DIAGNOSTISCHE WAARDE VAN BRONCHOALVEOLAIRE LAVAGE BIJ INTERSTITIËLE LONGZIEKTEN

door
Marjolein Drent

Aantonen van longaandoeningen door middel van het spoelen van de long

De bouw en ligging van de longen maken uniek contact met de buitenwereld mogelijk. De long vertakt zich als een boom, net als in de bladeren vindt in de longblaasjes (alveoli) de gaswisseling (zuurstof-opname, respectievelijk koolzuur-afgifte) plaats. Het oppervlak van de longen bedraagt ± 70 m^2, van de huid slechts 1,5-2 m^2. De long neemt een belangrijke plaats in het afweersysteem in. Het verdedigingssysteem van de long bevat zowel a-specifieke onderdelen - zoals hoesten en niezen - als specifieke componenten, zoals cellen en door cellen afgegeven produkten. Het afweersysteem kan actief worden onder invloed van bepaalde prikkels en schadelijke stoffen van buiten af zoals bacteriën, contact met dieren of schadelijke dampen, maar ook door van binnen uit via de bloedsomloop in de longen terecht gekomen stoffen.

Wanneer de 'eenvoudige', a-specifieke verdedigingsmechanismen niet afdoende effect hebben, treden de meer specifieke afweermechanismen in werking. De cellen betrokken bij de verdediging hopen zich dan op in het longweefsel en produceren ten gevolge van deze prikkels allerlei, soms ook voor het longweefsel schadelijke, stoffen. Deze cellen - en hun produkten - bevinden zich in het longweefsel en mede door beschadiging van het longoppervlak ook op het oppervlak van de longblaasjes.

Als een patiënt zich met longklachten bij een arts meldt, is het van belang uit te zoeken wat de oorzaak van die klachten is. Naast een uitgebreid interview vindt een lichamelijk onderzoek plaats. Vervolgens wordt meestal een röntgenfoto gemaakt en bloedonderzoek gedaan. Bepaalde aandoeningen, zoals infecties, kwaadaardige aandoeningen en allerlei andere longziekten kunnen aanvullend onderzoek noodzakelijk maken. Liefst wordt hiervoor zo betrouwbaar mogelijk, doch voor de patiënt zo min mogelijk belastend, onderzoek gebruikt.

Begin 70er jaren is er een techniek ontwikkeld - het spoelen van de long met een beperkte hoeveelheid voor de long onschadelijke vloeistof (fysiologisch zout): de bronchoalveolaire lavage (BAL) - die het mogelijk maakt cellen en niet-cellulaire bestanddelen uit de long te verzamelen en aan een nader onderzoek te onderwerpen. Via het spoelen van de long kan men een indruk krijgen van de cellen en stoffen, die zich op het oppervlak van de longblaasjes bevinden. Er is gebleken dat bepaalde aandoeningen, zoals duivenmelkerslong, sarcoïdose ofwel de ziekte van Besnier-Boeck, en bindweefselvorming in de long ten gevolge van schadelijke prikkels - die vooral de longblaasjes aantasten - hele karakteristieke kenmerken vertonen in de spoelvloeistof.

Aan de hand van een profielschets van de spoelvloeistof is het dikwijls mogelijk een uitspraak te doen over de aard van de bij de desbetreffende patiënt gevonden afwijkingen. Wel dient rekening gehouden te worden met een aantal verstorende factoren. Zo beïnvloedt bijvoorbeeld het rookgedrag van de patiënt de samenstelling van de spoelvloeistof enorm. Het spoelen van de long is een betrekkelijk eenvoudig uitvoerbare techniek, heeft nauwelijks bijwerkingen en kan in de toekomst met meer verfijnde analysemethoden wellicht voor de patiënt belastende ingrepen ter opheldering van het onderliggend lijden vervangen.

DIAGNOSTIC VALUE OF BRONCHOALVEOLAR LAVAGE IN INTERSTITIAL LUNG DISEASES

The research presented in this thesis was performed at the Departments of Pulmonary Diseases, Pathology, Immunology and Microbiology of the Sint Antonius Hospital, Nieuwegein, and the department of Epidemiology en Biostatistics of the Erasmus University and the Department Pulmonary Diseases of the Dijkzigt Hospital, Rotterdam, The Netherlands.

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DIAGNOSTIC VALUE OF BRONCHOALVEOLAR LAVAGE IN INTERSTITIAL LUNG DISEASES

Diagnostische waarde van bronchoalveolaire lavage bij interstitiële longziekten

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Erasmus Universiteit te Rotterdam op gezag van de rector magnificus Prof. Dr. P.W.C. Akkermans M. Lit. en volgens het besluit van het College van Dekanen. De openbare verdediging zal plaatsvinden op woensdag 8 december 1993 om 11.45 uur

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Ter nagedachtenis aan mijn oma

Contents

Preface

When the "fathers" of bronchoalveolar lavage (BAL), Reynolds and Newball, developed this technique as a research tool almost 20 years ago, they most likely did not foresee the further career of lavage in full detail. Meanwhile, the "child" has reached "adulthood", and its usefulness also for clinical applications has been appreciated worldwide in the field of interstitial lung diseases and particularly of opportunistic infections. BAL recovers cells and solutes from the lower respiratory tract and the alveolar spaces. It opens a window to the lung. It is thought that alterations in lavage fluid and cells reflect pathological changes in the corresponding parenchymal constituents. BAL has several advantages over biopsy procedures. It is safe, minimally invasive, and associated with virtually no morbidity.

No wonder that clinicians are increasingly fond of performing lavage for diagnostic purposes, although the results of BAL should not be used esoterically but should be considered in the context of other information from conventional investigative methods. Clearly, there are some diseases and conditions which can definitely be diagnosed by BAL, such as pulmonary infections, malignant infiltrates, alveolar proteinosis, alveolar haemorrhage syndromes, eosinophilic pneumonia, and dust exposure. In others, the BAL findings, such as an increase in lymphocytes or neutrophils, are rather nonspecific but can be helpful in certain clinical circumstances. For example, given a patient with slowly progressive interstitial lung disease, finger clubbing, and subpleural fibrosis with honeycombing found on computer tomography (CT) examination, lavage cytology with an increase in neutrophils with or without eosinophils strongly supports the diagnosis of idiopathic pulmonary fibrosis and excludes other diseases with high probability so, that open lung biopsy may not be necessary in this patient. Diseases with an increase in lavage lymphocytes can be further differentiated by measuring the $CD4^+/CD8^+$ ratio in BAL. This ratio may be increased, normal, or decreased, depending on the underlying condition. Particularly in sarcoidosis, an elevated ratio is of high diagnostic specificity.

It is with great pleasure that I deliver this short introduction to Marjolein Drent's thesis.

Essen, September 1993 *Ulrich Costabel*

Voor het kennen van de weg,
moeten we gewoon op weg.
Dingen doen, liefst met plezier,
heel simpel op de Poeh-manier.
Maar ga niet zoeken naar de weg,
want je zal zien, dan is hij weg!
Maar als je nu de dingen doet,
de dingen die jij kan,
dan vind je heus de weg vanzelf,
en de weg komt achter je an!

Uit: Tao van Poeh, Benjamin Hoff

General introduction

Although details hidden in bronchoalveolar lavage may add useful information about a patient's disorder, one should never forget the importance of the individual's unique history and physical examination.

Marjolein Drent

1.1 Historical introduction

Currently, bronchoalveolar lavage (BAL) is widely applied in both fundamental research and for clinical purposes in pulmonology.[1-3] Initially, this technique was used as therapeutic tool, *eg*, in the management of phosgene poisoning and as a means to remove abundant secretions.[4] Gradually, BAL became more widely applied in the treatment of various pulmonary disorders, such as cystic fibrosis, alveolar microlithiasis and alveolar proteinosis.[5,6]

The application of BAL for diagnostic purposes is only a relatively recent development.[7-9] In particular, BAL has significantly improved the diagnostic workup of interstitial lung diseases (ILD) and opportunistic infections in the immunocompromised host[10] and ventilated patients.[11,12]

When applied according to standardized protocols, BAL may be of value for the assessment of the diagnosis, course and prognosis, as well as for the evaluation of the effect of treatment in some pulmonary diseases.[13]

1.2 Bronchoalveolar lavage

After premedication and local anaesthesia of the larynx and bronchial tree, a fiberoptic bronchoscope is passed into the airways and gently impacted, or 'wedged', into a subsegmental bronchus.[1] Sterile saline, prewarmed to body temperature, is injected into the subsegment through the bronchoscope biopsy channel, and subsequently, aspirated and recovered.

From an anatomical point of view, the middle lobe is the most convenient lobe to lavage.[14] In general, the middle lobe or the lingula are used as standard sites for lavage. From these lobes, 20 percent or more fluid and cells are recovered than from the lower lobes.[15] The basic assumption is, that the injected fluid reaches the pathological area and that the aspirate will be a representative sample containing solutes and a population of cells from the lower respiratory tract, reflecting the pathophysiology of the disease process.

The major technical variation in the performance of BAL is the volume of fluid used. The volume infused usually ranges from 100 to 300 ml with aliquots of 20 to 50 ml. Reports by various groups performing lavage with different amounts of saline suggest that, at least in healthy individuals, the information about cell types obtained in volumes ranging from 100 to 250 ml are comparable.[9]

In general, results obtained at one site are representative for the whole lung. In patients with localized lesions, such as inflammatory infiltrates, malignant lesions etc, it is recommended to choose the area of greatest abnormality, as seen on the chest x-ray, as the preferred site for BAL.[1]

Though attempts have been made to standardize the BAL-technique, details such as the amount of fluid injected, the number of aliquots used, the 'dwelling time' and the aspiration pressure, still vary greatly among laboratories.[16-18]

1.2.1 Cellular and noncellular constituents in the bronchoalveolar space

Cells in BAL fluid samples of healthy controls are alveolar macrophages (AM; 80 to 90 percent of the total cell count), lymphocytes (5 to 15 percent); neutrophils (1 to 2 percent), eosinophils and mast cells (1 to 2 percent). These normal values may vary due to confounding factors, of which cigarette smoking is the most important.[16,19] The aforementioned cells all are potentially inflammatory cells. In patients with pulmonary inflammation marked differences in cell yield and cell differentiation may occur.

Alveolar macrophages

Macrophages play an important role in host defence, *eg*, phagocytosis of foreign materials (antigen engulfment) and killing of invading micro-organisms and the release of factors involved in inflammation.[20] Macrophage colony stimulating factor (M-CSF) stimulates the production of monocytes and macrophages from bone marrow progenitor cells and stimulates the secretion of cytokines (see table 1). Blood monocytes are the precursors of macrophages, but local proliferation of macrophages has also been demonstrated.[21] After antigenic stimulation, blood monocytes leave the vascular compartment to become tissue macrophages. In addition, the influx of monocytes increases, which contributes to an enhanced number of AM.[22]

Recent studies have demonstrated that, in the lung, under normal steady state conditions, local proliferation of AM is responsible for the maintenance of the major part of the AM population, whereas there is only a minor influx of monocytes from the interstitium.[23,24] The ultrastructure of the AM is characterized by a single, lobulated nucleus, and cytoplasm

containing vacuoles, mitochondria and lysosomes. Alveolar macrophages are the only macrophages which function under aerobic circumstances.[22]

Table 1—*Major Products Released by Alveolar Macrophages*

Cytokines	*Oxygen metabolites*
interleukin-1 α and ß	O_2-radicals, H_2O_2, OH^-
interleukin-6	*Proteins*
tumour necrosis factor α	antiproteases
α interferon	α_2-proteinase inhibitor
colony stimulating growth factors	α_2-macrogobulin
transforming growth factor ß	plasminogen activator inhibitor
fibroblast growth factor (insulin growth factor I)	collagenase inhibitor
neutrophil activating factor	other inhibitors
enzyme-releasing peptide	interleukin-1 inhibitor
neutrophil chemotactic factor	neutrophil migration inhibitor
platelet-derived growth factor	fibroblast growth inhibitor
histamine releasing factor	lipomodulin
Enzymes	glycoprotein
lysozyme, ß-glucuronidase	fibronectin
acid hydrolases	complement components:
angiotensin converting enzyme	C2, C4
elastase: serine and metalloenzyme	binding proteins
collagenase:	transferrin, ferritin
fibroblastlike	apolipoprotein E
type V (gelatinase)	*Free fatty acids*
plasminogen activator	*Antioxidants*
cysteine proteinase (cathepsin L)	glutathione
Biologically active lipids	*Coagulation factors*
cycloxygenase metabolites	factor V
thromboxane A_2	factor VII
prostaglandins (E_2, D_2, 6-keto-PGF- 1-α and F2-α)	*1,25 dihydroxyvitamin D_3*

(adapted from Sibille and Reynolds 1990[22])

During inflammatory processes, $CD4^+$ T lymphocytes release soluble factors, such as interferon (IFN)-γ and other macrophage-activating cytokines. Interferon-γ enhances the expression of major histocompatibility complex (MHC) molecules and thus enables primed AM to present antigen to T- and B lymphocytes.[25] In this way, antigen-activated $CD4^+$ T lymphocytes can activate phagocytes.

In addition to their role as phagocytic cells, AM are essential elements of the inflammatory response. They have the capacity to secrete a large number of molecules that regulate the immune response, release components of the complement cascade, procoagulants and anticoagulants, proteases, lipases, DNAses, metalloproteinases, reactive oxygen and nitrogen intermediates (see table 1).[22,26]

In the process of digesting an antigen, AM release interleukin-1 (IL-1). Interleukin-1 increases the recruitment of T lymphocytes, neutrophils and monocytes. Moreover, it induces T-lymphocyte proliferation and IL-2 production, as well as B lymphocyte proliferation and antibody synthesis. In this way, AM subserve a number of important effector functions including immunoregulation, selective removal of autologous cells and resolution of acute inflammation.[22] It is clear therefore that cytokines, acting alone or in concert, are capable of exerting a considerable effect in which components of the vast repertoire of macrophage functions are expressed in any given situation. Uncontrolled secretion of any of these mediators can produce extensive tissue damage.[27]

The increased levels of macrophage secretory products in BAL obtained from patients with various pulmonary diseases suggest a pathogenic role for AM in certain pulmonary disorders such as some ILD.[22]

Lymphocytes

Monoclonal antibodies specific for cell surface differentiation antigens, in combination with flow cytometry techniques, constitute useful tools to explore the phenotype of BAL lymphoid cells. Immunophenotyping allows to discern a great variety of lymphocyte subpopulations with distinct functional properties.[28] The various monoclonal antibodies used in immunophenotyping by various immunological laboratories, have been grouped together into so-called clusters of differentiation (CD), according to their specificity in antigen recognition.[29] Throughout their development from stem cell into mature effector cell, lymphocytes express different antigens. The expression of particular surface antigens reflects the stage of activation-differentiation attained by lymphocytes, such as the α chain (CD25) of the IL-2 receptor, the transferrin receptor (CD71), and human leukocyte antigen (HLA)-DR molecules.[30,31] Some of these structures have been characterized as receptors, involved in trophic functions during cell

activation.[28,31,32]

Under physiological circumstances lymphocytes constitute 5 to 15 percent of the cells present in the BAL fluid. Functionally, 3 distinct lineages of lymphocytes are to be distinguished: T and B lymphocytes, and natural killer (NK) cells. The numbers of T lymphocytes in BAL are similar to those found in peripheral blood (60 to 80 percent), the percentage of B lymphocytes is lower or similar (0.5 to 5 percent) and the percentage of NK cells usually is higher in BAL fluid (10 percent).[22,31]

T lymphocytes

T lymphocytes initiate and mediate immune responses and regulate the activity of other leukocytes by secreting soluble factors. A correlation exists between the expression of certain membrane antigens, *eg* HLA-DR, and the functional activity of T lymphocytes.[25,28] T lymphocytes can be divided into subsets based on their respective expression of $CD4^+$ or $CD8^+$ antigens on the cell surface. T lymphocytes recognize antigen(s) complexed with MHC molecules on antigen presenting cells (APC) through their T cell antigen receptor (TCR). Binding to the TCR and activation of related molecules induces the expression of a number of growth factor receptors, such as the IL-2 receptor. Several cytokines are involved in T-lymphocyte activation and proliferation. In addition, upon antigenic stimulation, T lymphocytes secrete soluble factors which regulate the activity of other leukocytes. Thus, antigen-specific stimulation of $CD4^+$ T lymphocytes results in the production of many cytokines (*eg*, IL-2, -3, -4, -5, and -6, granulocyte macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF) and IFN-γ).

Studies of $CD4^+$ T-lymphocyte clones have resulted, at least in mice, in differentiation of T helper (TH) lymphocytes into two subsets: TH1 and TH2 lymphocytes. These subtypes of TH lymphocytes produce different cytokines (see table 2).[27,33]

In humans, representatives of these two subtypes do exist. The molecules inducing the differentiation of TH precursors into TH1 or TH2 lymphocytes may be related to the nature of the antigenic trigger. Tuberculin favour a TH1 type of response, whereas allergens favour a TH2 differentiation. In addition, TH1 induces delayed hypersensitivity responses and favours the activation of specific cytotoxic $CD8^+$ T lymphocytes and NK cells by the production of IL-2 and IFN-γ. TH2

lymphocytes can control TH1 lymphocytes and vice versa. The TH2 lymphocytes therefore inhibit the production of cytokines by TH1 lymphocytes and NK cells by releasing IL-4 and IL-10. TH1 lymphocytes, by releasing IFN-γ, block the proliferation and differentiation of mast cells or eosinophils enhanced by the TH2 production of IL-3, IL-4, IL-5 and IL-10.

Table 2—*Cytokines Secreted by T Lymphocytes*

	$CD8^+$	$CD4^+$(TH1)	$CD4^+$(TH2)
Interferon-γ	+ +	+ +	—
Interleukin (IL)-2	+	+ +	—
Tumour necrosis factor (TNF) ß	+	+ +	—
Granulocyte macrophage colony-stimulating factor	+ +	+ +	+
TNF-α	+	+ +	+
IL-3	+	+ +	+ +
IL-4	—	—	+ +
IL-5	—	—	+ +
IL-6	—	—	+ +
IL-10	?	—	+ +

(adapted from Nicod 1993)[27]

TH lymphocytes ($CD4^+$) have the ability to augment B lymphocyte responses and to amplify the cell-mediated responses effected by $CD8^+$ T lymphocytes. Cytotoxic T ($CD8^+$) lymphocytes mediate most antigen-specific cytotoxity. In addition to their cytotoxic function, $CD8^+$ T lymphocytes can suppress the immune response functions.

A prominent accumulation and recruitment from the blood of activated T lymphocytes has been found in the lungs of patients with certain ILD, especially sarcoidosis and extrinsic allergic alveolitis (EAA), collagen-vascular diseases, tuberculosis and malignant lymphomas.[9] Furthermore, a differential increase in T-lymphocyte subsets, both in quality and quantity, has been found. It is widely accepted that, in contrast to sarcoidosis, predominant $CD8^+$ T-lymphocyte alveolitis is a feature of EAA at an acute phase. Also in pulmonary fibrosis different subsets of T lymphocytes have

been found. In several cases in pulmonary fibrosis $CD4^+$ T-lymphocyte-dominant BAL findings may occur, whereas $CD8^+$ T-lymphocyte dominance in BAL fluids may disappear.[34-36] These findings suggest that increased $CD4^+$ T-lymphocyte subtypes might play an important role in the pathogenesis of fibrosis according to cytokine production (see table 1). Since high numbers of $CD8^+$ T lymphocytes are present at time points when no severe fibrosis is present and $CD8^+$ T lymphocytes disappear when fibrosis occurs, it might well be that cytokines derived from $CD8^+$ T lymphocytes have a protective effect on the development of pulmonary fibrosis or that relatively increased $CD4^+$ T-lymphocyte subtypes might play an important role in the pathogenesis of pulmonary fibrosis according to different cytokine production (see table 1).[37]

This suggests that different pathogenic processes of T-lymphocyte activation and accumulation may occur in patients with ILD.[38] In addition, as mentioned before, a functional heterogeneity of T-lymphocyte subsets and their secretion products is considered to be present.[27,39,40]

B lymphocytes

B lymphocytes represent 5 to 15 percent of the circulating lymphoid pool and are classically defined by the expression of immunoglobulins on the cell surface membrane. These surface immunoglobulins function as antigen receptors. Following T- and B lymphocyte activation by mitogen or antigen, distinctive differentiation features are observed. TH2 lymphocytes induce a strong differentiation and proliferation of B lymphocytes by the production of IL-4, IL-5 and IL-10. Interleukin-2, IL-4 and IFN-γ are also capable of inducing proliferation and differentiation of B lymphocytes. Upon binding of the antigen to specific surface immunoglobulins, B lymphocytes may become activated and differentiate into clones of antibody-secreting plasma cells. The latter cells secrete large amounts of antibodies (2,000 molecules/cell/sec) into blood and tissues. The mature plasma cell can be recognized by an eccentric nucleus with a large amount of basophilic cytoplasm, due the abundant RNA required for protein synthesis.

Under physiological conditions, plasma cells, known as tissue cells, are not found in BAL fluid samples nor in the peripheral blood. However, plasma cells are present in BAL fluid of patients with pulmonary inflammatory processes with an antibody-mediated component, such as EAA.[36]

Derangements of the alveolar walls and loss of functional capillary units are characteristics of inflammation of the lower respiratory tract. Destruction of the basement membrane of alveolar walls causes a passive leak of plasma cells amongst other cells, which normally are only located within the alveolar interstitium. Thus, in BAL fluid, various differentiation stages of B lymphocytes may be present.

Neutrophils

In normal subjects, polymorphonuclear neutrophils represent the majority of the intravascular leukocytes, and only a few can be observed within the alveolar space. However, the lung vasculature and, more specifically, the capillary bed contains a large reservoir of neutrophils.[22] To perform their function in tissues, neutrophils adhere to vascular endothelium, diapedese through the walls of small blood vessels, and migrate towards infectious agents (chemotaxis).

The neutrophil has a characteristic segmented, multilobe nucleus, which contains densely clumped chromatin and no nucleoli, the cytoplasm contains fine granules. Like AM, neutrophils release potent mediators that may cause tissue damage via the release of toxic oxygen metabolites and proteolytic enzymes.[22] They are primarily responsible for maintaining normal host defences against invading micro-organisms, being the major cellular elements in most forms of acute inflammation. Neutrophil functions such as phagocytosis and migration are enhanced by various substances such as GM-CSF. GM-CSF is produced by various cell types such as AM, T lymphocytes and mast cells in response to numerous stimuli.

Evidence for the importance of neutrophils in the defence against many micro-organisms is provided by the occurrence of severe infectious complications in patients with neutropenia or selective functional defects in neutrophils.[22] Infiltration of the lungs by neutrophils is an early event in lung injury and tissue derangement. The AM release factors inhibitory for neutrophil migration and chemotaxis. Through these factors AM regulate neutrophil migration towards the alveolar space and thereby modulate alveolitis.[22] For example, increases in BAL neutrophils have been implicated in the derangement observed in ILD, such as idiopathic pulmonary fibrosis (IPF),[41] the early phase of EAA,[42] asbestosis, collagen vascular disorders and the adult respiratory distress syndrome (ARDS). Other

factors causing neutrophil alveolitis include viral infections and smoking.[43]

Eosinophils

Like neutrophils, eosinophils are bone-marrow derived granulocytes, which can be distinguished on the basis of morphology, staining properties, and mediator content. The eosinophilic nucleus is bilobed and lacks a nucleolus. The most distinctive feature of eosinophils is the presence of specific or secondary cytoplasmatic granules.[44] Chemotactic and activating factors derived from mast cells, monocytes, and especially T lymphocytes appear to regulate eosinophil functions. A large number of other eosinophilic chemotactic factors have lately been recognized.[27,44] Interleukin-3, GM-CSF, IL-4 and IL-5 induce the proliferation and differentiation of haematopoietic progenitors of eosinophils, only IL-5 appears to be specific for the eosinophilic lineage and IL-5 secretion. Interleukin-5 secretion may explain the simultaneous, delayed maximal accumulation of mononuclear cells and eosinophils 24 to 48 hours after allergen challenge of the skin.[44-46] Moreover, it is apparent that eosinophils by virtue of their granule-associated and membrane-derived products have considerable potential for tissue damage in several pulmonary disorders.[5,47,48] Normally, BAL fluid contains less than 1 percent eosinophils.[48] Increased numbers of eosinophils have been observed in several ILD, chronic eosinophilic pneumonia, drug-induced ILD, allergic bronchopulmonary aspergillosis (ABPA), acute eosinophilic pneumonia, *Pneumocystis carinii* pneumonia associated with the acquired immunodeficiency syndrome (AIDS), and filarial infection.[49-51] In most ILD, however, the BAL fluid shows either lymphocyte or neutrophil predominance with a normal or near-normal eosinophil count.

Mast cells

Mast cells are relatively large cells. They possess a single round or oval, eccentrically located nucleus and membrane-bound (histamine-containing) cytoplasmatic granules.[52] It has been suggested that lung mast cells play a role in the pathogenesis of pulmonary inflammation, the stimulation of collagen deposition and fibrosis.[53,54] In addition, mast cells release a variety of cytokines, lipid-derived mediators, amines, proteases, and proteoglycans, all of which can regulate adjacent cells and the turnover of the extracelluar matrix of connective tissue.[55] Mast cells are derived from multipotential stem cells in the bone marrow. Interleukin-3 and IL-4

stimulate the proliferation and differentiation of haematopoietic progenitor cells to generate mast cells, as well as neutrophils and macrophages. The release of inflammatory mediators by mast cells when exposed to known activators can be enhanced by IL-3, IL-5, IL-8 or GM-CSF. Mast cells themselves can produce chemotactic cytokines and cytokines with biological properties similar to those produced by TH2 lymphocytes. TH2 lymphocytes, however, produce much larger amounts of these cytokines (see also table 2). Increased numbers of mast cells and raised concentrations of mediators released from mast cells, such as histamine and tryptase, have been observed in BAL fluid from patients with fibrosing alveolitis.[56] Release of mediators from mast cells might increase lung capillary permeability and allow increased access of inflammatory cells into the interstitium. In this way, mast cells participate in chronic inflammation. The recent demonstration of an elevated number of mast cells and their degranulation products in BAL fluid, as well as the observed relation between cell numbers and the severity of fibrosis, implies a role for mast cells in modulating the deposition of collagen in fibrotic lung disorders.[57] In addition, the presence of mast cells was suggested to represent a marker of more advanced or progressive disease.[54,57] Also, findings of lymphocytosis or neutrophilia combined with mastocytosis in BAL fluid were regarded as having the highest specificity (100 percent) for prediction of deterioration in ILD.[53]

Noncellular constituents of bronchoalveolar lavage fluid

Soluble BAL fluid components may originate from various sources including passive transudation, active transport and local production. The various inflammatory cells discussed above, which mediate their responses by releasing or generating chemical compounds or by recruiting other cells to release or activate additional mediators, count for the local production. Analysis of the soluble components of BAL fluid has gained significant attention. A variety of methods for preparation have been developed and various assays for a large number of these components have been employed. Today, the number of species detected and quantified in the lower respiratory tract continues to increase. However, it is impossible to disguise completely the vast repertoire and clinical application of this potent group of effector molecules.[1,58] The majority of the

noncellular components are (glyco)proteins such as cytokines, complement components and immunoglobulins, and lipids.[1]

Immunoglobulins

After binding the antigen, immunoglobulins initiate biologic phenomena which are independent of antigen specificity. Each antibody molecule is composed of two identical heavy (H) chains and two identical light (L) chains. Typically, parts of both the H and L chains form the antigen-binding sites. The H chains form the Fc region of the antibody, which determines the biological properties of the antibody. There are five classes of immunoglobulins (IgM, IgG, IgA, IgD and IgE), each with a distinctive H chain. Only IgM, IgG and IgA will be discussed below since these immunoglobulins are relevant in ILD. IgM constitutes approximately 10 percent of the total serum immunoglobulins and normally exists as a pentamer with a high molecular weight, mainly intravascular. IgM antibody is prominent in early immune responses to antigens and predominates in certain antibody responses such as 'natural' blood group antibodies. IgM (with IgD) is the major immunoglobulin expressed on the surface of B lymphocytes. Surface IgM is, in contrast to serum IgM, expressed as a monomer. IgM is also the most efficient complement-fixing immunoglobulin. A single molecule bound to antigen can initiate the complement cascade. IgG is the predominant class in serum, which is produced in large quantities during secondary immune response. IgG molecules are the only antibodies that cross the placenta and can confer immunity on the fetus. IgG is also capable of fixing complement and promoting phagocytosis (opsonisation). There are 4 subclasses, IgG1, IgG2, IgG3 and IgG4, with different immunological functions.[21] IgA is produced in a monomeric or in a dimeric form. Monomeric IgA is present in serum, whereas dimeric IgA is the principal class of antibody in secretions. Secretory IgA provides specific defense against some local infections (bacteria, viruses).

Proteins in respiratory secretions come from two major sources: (1) local synthesis by airway luminal cells and by cells lining the air spaces or located in the submucosa and (2) transudation across the capillary endothelial-interstitial-alveolar epithelial interface, the so-called blood-air barrier. The major soluble constituents in BAL fluid are albumin and the aforementioned immunoglobulins.[59] The degree to which transudation of plasma proteins occurs, depends largely on molecular size and configur-

ation and integrity of the capillary-alveolar barrier, and is influenced by factors such as hydrostatic pressure and inflammation.[7] The concentrations of IgG and IgA reflect the rate of transport across the bronchial epithelium.[7] In contrast, IgM is only present in minute amounts. Normally, IgM cannot cross the epithelial barrier due to its high molecular weight. Local plasma cell accumulation can account for higher immunoglobulin levels in BAL fluid samples. Therefore, immunoglobulin levels in BAL fluid may be influenced by the agent causing alveolitis.

