogd, maar het percentage AM dat de cel adhesie eiwitten CD11a, CD11b, CD11c en CD18 tot expressie bracht, was verlaagd. Dit duidt erop, dat tabaksrook de expressie van deze cel adhesie eiwitten vermindert. Hoewel bij rokers het aantal AM is toegenomen, werd er noch in het percentage PBM dat deze markers tot expressie

bracht, noch in de intensiteit van expressie zoals die werd gemeten d.m.v. flow cytometrie (FACScan), een verschil gevonden. Dit duidt erop dat de toegenomen influx van PBM in de alveoli tijdens ontstekingen niet gepaard gaat met een toegenomen expressie van bovengenoemde cel adhesie eiwitten door PBM.

De invloed van het kweken op het immunologische fenotype van d.m.v. de elutriator gezuiverde PBM wordt beschreven in **hoofdstuk 6.3**. Er werd gevonden dat gedurende het kweken de expressie van CD11a niet veranderde en dat de expressie van CD13 in geringe mate toenam, terwijl de expressie van CD14 aanzienlijk veranderde en resulteerde in een CD14 positieve en CD14 negatieve subpopulatie. De expressie van RFD9 nam duidelijk toe gedurende het kweken. Toediening van GM-CSF of IL-4 aan het kweekmedium veroorzaakte een verdere toename van de hierboven beschreven veranderingen in expressie. Dexamethason daarentegen, kon deze veranderingen in expressie voorkomen. Tijdens het kweken van PBM veranderde de voorwaartse en zijwaartse lichtverstrooiing op de FACScan, hetgeen erop duidt dat het aantal granulae in de cellen toenam en het volume van de cellen groter werd. Na enkele dagen kweken lijken PBM op AM, niet alleen wat betreft hun morfologie en immunologisch fenotype, maar ook wat betreft de aanwezigheid van het enzym zure fosfatase in het cytoplasma.

Het immunologisch fenotype van PBM en AM bij patiënten met interstitiële longziekten wordt beschreven in **hoofdstuk 7.1**. Dit werd onderzocht met een uitgebreid panel myeloïde mAb. De mate van expressie van de verschillende markers op PBM werd onderzocht d.m.v. de FACScan. In alle groepen bleek dat het percentage PBM dat positief was voor de markers die door CD14, CD33 en Max3 worden herkend, hoger was dan het percentage positieve AM in de BAL. De marker die wordt herkend door Max24 kwam in gelijke mate tot expressie op PBM en AM, terwijl het percentage AM dat RFD9, CD68(Ki-M6), CD68(Ki-M7) en CD68(Y2/131) tot expressie bracht, hoger was dan het percentage PBM. Dus migratie en uitrijping gaan gepaard met een verminderde expressie van sommige markers, terwijl andere, zoals RFD9 en CD68, in verhoogde mate tot expressie worden gebracht. Tengevolge van het toegenomen aantal cellen in de BAL vloeistof bij patiënten met een EAA, was voor de meeste markers het absolute aantal positieve AM toegenomen.

De CD68(Ki-M6) marker is geassocieerd met de vorming van zuurstofradicalen gedurende de respiratoire burst en met een toegenomen chemiluminescentie. In patiënten met een IPF waren de percentages van CD68(Ki-M6) positieve cellen verhoogd in PB en BAL vloeistof.

De expressie van de cel adhesie eiwitten CD11/CD18 op PBM en AM van patiënten met interstitiële longziekten wordt beschreven in **hoofdstuk 7.2.** In het PB konden geen verschillen worden aangetoond in de percentages positieve monocyten. In IPF patiënten, maar niet in de andere patiëntengroepen, waren het percentage en het absolute aantal $CD11b^+$ AM significant toegenomen. Bovendien bleek dat bij IPF en EAA patiënten het absolute aantal $CD11c^+$ AM in de BAL vloeistof was toegenomen. Deze bevindingen suggereren dat de toegenomen influx van PBM in de alveoli van patiënten met interstitiële longziekten niet het gevolg is van een toename in expressie van CD11/CD18 cel adhesie eiwitten. De toegenomen expressie van deze cel adhesie eiwitten op AM bij sommige interstitiële longziekten suggereert activatie door lokaal geproduceerde cytokinen.

In **hoofdstuk 7.3** wordt de alveolitis van patiënten met een extrapulmonale sarcoïdose beschreven. De patiënten met een extrapulmonale sarcoïdose konden worden verdeeld in een

groep met een abnormale thoraxfoto (stadium I of II) en een groep met een normale thoraxfoto (stadium 0). Deze twee groepen werden vergeleken met een groep sarcoïdose patiënten met alléén pulmonale afwijkingen en een abnormale thoraxfoto (stadium I of II). In alle drie groepen werd een significant verhoogd percentage T-lymfocyten in de BAL vloeistof gevonden. Het percentage T-lymfocyten in de BAL vloeistof van patiënten met een extrapulmonale sarcoïdose en een normale thoraxfoto (stadium 0) was significant lager dan bij patiënten met een extrapulmonale sarcoïdose en een abnormale thoraxfoto (stadium I of II), terwijl de laatste groep niet verschilde van de patiënten met een pulmonale sarcoïdose. Dit suggereert dat bij patiënten met een extrapulmonale sarcoïdose een discrepantie kan bestaan tussen de bevindingen op de thoraxfoto en de eventuele aanwezigheid van een alveolitis zoals deze kan worden aangetoond d.m.v. immunologische marker analyse van cellen in BAL vloeistof.