1.2.2 Factors influencing the bronchoalveolar lavage fluid profile

There is a lack of data in the literature regarding normal values for the relative distribution of BAL cells and other BAL constituents. For the interpretation of findings in BAL fluid samples, details including age, cigarette smoking (see appendix) and the use of drugs by the subject and the lavage technique should be taken into account.[16] Moreover, as the BAL data obtained from normal volunteers are not normally distributed, nonparametric statistical methods are required for analysis.[60]

As mentioned above, many factors, all related to the BAL procedure and to the population under study, greatly influence both the yield and the composition of BAL fluid. The epithelial cell layer is extremely vulnerable to trauma. Damage caused by insertion of a fiberoptic bronchoscope into the airways may result in an increased number of neutrophils in BAL fluid samples.[61]

Various technical aspects of BAL are critical in the ultimate obtaining of representative samples. Smaller volumes of instilled fluid carry the risk that a more 'bronchial' washing component dominates the cellular picture.[62] As a consequence, the relative number of neutrophils is found increased in the BAL fluid, particularly in the first two aliquots. Several researchers have carried out cell differential analysis on sequential aliquots of the recovered lavage fluid. In nonsmokers, the proportions of alveolar cells in each bolus of 50 ml are roughly uniform. However, in smokers and patients with bronchial inflammation the first 'wash' is different from all subsequent boluses: the first bolus shows more characteristics of bronchial rather than alveolar sampling and should therefore be analyzed separately.[63] A total instilled lavage volume of 100 ml adequately samples

the airways and alveolar space and the larger total volume of lavage yields no additional information in normal subjects nor in patients with ILD.[64-66]

As the volume of fluid infused as part of the lavage technique has a variable dilutional effect on protein concentrations, no completely satisfactory method of standardizing protein recoveries has been developed.[67] Instead, protein values in BAL fluid are frequently expressed as ratios of the amount of a particular protein to the amount of albumin or as a percentage of total protein in the sample. Use of either method allows standard reporting of results and comparison with values obtained by others.[13,68,69] In general, the albumin level in BAL fluid is used as a reference to correct for the degree of dilution of BAL fluid.[59] Other markers, investigated to serve as reference, are methylene blue[70] and urea.[71] However, the usefulness of the latter markers has proved to be limited.[72]

Both in health and disease, smoking has been found to adversely affect the alveolar micro-environment.[73-75] Thus, when interpreting BAL fluid sample analysis results, smoking has to be considered as the major confounding factor. In the normal population, cigarette smoking has significant effects: the recovery and the viability of the cells decrease, the number of red cells contaminating BAL fluid samples increases, and the quantity of cellular and noncellular constituents changes.[16,76] In BAL fluid of normal smokers the cell number is increased approximately fourfold. An absolute and relative increase in the number of neutrophils is observed. Similarly, although the percentage of lymphocytes is diminished, the absolute number is actually elevated.[13,75] Therefore, both the absolute and relative values of the different cell populations recovered from BAL fluid samples should be given.[77] Obvious changes in AM morphology and function are reported. In smokers AM were found to be larger, showing pigmented cytoplasmatic inclusions ('smokers' inclusion bodies') and signs of a maximum phagocytosing capacity. Some functions of AM obtained from smokers are enhanced, such as the tissue-damaging cytotoxic function, production and release of lysosomal enzymes and oxygen radical production. Other AM functions, however, such as phagocytosis, microbicidal activity, immune and mitogenic responses, are suppressed by cigarette smoking (table 3). The mechanisms underlying these effects still need to be elucidated.

Age also is a factor which should be considered in the interpretation of BAL fluid sample analyses. First of all, the recovery changes with the increase of the age due to, among others, the known decrease in lung surface and changes in pulmonary functions. An age related increase in the number of AM in the lungs has been found.[78] Moreover, the percentage $CD4^+$ T lymphocytes is higher in individuals above fifty years than in individuals younger than thirty-five years. In addition, in the elderly, the concentrations of immunoglobulins increase.[16]

Table 3—*Effects and Consequences of Smoking on Bronchoalveolar Lavage (BAL) Fluid*

BAL fluid constituent	Effect
Alveolar macrophages	number ↑
	motility ↑
	responsiveness to chemotactic stimulants ↑
	metabolic activity ↑
	lysosomal enzyme release ↑
	oxygen radical production ↑
	tissue-damaging cytotoxic function ↑
	phagocytosis ↓
	microbicidal activity ↓
	immune responses ↓
	mitogenic responses ↓
T-cells	total number =
$CD4^+/CD8^+$ ratio	↓
Natural killer cells	activity ↓
Polymorphonuclear neutrophils	number ↑
Immunoglobulins	
IgG	level ↑
IgA	level ↑
Histamine	level ↑
Tryptase	level ↑
Phospholipids	level ↑
Cholesterol	level ↓

(adapted from Costabel 1986[79]; 1992[75])

1.2.3 Side-effects of bronchoalveolar lavage

Currently, BAL is regarded as a safe procedure. The risk of the occurrence of complications is comparable to regular fiberoptic bronchoscopy unless invasive procedures like transbronchial lung biopsy are performed (table 4).[2,13,80,81]

The overall complication rate of BAL is reported to be smaller than 3 percent in comparison to 7 percent with transbronchial lung biopsy and 13 percent when using open lung biopsy. To date, no lethal complication directly attributable to BAL has been reported.

Table 4—*Possible Consequences and Side Effects of Bronchoalveolar Lavage*

Side-effect	Occurrence
Alveolar infiltration	<10% of cases, usually subsides after 48 hours
Crackles	within 24 hours over dependent areas
Wheezing	in hyperreactive patients up to 1-2 weeks
Bronchospasm	rarely in normoreactive, more frequent in hyperreactive patients
Fever	10-30%, some hours after BAL
Lung function	transient decrease of FEV_1, VC, PEF, pO_2 transient rise of pCO_2 in patients with COPD
Bronchial reactivity	no change after BAL
Epithelial integrity	no effect on lung epithelial permeability 24 hours after BAL transient decrease of ciliary beat frequency
Bleeding	insignificant
Chest x-ray	abnormalities in the lavaged area 30 min after BAL (90%)
Heartrhythm irregularity	<2%

(adapted from Tiles et al. 1989[81]; Klech and Hutter 1990[2]; Baughman 1992[13])

1.3 Interstitial lung diseases

Interstitial lung diseases are a heterogeneous group of lung parenchymal inflammatory disorders, which often have a similar clinical presentation. This group includes disorders such as sarcoidosis, EAA and IPF. Although the pathogenesis and etiology of most of the disorders remain unknown, evidence has been obtained that, regardless of the inciting agent, interactions between immune complexes, inflammatory cells, and alveolar lining cells eventually lead to the formation of granulomata or extensive alveolar

wall fibrosis. Inflammatory cells damage connective tissue components and distort the normal architecture of the interstitium.[82] In ILD, specific immune mechanisms have also been found involved in perpetuating chronic interstitial inflammation by the influx and activation of lymphocytes. Smoking was suggested to protect against several ILD.[83,84]

The lungs are continuously exposed to a variety of air- and bloodborne substances that have the potential to trigger an inflammatory and immune response. The interaction of antigen with organized lymphatic tissue usually results in the generation of both cell-mediated and humoral-mediated effector mechanisms.[21] The magnitude of stimulation of each effector system depends on the physicochemical properties of the immunizing antigen. The basic architecture of the lungs' immune system includes areas where specialized epithelium covers bronchus-associated lymphoid tissue (BALT) in the proximal airways and the less-defined lymphoid aggregates in the distal airways. BALT consists of large collections of lymphocytes organized into lymphoid aggregates and follicles. Unlike BALT, the lymphoid aggregates in the distal airways are less organized and do not contain true germinal centres.[21,85,86] The majority of lymphocytes in BALT are B lymphocytes. Interactions among immunocompetent cells, cellular proliferation and differentiation characterize the immune response. The integrity of the alveolar wall is essential for the normal function of the pulmonary interstitium. An influx of inflammatory cells or damage to the basal membrane may lead to the development of ILD.

1.3.1 Sarcoidosis

Sarcoidosis is a disorder of unknown origin, characterized by the formation of noncaseating epithelioid cell granulomas, probably antigen driven, in various organ systems of which the lung is the most frequently involved.[6,69,87-89] Granuloma formation in the lung is preceded by a mononuclear cell alveolitis with increased numbers of activated T lymphocytes and AM.[38,90,91] A genetically-determined predisposition to the development of a granulomatous hypersensitivity has been proposed.[32]

Clinical manifestations of sarcoidosis depend on the intensity of the inflammation and organ systems affected.[92] In some sarcoidosis patients,

the alveolitis remains subclinical, whereas in others, both an alveolitis and granuloma formation are present resulting in specific pulmonary symptoms.[93] Also, extrapulmonary manifestations, such as erythema nodosum, may occur.[94] BAL is regarded as an important diagnostic procedure in sarcoidosis.[38] However, studies on BAL fluid profile characteristics in sarcoidosis patients hitherto reported in literature have produced conflicting data, especially concerning the use of BAL in the assessment of the prognosis of the disease.[95-97]

In general, the cellular profile in BAL fluid usually reflects the presence of an alveolitis as a local expression of a disseminated immunological disorder. In patients with sarcoidosis, the number of T lymphocytes in BAL fluid is usually increased, with a predominance of the number of $CD4^+$ T lymphocytes. As a consequence, an increase in the $CD4^+/CD8^+$ ratio in BAL fluid is observed in this patient group.[9,98] However, an intense alveolitis reflected by a high $CD4^+/CD8^+$ ratio is not an indicator of poor prognosis, but rather reflects an active immune response that may even protect the patient from further lung damage.[97] The activation of T lymphocytes in sarcoidosis is subset-specific.[32]

Interestingly, in other extrathoracic granulomatous diseases, such as M. Crohn and liver cirrhosis without pulmonary manifestations, also a latent T-lymphocyte alveolitis, similar to that occurring in sarcoidosis, has been reported. Patients with apparently localized Crohn's disease have generalized abnormalities in the distribution of immune effector cells in the lung similar to those found in patients with sarcoidosis.[99-101] Therefore, the lymphocytic alveolitis found in sarcoidosis patients is not pathognomic.

1.3.2 Extrinsic allergic alveolitis

Extrinsic allergic alveolitis or hypersensitivity pneumonitis is a disease initiated by repeated exposure to extrinsic organic antigens, mostly observed after inhalation, in susceptible individuals.[36] The clinical manifestation of EAA shows considerable variation as it is related to the frequency and intensity of exposure to the causative agent.[2,102] Commonly, the diagnosis EAA is based on clinical information, chest x-ray film, the presence of precipitins in peripheral blood, pulmonary function tests and disappearance of symptoms after avoidance of antigen exposure. The course of EAA may be divided into various phases according to subse-

quent immunological reactions in the lung.[35,103,104] Consequently, these phases of the immune response are reflected in a varying composition of BAL fluid profile.

Clinically, EAA may present itself in three forms: acute, subacute and chronic. The initial phase of EAA is characterized by an early increase in total cell count in BAL fluid, especially of neutrophils.[36,42,105] Subsequently, an increase in T lymphocytes has been found after inhalation challenge with antigen.[104] Twelve hours to several days following onset, the bronchiolo-alveolitis consists mainly of $CD8^+$ T lymphocytes[39] which, among others, modulate the B lymphocyte response and antibody production by plasma cells.[34,36] An increase in the $CD8^+$ T lymphocytes results in a relatively low $CD4^+/CD8^+$ ratio in BAL fluid. Weeks to months following antigen exposure, a slight predominance of $CD4^+$ T lymphocytes in BAL fluid is found (table 5).[106]

Various immune mechanisms, especially a combination of type III and type IV allergic reactions are involved in the pathogenesis of EAA.[105,107,108] Among these, immunoglobulins,[68,69,109,110] immune complexes,[26,111,112] complement, cytokines,[26] lipids and other biological modifiers have been described to play a role.[36] In addition, T lymphocytes have been implicated in the mechanism underlying tissue damage and repair in EAA.[36,40,113]

In contrast to the supposition that the presence of T lymphocytes is predictive for the activity and progression of the disease,[34] clinical symptoms were found to subside in longstanding EAA, whereas the number of T lymphocytes in BAL fluid remained increased.[39,42,114] Similar observations were made in asymptomatic pigeon breeders'.[115] Thus, the presence of T lymphocytes in the BAL fluid as such neither is a proof for disease activity nor a marker of progression. Rather, T-lymphocyte activation, the presence of distinct T-lymphocyte subpopulations,[40] and their respective secretory products, may be of importance.

Histopathologic analysis of lung biopsy material in EAA reveals noncaseating granulomas with foreign body giant cells, large numbers of lymphocytes, and foamy macrophages. Staining with fluorescein-labelled isotype specific antibodies reveals IgM-, IgG-, and IgA-positive plasma cells.

Table 5—*Time Course of the Immune Reactions of Alveolitis in EAA*

Time	Immune reaction	BAL feature	Histopathologic feature
4 to 48 hours	immune complex mediated	neutrophil influx	vasculitis edema neutrophil infiltration
12 hours to several days	cell mediated: -suppression of antibody production -cytotoxic effects	lymphocytes↑ -$CD8^+$ -natural killer cells -plasma cells	mononuclear infiltrate -lymphocytes -plasma cells -foamy histocytes
weeks to months	cell mediated: -delayed type hypersensitivity	lymphocytes↑ -$CD4^+$ -natural killer cells	mononuclear infiltrate and granulomas
months to years	repetition of the immune mediated injury the alveolar wall, fibroblast proliferation	lymphocytes↑ -$CD8^+$ neutrophils↑	fibrosis end-stage lung

(adapted from Costabel 1988[36])

1.3.3 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis, or cryptogenic fibrosing alveolitis is an ILD of unknown etiology, starting as an alveolitis and progressing into interstitial fibrosis.[116-118] The initial phase is characterized by parenchymal inflammation followed by the accumulation of collagen in the alveolar lumen and interstitium.[119,120] Diffuse injury to the alveolar type I cell epithelium and hyperplasia and hypertrophy of alveolar type II cells are early histopathologic findings in IPF.[121] Surfactant, secreted by type II cells, prevents alveolar collapse. It has been suggested that alterations in the composition of surfactant alters its biophysical activity, promotes lung fibrosis, and diminishes lung compliance in IPF.[122,123] Alterations in surface-active material may contribute to the pathogenesis of IPF both by increasing the elastic recoil due to surface forces and by promoting alveolar collapse and apposition of alveolar walls.[123] A current hypothesis

for the loss of gas-exchange units after lung injury is the irreversible fusion of damaged alveolar basement membranes.[124] In order to determine whether the fibrosing process is of idiopathic or cryptogenic origin, all known causes of fibrosis such as irradiation, pneumoconiosis, drug-induced pneumonitis, and collagen-vascular diseases should be excluded. In table 6 other causes of fibrosis are listed.

Table 6—*Some Causes of Diffuse Pulmonary Damage Proceeding to Interstitial Pulmonary Fibrosis*

Systemic diseases	*Dusts*	*Inhalants*
Rheumatoid arthritis	Organic	Oxygen
Systemic scleroderma	*eg*, those associated with	Nitrogen dioxide
Dermatomyositis	EAA	Cadmium
Polymyositis	Inorganic	Smoke
Sjögren's syndrome	coal; asbestos; silica;	Mercury vapour
Chronic active hepatitis	aluminium; etc.	Sulphur dioxide
Ulcerative colitis		Beryllium fumes
Coeliac disease		
Renal tubular acidosis		
Miscellaneous	*Cytotoxic drugs*	*Non-cytotoxic drugs*
Infections		
Viral pneumonia		
Mycoplasma		
Opportunistic (immunosuppressed patients)		
Ingested agents		
Paraquat		
Verosene		
Ionizing radiation		
'Shock'lung		
Uraemia		

(adapted from Dunnill 1990[121])

Idiopathic pulmonary fibrosis has characteristic clinical, roentgenologic, physiologic and pathologic features.[121] Patients present with dyspnea and bibasilar end-inspiratory dry rales. The chest x-ray reveals a diffuse reticulonodular infiltrate. Pulmonary function tests often demonstrate a restrictive process with a reduced diffusing capacity (D_{CO}) and abnormal

gas exchange. Histologically, the most important finding is the accumulation of inflammatory and immune effector cells within the alveolar structures and interstitium with fibrosis in varying amounts. End-stage fibrosis results in a so-called honeycomb pattern on the chest x-ray. An alveolitis, usually with increased numbers of neutrophils and eosinophils, but sometimes also with lymphocytes and mast cells, is demonstrated in BAL fluid samples.[57,117,125,126] In addition, AM and numerous inflammatory mediators produced by these cells are detected in BAL fluid of these patients (see also table 1).

Proliferation of fibroblasts and excessive deposition of extracelluar connective tissue matrix are essential features of the fibrotic process. Their activity is regulated by several soluble substances including cytokines, matrix proteins or their fragments, eicosanoids, and reactive oxygen species.[26,127-130] In addition, immune complexes may also be involved as the number of IgG-secreting cells is increased resulting in elevated IgG-levels. Also, collagenase and fibronectin are implicated in the development of fibrosis.[131] Depression of total phospholipids and the severity of alterations in phospholipid composition are correlated with more advanced fibrosis.[122]

Since BAL enables to obtain and examine cellular and noncellular components from small airways and alveolar spaces, this procedure may substitute more invasive diagnostic procedures in the evaluation of ILD.[13,132] In addition, many studies have resulted in major advances in the understanding of the cell biology of the lung and of the pathogenesis of many pulmonary diseases.

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Aims of the study

'Zeg dit nooit:
"Ik ken het niet,
dus het is niet waar."
Je moet leren voordat
je kunt weten;
weten voordat
je kunt begrijpen;
begrijpen voordat
je kunt oordelen.'

Apothegm van Narada

Aims of the study

The studies presented in this thesis are based on BAL fluid samples obtained from patients during a ten-year period between 1980 and 1990. The material was processed directly and the data were analyzed retrospectively.

Bronchoalveolar lavage (BAL) has been widely established to sample the lower respiratory tract, and represents an important clinical and research tool. This technique allows investigation of cells and mediators recovered from the airway lumen. The composition of BAL fluid is generally believed to reflect inflammatory processes in the lung. Patients with various interstitial lung diseases, such as sarcoidosis, extrinsic allergic alveolitis, and idiopathic pulmonary fibrosis may have many symptoms in common. Therefore it is often not possible to categorize these diseases accurately without an invasive procedure to obtain tissue. However, the composition of BAL fluid differs in interstitial lung diseases, and may be useful in distinguishing some of these disorders.

The most important aim of this study was to investigate whether a number of characteristic features derived from BAL fluid analysis would enable to distinguish between various interstitial lung diseases and, consequently, would influence the clinicians diagnostic considerations, resulting for example in avoidance of more invasive diagnostic procedures. A discriminant analysis with a number of selected BAL fluid variables in patients with sarcoidosis, extrinsic allergic alveolitis, or idiopathic pulmonary fibrosis is elaborated in chapter 3.

In the differential diagnosis of sarcoidosis with involvement of regional lymph nodes, tuberculosis and especially malignant lymphomas including non-Hodgkin's and Hodgkin's disease are important. The aim of the study presented in chapter 4 was to identify characteristic features in BAL fluid samples obtained from patients with tuberculosis, non-Hodgkin's or Hodgkin's disease and to investigate whether these differences facilitate the distinction of those disorders from sarcoidosis presenting with a similar clinical picture.

Clinical manifestations of sarcoidosis depend on the intensity of the inflammation and organ systems affected. The hitherto reported studies on BAL fluid analysis in sarcoidosis patients have given rise to conflicting

data, due to differences in sarcoidosis subpopulations studied and methodological variations. In addition, most studies do not distinguish between smokers and nonsmokers. The analysis presented in chapter 5 was performed to investigate whether the presentation of sarcoidosis is associated with differences in cellular profile in BAL fluid samples, especially with regard to the number of T lymphocytes and T lymphocyte subpopulations. Subsequently, the results were analyzed according to the smoking status of these patients.

Extrinsic allergic alveolitis is an interstitial lung disease initiated by exposure to extrinsic antigens in susceptible individuals. Based on subsequent immunological reactions in the lung, the course of extrinsic allergic alveolitis is characterized by various phases. Therefore, patients were divided into four categories based on the time period elapsed between the last antigen exposure and the performance of the lavage. The aim of the study presented in chapter 6 was to investigate whether there is a relationship between the timing of BAL and the last exposure to the causative antigen on BAL fluid characteristics in extrinsic allergic alveolitis and to detect specific features of the various categories.

To date, most interest has been focused on the identification of T lymphocytes and the $CD4^+/CD8^+$ ratios in BAL fluid samples obtained from patients with pulmonary disorders. In chapter 7 some attention is paid to B lymphocytes, especially plasma cells. Interestingly, in some cases we noticed the presence of plasma cells in BAL fluid samples, which are normally absent. The relationship between the presence of plasma cells in BAL fluid samples and the diagnosis of specific pulmonary disorders was evaluated.

Extrinsic allergic alveolitis has been associated with the presence of a few plasma cells in BAL fluid samples (chapter 7). In chapter 8 a study is presented regarding a possible relationship between the presence of plasma cells in BAL fluid samples and the severity of the alveolitis found in patients with extrinsic allergic alveolitis, who were recently exposed to the causative antigen. Differences between the profile of the BAL fluid of these patients with or without plasma cells, and the relationship of plasma cells with immunoglobulin levels in the lavage fluid were investigated.

Differences in BAL fluid variables in interstitial lung diseases evaluated by discriminant analysis

Differences in BAL fluid variables in interstitial lung diseases evaluated by discriminant analysis

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Abstract

The aim of this study was to investigate the possibility of distinguishing between patients with similarities in clinical presentation, suffering from three frequently occurring interstitial lung diseases, by means of discriminant analysis using a number of selected variables derived from bronchoalveolar lavage fluid (BALF) analysis.

The study involved all 277 patients, who had an initial bronchoalveolar lavage (BAL) in the period 1980-1990. These patients belonged to the following diagnostic groups: sarcoidosis (n=193), subacute extrinsic allergic alveolitis (EAA) (n=39) and idiopathic pulmonary fibrosis (IPF) (n=45). Thirty healthy volunteers were used as controls. Cellular and non-cellular constituents of BALF were evaluated.

Variables, which could be used to discriminate among the three diagnostic groups were: yield of recovered BALF, total cell count, and percentages of alveolar macrophages, lymphocytes, polymorphonuclear neutrophils, eosinophils, and plasma cells in BALF. When the set of data used to predict the membership of patients to diagnostic groups (test set) was the same as that in which the discriminant analysis was performed (learning set), 93 percent of the cases were correctly classified. This percentage decreased to 90 percent, however, when the test set was different from the learning set.

It is possible to discriminate among patients with sarcoidosis, EAA or IPF with these selected variables. It appears that BAL is useful as an adjunct in concert with other diagnostic methods.

Introduction

A biopsy of patients with interstitial lung diseases (ILD) is not always available, thus, other diagnostic features are needed. Bronchoalveolar lavage (BAL) has proved to be of diagnostic value for analysis of inflammatory processes in the lung, particularly in ILD.[1-4] These disorders usually show an alveolitis characterized by an accumulation of inflammatory and immune effector cells within the interstitium and the alveolar structures.[3-5] The increase in different cell types and immunoglobulins (Igs) in BAL fluid (BALF) may vary among the various ILD.[1-4] In order to discriminate between the ILDs, characteristic BAL features should be considered, together with unique clinical parameters.[6,7]

Sarcoidosis patients usually show an accumulation of activated, proliferating T-lymphocytes in the BALF, although some cases may show normal values.[8-10] Also, in extrinsic allergic alveolitis (EAA) lymphocytes are increased in the BALF. However, the composition of lymphocyte subpopulations in BALF obtained from EAA patients differs from that observed in other diseases, as is usually characterized by a low $CD4^+/CD8^+$ ratio.[11,12] In contrast, in sarcoidosis a high $CD4^+/CD8^+$ ratio is frequently found.[9,10] Also, in EAA, a mild increase of polymorphonuclear neutrophils (PMN) and mast cells can be found shortly after inhalation of antigen.[11,12] Plasma cells (0.1-2%) have been observed in BALF of EAA patients.[13,14] Idiopathic pulmonary fibrosis (IPF) has no specific diagnostic BAL features. Random increases of BALF lymphocytes, PMN and eosinophils occur in about two-thirds of IPF patients.[3,15,16]
In BALF of sarcoidosis, EAA and IPF patients in addition to changes in cellular constituents, Igs and Ig to albumin ratios are frequently increased.[1,3,4]

In order to evaluate whether a number of selected variables derived from BALF analysis can distinguish between the three diagnostic groups, we have performed a discriminant analysis in patients with sarcoidosis, EAA and IPF.

Materials and Methods

Patients

Retrospectively, the initial BAL of patients with sarcoidosis, EAA and IPF were selected out of all BAL analyses (n = 2,008) performed during a 10 yr period, between 1980 and 1990.

In this study, consecutive patients with sarcoidosis (n = 193) at time of diagnosis were included. The patient group consisted of patients detected on routine chest x-ray film (n = 37), patients with respiratory and general constitutional symptoms (n = 110) and patients with erythema nodosum and/or arthralgia and hilar lymphadenopathy (*ie*, Löfgren's syndrome; n = 46). All patients presented with a stage I or II x-ray film; none with a stage III x-ray film. The diagnosis was histologically proven, by biopsy of mediastinal lymph nodes, transbronchial biopsy, open lung biopsy, or liver biopsy. BAL was performed when the sarcoidosis patients were admitted to the hospital, to establish the diagnosis, and before corticosteroids were given.

The diagnosis EAA was based on clinical information, chest x-ray film, pulmonary function tests, the presence of precipitins in peripheral blood and the disappearance of the symptoms after avoidance of the causative antigen or, in some cases, after a short

treatment with corticosteroids. All EAA patients (n=39) had recent contact with the causative antigen, but not within the last 48 h before BAL.

Patients with IPF (n=45) commonly presented with an onset of breathlessness on exercise and non-productive cough and, sometimes, with constitutional symptoms. The diagnosis IPF was based on clinical information, chest x-ray film, pulmonary function tests, *ie*, decrease of lung compliance and diffusion capacity for carbon monoxide, hypoxaemia, especially on exercise, without hypercapnia. IPF was histologically proven by biopsy. The demonstrated alveolitis was characterized by an infiltration of mononuclear cells, interstitial pneumonitis and/or derangement of parenchymal structures, *ie*, fibrosis. No patient received corticosteroid treatment, or other medication, either at the time of lavage or before.

The control group consisted of 30 healthy volunteers, without any pulmonary history, having normal chest x-ray film and lung function tests. Table 1 lists the characteristics of the groups studied.

Table 1—*Characteristics of Controls and Patients with Interstitial Lung Diseases*

Groups	n	m[+]	Age*	Female	Male	NSm	Sm**
C	30	0	33 (21-55)	15	15	15	15 (14.9±8.8)
Sar	193	3	35 (18-79)	96	94	145	45 (15.7±7.8)
EAA	39	1	50 (23-78)	14	24	34	4 (12.8±6.9)
IPF	45	1	60 (30-79)	16	28	27	17 (18.0±5.1)

*n=number of cases; m=missings, [+]missing at least one discriminating variable and thus not used in analysis; *mean with range in parenthesis; NSm=Nonsmokers; Sm=Smokers; **number of smokers, mean number of cigarettes a day ± standard deviation in parenthesis; C=Controls; Sar=Sarcoidosis; EAA=Extrinsic allergic alveolitis; IPF=Idiopathic pulmonary fibrosis.*

Bronchoalveolar lavage

BAL was performed, as reported previously, during fibreoptic bronchoscopy.[11] In short, the procedure is as follows. After premedication (atropine and sometimes diazepam or codeine), and local anaesthesia of the larynx and bronchial tree (tetracaine 0.5%), BAL was performed by standardized washing of the right middle lobe, with four aliquots of 50 ml sterile saline (0.9% NaCl) at room temperature. Simultaneously, peripheral blood samples were taken.

Recovered BALF was kept on ice in a siliconized specimen trap and was separated from cellular components by centrifugation (5 min, at 350xg). Supernatants were directly stored at -70 °C after an additional centrifugation step (10 min, at 1,000xg).

Cells were washed twice, counted, and suspended in minimal essential medium (MEM; Gibco, Grand Island, New York, USA) supplemented with 1% bovine serum albumin (BSA; Organon, Teknika, Boxtel, the Netherlands).
Preparations of cell suspensions were made in a cytocentrifuge (Shandon). Cytospin slides of BAL cells were stained with May-Grünwald-Giemsa (MGG; Merck, Darmstadt, Germany) for cell differentiation. At least 1,000 cells were counted.

If more than 15 percent lymphocytes were present, T-cell (sub)populations were determined. Total T-cells and subpopulations were recognized by staining with monoclonal antibodies CD2(OKT11), CD3(OKT3), CD4(OKT4) and CD8(OKT8) (Orthopharmaceuticals, Beerse, Belgium). Identification of T-cells reacting with monoclonal antibodies was performed by means of a conventional indirect immunofluorescence technique using fluorescein isothiocynate (FITC)-labelled goat-antimouse-immunoglobulin-Ig (GAM, Nordic, Immunological Laboratories, Tilburg, the Netherlands and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands).

Albumin determinations were performed, according to a modification of the bromocresol purple (BCP) dye-binding method.[17,18] In short, this method is as follows: in the presence of a solubilizing agent, BCP binds to albumin at a pH 4.9. The amount of albumin-BCP complex is directly proportional to the albumin concentration. The complex absorbs at 600 nm. Albumin concentrations in serum and BALF are expressed as g/l and mg/l, respectively.
Immunoglobulin concentrations, *ie*, IgM, IgG and IgA in BALF were determined by an enzyme-linked-immunosorbent assay (ELISA) method; microtitre plates were coated with a rabbit anti-human-isotype antiserum (anti IgM, [CLB, Amsterdam, The Netherlands), anti IgG and anti IgA (Dako, Glostrup, Denmark]). Bound Igs from BALF were visualized by using a horseradish peroxidase (HRP) labelled rabbit anti-human-immunoglobulin antiserum (with anti-IgA, anti-IgG, anti-IgM, anti-kappa, anti-lambda reactivity [Dako, Glostrup, Denmark]) and a chromogenic substrate ortho-phenyl diamine (OPD, Baker, Chemicals BV, Deventer, The Netherlands). Ig-concentrations in BALF were expressed in mg/l using as a reference a commercial human standard serum, HOO-03 (CLB, Amsterdam, the Netherlands).

Statistical methods

In order to distinguish between the three diagnostic groups, a discriminant analysis was performed, according to the following procedure. Each of the 277 patients in the total study group belongs to one, and only one, diagnostic group. Five of the cases (three sarcoidosis patients; one EAA patient and one IPF patient) had at least one missing discriminating variable, therefore 272 cases were used for the analysis. Thus, 190 patients belong to the sarcoidosis group, 38 to the EAA group and 44 to the IPF group (table 1). Hence, an arbitrary patient out of the total study group has a probability of 190/272 = 0.70, 38/272 = 0.14 and 44/272 = 0.16, respectively, of belonging to either

one of the three diagnostic groups. These probabilities, which add up to 1 (as they should), are called "prior probabilities". If a set of predefined characteristics (so-called "explanatory variables") of a patient are known, then these characteristics can be involved in making these probabilities vary among patients. For example, if it is known that a patient is a smoker, then his probabilities may be different from the above prior probabilities, and so also from the probabilities of a nonsmoker. The latter probabilities, which can be calculated if we know the smoking status of a patient, are the so-called "posterior probabilities". A statistical technique with which these posterior probabilities can be calculated from the prior probabilities and from the patients characteristics is called discriminant analysis.[19]

By means of discriminant analysis, an allocation rule can be derived according to which patients i are allocated to one and only one diagnostic category j ($j=1,...,J$) on the basis of a number of explanatory characteristics $x_{i1},x_{i2},...,x_{in}$.
Taking diagnostic category 1 ($j=1$) as a basis, the explanatory characteristics are summarized in J-1 linear discriminant functions $Y_{ij}(j=2,...,J)$ for each patient i: $Y_{ij}=ß_{0j}+ß_{1j}X_{i1}+ß_{2j}X_{i2}+..\ .ß_{nj}X_{in}$, where the ß-coefficients are the same for all patients and are estimated by means of discriminant analysis. The estimated coefficients are presented in table 2.

The probability p_{ij} that patient i falls into diagnostic category j ($j=1,...,J$), given his discriminant function scores $Y_{ij}(j=2,...,Y)$, equals:

$$P_{ij} = \frac{1}{1 + \sum_{j=2}^{J} \exp(Y_{ij})} \quad \text{for } j=1;$$

$$P_{ij} = \frac{\exp(Y_{ij})}{1 + \sum_{j=2}^{J} \exp(Y_{ij})} \quad \text{for } j>1,$$

so that is guaranteed that $\Sigma_j p_{ij}=1$.
Patient i is allocated to that diagnostic category j for which p_{ij} is maximum.

This analysis is based on the formula of Bayes[19] and on the assumption that in each of three diagnostic groups the explanatory variables have a multivariate Gaussian distribution with different means. If it is also assumed that the variances and covariances differ between the diagnostic groups, then also quadratic terms of the explanatory variables have to be included in the discriminant analysis. In this study, a number of variables (*eg*, sex and smoking) are clearly non-Gaussian variables. However,

discriminant analysis is known to be rather robust for deviations from the Gaussian distribution. The variables are selected into the analysis according to a stepwise procedure. The stepwise selection procedure used is as follows. At each step, the variable with the smallest Wilks' lambda was selected with the significance level used as a criterion for entry ($p=0.05$) and removal ($p=0.10$). Age, sex, smoking and yield are included in the analysis as standard personal characteristics, and because of their possible confounding with other variables in the analysis. As it is supposed that the discriminatory power of explanatory variables may depend on the smoking status of a patient, the interaction terms of all explanatory variables (and their quadratic terms) with smoking is also eligible for stepwise inclusion in the model.

Table 2—*Variables Eligible for Discriminant Analysis with Unstandardized Canonical Discriminant Function Coefficients Mentioned only for those Variables and Higher Order Terms Eventually Selected in the Discriminant Functions* (the only Variable which had a Significant Interaction with Smoking was Age Squared)*

Explanatory variable	Linear effect		Quadratic effect		Interaction linear and smoking*		Interaction quadratic and smoking*	
	F1	F2	F1	F2	F1	F2	F1	F2
Constant	-20.53985	-15.49421						
Age yrs	0.04000	0.00219					0.00036	0.00001
Sex (Female)	-0.05678	-0.13240						
Smoking (yes)	0.65372	-0.13799						
BALF								
Yield(out/in)x100	0.01488	0.01539						
Cells $x10^4$/ml	-1.76927	-0.21222						
AM %	0.21780	0.49864	0.00002	-0.00344				
PMN %			0.00421	0.00131				
Lym %	0.27903	-0.13799	-0.00070	0.00343				
Eos %			0.00451	0.00292				
MC %								
PC %	-1.28392	-2.54227	0.38701	0.62018				

F1 = Function 1; F2 = Function 2; AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; Eos = Eosinophils; MC = Mast cells; PC = Plasma cells.

In order to test the goodness-of-fit of the discriminant analysis model, the predicted and the actual group membership were compared in a different testing set. Therefore, the total set was randomly and evenly split per diagnostic group in a testing and a learning set. The same variables as selected in the discriminant analysis on the total group were used to estimate the discriminant functions in the learning set. Next, these functions were used to compare predicted and actual group membership in the testing set.

Results

Tables 3-5 report the results of BALF cell and protein analyses of the controls and patients with sarcoidosis, EAA, or IPF.

Table 3—*Absolute Number of Cells in BALF of Controls and Patients with Interstitial Lung Diseases* *

Study group	AM	PMN	Lym	Eos	MC	PC
C(NSm)	8.7 ± 4.0	0.15 ± 0.12	1.2 ± 0.9	0.03 ± 0.05	0.006 ± 0.01	0.0 ± 0
C (Sm)	23.9 ± 12.0	0.25 ± 0.34	1.7 ± 3.5	0.10 ± 0.11	0.006 ± 0.02	0.0 ± 0
Sar(NSm)	11.7 ± 7.3	0.27 ± 0.57	6.8 ± 5.8	0.10 ± 0.15	0.03 ± 0.05	0.002 ± 0.001
Sar (Sm)	23.2 ± 17.0	0.34 ± 0.36	7.7 ± 9.6	0.16 ± 0.22	0.07 ± 0.13	0.0 ± 0
EAA(NSm)	12.1 ± 7.5	1.71 ± 2.00	26.3 ± 18.6	1.01 ± 1.52	0.40 ± 0.51	0.24 ± 0.41
EAA (Sm)	24.3 ± 10.5	3.22 ± 2.01	25.0 ± 22.3	1.23 ± 1.33	0.42 ± 0.28	0.47 ± 0.95
IPF(NSm)	15.7 ± 20.0	2.83 ± 3.92	3.7 ± 6.3	1.80 ± 3.11	0.09 ± 0.14	0.003 ± 0.001
IPF (Sm)	38.1 ± 25.6	6.91 ± 4.00	1.7 ± 1.9	3.04 ± 5.70	0.19 ± 0.40	0.001 ± 0.001

* *Data are expressed as mean absolute number of cells$x10^4$/ml ± standard deviation. AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; Eos = Eosinophils; MC = Mast cells; PC = Plasma cells; C = Controls; Sar = Sarcoidosis; EAA = Extrinsic allergic alveolitis; IPF = Idiopathic pulmonary fibrosis; NSm = Nonsmokers; Sm = Smokers.*

Since statistically significant differences were found among nonsmokers (NSm) and smokers (Sm) within the studied groups these data are shown separately.

Table 4—*Differential Cell Count in BALF of Controls and Patients with Interstitial Lung Diseases* *

Study group	AM	PMN	Lym	Eos	MC	PC
C (NSm)	87.0 ± 5.2	1.6 ± 1.4	11.0 ± 5.2	0.34 ± 0.52	0.07 ± 0.12	0.0 ± 0
C (Sm)	91.6 ± 8.8	1.2 ± 1.6	6.8 ± 8.6	0.41 ± 0.45	0.02 ± 0.06	0.0 ± 0
Sar(NSm)	63.2 ± 17.8	1.7 ± 5.4	34.3 ± 17.9	0.55 ± 0.73	0.16 ± 0.31	0.001 ± 0.001
Sar (Sm)	74.9 ± 16.7	1.2 ± 1.2	23.0 ± 16.7	0.72 ± 1.07	0.18 ± 0.25	0.0 ± 0
EAA(NSm)	38.2 ± 8.9	4.3 ± 3.4	58.1 ± 14.9	2.6 ± 3.0	0.85 ± 0.71	0.43 ± 0.77
EAA (Sm)	57.3 ± 11.2	5.8 ± 2.4	40.8 ± 15.0	3.3 ± 4.7	0.90 ± 0.66	0.45 ± 0.90
IPF(NSm)	67.8 ± 19.4	11.7 ± 10.1	13.8 ± 15.7	6.4 ± 9.3	0.31 ± 0.35	0.001 ± 0.003
IPF (Sm)	76.0 ± 25.5	11.5 ± 21.5	5.1 ± 4.8	7.1 ± 12.2	0.34 ± 0.56	0.0 ± 0

* *Data are expressed as mean percentage of the total cell count ± standard deviation. AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; Eos = Eosinophils; MC = Mast cells; PC = Plasma cells; C = Controls; Sar = Sarcoidosis; EAA = Extrinsic allergic alveolitis; IPF = Idiopathic pulmonary fibrosis; NSm = Nonsmokers; Sm = Smokers.*

Table 5—*Proteins in BALF of Controls and Patients with Interstitial Lung Diseases* *

Study group	l-alb mg/l	l-alb/s-alb x100	IgM/l-alb	IgG/l-alb	IgA/l-alb
C (NSm)	60.5 ± 25.6	0.16 ± 0.05	0.01 ± 0.00	0.11 ± 0.08	0.05 ± 0.04
C (Sm)	68.2 ± 46.8	0.14 ± 0.04	0.0 ± 0	0.21 ± 0.27	0.02 ± 0.01
Sar (NSm)	150.9 ± 198.8	0.39 ± 0.57	0.01 ± 0.01	0.42 ± 0.29	0.10 ± 0.14
Sar (Sm)	144.6 ± 150.6	0.37 ± 0.39	0.01 ± 0.01	0.41 ± 0.42	0.07 ± 0.07
EAA (NSm)	185.4 ± 152.3	0.49 ± 0.40	0.10 ± 0.12	1.62 ± 1.86	0.49 ± 0.86
EAA (Sm)	171.5 ± 163.8	0.44 ± 0.36	0.04 ± 0.05	0.77 ± 0.37	0.16 ± 0.11
IPF (NSm)	141.3 ± 98.9	0.37 ± 0.28	0.01 ± 0.01	0.40 ± 0.19	0.13 ± 0.10
IPF (Sm)	81.5 ± 38.9	0.22 ± 0.11	0.0 ± 0	0.35 ± 0.30	0.15 ± 0.16

* *Data are expressed as mean ± standard deviation. l-alb = lavage albumin; s-alb = serum albumin; C = Controls; Sar = Sarcoidosis; EAA = Extrinsic allergic alveolitis; IPF = Idiopathic pulmonary fibrosis; NSm = Nonsmokers; Sm = Smokers.*

The recovery or yield (out/in) x 100% of the BAL was 55.9±0.8% in the sarcoidosis group, 46.7±1.4% in the EAA group, 44.7±1.5% in the IPF group and 58.4±2.5% in the control group. These results were presented as mean ± standard error of the mean. No differences were found between Sm and NSm.

The explanatory variables used in the analysis are listed in table 2. The explanatory variables eventually selected according to the stepwise procedure are those in table 2 with an explicitly mentioned coefficient. Indeed, two discriminant functions appear to be necessary for appropriately discriminating between three diagnostic groups. Function 1 discriminates the three groups for 62% and function 2 for 38% (see also table 6 and figure 1). Variables used in both functions are total cell count, the percentage of alveolar macrophages (AMs), polymorphonuclear neutrophils (PMNs), lymphocytes, plasma cells and eosinophils.

The classification results for all cases used in the analysis are shown in table 7. These results are obtained by applying the allocation rule as described above. The percentage of patients correctly classified in all patients with a given actual diagnosis, called the "diagnostic effectiveness" is 100((188+28+36)/272)=92.6.[21] The diagnostic effectivity for sarcoidosis is 100(188/190)=98.9%, for EAA 100(28/38)=73.7% and IPF 100(36/44)=81.8% (table 7). The predicted value of a classification can be calculated as the probability that a patient actually belongs to the predicted group. For the prediction "sarcoidosis", the predicted value (PV^+) equals 100(188/204)=92.2%, and for the prediction "EAA" and "IPF" these values are 100(28/29)=96.6% and 100(36/39)=92.3%, respectively.

The three respective prior probabilities that an arbitrary patient (without using any additional information) actually belongs to a diagnostic group are for sarcoidosis 100(190/272)=69.9%, for EAA 100(38/272)=14.0% and for IPF 100(44/272)=16.2%.

The specificity, *ie*, the probability of the prediction "non-sarcoidosis" in the group without sarcoidosis is 100((28+1+1+36)/(38+44))=80.5%, for "non-EAA" 100((188+2+7+36)/(190+44))=99.6% and for "non-IPF" 100((188+0+9+28)/(190+38))=98.7%.

The predicted value of the negative result, *ie*, the predicted value of the group with "non-sarcoidosis" (PV^-) equals 100(28+1+1+36)/(29+39)=

97.1%, for "non-EAA" 100(188+7+2+36)/(204+39)=95.9% and for "non-IPF" 100 (188+0+9+28)/(204+29)=96.6% (table 7).

Table 6—*Canonical Discriminant Functions*

Fcn	Eigen-value	Percentage of variance	Cumulative percentage	Canonical correlation	After fcn	Wilks' lambda	Chi-squared	DF	Signifi-cance
1*	2.7879	62.14	62.14	0.8579	0	0.0978	584.588	28	0.0000
2*	1.6982	37.86	100.00	0.7933	1	0.3706	249.939	13	0.0000

* *marks the 2 canonical discriminant functions (DF) remaining in the analysis; fcn = function.*

Table 7—*Classification Results from the Patients with Interstitial Lung Diseases*

	Predicted Group Membership (n)				Percentage			
Actual Group	Sar	EAA	IPF	Total	SPEC	DE	PV^+	PV^-
Sar	188	0	2	190	80.5	98.9	92.2	97.1
EAA	9	28	1	38	99.6	73.7	96.6	95.9
IPF	7	1	36	44	98.7	81.8	92.3	96.6
Total	204	29	39	272				

n = number of cases; Sar = Sarcoidosis; EAA = Extrinsic allergic alveolitis; IPF = Idiopathic pulmonary fibrosis; SPEC = Specificity; DE = Diagnostic effectiveness (ie, sensitivity); PV^+ = Positive predicted value; PV^- = Negative predicted value.

The results of the goodness-of-fit test were as follows: in the learning set the discriminant functions were estaminated again, yielding a correct classification in 96.2% of the cases. Next, these functions were applied to the testing set in order to predict group membership, yielding 90.1% correctly classified cases.

A separate analysis was performed in a subset of 196 patients, whose BALF was assayed for Igs. In this subgroup, group membership is now correctly predicted in 28 out of 36 EAA patients (77.8%, data not shown). In contrast to the original discriminant analysis without including the Igs, the diagnostic effectiveness was 73.7% (table 7). In the original analysis, 10 EAA patients were incorrectly predicted. Inclusion of Igs in the discriminant analysis corrects the prediction in two out of these 10 cases. No such correction was found in IPF patients. In contrast, inclusion of T-cell subpopulations and $CD4^+/CD8^+$ ratio in the analysis did not result in a better prediction because of too many missing data.

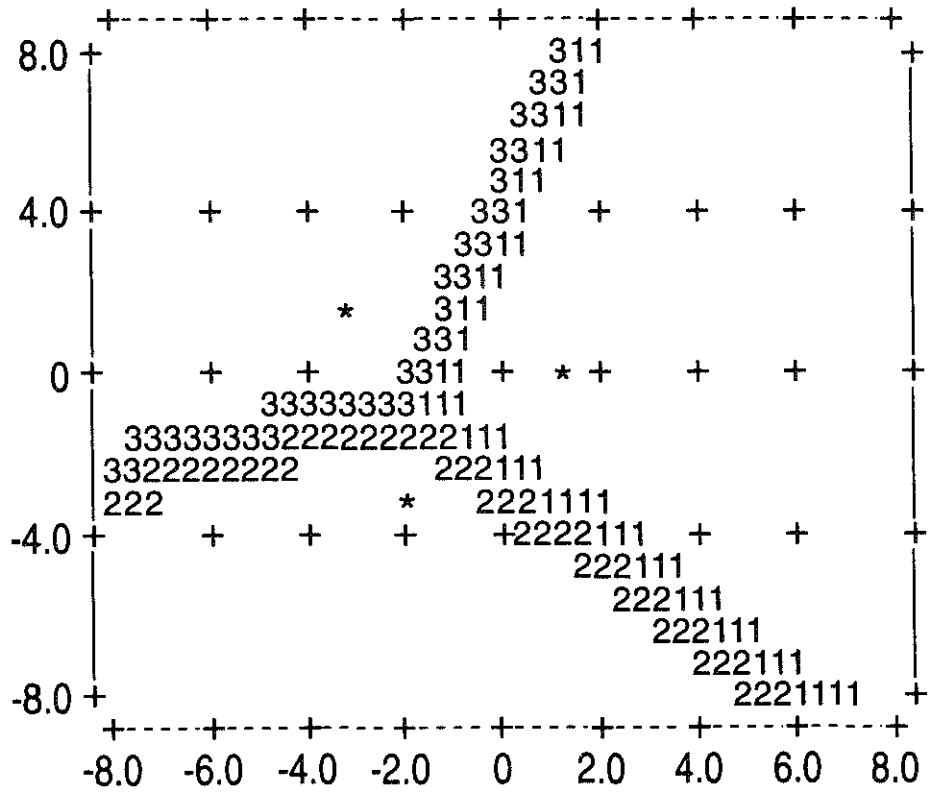

Figure 1. *Territorial map: clustering (* = group centroid) of the three groups in the plane scanned by the two discriminant functions. The symbols 1 represent sarcoidosis patients, 2 extrinsic allergic alveolitis patients and 3 idiopathic pulmonary fibrosis patients; Across function 1, down function 2.*

Discussion

The aim of this study was to investigate whether a number of selected variables derived from routine BALF analysis, made it possible to discriminate between three disorders belonging to the ILD group, all of which show striking similarities in their clinical presentation. To this end, a discriminant analysis was used to distinguish between sarcoidosis, EAA and IPF.

To be of diagnostic value, BAL should be performed using a standard procedure and BALF recovery, *ie*, yield, should be as comparable as possible. The recovery is, among others, related to BAL procedure and pulmonary function values, so standardization among varying diseases is very difficult. The recovery was included in the analysis, so the effects of the other variables in the model are adjusted for yield. In our studied patient population, the recovery was higher in sarcoidosis patients as compared to EAA and IPF patients. With regard to characteristic changes in cellular components of BALF in various ILD, our results confirm those described by others, showing high absolute and relative numbers of lymphocytes in the sarcoidosis and EAA groups[1-4], high numbers of plasma cells[13,14] and mast cells[11,12] in the EAA group, and an increased number of PMN and eosinophils in the IPF group.[15,16] Interestingly, by including the BALF-cell profile only, we have found a high percentage of cases classified correctly (92.7%) and, as such, a very high overall diagnostic effectiveness. This percentage may be somewhat too optimistic as we found the diagnostic effectiveness decreasing from 96.2 to 90.1% when comparing the learning with the testing set.

In previous studies, immunological parameters have been included in discriminant analysis of the different ILD.[6,21] Thus, Bertorelli et al[6] regard T lymphocyte subpopulations, PMN and eosinophils in BALF, immune complex determination and as gallium-67 lung scanning to possess the most important discriminant capacity. However, our study is not quite comparable with the above-mentioned study, because it included other variables and non-BALF parameters, and the use of different inclusion criteria. The EAA population of Bertorelli et al[6] was recently exposed to the causative antigen, and the authors do not state whether patients who had a BAL after provocation were also included. This is very important,

because of the reported influence of the timing of BAL related to the last antigen exposure on the results of BALF analyses.[11,12] It is also our experience, that the BALF profile after provocation differs from BAL at other times. Moreover, no correlation was made between the smoking status of a patient and the BALF analysis results.

In our study, we found that a little improvement of the diagnostic effectiveness for the EAA group can be obtained by including Igs in the discriminant analysis (77.8%) in addition to the BALF cell-profile. We found increased ratios of IgM, IgG and IgA to albumin in BALF in EAA patients. These increased ratios are supposed to be the result of the immune stimulation in the lung by inhalation of a causative antigen.[22] In contrast, Bertorelli et al[6] only described IgM in lung biopsy material both in EAA and IPF patients, but not in BALF. Our results suggest that the IgM ratio to albumin in BALF mainly differentiates EAA from the other two diagnostic groups. This observation was also reported by Reynolds et al.[22] The IgG and IgA ratios to albumin in BALF are only high in the nonsmoking EAA patients, compared to the two other groups. Bertorelli et al[6] did not include the patient smoking status as a variable, this may be due to the conflicting data. Reynolds et al[22] recently also reported the negative influence of smoking on total and functional lung Igs, and local immune response. In this study, and in a follow-up study (data not shown), we noticed that the demonstration of plasma cells in BALF is highly suggestive for the diagnosis EAA.[14] Local production of Igs by plasma cells has been suggested and could be an explanation of the increased ratios of Igs to BALF albumin, especially in EAA patients.[22] Plasma cells and Igs are, therefore, useful in discriminating EAA from other ILD.[13,14]

Previously, other BALF variables have been selected to discriminate sarcoidosis from other lung diseases (Costabel et al).[21] They also used a discriminant analysis. However, they selected other BALF variables, *ie*, percentage of lymphocytes, the $CD4^+/CD8^+$ ratio and Leu7+ natural killer cells. They also concluded that the determination of multiple BALF variables may be of diagnostic help in sarcoidosis. The diagnosis of sarcoidosis can be made by the determination of BALF lymphocyte subpopulations, and may avoid the need for more invasive biopsy procedures.[21] Our results confirm this hypothesis. Moreover, our first discrimination was based on cell differentiation only, without including the

$CD4^+/CD8^+$ ratio. However, our data are not comparable with those of Bertorelli et al[6] and Costabel et al[21], because of a different study protocol. It has been reported that the total cell count and the absolute and relative number of AM are increased in BALF of current smokers.[23-26] In addition, the percentage lymphocytes is decreased. These differences in the BALF profile suggest a modification of the inflammatory reactions in the lungs, due to smoking.[27]

Recently, increased mast cells in BALF in sarcoidosis patients were reported. Mast cells were related to a more active sarcoidosis by Bjermer et al[28], who did not differentiate between smokers and nonsmokers. This is in contrast with the results of Valeyre et al[29], who reported a lower incidence of sarcoidosis, with less severe symptoms, among smokers with a tendency to high numbers of mast cells in BALF. One has to take into account the fact that there is a profound effect of smoking on the number of mast cells in BALF.

This study reports only BALF features with regard to the presentation of the disease and not to the prognosis or the possible response to any therapy. Follow-up studies are needed to investigate the value of BAL to evaluate disease improvement or progression, and the possible influence of therapy on BALF profile.

Moreover, the differences between other disorders with similar clinical presentation to sarcoidosis should be included in the analysis to confirm the discriminant analysis. For instance, tuberculosis, important in the differential diagnosis of sarcoidosis, has a characteristic BALF profile, different from sarcoidosis.[30-33] Pulmonary Hodgkin's disease, also important in the differential diagnosis of sarcoidosis, has characteristic diagnostic cytologic features. Mononuclear Reed-Sternberg cells can be identified in BALF and fine needle aspiration.[34] Costabel et al[35] suggested that BAL should be considered as a non-invasive diagnostic approach in cases of pulmonary shadowing associated with malignant haematological disorders. We also found that malign lymphomas, *ie*, non-Hodgkin's lymphoma and Hodgkin's lymphoma, and tuberculosis have features in BALF profile which enables these disorders to be differentiated from other ILD, such as sarcoidosis (data to be published).

In the discriminant analysis, a number of 14 explanatory variables have been used, derived from 10 different variables measured in the studied patients. From the results so far, we can already conclude that, with a number of selected variables, it is possible to discriminate among patients with sarcoidosis, EAA or IPF. Furthermore, we demonstrated that there is an association between BALF profile and smoking. Therefore, smoking was included as a confounding variable in the discriminant analysis. BAL could serve as an adjunct in concert with other methods for establishing the diagnosis, especially with regard to differentiating between disorders with similar clinical presentation to the studied ILD.

Certainly, attempts still have to be made to develop a more general discriminant model, based on polytomous logistic regression analysis. Currently, we are developing a testing model, which can be used to predict the diagnosis of an arbitrary patient, using information provided only from BALF analysis.

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Bronchoalveolar lavage fluid profiles in sarcoidosis, tuberculosis, non-Hodgkin's and Hodgkin's disease: an evaluation of differences

Bronchoalveolar lavage fluid profiles in sarcoidosis, tuberculosis, non-Hodgkin's and Hodgkin's disease: an evaluation of differences

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Abstract

The aim of this study was to identify characteristic features in bronchoalveolar lavage fluid (BALF) samples of patients with tuberculosis, non-Hodgkin's or Hodgkin's disease and to investigate whether these differences facilitate the distinction of those disorders from sarcoidosis presenting with a similar clinical picture. Nonsmoker patients with histologically verified sarcoidosis (n=29), tuberculosis (n=6) proven by positive culture, non-Hodgkin's disease (n=6) or Hodgkin's disease (n=7), both histologically verified, were investigated by BAL. A control group consisted of subjects without any pulmonary history. The presence of $CD4^+$ and $CD8^+$ T lymphocytes, as well as the $CD4^+/CD8^+$ ratio in BALF, aided in the differentiation between the various groups. Patients with malignant lymphomas had the lowest $CD4^+/CD8^+$ ratio in BALF, as well as in peripheral blood, and occasionally, plasma cells were present in BALF samples. The most important feature of BALF analysis in tuberculosis was detection of the causal microbial agent. In conclusion, although malignant lymphomas and tuberculosis require histologic evaluation and a positive culture, respectively, for diagnosis, BALF analysis may be of additional value in distinguishing those disorders from sarcoidosis.

Introduction

The use of bronchoalveolar lavage fluid (BALF) analysis for diagnostic purposes in pulmonary disorders has been widely established.[1-3] Previously, we reported the possibility of distinguishing between interstitial lung diseases, *ie*, sarcoidosis, extrinsic allergic alveolitis and idiopathic pulmonary fibrosis by a number of selected variables derived from BALF analysis.[4]

In sarcoidosis, granuloma formation is preceded by a mononuclear cell alveolitis with increased numbers of activated T lymphocytes and alveolar macrophages.[5-9] Although the lung is the most commonly affected organ, extrapulmonary manifestations, such as erythema nodosum, arthralgia, and hilar lymphadenopathy, constituting a clinical picture referred to as Löfgren's syndrome, frequently occur.[10,11] Patients with Löfgren's syndrome, having the most severe alveolitis, show distinct characteristics in BALF sample analysis, among which increased numbers of lymphocytes

and high $CD4^+/CD8^+$ ratios.[11,12]

Tuberculosis and malignant lymphomas, *ie*, non-Hodgkin's, and Hodgkin's disease, especially the nodular-sclerosis type, also may present with bilateral mediastinal or hilar lymphadenopathy and alveolar mononuclear infiltration.[13,14] These disorders, requiring an even more rapid diagnosis and substantially different therapeutic regimens, should be readily differentiated from sarcoidosis.[13-15]

Recently, BALF sample analysis, in comparison with more conventional methods, has proven an even more sensitive technique in the diagnostic workup for tuberculosis detection.[16-20] In order to detect and further classify malignant lymphomas, histologic evaluation is required.[14,21,22] However, obtaining representative tissue samples may be a major problem. Pulmonary localization of Hodgkin's disease has been confirmed by identification of Reed-Sternberg cells in BALF specimen.[23-26] Also, the detection of non-Hodgkin's disease by BALF evaluation, using immunologic markers, has been described.[27,28]

The aim of this study was to investigate whether there are characteristic features in BALF samples obtained from patients with tuberculosis, non-Hodgkin's disease, or Hodgkin's disease and whether these differences assist in distinguishing these clinically similar disorders from sarcoidosis.

Materials and Methods

Patients and control subjects

Bronchoalveolar lavage was performed in 90 sarcoidosis patients, 6 tuberculosis patients, 6 patients with non-Hodgkin's disease, and 7 patients with Hodgkin's disease. The control group consisted of 28 healthy individuals who did not have chest x-ray film abnormalities or history of pulmonary disease. All patients and control subjects were nonsmokers. The characteristics of the patients and control subjects are described in table 1.

Our sarcoidosis patient population consisted of patients who had no symptoms (n = 11), those whose disease was detected on routine chest x-ray film, patients with respiratory and general constitutional symptoms (n = 50) and patients with Löfgren's syndrome (n = 29). All diagnoses were histologically proven. Unless otherwise stated, only the latter group was used in this comparative study.

The tuberculosis patient group consisted of six immunocompetent cases (five with pulmonary tuberculosis and one with lymph node tuberculosis). These patients initially presented with cough, dyspnea, erythema nodosum, chest pain, or fever. The chest x-ray film invariably showed infiltrates, pleural effusion, or enlarged mediastinal lymph nodes. Histologically, granulomas and necrosis were demonstrated. Five patients had proven infection with *Mycobacterium tuberculosis*, one patient with *M Bovis*.

All patients with malignant lymphomas initially presented with pulmonary manifestations. The chest x-ray films showed pulmonary infiltrates, interstitial involvement or enlarged mediastinal lymph nodes, or all three. All cases of non-Hodgkin's disease were histologically classified as low-grade B lymphocyte lymphomas of various stages (stages II, III or IV), and the cases of Hodgkin's disease, as the nodular sclerotic type.[14] The patients with Hodgkin's disease were in various stages of the disease (according to the Ann Arbor classification).[14,15] At the time of performance of bronchoalveolar lavage (BAL), chemotherapy was not yet started.

Table 1—*Characteristics of the Groups Studied*

Studied groups	n	Age, yr*	Female	Male
Control subjects	28	39 (19-70)	12	16
Sarcoidosis	29	43 (23-27)	14	15
Tuberculosis	6	41 (28-76)	2	4
non-Hodgkin's disease	6	57 (39-71)	2	4
Hodgkin disease's	7	35 (16-79)	4	3

**Mean with range in parentheses; n = number of cases.*

Bronchoalveolar lavage

The BAL was performed as previously reported during fiberoptic bronchoscopy.[4] Simultaneously, blood samples were taken. In short, the procedure was as follows: After premedication with atropine and sometimes diazepam or codeine and locally anaesthetizing the larynx and bronchial tree with 0.5 percent tetracaine, BAL was performed by standardized washing of the right middle lobe with four 50-ml aliquots of sterile saline solution (0.9 percent NaCl) at room temperature.

Sample Collection and Preparation

The first portion lavage fluid recovered was collected in a special test tube which was sent for culture. After centrifugation, the sediment was screened for acid-fast bacilli by both fluorescent auramine-rhodamine and Ziehl-Neelsen stains and cultures were performed on Löwenstein-Jensen medium.

Recovered BALF samples of the other three aliquots, kept on ice in a siliconized specimen trap, were centrifuged (5 min, 350 g) and separated from cellular compounds. Supernatants were directly stored at -70° C after an additional centrifugation step (10 min, 1,000 g). The cells were washed twice, counted, and suspended in minimal essential medium (Gibco, Grand Island, New York, USA) supplemented with 1 percent bovine serum albumin (Organon, Teknika, Boxtel, the Netherlands).

Preparations of the cell suspensions were made in a cytocentrifuge (Shandon). Cytospin slides of BALF sample cells were stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany) for cell differentiation. At least 1000 cells were counted. Reed-Sternberg cells were recognized by scanty to moderate amounts of finely vacuolated cytoplasm and a multilobulated nucleus with vesicular chromatin and large prominent eosinophilic-to-cyanophilic nucleolus.

If more than 15 percent lymphocytes were present, T lymphocyte subpopulations were determined. Total number of T lymphocytes and subpopulations were recognised by staining with monoclonal antibodies CD3(OKT3), CD4(OKT4) and CD8(OKT8) from Ortho-pharmaceuticals (Diagnostic Systems, Beerse, Belgium). Identification of T lymphocytes reacting with monoclonal antibodies was performed by means of a conventional indirect immunofluorescence technique using fluorescein isothiocyanate (FITC)-labelled goat-antimouse-Ig (Nordic, Immunological Laboratories, Tilburg, the Netherlands and from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands).