In **hoofdstuk 7.4** wordt de diagnostische waarde beschreven van bronchoalveolaire lavage bij patiënten met oogafwijkingen die op sarcoïdose zouden kunnen berusten. Van elf van de zestien patiënten toonden de cellen in de BAL vloeistof een beeld dat paste bij sarcoïdose. Bij twee patiënten toonde analyse van de BAL vloeistof een verhoogd percentage T-lymfocyten aan, terwijl de thoraxfoto normaal was. Blijkbaar kan bij deze patiënten een subklinische alveolitis bestaan. Daarom moeten deze patiënten, om verdere progressie te voorkomen, onder controle blijven. Deze gegevens wijzen erop dat analyse van cellen in BAL vloeistof een belangrijk aanvullend onderzoek kan zijn bij patiënten met oogafwijkingen t.g.v. een sarcoïdose.

Concluderend kan worden gezegd dat het onderzoek in dit proefschrift aantoont dat het beschikbare panel monocytaire mAb kan worden gebruikt om de verschillende stadia van uitrijping van monocyten/macrofagen te karakteriseren. Het lokale micromilieu beïnvloedt het immunologische fenotype van monocyten/macrofagen. Immunologische marker analyse van leukocyten in de BAL vloeistof kan worden gebruikt als aanvullend hulpmiddel in de diagnostiek van interstitiële longziekten. Daardoor kan het gebruik van meer invasieve ingrepen dikwijls worden vermeden.

ABBREVIATIONS

AA	Arachidonic acid
ACE	Angiotensin converting enzyme
AM	Alveolar macrophages
AMDGF	Alveolar macrophage derived growth factor
b.v.	Besloten vennootschap
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guérin
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
C3bi	Cleaved C3b
CD	Cluster of differentiation
CFU-GM	Colony forming unit, of the granulocyte-monocyte lineage
CME	Cystoid macular edema
CMI	Cell-mediated immunity
ConA	Concanavalin A
cpm	Counts per minute
CR	Complement receptor
CSF	Colony stimulating factor
CyCD3	Cytoplasmic expression of CD3 antigen
CyIg	Cytoplasmic Ig
DIP	Desquamative interstitial pneumonia
e.g.	Exempli gratia: for example
EAA	Extrinsic allergic alveolitis
EM	Electron microscopy
FACS	Fluorescence activated cell sorter
Fc	Constant fragment of immunoglobulin
FcR	Fc receptor
FCS	Fetal calf serum
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
FMLP	Formyl-methionyl-leucyl-phenylalanine
G-CSF	Granulocyte colony stimulating factor
GIP	Giant cell interstitial pneumonia
GM-CSF	Granulocyte/monocyte colony stimulating factor
GP	Glycoprotein
GpA	Glycophorin A
HETE	Hydroxyeicosatetraenoic acid
HLA	Human leukocyte antigen
i.e.	Id est: that is to say
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
kDa	Kilo Dalton
LC	Langerhans cell
LFA	Leukocyte function antigen
LIP	Lymphoid interstitial pneumonia
LPS	Lipopolysaccharide
M-CSF	Monocyte colony stimulating factor
mAb	Monoclonal antibody
MAF	Macrophage activating factor
MCF	Monocyte chemotactic factor
MFR	Mannosyl-fucosyl receptor
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MNC	Mononuclear cells
MPO	Myeloperoxidase
MW	Molecular weight
n.d.	Not determined
NK	Natural killer cell
NMS	Normal mouse serum
PAF	Platelet activating factor
PB	Peripheral blood
PBM	Peripheral blood monocytes
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PG	Prostaglandin
PHA	Phytohemagglutinin
PLC	Phospholipase C
PMN	Polymorphonuclear granulocyte
rIL	Recombinant interleukin
SD	Standard deviation
SmIg	Surface membrane Ig
TcR	T cell receptor
TcR-CD3	Complex of TcR and CD3 chains
TdT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
TX	Thromboxane
vs	Versus
[^{3}H]	Tritiated
[^{3}H]-TdR	Tritiated thymidine

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Rotterdam,
19 december 1990

Henk Hoogsteden

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One thing I have learned in a long life:
that all our science, measured against
reality, is primitive and childlike - and
yet it is the most precious thing we have.

Albert Einstein