For the quantitative determination of albumin in serum samples and BALF samples, the albumin method was used. The albumin method is an adaption of the bromocresol purple dye-binding method.[29,30] In short this method is as follows. In the presence of a solubilizing agent, bromocresol purple binds to albumin at a pH 4.9. The amount of albumin-bromocresol purple complex is directly proportional to the albumin concentration. The complex absorbs at 600 nm. Albumin concentrations in serum and BALF samples were expressed in grams per liter and milligrams per liter, respectively.

Immunoglobulin concentrations, *ie*, IgM, IgG and IgA in BALF samples were deter-

mined by an enzyme-linked immunosorbent assay method; microtitre plates were coated with a rabbit antihuman-isotype antiserum (anti-IgM, [CLB, Amsterdam, the Netherlands], anti-IgG, or anti-IgA [Dako, Glostrup, Denmark]). Bound immunoglobulins from BALF samples were visualized by using a horseradish peroxidase-labelled rabbit-antihuman-immunoglobulin antiserum (with anti-IgA, -IgG, -IgM, -kappa, -lambda reactivity [Dako, Glostrup, Denmark]) and a chromogenic substrate orthophenyl diamine (Baker, Chemicals BV, Deventer, the Netherlands). Immunoglobulin concentrations in BALF samples were expressed in milligrams per liter using as a reference a commercial human standard serum, HOO-03 (CLB, Amsterdam, the Netherlands).

Statistical analysis

Data are expressed as mean ± SEM and, if appropriate, as median with range. In order to detect statistically significant differences between the four patient groups, data were analyzed by the Kruskal-Wallis one-way analysis of variance (ANOVA) test. The Mann-Whitney *U* test was used for pairwise comparisons. Because 15 comparisons were made, a probability value smaller than $0.05/15 = 0.003$ was considered statistically significant (Bonferroni's correction). Logistic regression analysis was used to discriminate between sarcoidosis and malignant lymphomas, given the $CD4^+/CD8^+$ ratios in BALF.

Results

The cellular components and protein levels in BALF samples obtained from the patient groups and control subjects are summarized in tables 2 to 5. In the sarcoidosis group, the percentages of $CD4^+$ T lymphocytes were significantly higher, the percentage of $CD8^+$ T lymphocytes and mast cells lower, and the $CD4^+/CD8^+$ ratio in BALF samples higher, as compared with all other groups (figure 1, tables 2 to 4). The $CD4^+/CD8^+$ ratios in BALF samples of patients with Hodgkin's disease were decreased in comparison with all other groups including patients with non-Hodgkin's disease. Also, patients with either form of malignant lymphoma had significantly lower $CD4^+/CD8^+$ ratios in peripheral blood than sarcoidosis patients (table 4). Subsequent comparisons between the four patient groups revealed most prominent differences in the percentages of $CD4^+$ T lymphocytes ($p < 0.0001$), $CD8^+$ T lymphocytes ($p < 0.0001$), and the $CD4^+/CD8^+$ ratio ($p < 0.0001$) in BALF samples. The lowest BALF $CD4^+/CD8^+$ ratios were found in patients with Hodgkin's disease, and the highest in those with sarcoidosis. The $CD4^+/CD8^+$ ratios in the tuberculosis and non-Hodgkin's disease groups were similarly low (figure 1, table 4).

Table 2—*Total Cell Count and Differential Cell Count in Bronchoalveolar Lavage Fluid (BALF) Samples of Control Subjects (Cs), Patients with Sarcoidosis (Sar), Tuberculosis (Tbc), non-Hodgkin's Disease (NHL) or Hodgkin's Disease (HL)*

Groups	TCC x10^4/ml	Percentage of TCC					
		AM	PMN	Lym	PC	Eos	MC
Cs	10.3 ± 1.5	89.8 ± 0.7	1.3 ± 0.2	8.4 ± 0.7	0.0 ± 0.0	0.44 ± 0.10	0.09 ± 0.03
Sar	20.3 ± 1.7*	60.3 ± 3.4*	1.4 ± 0.2	37.9 ± 3.4*	0.0 ± 0.0#	0.32 ± 0.07	0.09 ± 0.03#
Tbc	26.9 ± 12.6*	72.2 ± 7.6*	1.2 ± 0.6	26.1 ± 7.8*	0.0 ± 0.0	0.33 ± 0.21	0.15 ± 0.04
NHL	23.9 ± 4.0*	65.7 ± 5.2*	2.5 ± 1.1	28.3 ± 3.1*	2.5 ± 2.4*	0.68 ± 0.49	0.33 ± 0.16*
HL	23.8 ± 7.4*	65.2 ± 4.8*	4.8 ± 2.2*	28.1 ± 2.8*	0.04 ± 0.04*	1.54 ± 0.70	0.34 ± 0.16
p-value**	0.85	...	...	...	...	...	...

*Data are expressed as mean ± SEM. TCC = Total cell count; AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; Eos = Eosinophils; MC = Mast cells; PC = Plasma cells. **Kruskal-Wallis ANOVA test.*
**p < 0.04, Mann-Whitney versus control group.*
#p < 0.04, Mann-Whitney versus malignant lymphomas (NHLs + HLs).

Table 3—*Absolute Number of Cells in Bronchoalveolar Lavage Fluid (BALF) Samples of Control Subjects (Cs), Patients with Sarcoidosis (Sar), Tuberculosis (Tbc), non-Hodgkin's Disease (NHL) or Hodgkin's Disease (HL)*

Groups	AM	PMN	Lym	PC	Eos	MC
Cs	9.3 ± 1.4	0.13 ± 0.03	0.8 ± 0.1	0.0 ± 0.0	0.07 ± 0.01	0.01 ± 0.005
Sar	12.1 ± 1.3*	0.27 ± 0.04*	7.9 ± 1.0*	0.0 ± 0.0#	0.05 ± 0.02*	0.02 ± 0.01#+
Tbc	22.0 ± 11.7	0.31 ± 0.20	4.5 ± 1.2*	0.0 ± 0.0	0.04 ± 0.02	0.04 ± 0.02*
NHL	14.8 ± 1.5*	0.61 ± 0.23*	7.2 ± 1.8*	1.0 ± 1.0*■	0.14 ± 0.09	0.11 ± 0.07*
HL	15.0 ± 0.5	1.06 ± 0.73*■	7.3 ± 2.6*	0.01 ± 0.01*	0.32 ± 0.21	0.06 ± 0.04*
p-value**	0.35	0.16	0.20	0.07	0.58	0.02

Data are expressed as mean absolute number of the total cell count x10^4/ml ± SEM.
AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils;
Lym = Lymphocytes; Eos = Eosinophils; MC = Mast cells; PC = Plasma cells.
***Kruskal-Wallis ANOVA test.*
**p < 0.04, Mann-Whitney versus control group.*
#p < 0.04, Mann-Whitney versus malignant lymphomas (NHLs + HLs).
+p < 0.05, Mann-Whitney versus tuberculosis, NHL and HL.
■p < 0.05, Mann-Whitney versus sarcoidosis.

Table 4—*Percentages of T Lymphocytes and T Lymphocyte Subpopulations in Bronchoalveolar Lavage Fluid (BALF) Samples of Control Subjects (Cs), Patients with Sarcoidosis (Sar), Tuberculosis (Tbc), non-Hodgkin's Disease (NHL) or Hodgkin's Disease (HL)*

Groups	n	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$ Ratio BALF	$CD4^+/CD8^+$ Ratio PB
Cs	6	73.0± 2.4	52.4±3.4	19.0±1.4	2.6±0.2	1.8±0.3
		74(68-81)	49(41-63)	20(14-34)	2.7(1.0-3.0)	1.8(1.1-2.9)
Sar	16	88.7± 2.0*	80.3±2.7*#	8.9±0.8*#	10.7±1.5*#	2.6±0.4$
		91(63-95)	85(48-90)	8(3-15)	9.3(4.4-30)	2.2(1.1-6.5)
Tbc	4	67.7±10.5&	42.0±7.6+	27.8±7.6+φ	1.8±0.3*■φ	1.9±0.3
		71(48-84)	47(21-53)	28(9-45)	1.9(0.9-2.3)	1.6(1.5-2.5)
NHL	6	83.0± 6.9	50.0±4.6■	34.2±4.9*■	1.7±0.4*■	1.2±0.3+
		91(54-99)	53(34-61)	34(18-54)	1.4(1.0-3.4)	1.1(0.8-2.2)
HL	7	81.6± 3.2	30.7±4.2*■&	52.0±4.9*■&	0.7±0.2*■&	1.1±0.5+
		79(70-96)	32(21-53)	55(29-67)	0.5(0.3-1.8)	1.0(0.2-2.2)
p-value**		...	<0.0001	<0.0001	<0.0001	...
Sar@	77	87.8± 0.9*	70.6±1.8*#	16.3±1.4*#	8.1±0.9*#	2.3±0.4$
		90(63-99)	80(33-97)	12(2-61)	6.3(0.5-48.5)	2.1(0.4-6.5)

Data are expressed as mean ± SEM; and median with range in parentheses. n = number of cases. @Pooled sarcoidosis population.

***Kruskal-Wallis ANOVA test.*

**p < 0.04, Mann-Whitney versus control group.*

#p < 0.001, Mann-Whitney versus malignant lymphomas (NHLs + HLs).

$p < 0.01, Mann-Whitney versus malignant lymphomas (NHLs + HLs).

■p < 0.001, Mann-Whitney versus sarcoidosis.

+p < 0.04, Mann-Whitney versus sarcoidosis.

&p < 0.05, Mann-Whitney versus NHL.

φp < 0.04, Mann-Whitney versus HL.

In two of the cases with low grade B lymphocytes lymphomas, one with paraproteins of the IgG-lambda and one of the IgM-kappa type, the diagnosis was made initially on BALF specimens. In the BALF samples of these latter patients plasma cells also were present. The IgM levels were lower in the sarcoidosis group, as compared with the non-Hodgkin's disease group, but the range was broad and SEM was high, due to some cases with high IgM levels in BALF samples and due to the presence of paraproteins. Reed-Sternberg cells were identified in the BALF sample of

one patient with Hodgkin's disease.

In tuberculosis patients, combined evaluation of Ziehl-Neelsen staining and culture for *Mycobacterium species* of BALF specimens yielded a sensitivity of 83.3 percent and a specificity of 100 percent, both of which were significantly higher than those obtained from sputum analysis (data not shown).

Table 5—*Protein Levels in Bronchoalveolar Lavage Fluid Samples of Control Subjects (Cs), Patients with Sarcoidosis (Sar), Tuberculosis (Tbc), non-Hodgkin's Disease (NHL) or Hodgkin's Disease (HL)*

Groups	l-alb	IgM	IgM/l-alb	IgG	IgG/l-alb	IgA	IgA/l-alb
Cs	71 ± 8.5	0.4 ± 0.1	0.01 ± 0.002	11.1 ± 2.0	0.16 ± 0.02	3.6 ± 0.7	0.05 ± 0.01
Sar	140 ± 20.9*	1.9 ± 1.3#	0.01 ± 0.0#	46.5 ± 12.3*	0.35 ± 0.04*	10.4 ± 3.2*	0.07 ± 0.01
Tbc	106 ± 29.9	1.3 ± 0.7	0.01 ± 0.01	35.0 ± 8.9*	0.37 ± 0.07*	10.7 ± 2.8*	0.12 ± 0.05*
NHL	163 ± 54.3*	8.8 ± 6.9	0.08 ± 0.07	287 ± 173*	1.47 ± 0.90*	23.2 ± 11.2*	0.19 ± 0.10
HL	182 ± 45.0*	3.1 ± 2.0	0.01 ± 0.01	99.2 ± 38.7*	0.48 ± 0.11*	19.3 ± 8.9	0.09 ± 0.04

Data are expressed as mean (mg/l) ± SEM. l-alb = lavage albumin.
**p < 0.01, Mann-Whitney versus control group.*
#p < 0.05, Mann-Whitney versus malignant lymphomas (NHLs + HLs).

The ranges of the $CD4^+/CD8^+$ ratio in the BALF in patients with Löfgren's syndrome (4.4 to 30.0) and in patients with non-Hodgkin's disease or Hodgkin's disease (0.3 to 3.4) are disjoint (figure 1, table 4). Thus, the $CD4^+/CD8^+$ ratio may serve as a 'perfect' testing variable with 100 percent sensitivity and specificity for distinguishing malignant lymphomas with pulmonary involvement from Löfgren's syndrome. In order to test this observation, a logistic regression analysis was performed including all 77 sarcoidosis patients (disregarding the clinical presentation), wherein T lymphocyte subpopulations were determined, and all patients with either malignancy (n = 13). The $CD4^+/CD8^+$ ratios were divided into three intervals, *ie*, values between 0 and 0.54 (interval A), between 0.54 and 3.39 (B), and those between 3.39 and 48.5 (C). Interval A contained 4 patients, all with malignant lymphomas, and interval B included 9 patients with lymphomas and 23 with sarcoidosis. In interval C, only sarcoidosis patients (n = 54) were found. Each unit

increase of the $CD4^+/CD8^+$ ratio decreases the odds of lymphomas by a factor 0.30 ($p<0.00005$). Based on the present data, the diagnosis malignant lymphoma was considered probably when a $CD4^+/CD8^+$ ratio below 1.85 was found. The sensitivity equals 12 of 13 or 92.3 percent and the specificity, 64 of 77 or 83.1 percent.

Figure 1

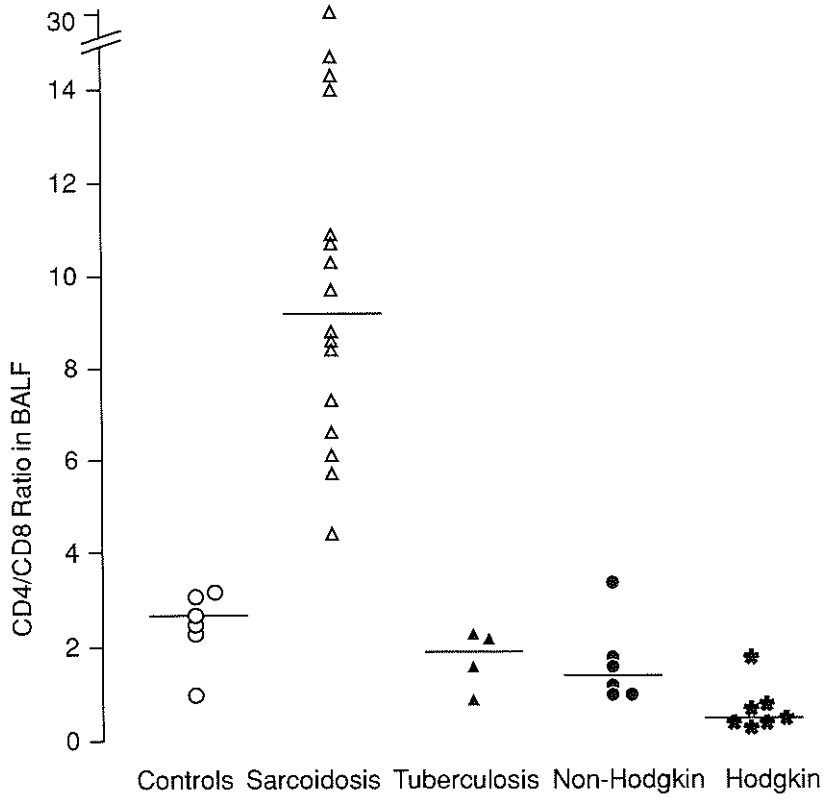

Figure 1. *Individual $CD4^+/CD8^+$ T lymphocyte ratios (with median values) in BALF samples obtained from control subjects, patients with sarcoidosis, tuberculosis, non-Hodgkin's or Hodgkin's disease.*

Table 6 summarizes the statistically significant differences between the diagnostic groups using the Mann-Whitney *U* test, a pairwise comparison.

Table 6—*Statistically Significant Differences in Bronchoalveolar Lavage Fluid (BALF) Samples between the Four Patient Groups*

BALF variable	Sar vs Tbc	Sar vs NHL	Sar vs HL	Tbc vs NHL	Tbc vs HL	NHL vs HL
PMN			↓ p=0.05*			
MC	↓ p=0.05*	↓ p=0.02*,**	↓ p=0.04*			
PC		↓ p=0.01*		↓ p=0.05**		
CD3+ T cells				↓ p=0.05		
CD4+ T cells	↑ p=0.04	↑ p=0.001	↑ p=0.0002			↑ p=0.008
CD8+ T cells	↓ p=0.01	↓ p=0.0004	↓ p=0.0002		↓ p=0.04	↓ p=0.003
$CD4^+/CD8^+$ ratio	↑ p=0.003	↑ p=0.0004	↑ p=0.0002		↑ p=0.02	↑ p=0.02

Data were analyzed by the Mann-Whitney U test. Sar=sarcoidosis; Tbc=tuberculosis; NHL=non-Hodgkin's disease; HL=Hodgkin's disease; vs=versus; PMN=Polymorphonuclear neutrophils; MC=Mast cells; PC=plasma cells.

↑=value first mentioned diagnostic group higher than second group.

↓=value first mentioned diagnostic group lower than second group.

**absolute number of the total cell count (TCC).*

***percentage of TCC.*

Discussion

Although bilateral mediastinal or hilar lymphadenopathy is most frequently caused by the benign and self-limiting disease sarcoidosis, disorders that require rapid diagnosis such as tuberculosis and malignant lymphoma should be excluded.[13,14] In the present study, differences in BALF cell profile and protein levels between patients suffering from sarcoidosis, tuberculosis, non-Hodgkin's disease or Hodgkin's disease were found.

As did Harf et al,[19] we observed high proportions of mast cells in BALF samples in tuberculosis, in contrast to sarcoidosis. In addition, $CD4^+/CD8^+$ ratios were lower in comparison with those of sarcoidosis patients and control subjects, which was in agreement with the findings of others.[31] The cell-mediated immune response to *M tuberculosis*, which plays a predominant role in host defence, involves subpopulations of specifically sensitized $CD4^+$ helper-inducer or cytolytic T lymphocytes.[31,32]

An initially increased number of lymphocytes is a feature of the histopathology of pulmonary tuberculosis with a $CD8^+$ T lymphocyte predominance, whereas during recovery, a $CD4^+$ predominance is found.[31] The $CD8^+$ T lymphocytes are believed to be involved in the production of $1,25(OH)_2D_3$.[33] This compound has been implicated in the improvement of the mycobacterial killing capacity of alveolar macrophages. In tuberculosis, $CD4^+$ T lymphocytes rather than $CD8^+$ T lymphocytes express receptors for $1,25(OH)_2D_3$, whereas a greater proportion of $CD8^+$ than of $CD4^+$ T lymphocytes in patients with sarcoidosis are $1,25(OH)_2D_3$ receptor-positive.[33-35] Thus, the various distribution of $1,25(OH)_2D_3$-receptors points to a different role for the potent immunoregulatory molecule in the granulomatous inflammatory reactions in sarcoidosis and tuberculosis, respectively.[33] The diagnosis of tuberculosis can only be confirmed by culture. In this study, combined evaluation of Ziehl-Neelsen-staining and culture for *Mycobacterium species* of BALF specimens was more sensitive and specific than that of sputum, which was in agreement with studies by others.[16-18]

Lymphocytic lymphomas are immunologically defined by the monoclonal proliferation of T or B lymphocytes.[14] Tumor cells derived from B lymphocytes produce immunoglobulins of one single light chain type.[36]

The majority of lymphomas with pulmonary manifestations are non-Hodgkin's disease derived from B lymphocytes.[14,21,22] However, to date, the diagnostic value of BALF cellular analysis in malignant lymphomas has not been established. In our study, the cellular BALF profile differed between sarcoidosis patients and patients with non-Hodgkin's or Hodgkin's disease. All patients with either malignant lymphoma showed a lymphocytosis in BALF samples. However, a high proportion of lymphocytes is not a characteristic finding, since this has been found in BALF specimens in many pulmonary disorders.[3] The presence of plasma cells in BALF was found to be highly suggestive for malignant lymphomas, especially for non-Hodgkin's disease with paraproteins in their BALF samples. Recently, plasma cells in BALF were associated with extrinsic allergic alveolitis and other antibody-mediated inflammatory processes of the lung, as well as with non-Hodgkin's disease.[37] Increased proliferation of B lymphocytes has been found in lymphocytic lymphomas,[14,36,38] which may account for the presence of plasma cells in BALF samples of patients with maligne lymphomas. Also, in these patients, paraproteins were detected (data not shown). Therefore, our results indicate that BALF studies (B lymphocyte marker and paraprotein analysis) to detect monoclonality can be of additional value in distinguishing between malignant lymphomas and other pulmonary disorders in patients with plasma cells present in BALF samples.

The most important characteristic features in BALF, which allowed the differentiation between malignant lymphomas and sarcoidosis, were differences in T lymphocyte subpopulations and the $CD4^+/CD8^+$ ratios. Moreover, patients with malignant lymphomas, in particular patients with Hodgkin's disease, also demonstrated a decreased $CD4^+/CD8^+$ ratio in peripheral blood, most likely as a consequence of an advanced, disseminated disease.[14] A permanent immunologic defect, both in number and function of T lymphocytes, has been reported to be a concomitant of Hodgkin's disease.[14] However, occasionally, also low $CD4^+/CD8^+$ ratios in BALF were found in sarcoidosis patients.

In this study, the number of mast cells were high in the BALF in patients with tuberculosis and those with maligne lymphomas, in contrast to patients with active sarcoidosis. Recently, Pesci et al[39] suggested that

mast cells participate in chronic inflammation and that their presence is related to interstitial fibrosis in fibrotic lung disorders. Therefore, in addition to assessing $CD4^+/CD8^+$ ratios, determinations of other BALF constituents, such as plasma cells, mast cells and immunoglobulins, may provide additional information to discriminate among the studied disorders besides the $CD4^+/CD8^+$ ratios.[40-43]

Although the patient populations in this study are small, the study illustrates that a limited invasive technique, such as BAL, may be of additional value to distinguish between sarcoidosis and other disorders with similar clinical manifestations, such as tuberculosis and malignant lymphomas with pulmonary involvement, provided that simultaneous careful clinical and pathologic staging is performed. The $CD4^+/CD8^+$ ratio may facilitate the differentiation between sarcoidosis, tuberculosis and malignant lymphomas. In addition, the presence of plasma cells in BALF may permit detection of malignant lymphomas, highly likely to be non-Hodgkin's disease. Future BALF studies, including immunologic marker analyses, are needed to investigate the reliability of BAL in diagnosing malignant lymphomas with pulmonary involvement.

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Relationship between presentation of sarcoidosis and T lymphocyte profile: a study in bronchoalveolar lavage fluid

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Abstract

One hundred patients with histologically verified sarcoidosis were studied. They were divided into three groups, based on their clinical presentation and smoking status. Group A consisted of patients whose disease was detected by routine chest x-ray film, without symptoms; group B included those with respiratory and general constitutional symptoms; and group C included patients with erythema nodosum and/or arthralgia and hilar lymphadenopathy. Group A showed an increased $CD4^+/CD8^+$ ratio of 4.7 ± 1.1; group B, 8.0 ± 1.2; and group C counted for the highest ratio of 10.7 ± 1.5. Cigarette smoking modifies the immunologic bronchoalveolar lavage (BAL) fluid sample profile, since alveolitis was less pronounced in smokers. In addition, BAL fluid samples obtained from sarcoidosis patients with hilar lymphadenopathy showed the most characteristic features of alveolitis, suggesting a disseminated instead of a local immune response. Therefore, the clinical presentation of sarcoidosis and the smoking status of a sarcoidosis patient are crucial for interpreting individual lavage analysis results.

Introduction

Sarcoidosis is a disorder of unknown origin, characterized by the formation of noncaseating epithelioid cell granulomas, probably antigen driven, most frequently occurring in the lungs.[1-4] In addition to granuloma formation, there is often an extensive vascular disease as seen by the appearance of microangiopathies.[4] Granuloma formation in the lungs is preceded by a mononuclear cell alveolitis with increased numbers of activated T lymphocytes and alveolar macrophages.[5-7] Besides changes in T lymphocyte and alveolar macrophage populations, changes in the humoral immunity have been reported.[8]

Clinical manifestations of sarcoidosis depend on the intensity of the inflammation and organ systems affected.[9-11] In some sarcoidosis patients, the alveolitis remains subclinical, whereas in others both alveolitis and granuloma formation are present, resulting in specific pulmonary symptoms.[12-14] Although the lung is the most frequently affected organ, extrapulmonary manifestations such as erythema nodosum commonly occur.[1,3,10]

Bronchoalveolar lavage (BAL) is regarded as an important diagnostic method in sarcoidosis.[15-17] However, conflicting results have been reported in studies evaluating the utility of BAL in assessing the prognosis of the disease. The cellular profile in BAL fluid samples reflects the presence of alveolitis as a local expression of a disseminated immunological disorder.[8,15,18] Lymphocytes recovered in BAL fluid are predominantly T lymphocytes and, there is no more than a 5 percent proportion of B lymphocytes.[8,9] Activation of alveolar T lymphocytes is a characteristic feature of sarcoidosis [19,20] and is demonstrated not only by an increased expression of typical activation markers on the cell surface (immunophenotypic markers, such as HLA-DR antigens expression, T lymphocyte antigen receptor decrease and interleukin-2 (IL-2) receptors), but also by the release of specific mediators (functional markers, such as IL-2, interferon gamma, and other T lymphocyte mediators).[8,9,21-23] Moreover, activation of T lymphocytes in sarcoidosis is subset-specific. Also, inhibition of responsiveness of memory T lymphocytes to recall antigens is part of the immune response in active sarcoidosis, which has been suggested possibly to contribute to the anergy observed in these patients.[24] According to current concepts, the process of cell-mediated immunity is thought to mediate the pathogenesis of sarcoidosis.[8]

Studies on BAL fluid samples profile characteristics in sarcoidosis patients hitherto reported in literature give rise to conflicting data. These controversial results and disparity between conclusions may be explained by differences in the sarcoidosis subpopulations studied and methodologic variations, as well as the fact that sarcoidosis does not present as an entity. Only a few reports regarding the clinical presentation of the disease associated with alveolitis are available. Furthermore, many studies do not differentiate between smoker (Sm) and nonsmoker (NSm) patients.

The aim of this study was to investigate whether the way in which sarcoidosis presents is associated with differences in cellular profile in BAL fluid samples, especially with regard to the number of T lymphocytes and T lymphocyte subpopulations and the smoking status in sarcoidosis patients.

Materials and Methods

Patients and Control Subjects

Bronchoalveolar lavage was performed in 100 patients with histologically proven sarcoidosis and 14 control subjects. The characteristics of the patients and control subjects are described in table 1. The patients were divided into the three groups based on their clinical presentation.

Group A consisted of patients whose disease was detected on routine chest x-ray film, without symptoms or knowledge on the exact duration and the time onset of the disease (11 NSm and 7 Sm); group B included those with respiratory and general constitutional symptoms (50 NSm and 10 Sm) and group C included patients with erythema nodosum and/or arthralgia and hilar lymphadenopathy (*ie*, Löfgren's syndrome; 16 NSm and 6 Sm). All patients had stage I or II disease and none had stage III disease, as evidenced on x-ray films. The majority of the patients were NSms, 77 of 100 (table 1).

Table 1—*Characteristics of the Groups Studied*

Group	n	Age*, yr	Female	Male
Nonsmokers				
Control subjects	11	39.4 (27-66)	7	4
Patients				
A	11	38.2 (27-69)	5	6
B	50	36.6 (22-79)	28	22
C	16	37.0 (23-57)	9	7
Smokers				
Control subjects	3	41.0 (30-60)	2	1
Patients				
A	7	39.0 (24-67)	3	4
B	10	32.3 (19-48)	5	5
C	6	29.7 (21-36)	3	3

** Mean with range in parenthesis. A = sarcoidosis patients, no symptoms; B = sarcoidosis patients, respiratory and general constitutional symptoms; C = sarcoidosis patients, erythema nodosum and/or arthralgia and hilar lymphadenopathy (ie, Löfgren's syndrome); n = number of cases.*

The initial BAL fluid samples of consecutive sarcoidosis patients obtained at the time of the diagnosis, within 2 weeks after admission to our hospital, were used for this study. No patient was receiving corticosteroid or other treatment either at the time of

or before the lavage. The control group consisted of individuals without chest abnormalities or a history of pulmonary abnormalities or disease (11 NSm; 3 Sm). The studied groups were subdivided according to their smoking status (table 1).

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously reported during fibreoptic bronchoscopy.[22] At the same time, blood samples were taken. In short, the procedure was as follows. After premedication with atropine and sometimes diazepam or codeine, and local anaesthesia of the larynx and bronchial tree with 0.5 percent tetracaine, BAL was performed by standardized washing of the right middle lobe with four 50-ml aliquots of sterile saline solution (0.9 percent NaCl) at room temperature. Lavage fluid samples, kept on ice in a siliconized specimen trap, were centrifuged (10 min, 350 g) and separated into cells and supernatant. The cell pellet was washed twice, counted and suspended in minimal essential medium (Gibco, Grand Island, NY) supplemented with 1 percent bovine serum albumin (Organon, Teknika, Boxtel, the Netherlands). Preparations of the cell suspension were made in a cytocentrifuge (Shandon). Cytospin slides of BAL cells were stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany) for cell differentiation. At least 1,000 cells were counted.

If more than 15 percent lymphocytes were present in the BAL fluid samples, T lymphocyte subpopulations were determined. Identification of T lymphocytes reacting with monoclonal antibodies was performed by means of a conventional indirect immunofluorescence technique. Total T lymphocytes and subpopulations were recognised by staining with monoclonal antibodies ($CD3^+$, $CD4^+$ and $CD8^+$). Monoclonal antibodies, CD3 (OKT3), CD4 (OKT4) and CD8 (OKT8) were obtained from Ortho-pharmaceuticals (Beerse, Belgium) and subsequently labelled with fluorescein isothiocyanate (FITC)-conjugated goat-antimouse-immunoglobulin (Nordic, Immunological Laboratories, Tilburg, the Netherlands and from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, the Netherlands). Results were expressed as a percentage of lymphocytes.

Statistical analysis

To investigate whether there were statistically significant differences between the three categories of sarcoidosis patients, the Kruskal-Wallis one-way analysis of variance test was used. Each category; group A (no symptoms), group B (respiratory and general constitutional symptoms), and group C (erythema nodosum and hilar lymphadenopathy [Löfgren's syndrome]), respectively, denoted the clinical presentation of the patient.

The Mann-Whitney U test, a pairwise comparison, was used to evaluate any differences between Sms and NSms in each group, as well as the differences in each category with the control subjects. The probability values less than 0.05 were considered to be significant.

Results

The mean values ± SEM of the immunologic marker analysis of the cells in BAL fluid and peripheral blood (PB) samples of the groups studied are summarized in table 2. In general, the cellular components of BAL fluid samples obtained from all patient groups differed significantly from those obtained from control subjects. The mean percentages of $CD4^+$ and $CD8^+$ T lymphocytes and the $CD4^+/CD8^+$ ratio in BAL fluid samples showed statistically significant differences in the NSm sarcoidosis patients (figure 1, table 2).

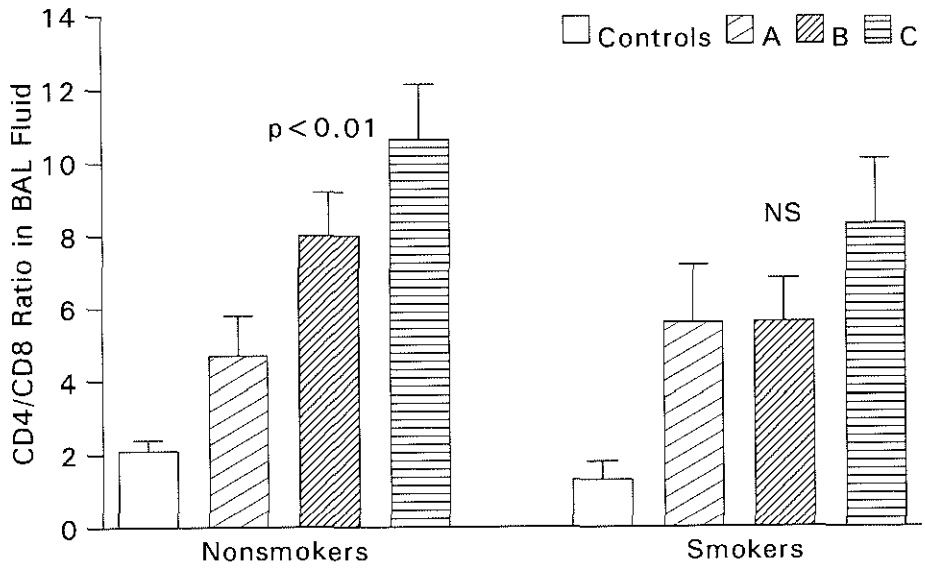

Figure 1. *$CD4^+/CD8^+$ T lymphocyte ratio in BAL fluid samples in three different clinical presentations of sarcoidosis patients (A = no symptoms; B = respiratory and general constitutional symptoms; C = erythema nodosum and/or arthralgia and hilar lymphadenopathy [ie, Löfgren's syndrome]; mean ± SEM) and control subjects. The general effect of smoking is a reduction of the $CD4^+/CD8^+$ ratio.*

No significant difference could be demonstrated between the three categories of Sm patients, except the mean percentage of $CD3^+$ T

lymphocytes in the BAL fluid samples, which appeared to be the highest in patients with erythema nodosum and/or arthralgia, *ie,* Löfgren's syndrome (table 2). No plasma cells were demonstrated in the BAL fluid samples from the sarcoidosis patient groups or from the control subjects. In BAL fluid samples of all categories sarcoidosis patients, the total cell count was significantly increased compared with that of the control subjects ($p < 0.04$). The absolute and relative number of lymphocytes were increased, as well as the percentages of $CD3^+$ and $CD4^+$ T lymphocytes; in contrast, the percentage of $CD8^+$ T lymphocytes was decreased most of all in patients with Löfgren's syndrome (category C). The $CD4^+/CD8^+$ ratios in the BAL fluid samples were increased in all sarcoidosis patient categories, most prominent in both NSm and Sm patients with Löfgren's syndrome. The $CD4^+/CD8^+$ ratios in the PB samples showed no statistical differences, except in category A of the NSm patients, who demonstrated a decreased ratio compared with that of the control subjects.

Significant differences were demonstrated between Sm and NSm subjects of the studied categories. In general, total cell count and the number of alveolar macrophages were increased, while the percentages of lymphocytes were decreased in the Sm groups. In the sarcoidosis Sm groups studied, also a significant increase in the number of polymorphonuclear neutrophils and a decrease in the number of mast cells were found in BAL fluid samples (data not shown).

Smoker patients with respiratory or constitutional symptoms (*ie,* group B) showed a decreased percentage of $CD3^+$ T lymphocytes in the PB samples (data not shown) and decreased $CD4^+/CD8^+$ ratios in BAL fluid and PB samples compared with NSm patients of category B. Smoker patients, presenting with Löfgren's syndrome (*ie,* group C) showed an increased percentage of $CD3^+$ lymphocytes and less increased $CD4^+/CD8^+$ ratio in BAL fluid and lower $CD4^+/CD8^+$ ratio in PB samples in comparison with NSm patients. In general, most significant changes were demonstrated in the BAL fluid samples of patients with Löfgren's syndrome both in NSm and Sm patients compared with the other categories and control subjects (figure 1, table 2).

Table 2—*Total Cell Count, Lymphocytes, T Lymphocyte Subpopulations and the $CD4^+/CD8^+$ Ratios in Bronchoalveolar Lavage Fluid Samples and Peripheral Blood Samples in Three Different Clinical Presentations of Sarcoidosis Patients and Control Subjects*

Group	Total Cell	Lym	Lym	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$ ratio	
	Countx10^6	x10^4/ml	%	%	%	%	BAL	PB
Nonsmokers								
Controls	11.7 (2.7)	1.0(2.8)	10.8(1.7)	75.8(2.3)	49.5(3.5)	29.6(4.8)	2.1(0.3)	2.3(0.5)
A	19.2 (3.5)	4.7(1.1)#	31.3(5.1)#	83.5(2.4)#	60.9(4.7)*	19.9(3.5)*	4.7(1.1)*	1.3(0.2)*
B	20.8 (1.8)*	7.7(0.9)#	38.1(2.6)#	88.3(1.0)#	69.5(2.2)#	17.9(2.0)#	8.0(1.2)#	2.4(0.4)
C	23.5 (3.2)*	8.7(1.4)#	40.2(4.5)#	88.8(2.1)#	80.3(2.7)#$	8.9(0.8)#$	10.7(1.5)#$	2.6(0.4)
p-value +	NS	NS	NS	0.05	<0.001	0.01	0.008	0.06
Smokers								
Controls	33.3(12.7)	8.9(3.5)	4.2(2.1)	82.3(5.4)	45.7(7.2)	40.7(7.3)	1.3(0.5)	1.4(0.6)
A	31.0 (5.5)	6.1(1.4)*	21.3(3.6)#	89.1(2.6)*	69.4(6.8)#	19.4(5.6)#	5.6(1.6)*	2.1(0.5)
B	43.9(14.9)*	10.8(4.1)	27.1(6.4)#	86.1(3.4)	66.2(6.4)*	22.0(6.8)#	5.6(1.2)#	1.7(0.3)
C	31.2 (7.0)	4.7(1.8)*	18.7(5.9)#	95.3(1.2)#$	81.0(4.0)#$	12.5(2.8)#$	8.3(1.8)#$	1.8(0.5)
p-value +	NS	NS	NS	0.03	NS	NS	NS	NS

*Data are expressed as mean with SEM in parentheses. Lym = Lymphocytes; A = Sarcoidosis patients, no symptoms; B = Sarcoidosis patients, respiratory and general constitutional symptoms; C = Sarcoidosis patients, erythema nodosum and/or arthralgia and hilar lymphadenopathy (Löfgren's syndrome). NS = not significant. *$p<0.04$ Mann-Whitney versus control group. #$p<0.02$ Mann-Whitney versus control group. $$p<0.05$ Mann-Whitney C versus A and B. + = p-value Kruskal-Wallis test for differences between group A, B and C.*

Discussion

The results presented in this study confirm previous observations of signs of a T lymphocyte alveolitis and the influence of smoking on T lymphocyte subsets in patients with sarcoidosis. Most remarkably, we demonstrated that the BAL fluid T lymphocyte profiles are related to the differential clinical presentation of sarcoidosis patients. This relationship was found to be less pronounced in Sms. However, comparison between the different studied categories of sarcoidosis patients is rather difficult, whereas estimating the exact time of onset of the asymptomatic patients and those presenting with respiratory symptoms is much more difficult than assessing the time of onset of erythema nodosum.[14] Although the influence of smoking on BAL fluid cell profile has been studied extensively, only a few reports concerning the influence of clinical presentation of sarcoidosis on the cell profile in BAL fluid samples have hitherto been published.

Costabel et al[25] suggested earlier that cellular immunoregulation may be disturbed in the lungs of cigarette smokers, and as such, may influence pulmonary host defence. In the present study, we confirm the data previously reported by others, by showing that smoking results in increased total cell counts, less increased percentages of T lymphocytes, and less increased $CD4^+/CD8^+$ ratios in the BAL fluid samples in both sarcoidosis patients.[26-32] Thus, alveolitis, as determined by immunologic marker analysis, is less significant in smokers.[27]

Although all sarcoidosis patient groups, both Sms and NSms, in our study appeared to have an increased $CD4^+/CD8^+$ ratio in BAL fluid samples, NSm asymptomatic patients (group A) had the lowest, less increased ratio (4.7) in comparison with that of NSm symptomatic patients with respiratory and general symptoms (group B). These latter patients had a mean $CD4^+/CD8^+$ ratio of 8.0, while NSm patients presenting with acute onset sarcoidosis (*ie*, Löfgren syndrome), had the highest ratio (10.7) in the BAL fluid samples. These significant changes in the $CD4^+/CD8^+$ ratio seem to be primarily due to an increased influx of $CD4^+$ T lymphocytes to the alveoli, indicating an active immune response.[2-4] The reported decreased influx of $CD8^+$ T lymphocytes also determines the $CD4^+/CD8^+$ ratio in sarcoidosis, most prominently demon-

strated in patients with Löfgren's syndrome, who showed significant lower percentages $CD8^+$ T lymphocytes both in Sms and NSms. Interestingly, patients with pronounced alveolitis, as reflected in a high $CD4^+/CD8^+$ ratio in BAL fluid samples, who clinically present with acute onset such as Löfgren's syndrome, do not need corticosteroid or any other treatment and have a short recovery time period.[14] In contrast, sarcoidosis patients with a relatively low $CD4^+/CD8^+$ ratio in BAL fluid samples more frequently develop permanent lesions, such as pulmonary fibrosis.[33-36] These data confirm the previously suggested poor prognostic significance of "isolated" BAL fluid analysis results.[10,14] The aforementioned best prognosis and spontaneous resolution of the lesions in almost all cases suffering from Löfgren's syndrome, compared with other subgroups, suggests the beneficial role of especially $CD4^+$ T lymphocytes in the immune response in sarcoidosis and their essential function in defense.[14,20,36]

Lymphocytic alveolitis is an early event in the evolution of pulmonary involvement in sarcoidosis.[14,15] This inflammatory response precedes granuloma formation in the lung and may be latently present without clinical or physiologic impairment, while remaining undetected by radiological investigation.[37,38] It has been demonstrated by Valeyre et al[10] that the alveolitis in sarcoidosis patients with erythema nodosum precedes the increase in serum angiotensin-converting enzyme. Furthermore, they showed that the serum IgG levels were correlated to the lymphocyte count in BAL fluid samples. Increased levels of serum IgG are probably under the influence of T lymphocytes through the activation of B lymphocytes present in sarcoid lesions, as have been demonstrated by Rankin et al[39] (1983) and recently by Fazel et al[8] (1992). Also, the existence of alveolitis in patients with extrapulmonary sarcoidosis with normal chest x-ray film findings has been reported by others.[10,12,37] Our findings that patients with Löfgren's syndrome show the most prominent alveolitis and the highest $CD4^+/CD8^+$ ratio in BAL fluid samples suggest the involvement of T lymphocytes activated by an unknown stimulus in the initiation of granulomatous inflammation in sarcoidosis.[20,38] In addition, the characteristic findings of Löfgren's syndrome, *ie*, erythema nodosum and arthralgia, early in the course of sarcoidosis, histologically resembling a nonspecific vasculitis, suggest a disseminated rather than a local

immune response, probably antigen driven.[8,20,37,40] This speculated involvement of an antigen as a stimulus underlying the granulomatous response has been demonstrated by the so-called Kveim-Siltzbach test.[18,41] Recently, du Bois et al[20] provided evidence of recent stimulation of the T lymphocyte antigen receptor of T lymphocytes accumulating in the lung in sarcoidosis. Fazel et al[8] found large numbers of B lymphocytes in sarcoidosis pulmonary lesions. The B lymphocytes at these sites were suggested to be the possible origin of some of the humoral changes in serum and lesions of sarcoidosis patients. They might also influence the pathogenesis of the disorder by presenting antigens and forming immune complexes at sites of disease activity.[8] Therefore, analysis of the antigen specificity of these expanded populations is likely to provide insight into the pathogenesis of the disease.

In conclusion, patients with different clinical presentation of sarcoidosis have various T lymphocyte profiles in BAL fluid samples. Patients with erythema nodosum and/or arthralgia and hilar lymphadenopathy (*ie*, Löfgren's syndrome) show the most marked characteristics of alveolitis, including the highest $CD4^+/CD8^+$ ratios in BAL fluid samples, suggesting a disseminated instead of a local immune response. Furthermore, cigarette smoking modifies the immunologic BAL fluid sample profile, and in addition, alveolitis is found to be less pronounced in Sms. Therefore, disease presentation or activity at the time of onset and smoking status are crucial for interpretation of individual BAL fluid sample analysis results.

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Bronchoalveolar lavage in extrinsic allergic alveolitis: effect of time elapsed since antigen exposure

Bronchoalveolar lavage in extrinsic allergic alveolitis: effect of time elapsed since antigen exposure

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Abstract

The aim of the present study was to investigate whether bronchoalveolar lavage (BAL) cell profile and immunoglobulin levels from patients with extrinsic allergic alveolitis (EAA) were related to the time elapsed between last antigen exposure and BAL.

For this purpose, an analysis was performed of BAL fluid (BALF) obtained from 59 nonsmoking EAA patients at various time-points after termination of antigen exposure and BAL.

BALF obtained early after antigen provocation (group 1: < 24 hours) contained high absolute and relative numbers of lymphocytes, neutrophils, eosinophils and mast cells, and a low relative number of alveolar macrophages. When obtained after recent antigen exposure (group 2: 2-7 days), BALF showed high numbers of lymphocytes, plasma cells and mast cells, and high levels of immunoglobulins M, G and A (IgM, IgG and IgA). In BALF obtained one week or more after the final antigen exposure, the distribution of all constituents showed a tendency to return to normal values, with the exception of the lymphocytes.

In summary, these results demonstrate that BAL cell profile and immunoglobulin levels in EAA are highly dependent on the time-point at which the material is obtained in relation to the last exposure to the causative antigen.

Introduction

Extrinsic allergic alveolitis (EAA), or hypersensitivity pneumonitis, is a disease initiated by repeated exposure to extrinsic organic antigens in susceptible individuals.[1,2] The clinical manifestation of EAA shows considerable variation as it is related to the frequency and intensity of exposure to the causative agent.[3,4]

The initial phase of EAA is characterized by an early increase in bronchoalveolar lavage fluid (BALF) polymorphonuclear neutrophils (PMNs).[1,2,5,6] Twelve hours to several days following onset, the bronchioloalveolitis consists mainly of $CD8^+$ T-cells,[7] which among others, modulates the B-cell response and antibody production by plasma cells.[1,7,8] Increase in the $CD8^+$ T-cells in BALF results in a relatively low $CD4^+/CD8^+$ ratio.[1,2,7] After weeks to months following antigen exposure, a slight predominance of $CD4^+$ T-cells is found in BALF.[1,7,9]

Various immune mechanisms are involved in the pathogenesis of EAA.[1] Among these, immunoglobulins (Igs),[10-12] immune complexes,[13,14] complement, cytokines,[15] lipids and other biologic modifiers have been described to play an important role.[1] In addition, T-cells have been implicated in the mechanism underlying tissue damage and repair in EAA.[1]

As the relative number of lymphocytes and the balance of the various T-cell subsets present in BALF may be of predictive value with regard to nature (and outcome) of the pathological process,[16-20] the timing of the BAL seems to be critical for the detection of characteristic constituents.

The aim of this study was to establish the relationship between the timing of BAL and the last exposure to the causative antigen on BAL-profiles of T-cells, other cells and proteins in EAA patients and to detect specific features of the various categories.

Patients and controls

Bronchoalveolar lavage fluid samples, obtained from patients suffering from EAA (n=67) during a ten year period between 1980 and 1990, were studied. The EAA patients presented with generalized constitutional and pulmonary symptoms, *ie*, cough, dyspnea and sometimes fever and chills. Although the symptoms mostly were transient, initial complaints and exacerbations occurred with repeated exposure or provocation to the causative antigen. The major physical findings were tachypnea, cyanosis, and bilateral crepitant rales. Both restrictive and obstructive defects in pulmonary function were observed. In most cases, the carbon monoxide diffusion capacity, measured when the patient was admitted to the hospital, was seriously disturbed. In some patients, hypoxemia showed a substantial worsening with exercise. The diagnosis EAA was based with care on clinical information, chest x-ray film, the presence of precipitating serum antibodies against the suspected antigens, pulmonary function tests and disappearance of symptoms after avoidance of antigen exposure. An open lung biopsy was performed in six patients, without sufficient clinical criteria to make a definitive diagnosis, and the diagnosis EAA was verified histologically.

Fifty nine nonsmoking EAA patients were included, all of whom were frequently exposed to birds, *ie*, pigeons, parrots, budgerigars or canaries (table 1). No patient was receiving corticosteroid treatment before BAL.

At the time of the lavage, these patients were divided into four categories, based on the time period between the presumed termination of antigen exposure and the BAL: group 1: < 24 hours; group 2: 2-7 days; group 3: 8-30 days; and group 4: 1-12 months. Antigen provocation was achieved by bringing the patient into contact with the birds, followed by a BAL within 24 hours. The control group consisted of 28 nonsmoking healthy volunteers, without chest abnormalities or a history of pulmonary

abnormalities or disease, and without contact to EAA inducing antigens (table 1).

Table 1—*Characteristics of the Groups Studied: Nonsmoking Extrinsic Allergic Alveolitis (EAA) Patients and Controls*

Groups	n	Age, yr*	Female	Male	Precipitins
EAA patient categories					
1) < 24 hours	14	53 (23-75)	4	10	$7^{A};3^{C};4^{D}$#
2) 2-7 days	28	51 (27-70)	7	21	$18^{A};5^{B};2^{C};3^{D}$#
3) 8-30 days	11	45 (19-71)	4	7	$8^{A};3^{C}$#
4) 1-12 months	6	55 (25-73)	2	4	$4^{A};1^{B};1^{C}$#
Controls	28	39 (19-60)	12	16	Not done

** Data indicate mean with range in parentheses; n = number of cases; # avian proteins, all birds: A = pigeon; B = budgerigar; C = canary and D = parrot.*

Methods

Bronchoalveolar lavage

BAL was performed as reported previously during fibreoptic bronchoscopy.[2] The procedure is briefly described. After premedication (atropine and sometimes diazepam or codeine), and local anaesthesia of the larynx and bronchial tree (tetracaine 0.5 %), BAL was performed by standardized washing of the right middle lobe with four aliquots of 50 ml sterile saline (0.9% NaCl) at room temperature.

Recovered BALF was kept on ice in a siliconized specimen trap, and was separated from cellular compounds by centrifugation (for 5 minutes with a force of 350xg). Supernatants were directly stored at -70 °C after an additional centrifugation step (for 10 minutes with a force of 1,000xg). The cells were washed twice, counted and suspended in minimal essential medium (MEM; Gibco, Grand Island, New York, USA) supplemented with 1% bovine serum albumin (BSA; Organon, Teknika, Boxtel, the Netherlands).

Preparations of the cell suspensions were made in a cytocentrifuge (Shandon). Cytospin slides of BAL cells were stained with May-Grünwald-Giemsa (MGG; Merck, Darmstadt, Germany) for cell differentiation. At least 1,000 cells were counted.

Simultaneously with the BAL, peripheral blood samples were taken. Mononuclear cells were isolated from heparinized blood by Ficoll isopaque density centrifugation (Pharmacia, Uppsala, Sweden).

If more than 15 percent lymphocytes were present in BALF, T-cell (sub)populations in BALF and also in blood were determined. Total T-cells, and subpopulations were recognized by staining with monoclonal antibodies CD2(OKT11), CD3(OKT3), CD4(OKT4) and CD8(OKT8) (Ortho-pharmaceuticals, Diagnostic systems, Beerse, Belgium). Identifi-

cation of T-cells reacting with monoclonal antibodies was performed by means of a conventional indirect immunofluorescence technique using fluorescein isothiocyanate (FITC)-labelled goat-antimouse-Ig (GAM, Nordic, Immunological Laboratories, Tilburg, the Netherlands and from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands).

For the quantitative determination of albumin in serum and BALF the albumin method was used. The albumin method is an adaption of the bromocresol purple (BCP) dye-binding method.[21,22] In short this method is as follows. In the presence of a solubilizing agent, BCP binds to albumin at a pH 4.9. The amount of albumin-BCP complex is directly proportional to the albumin concentration. The complex absorbs at 600 nm. Albumin concentrations in serum and BALF were expressed in g/l and mg/l, respectively.

IgM, IgG and IgA concentrations in BALF were determined by an enzyme-linked immunosorbent assay (ELISA) method; microtitre plates were coated with a rabbit anti-human-isotype antiserum (anti-IgM, (CLB, Amsterdam, the Netherlands), anti-IgG and anti-IgA (Dako, Glostrup, Denmark)). Bound Igs from BALF were visualised by using a horseradish peroxidase (HRP)-labelled rabbit anti-human-Ig antiserum (with anti-IgA, IgG, -IgM, -kappa, -lambda reactivity (Dako, Glostrup, Denmark)) and a chromogenic substrate orthophenyl diamine (OPD; Baker, Chemicals BV, Deventer, the Netherlands). Concentrations in BALF were expressed in mg/l using as a reference a commercial human standard serum, HOO-03 (CLB, Amsterdam, the Netherlands).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). In order to investigate whether there were statistically significant differences between the four categories of EAA patients data were analyzed by the Kruskal-Wallis one-way analysis of variance (ANOVA) test. Each category denoted a certain moment of observation after the last antigen exposure. The Mann-Whitney test was subsequently used to evaluate the differences between each category and the control group. A p-value of less than 0.05 was considered to be significant.

Results

The mean numbers of cells (±SEM) in BALF samples obtained from EAA patients and control subjects, as well as the $CD4^+/CD8^+$ ratio and protein levels in BALF and peripheral blood are summarized in tables 2-4 and figures 1 and 2.

Category 1: < 24 hours

Category 1 (BAL after antigen provocation) was characterized by the highest absolute and relative numbers of polymorphonuclear neutrophils (PMNs), as well as the highest percentage of eosinophils, and a low percentage of alveolar macrophages (AMs). As compared with the control

group, the total cell count, lymphocytes, plasma cells, and mast cells were also increased (figure 1 and table 2). The percentage of T-cells ($CD3^+$) and of $CD8^+$ T-cells were also significantly increased compared with the controls (table 3). Of all groups studied, the percentage of $CD4^+$ T-cells was highest in group 1, whereas the percentage of $CD8^+$ T-cells was relatively low in this group (table 3). The $CD4^+/CD8^+$ ratio in BALF was low compared with the control subjects (figure 2).

In BALF, the levels of albumin, Igs and Ig ratios to albumin were increased in category 1, and in the other categories (2, 3 and 4), as compared with the control subjects (table 4).

Category 2: 2-7 days

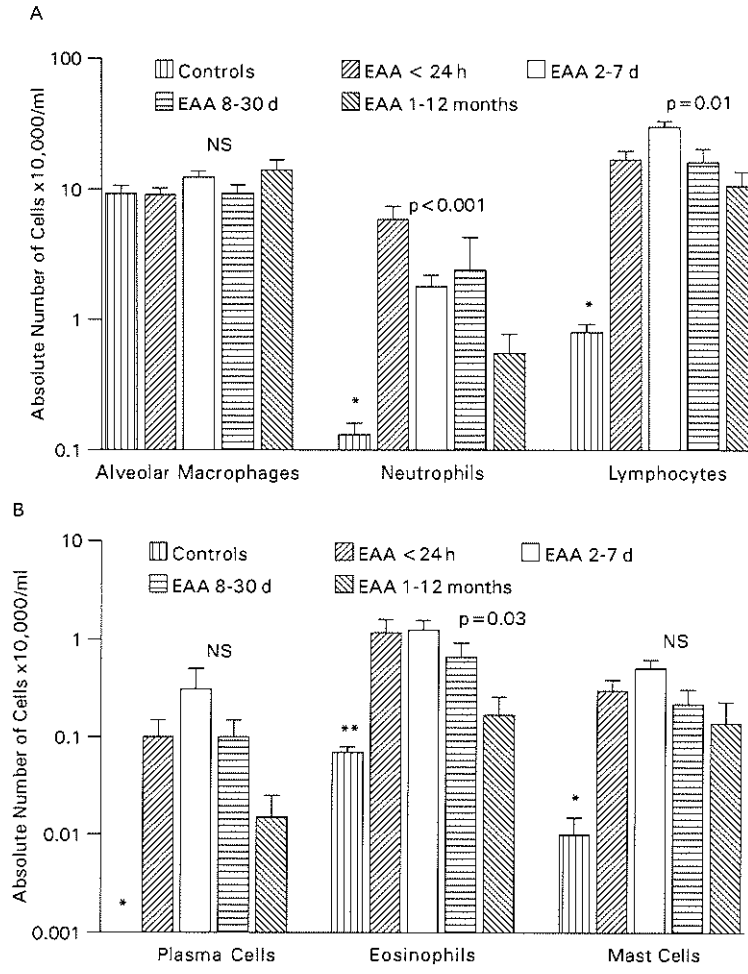
Category 2 (BAL performed within 2-7 days after the last antigen exposure) showed, as the most characteristic feature, a drop in the percentage of PMNs compared with category 1 (table 2). Category 2 patients demonstrated the highest absolute and relative numbers of lymphocytes, plasma cells, eosinophils and mast cells, and the lowest $CD4^+/CD8^+$ ratio in BALF. The $CD4^+/CD8^+$ ratio was also low (1.43 ± 0.13) in peripheral blood obtained from these patients. In addition, in BALF, the highest levels of the respective immunoglobulins IgM, IgG and IgA were found, and the IgM ratio to albumin in BALF samples obtained from patients of this category was the highest (table 4).

Category 3: 8-30 days

The median $CD4^+/CD8^+$ ratio in BALF was low compared with the control group (figure 2). In contrast, the mean values showed no differences, because of bias due to the nonparametric distribution (table 3). Furthermore, no specific features were demonstrated in BALF samples obtained from patients with EAA which belong to this category in comparison with category 2, except for a lower total cell count (table 2).

Category 4: 1-12 months

Category 4 (BAL performed within 1-12 months after termination of the antigen exposure) had no specific characteristics, the numbers of eosinophils, plasma cells and PMNs in BALF were returning to normal, in contrast to the absolute and relative number of lymphocytes and mast cells, which appeared to be still increased. As a consequence, the percentage AMs was also still decreased (figure 1 and table 2).



Figures 1A and B. *The effect of time elapsed between bronchoalveolar lavage (BAL) and termination of antigen exposure on the absolute numbers of cell types in BALF of extrinsic allergic alveolitis (EAA) patients and control subjects (the Y-axis has a logarithmic scale); p=Kruskal-Wallis p-value between the four categories of EAA-patients; NS=not significant; * p<0.05 Mann-Whitney U-test all EAA patient groups vs controls; ** p<0.05 Mann-Whitney U test EAA patients belonging to category 1, 2 and 3 vs controls.*

A. - Alveolar macrophages; neutrophils and lymphocytes.

B. - Plasma cells (value controls 0.0±0.0), eosinophils and mast cells.

Table 2—*Cellular Components in Bronchoalveolar Lavage Fluid (BALF) of Extrinsic Allergic Alveolitis (EAA) Patients and Controls: Differential Cell Counts*

	TCC	Percentage of TCC					
Groups	x10^4/ml	AM	PMN	Lym	PC	Eos	MC
EAA patient categories							
1) < 24 hours	33.6 ± 4.0#	29.0 ± 3.0#	18.1 ± 4.5#	48.2 ± 4.7#	0.34 ± 0.16#	3.55 ± 1.14#	0.85 ± 0.20#
2) 2-7 days	46.4 ± 4.8#	30.2 ± 2.6#	4.2 ± 0.7#	61.3 ± 2.7#	0.57 ± 0.16#	2.70 ± 0.60#	1.01 ± 0.14#
3) 8-30 days	29.0 ± 8.7#	41.3 ± 5.6#	4.2 ± 2.2#	52.0 ± 4.5#	0.21 ± 0.09#	1.70 ± 0.49#	0.60 ± 0.20#
4) 1-12 months	25.5 ± 4.1#	55.5 ± 8.3#	2.1 ± 0.6	41.2 ± 8.6#	0.05 ± 0.03#	0.60 ± 0.30	0.48 ± 0.19#
p-value*	0.03	0.005	< 0.001	0.008	0.05	0.05	NS
Controls	10.3 ± 1.5	89.8 ± 0.7	1.3 ± 0.2	8.4 ± 0.7	0.0 ± 0.0	0.44 ± 0.10	0.09 ± 0.03

TCC = Total cell count; AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; PC = Plasma cells; Eos = Eosinophils; MC = Mast cells; p = Kruskal-Wallis p-value between the 4 categories of EAA-patients; NS = not significant; #p < 0.05 Mann-Whitney U test EAA patient groups vs controls.*

Table 3—*T-Cell Subpopulations Recovered from Bronchoalveolar Lavage Fluid (BALF) and $CD4^+/CD8^+$ Ratios in BALF and Peripheral Blood (PB) of Extrinsic Allergic Alveolitis (EAA) Patients and Controls*

		BALF				PB
Groups	n	$CD3^+$(%)	$CD4^+$(%)	$CD8^+$(%)	$CD4^+/CD8^+$	$CD4^+/CD8^+$
EAA patient categories						
1)<24 hours	10	83.7±3.1#	49.6±6.3	36.9±5.4#	1.75±0.34	1.62±0.45
2)2-7 days	22	85.6±1.7#	44.3±4.1	40.6±2.9#	1.32±0.20#	1.43±0.13#
3)8-30 days	8	86.0±1.9#	46.1±8.7	42.6±8.2#	2.07±0.82	3.28±0.64
4)1-12months	5	88.0±1.2#	46.6±9.9	41.6±9.8#	2.28±1.03	1.99±0.37
p-value*		NS	NS	NS	NS	0.01
Controls	6	72.8±2.0	50.5±3.4	23.0±4.2	2.60 ±0.17	1.75 ±0.33

n=number of cases; p=Kruskal-Wallis p-value between the four categories of EAA patients; NS=not significant; # p<0.05 Mann-Whitney U-test EAA patient groups vs controls.*

Table 5—*Statistically Significant Differences of Bronchoalveolar Lavage Fluid (BALF) Constituents between Four Studied Categories of Extrinsic Allergic Alveolitis (EAA) Patients*

	EAA patient categories			
BALF constituents	<24 hours	2-7 days	8-30 days	1-12 months
Alveolar macrophages (%)	↓			↑
Lymphocytes*		↑		↓
Plasma cells*		↑		↓
Neutrophils*	↑			↓
Eosinophils*	↑			↓
IgM-, IgG-, and IgA-levels		↑		↓
Ratio IgM/albumin		↑		↓

**Absolute and relative number; ↑:highest value; ↓:lowest value (for the exact values see tables 2-4 and figure 1). IgM,G and A: immunoglobulin M, G and A.*

Table 5 gives a summery of most important significant differences between the four categories of EAA patients studied.

Table 4—*Protein Levels in Bronchoalveolar Lavage Fluid (BALF) of Extrinsic Allergic Alveolitis (EAA) Patients and Controls*

Groups	l-alb mg/l	l-alb/s-alb x100	IgM mg/l	IgM/l-alb	IgG mg/l	IgG/l-alb	IgA mg/l	IgA/l-alb
EAA patient categories								
1) < 24 h	187 ± 32#	0.45 ± 0.08#	10.3 ± 2.8#	0.06 ± 0.01#	242 ± 39#	1.62 ± 0.35#	41.6 ± 11.7#	0.22 ± 0.04#
2)2-7 days	180 ± 20#	0.47 ± 0.05#	19.0 ± 4.2#	0.12 ± 0.03#	243 ± 43#	1.89 ± 0.41#	59.7 ± 13.2#	0.57 ± 0.20#
3)8-30days	187 ± 53#	0.52 ± 0.15#	15.0 ± 7.8#	0.08 ± 0.01#	150 ± 60#	0.87 ± 0.23#	35.3 ± 10.2#	0.17 ± 0.04#
4)1-12 mth	117 ± 19#	0.25 ± 0.41#	3.6 ± 1.2#	0.03 ± 0.01#	85 ± 25#	0.70 ± 0.29#	23.9 ± 8.4#	0.23 ± 0.09#
p-value*	NS	NS	NS(0.06)	0.01	0.05	NS(0.07)	NS(0.06)	NS
Controls	71 ± 8	0.19 ± 0.02	0.4 ± 0.1	0.01 ± 0.002	11 ± 2	0.16 ± 0.02	3.6 ± 0.7	0.05 ± 0.01

alb = albumin; l-alb = BALF alb; s-alb = serum alb; p = Kruskal-Wallis p-value between the four categories of EAA patients; NS = not significant; # p < 0.05 Mann-Whitney U-test EAA patient groups vs controls.*

Figure 2

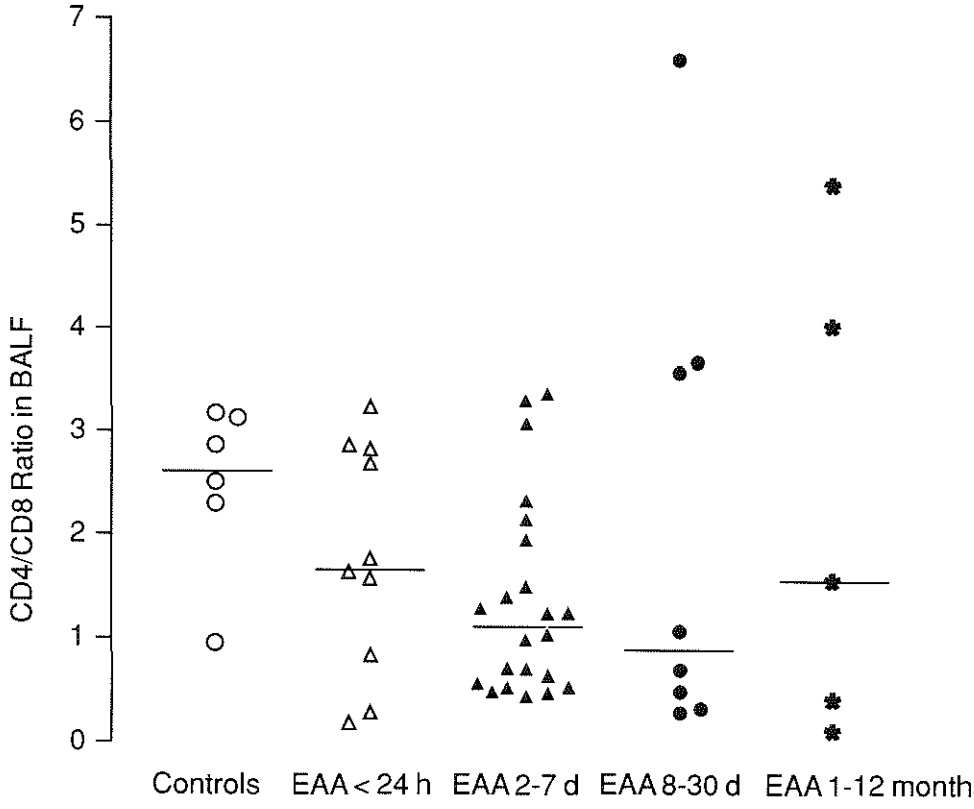


Figure 2. *Individual $CD4^+/CD8^+$ ratios (with median values) in bronchoalveolar lavage fluid (BALF) obtained from the four categories of extrinsic allergic alveolitis (EAA) patients studied and control subjects.*

Discussion

In the present study, the cellular components and protein levels in BALF obtained from EAA patients were found to be related to the time elapsed between termination of antigen exposure and the actual performance of BAL for obtaining the material for analysis. The course of EAA may be divided into various phases according to subsequent immunological reactions in the lung.[1,20,23] Consequently, these phases of the immune

response are reflected in a varying composition of BALF samples.[1,2,20] Patients in our study, lavaged within the first 24 hours after antigen inhalation, showed the highest values of PMNs in BALF, in agreement with other studies.[2,5,17] When BAL was performed in the subsequent 2-7 days after the exposure to antigens, not only total cell count, but also the absolute and relative number of lymphocytes, plasma cells, eosinophils and Ig levels were found to be elevated. In view of this time-related effect on the composition of BALF in EAA, an analysis of a BALF sample may make it possible to determine whether antigen exposure has recently occurred.

The mechanisms underlying the rapid recruitment of inflammatory cells into the alveoli, finally resulting in an accumulation of lymphocytes,[1,2] are as yet not clear. It has been suggested that cell migration is triggered by antigen-antibody complexes and the release of cytokines by antigen specific T-cells in the course of the immune response.[24,25]

In contrast to the presumption that the presence of T-cells is predictive for the activity and progress of the disease,[8] clinical symptoms were found to subside in longstanding EAA, whereas the number of T-lymphocytes in BALF remained increased.[1,5,7] Furthermore, in asymptomatic EAA patients and normal farmers the high numbers of T-lymphocytes in BALF can persist for 2 years or more.[23,26] Thus, the increased numbers of T-cells, as such, in the BALF is not necessarily a marker for disease activity or progression,[8] but subpopulations of T-cells may be of importance.[26,27] Recently, Trentin et al[17] showed a shift from the $CD8^+$-predominant cellular profile in BALF toward the normal $CD4^+$-predominant profile after termination of antigen exposure. Interestingly, in the present study, no such $CD8^+$ predominance was observed in the BALF obtained immediately after challenge, but rather a higher percentage of $CD4^+$ T-cells was demonstrated. However, when gathered during the subsequent 2-7 day time period, the mean $CD4^+/CD8^+$ ratio was lowest in both BALF and peripheral blood (PB), as compared with the time intervals. Our data are not necessarily contradictory with those of Trentin et al[17], because the latter authors did not perform a BAL within 1 week after antigen provocation. In our present study, an increase of $CD8^+$ T-cells was demonstrated in patients of category 2 and 3 (BAL within, respectively, 2-7 and 8-30 days after termination of antigen exposure), this tendency continued in category 4 (BAL within 1-12 months after antigen exposure), which is in

agreement with Trentin et al.[17] This might also explain the course of the $CD4^+/CD8^+$ ratio, which still decreased after challenge, and returned to the normal range starting 1 month after the last exposure. Thus, the timing of observation is an important variable accounting for the heterogeneous data reported from different authors.

The presence of plasma cells in BALF suggests recent antigen exposure and, as such, EAA.[28,29] Following an initial increase, more than a week after the last antigen exposure, both the number of plasma cells and Ig levels were found to decrease, indicating to a relationship between plasma cells and Igs in BALF. Recently, Reynolds et al[30] suggested the concept of local production of Igs within the lung after inhaling antigen, in addition to diffusion of Igs from the vessels. They also found significantly lower Ig levels in asymptomatic pigeon breeders. A rise of the concentrations of Igs was observed immediately after antigen exposure, parallelling the increase of plasma cells in BALF. However, Ig levels became maximum in category 2 (2-7 days following final antigen exposure). This may be due to the high molecular weight of Igs. Initially, Igs remain intravascularly and in the interstitium.[31,32] Bronchioloalveolitis in EAA caused by inflammation and antigenic stimulation is characterized by a rapid influx of PMNs into the alveolar space, just after challenge. PMNs are important mediators of tissue damage, and are able to enhance the permeability of capillary and alveolar membranes.[31] Due to this damage, Igs can easily diffuse across these no longer semipermeable membranes and enter the interstitium. The high levels of Igs and high numbers of plasma cells support the hypothesis that antibodies, presumably as antigen-antibody immune complexes, are involved in the pathogenesis of the early reaction in EAA.[20,30,33-35]

The presence of high numbers of eosinophils in BALF, confirmed in the present study, is considered to be a parameter of a hypersensitivity reaction[2,36] and occurs as part of the immune response to antigens.[10,36] Laviolette et al,[26] found increased numbers of mast cells in BALF, parallel to lymphocytosis, in farmers without clinical symptoms. However, their role remains obscure.[26,37]

In summary, our findings demonstrate that interpretation of BALF analysis in EAA greatly depends on the time elapsed between final antigen exposure and the performance of BAL, and therefore affects the results of BALF analyses. Thus, no support was found for the existence of a so called 'standard' BALF profile in EAA.

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Differential diagnostic value of plasma cells in bronchoalveolar lavage fluid

Differential diagnostic value of plasma cells in bronchoalveolar lavage fluid

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Abstract

The aim of this study was to investigate whether the demonstration of plasma cells (PC), which are normally absent in in bronchoalveolar lavage (BAL) fluid, facilitates differentiation among pulmonary disorders. Initial BAL fluid samples of 1,260 patients were analyzed. In 83 of these, PC were found. Of these 83, 47 were obtained from individuals suffering from extrinsic allergic alveolitis (EAA). The number of PC in BAL fluid from EAA patients was found related to the time between antigen exposure and BAL. Drug-induced pneumonitis appeared to be another disorder with a high percentage of cases with PC in the BAL fluid (35.7 percent). Therefore, we conclude that determination of PC in BAL fluid has differential diagnostic value in discriminating among interstitial lung diseases of various origins. However, the exact role of PC in BAL fluid and the link to clinical manifestations of these diseases needs further investigation.

Introduction

Bronchoalveolar lavage (BAL) fluid provides a sample of cells present in the intra-alveolar space. Many disorders have specific features evidenced in BAL fluid.[1-5] By the identification of the cellular constituents, inflammatory processes with predominant lymphocytes (*eg*, extrinsic allergic alveolitis (EAA) and sarcoidosis) can be distinguished from those in which neutrophils or alveolar macrophages predominate (*eg*, idiopathic pulmonary fibrosis).[1,4-8] Alveolar macrophage and neutrophil activation, and subsequent production of cytokines have been implicated in the pathogenesis of tissue destruction and fibrosis. The presence of lymphocytes in BAL fluid equivocally reflects immune stimulation.[5] To date, most interest has been focused on the identification of BAL fluid T lymphocytes and the $CD4^+/CD8^+$ ratio in various pulmonary disorders.[1,5] Interestingly, few data are available on the presence of B lymphocytes and plasma cells (PC). Under normal circumstances, PC are not found in blood or in BAL fluid.[9-12] Following antigenic stimulation, *eg*, vaccination and viral and bacterial infections, an increase in PC in both blood and lymph is observed.[13-17] The occurrence of a low percentage (0.1 to 2.0 percent) of PC in BAL fluid in EAA patients already has been reported by Costabel et al[9] (1985). However, little attention has been paid to this

observation. These authors claim that the presence of PC in BAL fluid is a highly diagnostic criterion for EAA, since no PC were found in BAL fluid from sarcoidosis patients.[9] Other reports describe the inconsistent finding of low numbers of B lymphocytes or PC in the interstitium of the lung in patients with interstitial infiltrates associated with collagen vascular disease or malignant lymphomas.[1,13]

The aim of this study was to investigate whether the presence of PC in BAL fluid has predictive value for the diagnosis of specific pulmonary disorders.

Methods and materials

Patients

Retrospectively, the initial BAL fluid samples of 1,260 patients with a great variety of pulmonary diseases were selected for study out of all BAL fluid analyses (n=2,008) performed over a ten year period between 1980 and 1990.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously reported during fiberoptic bronchoscopy.[3] At the same time, blood samples were drawn for differential cell counts. In short, the procedure is as follows. After premedication with atropine and sometimes diazepam and codeine, and local anaesthesia of the larynx and bronchial tree with 0.5 percent tetracaine, BAL was performed by standardized washing of the right middle lobe with four aliquots of 50-ml of sterile saline solution kept at room temperature. Lavage fluid samples, kept on ice in a siliconized specimen trap, were centrifuged (10 min, 350g). Cells were washed twice, counted and suspended in minimal essential medium (Gibco, Grand Island, NY) supplemented with 1 percent bovine serum albumin (Organon, Teknika, Boxtel, the Netherlands). Preparations of the cell suspension were made in a cytocentrifuge and stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). At least 1,000 cells were counted. The PC were morphologically identified in a routine May-Grünwald-Giemsa-stained slide.

Data analysis

Patients with PC in the BAL fluid were divided into groups according to various categories of pulmonary disorders (table 1). The EAA population was divided into two groups: In the first group (EAA-group A; n=69), the diagnosis EAA was based on clinical information, radiologic picture, the presence of precipitins in the blood, pulmonary function and disappearance of symptoms after avoidance of antigen exposure. The second group (EAA-group B; n=26) consisted of patients who satisfied the aforementioned diagnostic criteria with the exception of the serologic parameter.

For further analysis, nonsmoker EAA patients from the first group (n=61) were selected. All EAA patients were frequently exposed to birds. No patient was receiving corticosteroid or other treatment either at the time of or before the lavage. The patients were divided into four categories, based on the time period between termination of antigen exposure and initial BAL: group 1, less than 24 h; group 2, 2 to 7 days; group 3, 8 to 30 days; group 4, 1 to 12 months. Furthermore, patients with PC-positive BAL fluid were separated from those without PC in BAL fluid. Provocation was realized by providing contact between the patient and the birds, followed by a BAL within 24 h. The first BAL fluid sample was used for statistical analysis. If a patient had a BAL after provocation as well, this BAL fluid sample was included instead of the initial lavage fluid sample.

The control group consisted of 37 nonsmoker control subjects without any pulmonary history or chest x-ray film abnormalities.

Statistical Analysis

The data are presented as the mean ± SEM. The Mann-Whitney *U* test was used to evaluate the statistically significant differences between each category of EAA patients with or without PC in the BAL fluid and the control group. The probability values less than 0.05 were considered to be significant.

Results

In 6.6 percent (n=83) of the cases, PC were detected in the BAL fluid (figure 1; table 1). Patients with PC in BAL fluid were most frequently found among those with verified EAA (EAA-group A: 36 of 69 [52.1 percent]). Also, patients with clinical symptoms of EAA (EAA-group B: 11 of 26 [42.3 percent]) and those with drug-induced pneumonitis (5 of 14 [35.7 percent]) showed PC in BAL fluid (table 1). Among the remaining interstitial lung diseases, PC were found in 7 of 100 patients with pulmonary manifestations of collagen-vascular diseases, 3 of 26 patients suffering from chronic eosinophilic pneumonia, 1 of 47 patients with idiopathic pulmonary fibrosis as well as 1 of 7 patients with bronchiolitis obliterans and organizing pneumonia. In contrast, PC in BAL fluid was demonstrated in only 5 of 401 patients with histologically verified sarcoidosis (1.2 percent) of whom all were known to be frequently exposed to birds. A minor number of patients with PC in BAL fluid was found among those with microbial pulmonary diseases and malignancies (table 1).

Table 1—*Occurrence of Plasma Cells in BAL Fluid in Various Pulmonary Diseases*

Disorders	Σn	n	%
Interstitial lung diseases			
Extrinsic allergic alveolitis (EAA)	69	36	52.1
Suspected of having EAA	26	11	42.3
Pneumonitis induced by drugs (*)	14	5	35.7
Idiopathic pulmonary fibrosis	47	1	2.1
Idiopathic pulmonary fibrosis (not proven by biopsy)	68	0	0
Sarcoidosis	401	5[+]	1.2
Bronchiolitis obliterans and organizing pneumonia	7	1	14.2
Chronic eosinophilic pneumonia	26	3	11.5
Pulmonary manifestations of collagen-vascular diseases	100	7	7.0
Occupational induced disorders	40	0	0
Other interstitial lung diseases	18	0	0
Microbial pulmonary diseases			
Pulmonary aspergillosis/allergic bronchopulmonary aspergillosis	12	2	16.7
Pneumonia/infiltration	100	4	4.0
Tuberculosis	14	0	0
Obstructive diseases			
Bronchitis/COPD	126	0	0
Neoplasms/malignancies			
Malignant lymphomas	21	3	14.3
Haematologic malignancies	23	3	13.0
Neoplasms of the lung	38	2	5.3
Other disorders			
Pulmonary circulatory disorders	18	0	0
Diagnosis unclear	16	0	0
Digestive tract disorders	3	0	0
Control subjects	73	0	0
Total	1260	83	6.6

*Σn = total number of cases; n = number of cases with PC in BAL fluid; % = n/Σn x 100; *Amiodarone, nitrofurantoin or methotrexate; [+]patients exposed to EAA-inducing antigens (birds); COPD = Chronic Obstructive Pulmonary Disease.*

The relative number of PC in BAL fluid varied from 1 to 144 (per 1,000 cells counted), *ie*, 0.1 to 14.4 percent of the total cell count. The highest percentage PC of the total cell count (14.4 percent) was found in a patient with non-Hodgkin's lymphoma. In the BAL fluid of all the other patients (n=82), the PC varied from 1 to 39. The overall mean percentage of PC was 0.74 ± 0.06 in the 83 patients who had PC in BAL fluid (data not shown). No PC were found in peripheral blood.

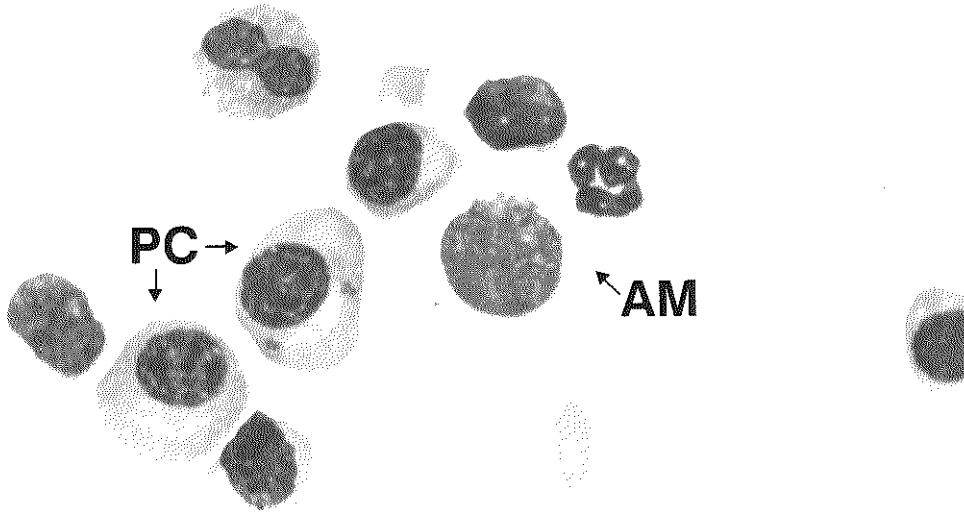


Figure 1. *Photomicrograph of bronchoalveolar lavage fluid showing plasma cells (PC); AM=alveolar macrophage (May-Grünwald-Giemsa stain, original magnification x100).*

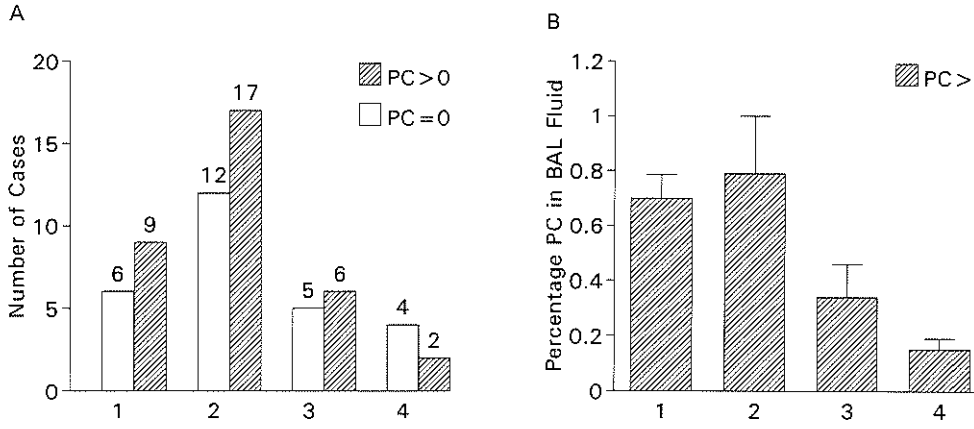
In the nonsmoker serologically verified EAA patients (EAA-group A) with PC in BAL fluid (34 of 61), the overall mean PC count of the total cell count was 0.67 ± 0.13. Total and differential cell counts of 61 EAA patients and 37 control subjects are listed in table 2. Both total cell count and the various cellular components in BAL fluid of the EAA patient population differed significantly from those of the control subjects.

There are statistically significant differences between the EAA patient group in whom PC were and were not found in BAL fluid. In a follow-up study, we will further analyze these differences.

Table 2—*Total Cell and Differential Cell Count in BAL Fluid of Extrinsic Allergic Alveolitis Patients and Control Subjects*

Groups	TCCx10^4/ml	AM	PMN	Lym	PC	Eos	MC
n=61							
EAA	36.3±3.3	37.6±2.1	4.7±0.8	54.3±2.4	0.37±0.01	2.3±0.01	0.76±0.09
n=37							
Cs	10.7±1.2	87.0±1.0	1.3±0.2	10.8±0.9	0.0 ±0.0	0.8±0.40	0.10±0.02

Data are expressed as mean ± SEM. TCC=Total cell count; AM=Alveolar macrophages; PMN=Polymorph neutrophils; Lym=Lymphocytes; PC=Plasma cells; Eos=Eosinophils; MC=Mast cells; All values of EAA-patients were found significantly different from control subjects ($p<0.01$).



Figures 2A and B. *The effect of time elapsed between termination of antigen exposure and initial BAL on the occurrence of plasma cells (PC) in BAL fluid.*
A. - The number of EAA-patients with PC versus number of EAA-patients without PC in BAL fluid [group 1) < 24 hours; 2) 2 to 7 days; 3) 8 to 30 days; 4) 1 to 12 months].
B. - The relative number of PC in BAL fluid from EAA-patients with PC in the BAL fluid only. Data of figure 2B are expressed as percentage PC ± SEM of total cell count in BAL fluid.

Taking into account the critical time relationship between the appearance of PC in the alveolar space and the moment of final antigen exposure, the timing of BAL is essential for detection of these cells. The PC mainly seemed to appear in the alveolar space during the first week following termination of antigen exposure (group 1 and 2) in 26 of 44 EAA patients (59.1 percent) with a relatively high percentage PC of the total cell count (0.76 ± 0.15) demonstrated in the BAL fluid (figure 2). Accordingly, the highest percentage of PC (3.9 percent) was found in one patient belonging to group 2. After avoidance of the antigen exposure for more than a week (groups 3 and 4), a lower number of PC (0.30 ± 0.12) was detected in only 8 out of 17 (47.1 percent) of EAA patients (figure 2). To compare, the mean PC count was 0.12 ± 0.02 in the PC-positive sarcoidosis patients ($p < 0.05$, data not shown).

Discussion

Plasma cells are seldom found in BAL fluid. Morphologically, these cells can be identified in a routine May-Grünwald-Giemsa-stained slide. The incidence of PC in BAL fluid has been reported in a number of pulmonary disorders, especially in EAA.[9] In the present study, PC were found in the BAL fluid obtained from 36 of 69 patients with serologically proven EAA and in 11 of 26 patients with clinical manifestations of the disease. Since nearly no BAL fluid PC were found in other interstitial lung diseases, such as sarcoidosis and idiopathic pulmonary fibrosis, both of which may be difficult to differentiate from EAA due to similarities in clinical presentations, our results suggest that the presence of PC are highly specific for EAA or EAA-like pulmonary disorders. These findings are in agreement with Costabel et al[9] (1985). There were only 5 of 401 patients with sarcoidosis and PC in the BAL fluid and they frequently were exposed to birds, which was believed to be a potential EAA-inducing antigen. Although these findings seem controversial, these individuals may be susceptible to EAA-inducing antigens and, as a consequence, have PC in the BAL fluid.

In the pathogenesis of EAA, several immune mechanisms are involved.[7,18-28] Alveolitis is characterized by an accumulation of inflammatory cells at the distal structures of the lung.[7,8,26] Our results confirmed this, showing an increase in lymphocytes, polymorphonuclear

neutrophils, eosinophils and mast cells with a simultaneous decrease in alveolar macrophages in the BAL fluid samples from EAA patients. The presence of PC supports the concept of local production of antibodies. Antigen-antibody complexes may lead to lung damage due to the interaction with other constituents of the interstitium of the lung.[10,29] The relative number of PC in BAL fluid samples was related to the time latency from termination of antigen exposure and performance of BAL. The PC mainly seem to appear in the alveolar space during the first week following termination of antigen exposure. When BAL is performed within the first week after antigen contact, a relatively high percentage of PC of the total cell count can be demonstrated in BAL fluid. After avoidance of exposure to the antigen for more than a week, the number of PC was found to gradually decrease in EAA patients. Therefore, the presence of PC is considered to be a feature of recent antigen exposure. These findings are in line with the etiology of EAA as an inflammatory reaction induced by repeated exposure to extrinsic antigens in susceptible individuals and the reported involvement of immunoglobulins, locally secreted by PC.[5,8,18-22] In view of this, PC may be expected to occur in BAL fluid samples from patients with drug-induced pneumonitis, a disease of similar pathogenesis as EAA, which was indeed confirmed by the present data.[27-35] The increased number of eosinophils found in the BAL fluid of these patients similarly points to hypersensitivity states.[27,35,36] Recently, eosinophilia has been described in a patient with aspergillosis.[35] In our patient population, we also found two aspergillosis patients with PC in the BAL fluid. A patient with eosinophilic pneumonia with PC in BAL fluid was described.[36] In three cases with chronic eosinophilic pneumonia, PC were detected in BAL fluid. These findings suggest that in inflammatory pulmonary diseases with an antibody-mediated component involved, the presence of PC may coincide with high numbers of eosinophils in BAL fluid. The absence of PC in the BAL fluid of some EAA patients remains unclear. Apparently, other mechanisms as well are involved in the pathogenesis of allergic alveolitis.[4,5,7] In accordance with previous reports, PC were found in BAL fluid obtained from patients with malignant lymphomas, haematologic malignancies, and pulmonary manifestations of collagen-vascular diseases.[1,37]

In conclusion, counting PC in BAL fluid has differential diagnostic value, especially in disorders with an increased absolute and relative number of lymphocytes based on allergic, inflammatory processes with an antibody-mediated component involved. This patient group is characterized by an increased number of eosinophils as well. In discriminating among interstitial lung diseases of various origins, the demonstration of PC in BAL fluid is highly suggestive of the diagnosis EAA. Furthermore, a relatively high number of PC in BAL fluid of EAA patients suggests recent antigen exposure. The exact role of BAL fluid PC in EAA patients and the link to clinical manifestation of the disease needs further investigation.

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Relationship between plasma cell levels and profile of bronchoalveolar lavage fluid in patients with subacute extrinsic allergic alveolitis

Relationship between plasma cell levels and profile of bronchoalveolar lavage fluid in patients with subacute extrinsic allergic alveolitis

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Abstract

Background—Plasma cells are usually absent in bronchoalveolar lavage fluid (BAL) fluid. Extrinsic allergic alveolitis (EAA) is associated with increased numbers of T and B lymphocytes in BAL fluid, as well as the presence of a few plasma cells. The aim of this study was to investigate whether there is a relationship between the presence of plasma cells and other cells, and immunoglobulin levels in BAL fluid of patients with EAA.

Methods—Thirty non-smoking patients with EAA, who had a BAL 2-7 days after their last exposure to the causative antigen were selected, retrospectively.

Results—Patients suffering from EAA with plasma cells in the BAL fluid (n = 18) had increased absolute numbers of lymphocytes, eosinophils and mast cells, a decreased percentage of alveolar macrophages and lower $CD4^+/CD8^+$ ratio, as well as higher immunoglobulin levels, when compared with patients with EAA having no plasma cells in the BAL fluid (n = 12).

Conclusions—The results suggest a relationship between the presence of plasma cells and the other constituents in BAL fluid and a more intense alveolitis. In addition there was a positive relationship between the number of plasma cells in BAL fluid and immunoglobulin levels. These data support the concept of local production of immunoglobulins by plasma cells in the lung following antigen exposure in susceptible individuals.

Introduction

The lung is capable of local immunological reactions by cell as well as antibody mediated mechanisms.[1] In interstitial lung diseases bronchoalveolar lavage (BAL) has become an important method of gaining information about disease processes in the lung.[2-4]

Extrinsic allergic alveolitis (EAA) is initiated by repeated exposure to specific antigens in susceptible individuals.[5-8] The immunological mechanisms underlying the pathological changes in the lung appear to be related to the dose of the causative antigens and the duration of exposure.[9-11]

In EAA, initial non-specific inflammation is followed by sensitisation causing a granulomatous inflammatory response modulated by T cell and macrophage derived cytokines.[5,12] This initial phase—that is, immediately

after antigen inhalation—is characterised by an increase in the number of neutrophils in BAL fluid.[5,13] In the subacute phase, 2-7 days after antigen exposure, the numbers of $CD8^+$ lymphocytes and natural killer cells are increased. A few plasma cells are occasionally found in BAL fluid,[5,14,15] but are not generally found in peripheral blood or BAL fluid. If present in BAL fluid, B lymphocytes vary in maturation from small lymphocytes to mature plasma cells, supporting the concept that antigen specific B lymphocytes enter the immunised lung (possibly as lymphoblasts) and mature to plasma cells.[16-18] Moreover, plasma cells in BAL fluid render allergic or inflammatory processes with an antibody mediated component highly likely.[14,15,19,20] In addition, immunoglobulin levels in BAL fluid are elevated because of antigenic stimulation in patients with EAA compared with control subjects.[5,20] These high immunoglobulin levels may be caused by an increase in pulmonary vascular permeability, or local production of immunoglobulins, or both.[20] Plasma cells synthesise and secrete immunoglobulins, but no relationship has been shown between plasma cells and immunoglobulin levels in BAL fluid.

In this study we have investigated differences between the profile of the BAL fluid in patients with EAA, with or without plasma cells and the relationship of plasma cells with immunoglobulin levels in the lavage fluid.

Methods

Patients and controls

Samples of BAL fluid obtained from patients suffering from EAA (n=67; 59 non-smokers and eight smokers) during a 10 year period between 1980 and 1990 were studied. The patients with EAA presented with generalised constitutional and pulmonary symptoms—that is, cough, dyspnoea and sometimes fever and chills. Although the symptoms were mostly transient, exacerbations occurred with repeated exposure to the causative antigen. The diagnosis of EAA was made from clinical information, chest radiology, the presence of serum antibodies (precipitins, table 1) against the suspected antigens in peripheral blood, pulmonary function tests and disappearance of symptoms after avoidance of antigen exposure. An open lung biopsy was performed in five patients without sufficient clinical criteria to make a definitive diagnosis. To exclude any influence of smoking on the profile of the BAL fluid only non-smoking patients (n=59) were studied, all of whom had been frequently exposed to birds including pigeons, parrots, budgerigars or canaries (table 1). No patient was on corticosteroid treatment before or at the time of the lavage. The patients were divided into four categories based on the time period between the presumed termination of

antigen exposure and the lavage: group 1) < 24 hours; group 2) 2-7 days; group 3) 8-30 days; group 4) 1-12 months. The patients with EAA last exposed 2-7 days before BAL (group 2; n=30), having the highest number of plasma cells in their lavage fluid were further divided into two subgroups according to the presence or absence of plasma cells in the lavage fluid (table 1) and form the basis of this study.

A control group of 28 non-smoking healthy volunteers with no contact with EAA inducing antigens was also studied (table 1). This study was approved by the Ethical Committee of our hospital.

Table 1-*Characteristics of the Groups Studied: the Non-smoking Patient Population with Extrinsic Allergic Alveolitis (EAA) and Non-smoking Control Subjects*

	Time since last exposure of EAA patients to birds				
	<24 hours	2-7 days	8-30 days	1-12 months	Controls
number of cases (n)	12	30	11	6	28
avian precipitins	$6^{A};3^{C};3^{D}$	$18^{A};6^{B};2^{C};4^{D}$	$8^{A};3^{C}$	$4^{A};1^{B};1^{C}$	not done
PC, % of TCC*	0.34 ± 0.14	0.58 ± 0.15	0.21 ± 0.09	0.05 ± 0.03	0.0 ± 0.0
PC=0; n	5	12	5	4	28
age yr (range)	52 (23-75)	54 (27-75)	42 (19-65)	56 (28-73)	39 (19-60)
sex	2F; 3M	5F; 7M	1F; 4M	2F; 2M	12F; 16M
PC>0; n	7	18	6	2	0
age yr (range)	54 (33-65)	50 (27-70)	47 (22-71)	52 (25-70)	-
sex	2F; 5M	6F; 12M	2F; 4M	0F; 2M	-

*F=Female; M=Male; A=pigeon; B=budgerigar; C=canary and D=parrot; PC=Plasma cells; TCC=Total cell count (x10^{4}/ml). *Data indicate mean ± SEM.*

Bronchoalveolar lavage

BAL was performed during fibreoptic bronchoscopy.[21] Following premedication with atropine and local anaesthesia of the larynx and bronchial tree with tetracaine 0.5 %, the right middle lobe was lavaged with four aliquots each of 50 ml sterile saline (0.9% NaCl) at room temperature. Simultaneous peripheral blood samples were taken.

The recovered BAL fluid was kept on ice in a siliconised specimen trap and separated from its cellular compounds by centrifugation (5 minutes at 350g). Supernatants were directly stored at -70 °C after additional centrifugation (10 minutes at 1,000g). Cells were washed twice, counted, and suspended in minimal essential medium (MEM; Gibco, Grand Island, New York, USA) supplemented with 1% bovine serum albumin (BSA; Organon, Teknika, Boxtel, the Netherlands).

Preparations of cell suspensions were made in a cytocentrifuge (Shandon). Cytospin slides were stained with May-Grünwald-Giemsa (MGG; Merck, Darmstadt, Germany) for

cell differentiation and at least 1,000 cells were counted. Morphologically, plasma cells were identified in a routine MGG stained slide by light microscopy. Only mature plasma cells recognised by an eccentric nucleus with a large amount of basophilic cytoplasm were included.

If more than 15 % of lymphocytes were present, T cell (sub)populations were determined. Total T cells and subpopulations were recognised by staining with monoclonal antibodies CD2(OKT11), CD3(OKT3), CD4(OKT4) and CD8 (OKT8) from Ortho Pharmaceuticals (Beerse, Belgium). Identification of T cells reacting with monoclonal antibodies was performed by means of a conventional indirect immunofluorescence technique using fluorescein isothiacyanate (FITC)-labelled goat antimouse (GAM) immunoglobulin, (Nordic, Immunological Laboratories, Tilburg, the Netherlands and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands). Albumin determinations were performed according to the modified adaptation of the bromocresol purple dye binding method.[22,23] Albumin concentrations in serum and lavage fluid were expressed in g/l and mg/l, respectively.

Immunoglobulin concentrations, (IgM, IgG and IgA) in BAL fluid were measured by an enzyme linked immunosorbent assay (ELISA) method; microtitre plates were coated with a rabbit antihuman isotype antiserum [anti-IgM, (CLB, Amsterdam, the Netherlands), anti-IgG and anti-IgA (Dako, Glostrup, Denmark)]. Bound immunoglobulins from BAL fluid were visualised with a horseradish peroxidase labelled rabbit antihuman immunoglobulin antiserum [with anti-IgA, -IgG, -IgM, -kappa, -lambda reactivity (Dako, Glostrup, Denmark)] and a chromogenic substrate orthophenyl diamine (OPD) (Baker, Chemicals BV, Deventer, the Netherlands).

Immunoglobulin concentrations in BAL fluid were expressed in mg/l using as a reference a commercial human standard serum, HOO-03 (CLB, Amsterdam, the Netherlands).

Statistical analysis

To investigate whether there were statistically significant differences between the profile of BAL fluid of patients suffering from EAA, with or without plasma cells, the Mann-Whitney *U* test was used.

Spearman rank correlation coefficients were estimated in order to test against a monotonic relationship between the absolute and relative number of plasma cells in BAL fluid on one hand, and the levels of albumin, IgM, IgG, IgA, and the ratios IgM to albumin, IgG to albumin, and IgA to albumin in BAL fluid on the other. A p value of <0.05 was considered to be significant.

Results

Patients with EAA who underwent a BAL within 2-7 days of antigen exposure (group 2) showed the highest percentage of plasma cells in the BAL fluid (table 1). Moreover, this largest subgroup ($n=30$), contained

most patients with plasma cells in the lavage fluid (n = 18). Furthermore, in this subgroup of patients the lowest value of plasma cells in the lavage fluid was 0.1 % and the highest was 3.9 % in one patient who had a lavage five days after antigen exposure. In none of the groups studied were plasma cells found in peripheral blood. In the patients in group 2 with plasma cells the total cell count ($p < 0.01$), the absolute and relative number of lymphocytes were increased ($p < 0.005$), and the percentage of alveolar macrophages was decreased ($p < 0.005$) compared with patients without plasma cells (table 2A and 2B). The absolute number of eosinophils and mast cells were higher in the patients with plasma cells ($p < 0.05$) than in those without (table 2B). Both groups had an increased total cell count ($p < 0.001$), absolute and relative number of lymphocytes ($p < 0.001$), neutrophils ($p < 0.005$), eosinophils ($p < 0.01$) and mast cells ($p < 0.005$), and a decreased relative number of alveolar macrophages ($p < 0.001$) compared with the control subjects.

The patients with plasma cells had a lower percentage of $CD4^+$ T cells ($p < 0.05$) and a significantly, lower $CD4^+/CD8^+$ ratio (median 0.7; range 0.4-3.0) ($p < 0.05$) than the patients without plasma cells (median 1.9; range 0.5-3.9) (table 3). The measured percentages of T cells and T cell subpopulations of both patient groups (with and without plasma cells) showed significant differences when compared with the control group in whom T cell (sub)population determinations were performed (n = 6; $p < 0.01$), except for the percentage $CD4^+$ T cells and $CD4^+/CD8^+$ ratio. The percentage $CD4^+$ T cells was higher in the patients without plasma cells than in the control subjects ($p < 0.05$) and patients with plasma cells ($p < 0.05$). The $CD4^+/CD8^+$ ratio was lower in the patient group with plasma cells than in the control group ($p < 0.01$). In BAL fluid of patients with plasma cells the ratio of IgG to albumin was higher (2.33 versus 0.95, ($p < 0.05$); the ratios of IgM and IgA to albumin in the lavage fluid tended to be higher than in the patients without plasma cells.

Table 2A-*Yield, Total Cell Count (TCC) and Differential Cell Count (Percentage TCC) in BAL Fluid of Patients Suffering from Extrinsic Allergic Alveolitis (EAA) last Exposed 2-7 Days before the Lavage (group 2; n=30) with or without Plasma Cells (PC) in BAL Fluid and Control Subjects (mean ± SEM)*

Groups	n	Yield %	TCC x10^4/ml	Percentage of TCC					
				AM	PMN	Lym	PC	Eos	MC
Controls	28	58.4±2.8	10.3±1.5	89.8±0.7	1.3±0.2	8.4±0.7	0.0 ±0.0	0.44±0.1	0.09±0.03
EAA(PC=0)	12	47.0±2.9	27.6±5.8	41.4±4.9	4.4±0.9	50.2±5.1	0.0 ±0	3.09±0.8	0.88±0.23
EAA(PC>0)	18	48.2±2.3	52.7±5.6	25.4±1.6	4.0±0.9	66.1±2.0	0.83±0.20	2.66±0.8	0.97±0.17
p-value*		NS	<0.01	<0.005	NS	<0.005	<0.001	NS	NS

n=number of cases; AM=Alveolar macrophages; PMN=Polymorphonuclear neutrophils; Lym=Lymphocytes; Eos=Eosinophils; MC=Mast cells; PC=Plasma cells; NS=not significant; p-value: Mann-Whitney test EAA patient group with PC vs without PC in the BAL fluid.*

Table 2B-*Absolute Numbers of Cells in BAL Fluid of Patients Suffering from Extrinsic Allergic Alveolitis (EAA) last Exposed 2-7 Days before the Lavage (group 2) with or without Plasma Cells (PC) in BAL Fluid and Control Subjects**

Groups	n	AM	PMN	Lym	PC	Eos	MC
Controls	28	9.3±1.4	0.13±0.03	0.8±0.12	0.0 ±0.0	0.07±0.01	0.01±0.005
EAA(PC=0)	12	9.6±1.7	1.01±0.35	15.9±4.4	0.0 ±0.0	0.77±0.20	0.29±0.12
EAA(PC>0)	18	13.1±1.8	2.14±0.57	35.1±3.8	0.45±0.11	1.38±0.45	0.56±0.14
p-value**		NS	NS	<0.005	<0.001	0.05	<0.05

Data are expressed as mean absolute numbers of cells$x10^4$/ml ± SEM. n=number of cases; AM=Alveolar macrophages; PMN=Polymorphonuclear neutrophils; Lym=Lymphocytes; Eos=Eosinophils; MC=Mast cells; PC=Plasma cells; NS=not significant; p-value: Mann-Whitney test EAA patient groups with PC vs without PC in the BAL fluid.*

Table 3-*Percentages of T Lymphocytes and T Cell Subpopulations in BAL Fluid of Patients Suffering from Extrinsic Allergic Alveolitis (EAA) last Exposed 2-7 Days before the Lavage (group 2) with or without Plasma Cells (PC) in BAL Fluid and Control Subjects (mean ± SEM)**

Groups	n	$CD2^+$	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$ *Ratio*
Controls	6	67.0±9.0	73.0±2.4	52.4±3.4	19.0±1.4	2.60±0.17
EAA (PC=0)	9	86.6±2.8	87.9±2.8	57.2±5.9	34.3±4.1	2.04±0.41
EAA(PC>0)	18	87.7±1.8	84.9±2.0	38.6±4.3	43.4±3.8	1.03±0.19
p-value**		NS	NS	<0.05	NS	<0.05

Data are expressed as mean ± SEM. n=number of cases; p-value: Mann-Whitney test EAA patient groups with vs without PC in BAL fluid; NS=not significant.*

The immunoglobulin levels (figure 1) and their ratios to albumin in BAL fluid were higher in both patient groups than in the control group ($p < 0.01$) (table 4). The Spearman rank correlation coefficients showed a significant monotonic relationship between the absolute and relative number of plasma cells in BAL fluid and the levels of immunoglobulins and their ratios to albumin (table 5).

Table 4-*Protein Levels in BAL Fluid of Patients Suffering from Extrinsic Allergic Alveolitis (EAA) last Exposed 2-7 Days before the Lavage (group 2) with or without Plasma Cells (PC) in BAL Fluid and Control Subjects (mean ± SEM)*

Groups	l-alb mg/l	IgM mg/l	IgM/l-alb	IgG mg/l	IgG/l-alb	IgA mg/l	IgA/l-alb
Controls n=21	71.0± 8.5	0.4±0.1	0.01±0.002	11.1± 2.0	0.16±0.02	3.6± 0.7	0.05±0.01
EAA(PC=0) n=12	137.3±24.9	10.0±3.6	0.07±0.02	115 ±30.3	0.95±0.29	28.3± 6.7	0.24±0.05
EAA(PC>0) n=18	181.6±25.3	22.8±5.6	0.14±0.03	303 ±55.2	2.33±0.53	75.2±17.9	0.72±0.27
p-value*	NS	0.05	NS	<0.01	<0.05	<0.05	NS

l-alb=lavage albumin; p-value: Mann-Whitney test EAA patient groups with vs without PC in the BAL fluid; n=number of cases; NS=not significant.*

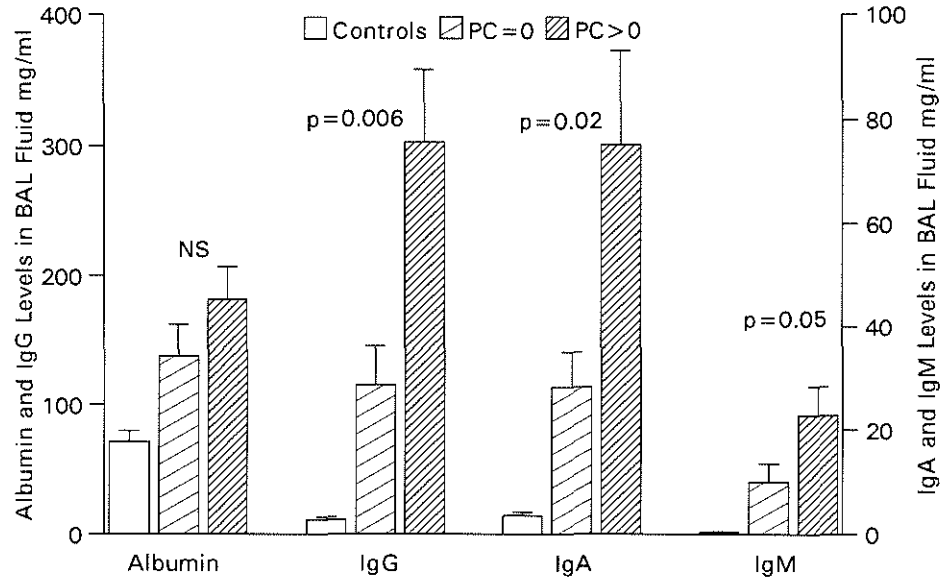

Figure 1. *Protein levels in brochoalveolar lavage (BAL) fluid of patients suffering from extrinsic allergic alveolitis with (PC>0) or without plasma cells (PC=0) in BAL fluid and controls.*

Table 5-*Spearman Rank Correlation Coefficients Testing a Monotonic Relationship between the Absolute and Relative Number of Plasma Cells (PC) in BAL Fluid and the Levels of Immunoglobulins (Igs) and their Ratios to Albumin*

	l-alb	IgM	IgM/l-alb	IgG	IgG/l-alb	IgA	IgA/l-alb
Correlation between percentage PC in BAL fluid and Igs							
cc	0.1878	0.4206	0.3920	0.5796	0.4092	0.5719	0.3329
p-value	NS	<0.01	<0.05	0.001	<0.05	<0.001	<0.05
Correlation between absolute number PC in BAL fluid and Igs							
cc	0.2356	0.4566	0.3920	0.5687	0.3671	0.6031	0.3235
p-value	NS	<0.01	<0.05	0.001	<0.05	<0.001	<0.05

l-alb = lavage albumin; cc = Spearman rank correlation coefficient; NS = not significant.

The light microscopic evaluation of the five patients who underwent open lung biopsy showed many plasma cells within the alveolar interstitium all specimens, irrespective of the presence or absence of plasma cells in the lavage fluid.

Discussion

Extrinsic allergic alveolitis (EAA) is thought to result from a combination of a type III immune complex and type IV cell mediated immunological reaction, although the mechanism remains unknown.[5] Plasma cell derived antibodies have been found in the serum and BAL fluid of patients with EAA.[5,21] Under normal circumstances plasma cells, known as tissue cells, are not found in BAL fluid nor in peripheral blood. However, in the alveolar interstitium of the immunised lung plasma cells frequently occur.[18] Recently we have shown the presence of plasma cells in the lavage fluid of patients with EAA and other antibody mediated inflammatory processes of the lung such as drug induced pneumonitis.[15] This uncommon motile behaviour of plasma cells can probably be explained by damage of the alveolar membranes and non-specific changes in vascular permeability produced by antigen exposure.[15,18] In addition, the presence of plasma cells in lavage fluid was considered to be a feature of recent antigen exposure, suggesting an active alveolitis.[15]

In the present study the cellular profile of the BAL fluid differed signifi-

cantly between patients with EAA last exposed 2-7 days before the lavage (group 2; n=30) who had plasma cells in the lavage compared with those who did not.

The patients with plasma cells had an even more active alveolitis with an increased total cell count and absolute and relative numbers of lymphocytes. In addition, the pattern of T cell subpopulations differed between both groups. The $CD4^+/CD8^+$ ratio in BAL fluid was decreased in the group with plasma cells suggesting a more active alveolitis in these patients. This finding agrees with Trentin et al[6] who also found a more active alveolitis in patients suffering from EAA with a decreased $CD4^+/CD8^+$ ratio, although in their study no differentiation was made between patients with or without plasma cells in the lavage fluid. Trentin et al[6] also showed a shift from the $CD8^+$ predominate cellular profile of BAL fluid of patients with EAA towards the normal $CD4^+$ predominant profile after removal from exposure to the causative antigen, suggesting a change in the alveolitis. In our study we found that the $CD4^+$ T cells predominated over $CD8^+$ T cells in the EAA patient group without plasma cells, whereas the percentage of $CD4^+$ and $CD8^+$ T cells were equal in the patients with plasma cells in the lavage fluid.

A decrease in $CD8^+$ T cell suppressor activity in EAA may cause augmented $CD4^+$ T cell reactions.[24] Activated $CD4^+$ T cells have been implicated in the cellular and humoral immune responses to antigenic stimulation by the production of cytokines.[25,26] These cytokines recruit alveolar macrophages (gamma interferon), neutrophils (interleukin (IL)-8), eosinophils (IL-5), mast cells (IL-3) and natural killer cells (IL-2) into immune responses.[27,28] Activated $CD4^+$ T cells, especially IL-5 producing $CD4^+$ T subsets, may be involved in the mediation of eosinophilic recruitment into the airways, as suggested by the preventive effect of depletion of $CD4^+$ T cells, but not of $CD8^+$ T cells, on antigen induced eosinophilic infiltration in mice.[25] We found an increase in the absolute number of eosinophils in group with plasma cells compared with patients without plasma cells, although the absolute numbers of $CD4^+$ T cells were equal in both groups (data not presented). This difference in eosinophilia might be caused by different subpopulations $CD4^+$ T cells causing different IL-5 production. Like Laviolette et al[29] we also found an increased absolute

number of mast cells in both groups, particularly in the patients with EAA with plasma cells. This might be caused by activation of the same $CD4^+$ T cell subpopulation, as this subpopulation of T cells produces both IL-3 (mast cell recruitment), IL-5 and also IL-4 (B cell stimulation).[30] Additional studies to detect cytokines in both groups of patients with EAA with or without plasma cells, are needed to determine the mechanisms leading to local augmentation of antigen sensitive cells in the lung and more or less severe EAA.

This study is the first report to show increased immunoglobulin levels in BAL fluid in patients suffering from EAA with plasma cells in BAL fluid. Synthesis of immunoglobulin has been reported in the lower respiratory tract.[29,31-33] These findings and those reported in our study, show that immunoglobulin levels and their ratios to albumin in BAL fluid are positively related to the presence of immunoglobulin producing plasma cells in lavage fluid. In this study patients without plasma cells also showed increased levels of immunoglobulins, the levels among patients with plasma cells being higher than in patients without plasma cells. These higher immunoglobulin levels in BAL fluid of patients with plasma cells might be the result of the local production of immunoglobulins by plasma cells in BAL fluid, in addition to enhanced diffusion from the interstitium due to destruction of the basement membrane.

In conclusion, patients suffering from EAA with plasma cells in BAL fluid show signs of a more active alveolitis. They have increased absolute and relative numbers of lymphocytes, higher absolute numbers of eosinophils and mast cells, higher immunoglobulin levels and IgG to albumin ratios, as well as a decreased $CD4^+/CD8^+$ ratio in comparison with patients without plasma cells in BAL fluid. In addition, there is a positive relation between the number of plasma cells in BAL fluid and immunoglobulin levels. These data promote the concept of local production of immunoglobulins by plasma cells within the lung following antigen exposure in susceptible individuals. Studies on the relation of time elapsed between antigen exposure and BAL procedure, and the production by plasma cells of IgM, IgG and IgA, are currently being conducted.

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Summary and conclusions

So einen Arbeit wird eigentlich nie fertig.
Man muss sie für fertig erklären,
wenn man nach Zeit und Umständen
das Mögliche getan hat.

J.W. von Goethe

Summary

Bronchoalveolar lavage (BAL) is currently widely applied to sample cells and proteins present in the bronchoalveolar space for subsequent studies. Moreover, this limited invasive technique is a sensitive indicator of infectious and non-infectious inflammatory disorders, such as interstitial lung diseases.

The aim of this study was to investigate the clinical applications of BAL, in particular the diagnostic value of this method with a view to preventing more invasive procedures. The studies presented in this thesis are based on BAL fluid data obtained from patients during a ten-year period between 1980 and 1990. Cells and proteins, *ie*, albumin and immunoglobulins, determined in those BAL fluid samples, have been analyzed. Retrospectively, we searched for specific features in the data from the BAL fluid analyses, which distinguish between various interstitial lung diseases. This thesis describes the diagnostic value of BAL fluid sample analyses in patients suffering from various interstitial lung diseases. Emphasis is put on patients with sarcoidosis, extrinsic allergic alveolitis (EAA), or idiopathic pulmonary fibrosis.

In *chapter 1*, the general introduction, the BAL procedure and some cellular and noncellular constituents present in the bronchoalveolar space are described. In addition, the most important confounding factors influencing the composition of the BAL fluid profile and side-effects of the BAL procedure are discussed. Finally, a brief summary of the clinical picture and pathogenesis of three types of interstitial lung diseases, namely sarcoidosis, EAA alveolitis and idiopathic pulmonary fibrosis is given.

Chapter 2, in which the aims of the study are described, provides an introduction to the experimental work described in the chapters 3 to 8.

A biopsy of patients with interstitial lung diseases was not always available. Therefore, other diagnostic methods were required. The aim of the study presented in *chapter 3* was to investigate, by means of discriminant analysis using a number of selected variables derived from BAL fluid sample analysis, whether BAL allows to distinguish between patients with three frequently occurring interstitial lung diseases, who often show a similar clinical presentation. The study involved all patients who had an

initial BAL in the period between 1980 and 1990. These patients belonged to the following diagnostic groups: sarcoidosis, subacute EAA and idiopathic pulmonary fibrosis. Cellular and non-cellular constituents of BAL fluid were evaluated. Variables, which could be used to discriminate among the three diagnostic groups were: yield of recovered BAL fluid, total cell count, percentages of alveolar macrophages, lymphocytes, neutrophils, eosinophils, and plasma cells in BAL fluid. When the set of data used to predict the membership of patients to diagnostic groups (test set) was the same as for the discriminant analysis (learning set), 93 percent of the cases was classified correctly. This percentage decreased to 90 percent, however, when the test set was different from the learning set.

These data show that it is possible to discriminate among patients with sarcoidosis, EAA or idiopathic pulmonary fibrosis on the basis of the selected variables. In view of this, it appears that BAL is useful as an adjunct in concert with other diagnostic methods.

Patients with Löfgren's syndrome, who show the most distinguished BAL fluid characteristics of sarcoidosis and parenchymal involvement from regional lymph nodes, may mimic pulmonary or lymph node tuberculosis, as well as pulmonary manifestations of malignant lymphomas. Therefore, the respective BAL fluid profiles from patients with sarcoidosis, tuberculosis, and malignant lymphomas, *ie*, non-Hodgkin's and Hodgkin's disease were compared in *chapter 4*. The presence of $CD4^+$ and $CD8^+$ T lymphocytes, as well as the $CD4^+/CD8^+$ ratio in BAL fluid aided in the differentiation between the various groups. Patients with malignant lymphomas had the lowest $CD4^+/CD8^+$ ratio in BAL fluid, as well as in peripheral blood, and occasionally, plasma cells were present in BAL fluid samples. The most important feature of BAL fluid analysis in tuberculosis was detection of the causal microbial agent. It was concluded that, although malignant lymphomas and tuberculosis require histologic verification and a positive culture, for diagnosis, respectively. BAL fluid analysis may be of additional value in distinguishing those disorders from sarcoidosis.

Clinical manifestations of sarcoidosis depend on the intensity of the inflammation and the organ systems affected. Patients with sarcoidosis

represent a heterogeneous population. In some sarcoidosis patients, however, the alveolitis remains subclinical, whereas in others both an alveolitis and granuloma formation are present, resulting in specific pulmonary symptoms. Although the lung is the organ most frequently affected, extra-pulmonary manifestations such as erythema nodosum commonly occur. Factors such as the duration of the disease and mode of presentation are reflected in the composition of BAL fluid. In *chapter 5* the relationship between the T lymphocyte profile in BAL fluid samples and disease presentation of sarcoidosis was studied. To this end, one hundred patients with histologically verified sarcoidosis were evaluated. They were divided into three groups, based on their clinical presentation and smoking status. Group A consisted of patients whose disease was detected by routine chest x-ray film, without symptoms; group B included those with respiratory and general constitutional symptoms; and group C included patients with erythema nodosum and/or arthralgia and hilar lymphadenopathy. BAL fluid samples obtained from sarcoidosis patients with hilar lymphadenopathy showed the most characteristic features of alveolitis, suggesting a disseminated instead of a local immune response. The last group (group C) was found to have the highest $CD4^+/CD8^+$ ratios, whereas in asymptomatic patients (group A) BAL fluid $CD4^+/CD8^+$ ratios were lowest. Patients with respiratory symptoms (group B) counted for intermediate $CD4^+/CD8^+$ values. In addition, cigarette smoking modifies the immunologic BAL fluid sample profile, since alveolitis was less pronounced in smokers. Therefore, the clinical presentation of sarcoidosis and the smoking status of a sarcoidosis patient are crucial for interpreting individual lavage analysis results.

Besides smoking, past and present medical treatment and clinical stage or presentation of a disease are important confounding factors which influence the BAL fluid profiles (see also chapter 5). In *chapter 6* lavage fluid cell profiles and immunoglobulin levels from patients with EAA in relation to the time elapsed between last allergen exposure and BAL, were investigated to this purpose. An analysis was performed of BAL fluid obtained from nonsmoking patients with EAA at various time-points after termination of allergen exposure. BAL fluid early after allergen provocation (group 1: less than 24 hours) contained high absolute and relative num-

bers of lymphocytes, neutrophils, eosinophils and mast cells, and a low relative number of alveolar macrophages. When obtained after recent allergen exposure (group 2: 2 to 7 days), BAL fluid showed high numbers of lymphocytes, plasma cells and mast cells, and high levels of IgM, IgG and IgA. In BAL fluid samples obtained one week or more after the final allergen exposure, the distribution of all constituents showed a tendency to return to normal values, with the exception of the lymphocytes. In summary, these results demonstrate that BAL fluid cell profile and immunoglobulin levels of patients with EAA greatly depend on the time-point at which the material is obtained in relation to the last exposure to the causative allergen. As a consequence of this time-related effect on the composition of BAL fluid in EAA, analysis of a randomly obtained diagnostic BAL fluid sample may enable to determine whether there has been recent allergen exposure.

BAL fluid provides a sample of cells present in the intra-alveolar space. Many disorders have specific features evidenced in BAL fluid. To date, most interest has been focused on the identification of BAL fluid T lymphocytes and the $CD4^+/CD8^+$ ratios in various pulmonary disorders, instead of plasma cells in BAL fluid. In *chapter 7,* initial BAL fluid samples of 1,260 patients were analyzed. In 83 of these (6.6 percent), plasma cells were found. Of these 83, 47 were obtained from individuals suffering from EAA. The number of plasma cells in BAL fluid from these patients was found to be related to the time between the last allergen exposure and the lavage. Drug-induced pneumonitis appeared to be another disorder with a high percentage of cases with plasma cells in the BAL fluid (35.7 percent). Therefore, determination of plasma cells in BAL fluid has differential diagnostic value in discriminating among interstitial lung diseases of various origins. However, the exact role of plasma cells in BAL fluid and the link to clinical manifestations of these diseases need further investigation.

Extrinsic allergic alveolitis was reported to be associated with increased numbers of T- and B lymphocytes in BAL fluid samples, as well as the presence of a few plasma cells. In *chapter 8* we investigated whether there was a relation between the presence of plasma cells and other cells, as well as immunoglobulin levels in BAL fluid of patients with EAA. Thirty

non-smoking patients with EAA who had a BAL between 2 to 7 days after their last exposure to the causative allergen were selected retrospectively. Patients suffering from EAA with plasma cells in the BAL fluid had increased absolute numbers of lymphocytes, eosinophils and mast cells, a decreased percentage of alveolar macrophages and lower $CD4^+/CD8^+$ ratios, as well as higher immunoglobulin levels, when compared with patients having no plasma cells in the BAL fluid. The results suggest a relationship between the presence of plasma cells and other constituents in BAL fluid and a more intense alveolitis. In addition, there was a positive relationship between the number of plasma cells in BAL fluid and immunoglobulin levels. These data support the concept of local production of immunoglobulins by plasma cells in the lung following allergen exposure in susceptible individuals.

Conclusions

Although the studies described in this thesis were performed retrospectively, these studies show that BAL fluid analysis is a sensitive and useful technique in distinguishing between various interstitial lung diseases, in particular sarcoidosis, EAA and idiopathic pulmonary fibrosis. The present data suggest that the performance of BAL may prevent more invasive diagnostic procedures, such as tissue biopsies. In addition, cigarette smoking appears to be an important confounding factor which has to be taken into account when interpreting BAL fluid analysis results. Moreover, clinical presentation and time course of the disease were found to influence the BAL fluid composition.

Although malignant lymphomas and tuberculosis necessitate histological verification and a culture, respectively, BAL fluid analysis may be of additional diagnostic value.

The presence of plasma cells in BAL fluid is highly suggestive of the diagnosis EAA. A relatively high number of plasma cells, positively related to immunoglobulin levels in BAL fluid samples, suggests recent allergen exposure. Moreover, patients with EAA with plasma cells in BAL fluid show signs of a more prominent alveolitis. Finally, no support was found for the existence of a so-called 'standard' BAL fluid profile in any aforementioned lung disorder.

Directions for future research

The findings in this thesis demonstrate the diagnostic value of BAL in the management of interstitial lung diseases. However, further prospective, standardized studies are needed to clarify the many remaining questions. Therefore, it would be of interest to investigate, *eg*, the prognostic value of BAL fluid parameters, such as the $CD4^+/CD8^+$ ratio, in sarcoidosis patients; to assess the link between the presence of plasma cells in BAL fluid obtained from patients with EAA and the severity and prognosis of the disease; and to examine the differential cell count in BAL fluid of patients suffering from idiopathic pulmonary fibrosis in relation to their survival. Also, the data on pulmonary manifestations of malignant lymphomas need to be expanded. By studying the changes in BAL fluid parameters, insight in the efficacy of medical treatment in various pulmonary diseases may be gained.

Future research should also focus on the detection of new markers of disease activity and further elucidation of pathogenetic mechanisms of the various pulmonary disorders. The usefulness of BAL as a clinical, diagnostic and research tool will further increase.

Samenvatting en conclusies

Samenvatting

Bronchoalveolaire lavage (BAL) wordt tegenwoordig aangewend om cellen en eiwitten, aanwezig in de bronchoalveolaire ruimte, te verzamelen voor aanvullend onderzoek. Deze in beperkte mate invasieve techniek is een gevoelige methode om infectieuze en niet-infectieuze aandoeningen, zoals interstitiële longaandoeningen, te diagnostiseren.

Het doel van deze studie was het nader onderzoeken van de mogelijke klinische toepassingen van de BAL, met name de mogelijkheid om meer invasieve diagnostiek te vermijden. Voor de in dit proefschrift beschreven studies is gebruik gemaakt van lavage-materiaal van patiënten verzameld gedurende een periode van tien jaar: van 1980 tot 1990. Cellen en eiwitten, te weten albumine en immunoglobulines, aanwezig in de betreffende lavage-vloeistofmonsters, werden nader onderzocht. Retrospectief werden de verzamelde gegevens geanalyseerd en werd gezocht naar specifieke kenmerken in de BAL-vloeistof, welke het mogelijk maken de verschillende interstitiële longaandoeningen te onderscheiden. Dit proefschrift beschrijft de diagnostische waarde van de analyse van monsters verkregen door middel van BAL bij patiënten met interstitiële longaandoeningen. De nadruk werd gelegd op patiënten met sarcoïdose, extrinsieke allergische alveolitis (EAA) en idiopathische longfibrose.

In *hoofdstuk 1*, de algemene introductie, worden de procedure van de BAL, de cellulaire en enkele niet-cellulaire bestanddelen, aanwezig in de bronchoalveolaire ruimte, beschreven. Tevens wordt ingegaan op de factoren, die de samenstelling van de BAL-vloeistof beïnvloeden en de neven-effecten van de BAL-procedure. Tot slot wordt een korte samenvatting gegeven van de klinische presentatie en pathogenese van drie vormen van interstitiële longaandoeningen, te weten sarcoïdose, EAA, en idiopathische longfibrose.

De doelstellingen van de studies, beschreven in *hoofdstuk 2*, vormen een introductie tot het experimentele werk beschreven in de hoofdstukken 3 tot en met 8.

Een biopsie van patiënten met interstitiële longaandoeningen was niet altijd beschikbaar. Daarom waren aanvullend soms andere diagnostische methoden noodzakelijk. In *hoofdstuk 3* is de mogelijkheid onderzocht om patiënten met drie vergelijkbare frequent voorkomende interstitiële longaandoeningen, te onderscheiden met behulp van een discriminantie-

analyse. Er werd gebruik gemaakt van een aantal in de BAL-vloeistof geselecteerde variabelen. De studie bevatte alle patiënten welke een BAL ondergingen in de periode tussen 1980 en 1990. Deze patiënten behoorden tot de volgende diagnostische groepen: sarcoïdose, subacute EAA en idiopathische longfibrose. Cellulaire en niet-cellulaire componenten aanwezig in de BAL-vloeistof werden onderzocht. Variabelen, die gebruikt werden om tussen de drie diagnostische groepen te discrimineren, waren: opbrengst van de BAL-vloeistof, het totale celaantal, en de percentages alveolaire macrofagen, lymfocyten, neutrofielen, eosinofielen en plasmacellen. Wanneer de geselecteerde data, die gebruikt werden om te voorspellen tot welke diagnostische groep de patiënten behoorden (test-set), afkomstig waren uit dezelfde groep waarin de discriminantie-analyse werd uitgevoerd (learning-set), werd de diagnose in 93 procent van de patiënten correct voorspeld. Wanneer de test-set ongelijk was aan de learning-set, daalde dit percentage naar 90 procent. Het is mogelijk om patiënten met sarcoïdose, EAA of idiopathische longfibrose te onderscheiden met de geselecteerde variabelen. Hieruit blijkt dat de BAL een waardevolle aanvullende diagnostische methode is.

Patiënten met het syndroom van Löfgren gekenmerkt door mediastinale en/of hilus lymfadenopathie hebben de meest uitgeproken karakteristieken van sarcoïdose in de BAL-vloeistof. Zij kunnen moeilijk te onderscheiden zijn van patiënten met pulmonale manifestaties van tuberculose of maligne lymfomen. In *hoofdstuk 4* werd de cellulaire samenstelling van de BAL-vloeistof van patiënten met sarcoïdose, tuberculose en maligne lymfomen, te weten non-Hodgkin's lymfoom en de ziekte van Hodgkin, vergeleken. De verschillende groepen waren te onderscheiden aan de hand van $CD4^+$- en $CD8^+$-T-lymfocyten, en de $CD4^+/CD8^+$-ratio's in de BAL-monsters. Patiënten met maligne lymfomen hadden de laagste $CD4^+/CD8^+$-ratio's, zowel in de BAL-monsters, als in het perifere bloed. Soms werden plasmacellen aangetroffen in de BAL-vloeistof. Het belangrijkste van de BAL-vloeistofanalyse bij tuberculosepatiënten was het aantonen van het microbiële agens. Hoewel bij maligne lymfomen histologische evaluatie, en bij tuberculose een positieve kweek noodzakelijk zijn, kunnen we concluderen dat BAL-vloeistofanalyse een extra hulpmiddel kan zijn om deze aandoeningen van sarcoïdose te onderscheiden.

Klinische manifestaties van sarcoïdose zijn afhankelijk van de intensiteit van de ontsteking en de orgaansystemen welke zijn aangedaan. Patiënten met sarcoïdose vertegenwoordigen een heterogene populatie. Bij sommige sarcoïdosepatiënten verloopt de alveolitis subklinisch, terwijl bij anderen zowel alveolitis als granuloomvorming optreden, wat gepaard kan gaan met specifieke pulmonale symptomen. Hoewel de long het meest aangetaste orgaan is, komen ook extrapulmonale manifestaties, zoals erythema nodosum, regelmatig voor. De ziekteduur en wijze van presentatie worden weerspiegeld in de samenstelling van de BAL-vloeistof. In *hoofdstuk 5* werd de relatie tussen het T-lymfocytenprofiel in de BALvloeistof en de ziektepresentatie van sarcoïdose bestudeerd. Met dit doel werden honderd patiënten met histologisch bewezen sarcoïdose onderzocht. Ze werden verdeeld in drie groepen, afhankelijk van hun klinische presentatie en rookgedrag. Groep A bestond uit patiënten zonder symptomen, bij wie de aandoening bij toeval was ontdekt op een thoraxfoto; groep B bevatte patiënten met algemene en pulmonale symptomen; groep C vertegenwoordigde patiënten met erythema nodosum en/of arthralgieën en lymfadenopathie. In de BAL-vloeistof van patiënten met hilaire lymfadenopathie werden de meest karakteristieke kenmerken van een alveolitis gevonden, hetgeen een systemische in plaats van een locale immuunrespons suggereert. De laatste groep (groep C) had de hoogste $CD4^+/CD8^+$-ratio's, terwijl de $CD4^+/CD8^+$-ratio's in de BAL-vloeistof van de asymptomatische patiënten (groep A) het laagst waren. De waarden van deze ratio's van patiënten met pulmonale afwijkingen (groep B) lagen tussen die van groep A en groep C in. Roken beïnvloedt bovendien het immunologische BAL-vloeistofprofiel, daar bij rokers een minder uitgesproken alveolitis werd aangetroffen.

Naast roken vormen het medicamentgebruik in het heden en verleden als ook het klinische stadium en verloop van de ziekte in de tijd belangrijke factoren, die het BAL-vloeistofprofiel verstoren (zie ook hoofdstuk 5). In *hoofdstuk 6* werd de relatie bestudeerd tussen enerzijds de cellulaire samenstelling en immunoglobulinespiegels in de lavagevloeistof van patiënten met EAA en anderzijds de tijdsduur, verstreken tussen de laatste allergeen-blootstelling en de BAL. Met dit doel werd een analyse gedaan van BAL-vloeistof afkomstig van niet-rokende patiënten met EAA op verschillende tijdstippen na het beëindigen van de allergeen-blootstelling.

Na allergeen-provocatie (groep 1: minder dan 24 uur) werden in de BAL-vloeistof hoge aantallen lymfocyten, neutrofielen, eosinofielen en mestcellen gevonden, en een laag percentage alveolaire macrofagen. BAL-vloeistof verzameld na recent allergeen-contact (groep 2: tussen 2 en 7 dagen) bevatte grote aantallen lymfocyten, plasmacellen en mestcellen, naast hoge IgM-, IgG-, en IgA-spiegels. De samenstelling van de BAL-vloeistof, verzameld één week of langer na de laatste allergeen-blootstelling, normaliseerde geleidelijk, met uitzondering van het aantal lymfocyten. Samenvattend demonstreren deze resultaten dat het cellulaire BAL-vloeistofprofiel en de immunoglobulinespiegels bij patiënten met EAA sterk afhankelijk zijn van het interval sinds de laatste allergeen-blootstelling. Aan de hand van deze tijdsafhankelijke samenstelling van de BAL-vloeistof bij patiënten met EAA geeft de analyse van een 'at random' verkregen BAL-vloeistofmonster een aanwijzing of er recent allergeen-contact is geweest.

In BAL-vloeistof bevindt zich een verzameling cellen die afkomstig zijn uit de intra-alveolaire ruimte. Vele aandoeningen hebben specifieke kenmerken in de BAL-vloeistof. Opmerkelijk is dat de meeste belangstelling is uitgegaan naar de identificatie van T-lymfocyten en de $CD4^+$/$CD8^+$-ratio's in de BAL-vloeistof van patiënten met verschillende longaandoeningen, en niet naar plasmacellen. In *hoofdstuk 7* werden de eerste BAL-vloeistofmonsters van 1260 patiënten nader geanalyseerd. In 83 van deze monsters (6.6 procent) werden plasmacellen aangetroffen. Van deze 83 monsters waren er 47 afkomstig van patiënten met EAA. Het aantal plasmacellen in de BAL-vloeistof van deze patiënten bleek gerelateerd te zijn aan de tijd tussen het laatste allergeen-contact en de BAL.

Een door medicamenten geïnduceerde pneumonitis is een aandoening, waarbij men ook in een hoog percentage van de gevallen plasmacellen in de BAL-vloeistof aantreft (35.7 procent). Het herkennen van plasmacellen in BAL-vloeistof heeft dus differentiaal-diagnostische waarde wanneer men moet discrimineren tussen interstitiële longaandoeningen van verschillende origine. Echter, de precieze betekenis van de aanwezigheid van plasmacellen in de BAL-vloeistof en de samenhang met de klinische presentatie van deze aandoeningen dient nader onderzocht te worden.

Extrinsieke allergische alveolitis is gerelateerd aan hoge aantallen T- en

B-lymfocyten in de BAL-vloeistof, naast de aanwezigheid van een aantal plasmacellen. In *hoofdstuk 8* werd onderzocht of er een relatie bestond tussen de aanwezigheid van plasmacellen en andere cellen en de hoogte van de immunoglobulinespiegel in de BAL-vloeistof van patiënten met EAA. Retrospectief werden dertig niet-rokende patiënten geselecteerd met EAA, welke een BAL ondergingen 2 tot 7 dagen na hun laatste blootstelling aan het oorzakelijke allergeen. Patiënten met EAA met plasmacellen in de BAL-vloeistof hadden een verhoogd absoluut aantal lymfocyten, eosinofielen en mestcellen, een verlaagd percentage alveolaire macrofagen en lagere $CD4^+/CD8^+$-ratio's, naast hogere immunoglobulinespiegels, vergeleken met patiënten zonder plasmacellen in de BAL-vloeistof. Deze resultaten suggereren een relatie tussen de aanwezigheid van plasmacellen en andere bestanddelen van de BAL-vloeistof, en een meer uitgesproken alveolitis. Bovendien was er een positieve relatie tussen het aantal aanwezige plasmacellen en de immunoglobulinespiegels in de BAL-vloeistof. Deze bevindingen ondersteunen de veronderstelling dat bij daarvoor gevoelige personen in de long locaal immunoglobulines worden geproduceerd door plasmacellen na allergeen-blootstelling.

Conclusies

Hoewel de studies beschreven in dit proefschrift retrospectief zijn uitgevoerd, tonen deze aan dat analyse van BAL-vloeistof een gevoelige en nuttige methode is om verschillende interstitiële longaandoeningen, met name sarcoïdose, EAA, en idiopathische longfibrose te onderscheiden. De gepresenteerde gegevens suggereren dat het gebruik maken van BAL meer invasieve diagnostische methoden, zoals biopsieën, in een aantal gevallen kan voorkomen. Het roken van sigaretten blijkt een belangrijke verstorende variabele te zijn, waarmee rekening gehouden dient te worden wanneer men de resultaten van de BAL-vloeistofanalyse interpreteert. Bovendien beïnvloeden de presentatie - en het verloop van de ziekte in de tijd - de samenstelling van de BAL-vloeistof. Hoewel de diagnose maligne lymfoom histologisch bewezen dient te worden, en de diagnose tuberculose met een positieve kweek bevestigd dient te worden, kan de analyse van BAL-vloeistof van aanvullende diagnostische waarde zijn.

De aanwezigheid van plasmacellen in de BAL-vloeistof maakt de diagnose EAA hoogst waarschijnlijk. Een relatief hoog aantal plasmacellen, dat

positief gerelateerd is aan de immunoglobulinespiegels in de BAL-vloeistof, is een aanwijzing dat er recent allergeen-contact heeft plaatsgevonden. Tevens vertonen patiënten met EAA met plasmacellen in de BAL-vloeistof tekenen van een meer uitgesproken alveolitis. Tenslotte dient opgemerkt te worden dat er geen 'standaard' BAL-vloeistofprofiel bestaat voor één van de eerder genoemde longaandoeningen.

Richtlijnen voor toekomstig onderzoek

De bevindingen van dit proefschrift ondersteunen de mogelijke diagnostische waarde van de BAL ten aanzien van interstitiële longaandoeningen. Echter, verdere, prospectieve, goed gestandaardiseerde studies zijn noodzakelijk om de vele overgebleven vragen te beantwoorden. Het zou interessant zijn om de prognostische waarde van BAL-vloeistofparameters, zoals de $CD4^+/CD8^+$-ratio van sarcoïdosepatiënten te onderzoeken; bij patiënten met EAA de relatie tussen de aanwezigheid van plasmacellen in de BAL-vloeistof en zowel de ernst als ook de prognose van de aandoening te beoordelen; en de relatie tussen de celverdeling in de BAL-vloeistof van patiënten met idiopathische longfibrose te analyseren en de eventuele relatie met de overleving. De gegevens betreffende de pulmonale manifestaties van maligne lymfomen dienen verder uitgebreid te worden. Wanneer men de veranderingen in de BAL-vloeistofparameters bestudeert, zou men inzicht kunnen verkrijgen in de doeltreffendheid van medicamenteuze therapie van verschillende longaandoeningen.

Toekomstige studies dienen zich ook ten doel te stellen te zoeken naar nieuwe parameters van ziekte-activiteit en verdere opheldering van de pathogenese van de verschillende longaandoeningen na te streven. De toepasbaarheid van de BAL als een klinische, diagnostische en wetenschappelijke onderzoeksmethode zal zich verder uitbreiden.

Abbreviations

Niet alle ziekten zijn geneeslijk,
dit behoort tot het levensdrama,
maar het is de taak van de arts
mèt de patiënt te zoeken naar
de zin ook van dit drama.
Het kunnen begeleiden van een
sterfproces behoort tot de meest
menselijke kunst van de arts.

Uit: Medisch contact 1975,
Prof. Dr. B.C.J. Lievegoed

List of abbreviations

ABPA = Allergic bronchopulmonary aspergillosis
AD = Anno Domini
AIDS = Acquired immunodeficiency syndrome
AM(s) = Alveolar macrophage(s)
ANOVA = Analysis of variance
APC = Antigen-presenting cell
ARDS = Adult respiratory distress syndrome
BAL = Bronchoalveolar lavage
BALF = BAL fluid
BALT = Bronchus-associated lymphoid tissue
BC = Before Christ
BCP = Bromocresol purple
BMDP = Biomedical package
BOOP = Bronchiolitis obliterans and organizing pneumonia
BSA = Bovine serum albumin
C = Controls
cc = Spearman rank correlation coefficient
CD = Cluster of differentiation
CLB = Central Laboratory of the Netherlands Red Cross Blood Transfusion Service
COPD = Chronic obstructive pulmonary disease
Cs = Control subjects
d = Day
D_{CO} = Carbon monoxide diffusing capacity
DE = Diagnostic effectiveness
DF = Discriminant functions
DNA = Deoxyribonucleic acid
EAA = Extrinsic allergic alveolitis
eg = Exempli gratia: for example
ELISA = Enzyme-linked immunosorbent assay
Eos = Eosinophils
et al = Et alia: and others
etc = et cetera
ESm = Exsmokers
F = Female
F1 = Function 1
F2 = Function 2
FEV_1 = Forced expiration volume in one second
FITC = Fluorescein isothiocynate
fnc = Function
g = Gram
GAM = Goat-antimouse-immunoglobulin
GM-CSF = Granulocyte macrophage colony-stimulating factor
h = Hour
H = Heavy
HL = Hodgkin's disease
HLA = Human leukocyte antigen
HRP = Horseradish peroxidase
ie = Id est: that is to say
IFN = Interferon

Ig(s) = Immunoglobulin(s)
IL = Interleukin
ILD = Interstitial lung diseases
IPF = Idiopathic pulmonary fibrosis
l = Liter
L = Light
l-alb = Lavage albumin
LT = Leukotriene
Lym = Lymphocytes
m = Missings
M = Male
MC = Mast cells
M-CSF = Macrophage colony stimulating factor
MEM = Minimal essential medium
MGG = May-Grünwald-Giemsa
MHC = Major histocompatibility complex
ml = Milliliter
n = Number of cases
NAF = Neutrophil-activating factor
NHL = non-Hodgkin's disease
NK = Natural killer
NS = Not significant
NSm = Nonsmokers
O_2 = Oxygen
OPD = Ortho-phenyl diamine
p = Pressure/probability
PAF = Platelet-activating factor
PB = Peripheral blood
PC = Plasma cell
PEF = Peak expiratory flow
PG = Prostaglandine
PMN(s) = Polymorphonuclear neutrophil(s)
PR = Polychotomous logistic regression
PV = Predicted value
RNA = Ribonucleic acid
s-alb = Serum albumin
Sar = Sarcoidosis
SE = Standard error
SEM = Standard error of the mean
Sm = Smokers
Spec = Specificity
Tbc = Tuberculosis
TCC = Total cell count
TCR = T cell receptor
TH = T helper
TNF = Tumor necrosis factor
VC = Vital capacity
vs = Versus
yr(s) = Year(s)
ZN = Ziehl-Neelsen

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Iemand die
dankbaarheid mist,
is verstoken van
die schoonheid
van karakter,
die de
persoonlijkheid
sfeer geeft.

Inayat Khan

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Arnhem, 8 december 1993 *Marjolein Drent*

Curriculum vitae

Er zijn vele wegen,
Maar de juiste weg
Is de weg ertegen;
Niet de weg eronder,
Dat is onderkruiperij,
Niet de weg erover,
Dat is pluimstekerij
Maar de weg ertegen,
Tegen alle wegen in,
Dat is van de wijsheid
Nog maar het begin.

Bertus Aafjes

Curriculum vitae

The author of this thesis was born in Bennekom, the Netherlands, on 23 December 1955. In 1975 she graduated at the Gymnasium ß department of the Wagenings Lyceum. She then studied physiotherapy at the Arnhem Academy of Physiotherapy; she graduated in 1979. She worked as a full-time physiotherapist at the St. Elisabeth Hospital in Arnhem till 1981, and on a part-time basis in a private practice, also in Arnhem, from 1982 to 1987.

In the autumn of 1981 she started her study of medicine at the Catholic University of Nijmegen, which she finished in 1985. After her general training she fulfilled a four-month fellowship doing research at the Department of Anaesthesia of the St. Radboud Hospital in Nijmegen (under the supervision of Dr. H.G. Kho en Dr. J. van Egmond). She conducted a literature search for the possible influence of electro-acupuncture-anaesthesia on the human immune system.

In 1988, after passing her final examinations in medicine, she started her training in internal medicine at the Rijnstate Hospital, located at the Gemeente Ziekenhuis (Community Hospital) in Arnhem (trainers: Dr. K-H. Brandt and Dr. J.M. Werre). During this period she decided to transfer to the department of Pulmonary Diseases and Tuberculosis. Her training as a pulmonary physician was continued at the St. Antonius Hospital in Nieuwegein in 1990 (trainer: Dr. R.G.J.R.A. Vanderschueren). From 1990 till now she has been doing research in the field of bronchoalveolar lavage supported by a grant of Glaxo BV, the Netherlands, under the supervision of Dr. J.M.M. van den Bosch (co-examiner).

Curriculum vitae

De auteur van dit proefschrift werd op 23 december 1955 te Bennekom geboren. In 1975 behaalde zij het diploma Gymnasium ß aan het Wageningse Lyceum. Vervolgens studeerde zij fysiotherapie aan de Arnhemse Academie voor Fysiotherapie. Na het behalen van het diploma in 1979 werkte zij als fysiotherapeut achtereenvolgens van 1979 tot september 1981 full-time in het St. Elisabeth Gasthuis te Arnhem en van 1982 tot 1987 part-time in een particuliere praktijk te Arnhem.

In het najaar van 1981 werd een aanvang gemaakt met de studie geneeskunde aan de Katholieke Universiteit te Nijmegen, zij behaalde het doctoraalexamen in 1985. In de postdoctoraal fase vervulde zij gedurende een periode van 4 maanden haar wetenschappelijke keuzestage op de afdeling Anaesthesie van het St. Radboud Ziekenhuis te Nijmegen (onder leiding van Dr. H.G. Kho en Dr. J. van Egmond). Zij verrichtte een literatuurstudie naar de mogelijke invloed van electro-acupunctuur-anaesthesie (EAA) op het menselijke immuunsysteem.

Na het behalen van het artsexamen in 1988 startte zij de opleiding Interne Geneeskunde in het Rijnstate Ziekenhuis, locatie Gemeente Ziekenhuis te Arnhem (opleiders: Dr. K-H. Brandt en Dr. J.M. Werre). Gedurende deze periode besloot zij van de opleiding Interne Geneeskunde over te stappen naar de opleiding Longziekten en Tuberculose. De opleiding tot longarts werd voortgezet in 1990 in het St. Antonius Ziekenhuis te Nieuwegein (opleider: Dr. R.G.J.R.A. Vanderschueren). Van 1990 tot heden deed zij aldaar onderzoek op het gebied van de bronchoalveolaire lavage met financiële ondersteuning van Glaxo BV onder begeleiding van Dr. J.M.M. van den Bosch (co-promotor).

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Appendix

Normal values of several bronchoalveolar lavage fluid components

Table 1—*Cellular Components in Bronchoalveolar Lavage Fluid of Normal Subjects*

	NSm	Sm	ESm	NSm:Sm
Yield %	63 (58.4±2.5)	62 (57.8±2.3)	59	1:1 (1.0)
Viable cells %	86 (92.1±0.9)	92 (93.8±0.8)	86	1:1.1*(1.0)
Total cell count x10^6	18 (11.1±1.7)	59 (33.2±3.9)	20	1:3.3*(3.0)
Cells x10^4/ml BAL fluid	13 (10.3±1.6)	42 (29.2±3.3)	14	1:3.2*(2.8)
Alveolar macrophages				
cells x10^4/ml	10 (9.3±1.4)	39 (27.9±3.2)	12	1:3.9*(3.0)
percentage	85 (89.8±0.7)	93 (94.5±0.6)	86	1:1.1 (1.1)
Polymorphonuclear neutrophils				
cells x10^4/ml	0.2 (0.1±0.03)	0.8 (0.35±0.9)	0.3	1:4* (3.5)
percentage	1.6 (1.3±0.2)	1.6 (1.2±0.2)	2.1	1:1 (0.9)
Lymphocytes				
cells x10^4/ml	1.5 (0.8±0.1)	1.7 (0.8±0.1)	1.7	1:1.1 (1.0)
percentage	11.8 (8.4±0.7)	5.2 (3.8±0.5)	11.5	1:0.4*(0.5)
T lymphocytes(CD3) %	70 (73.0±2.4)	69 (81.0±9.0)	74	1:1 (1.1)
$CD4^+$ %	41.4 (52.4±3.4)	32 (38.5±0.5)	46.8	1:0.8*(0.7)
$CD8^+$ %	20.7 (19.0±0.9)	29 (48.0±1.0)	20.7	1:1.4*(2.5)
$CD4^+/CD8^+$ ratio	2.6 (2.7±0.2)	1.6 (0.8±0.01)	2.8	1:0.6*(0.3)
B lymphocytes (CD21)%	3.2	6.5	3.5	1:2
Plasma cells	0.0 (0.0±0.0)	0.0 (0.0±0.0)		
Mast cells				
cells x10^4/ml	0.02(0.01±0.006)	0.30 (0.16±0.06)	?	1:15* (16)
percentage	0.03 (0.09±0.03)	0.07 (0.43±0.08)	?	1:2.5*(4.8)
Eosinophils				
cells x10^4/ml	0.02 (0.07±0.04)	0.31 (0.14±0.03)	0.08	1:15*(2.0)
percentage	0.20 (0.44±0.1)	0.60 (0.43±0.08)	0.21	1:3 (1.0)

*Data are expressed as mean values. NSm = Nonsmokers; Sm = Current Smokers; and ESm = Exsmokers. *p-value < 0.05, effect of smoking by multifactorial linear, logistic or Poisson regression analysis after accounting for other demographic factors.(adapted from Reynolds 1987[1]; Valberg et al 1990[2]; the BAL Cooperative Steering Committee 1990[3]; In parenthesis data ± SEM (NSm: n = 28; Sm: n = 32) and ratio respectively, Drent et al 1993[4]).*

Table 2—*Proteins in Serum and Bronchoalveolar Lavage Fluid of Normal Subjects*

Protein	NSm		Sm		ESm	NSm:Sm
Serum (g/l)						
Total protein	75.6		77.3		75.7	1:1.0
Albumin	41.3	(41.4±0.9)	43.6	(41.6±1.1)	41.4	1:1.1*(1.0)
Immunoglobulins (Ig)						
IgM	2.0	(1.74±0.16)	1.7	(1.82±0.14)	1.9	1:0.9 (1.0)
IgG	11.2	(12.0±0.59)	10.0	(10.2±0.34)	10.9	1:0.9*(0.9)*
IgA	2.9	(2.20±0.17)	2.7	(1.93±0.13)	3.3	1:0.9 (0.9)
BAL fluid (mg/l)						
Total protein	78		95		98	1:1.2
Albumin	34	(71.0±8.5)	43	(68.3±7.5)	43.3	1:1.3(1.0)
Immunoglobulins (Igs)						
IgM	0.20	(0.41±0.1)	0.23	(0.37±0.1)	0.33	1:1.2(0.9)*
ratio to albumin	0.006	(0.01±0.00)	0.005	(0.01±0.00)	0.003	1:0.8*(1.0)
IgG	8.0	(11.1±2.0)	10.2	(14.6±3.4)	9.1	1:1.3(1.3)*
ratio to albumin	0.23	(0.16±0.03)	0.24	(0.22±0.04)	0.21	1:1.0(1.4)*
IgA	6.2	(3.6±0.7)	5.9	(3.5±0.9)	7.6	1:1.0(1.0)
ratio to albumin	0.19	(0.05±0.01)	0.14	(0.05±0.01)	0.18	1:0.7(1.0)

*Data are expressed as mean values. NSm = Nonsmokers; Sm = Current Smokers; and ESm = Exsmokers. *p-value < 0.05, effect of smoking by multifactorial linear, logistic or Poisson regression analysis after accounting for other demographic factors. (adapted from the BAL Cooperative Steering Committee 1990[3]; Baughman 1992[5]; In parenthesis data ± SEM (NSm: n = 28; Sm: n = 32) and ratio respectively, Drent et al 1993[4]).*

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Predicting diagnostic group membership

In order to distinguish between the three diagnostic groups (see chapter 3 for patient selection and bronchoalveolar lavage (BAL) procedure), a polychotomous logistic regression analysis was performed, according to the following procedure. Each of the 277 patients in the total study group belongs to one, and only one, diagnostic group. Five of the cases (three sarcoidosis patients; one extrinsic allergic alveolitis (EAA) patient and one idiopathic pulmonary fibrosis (IPF) patient) had at least one missing discriminating variable, therefore 272 cases were used for the analysis. Thus, 190 patients belong to the sarcoidosis group, 38 to the EAA group and 44 to the IPF group (see chapter 3; table 1). Hence, an arbitrary patient out of the total study group has a probability of 190/272 = 0.70, 38/272 = 0.14 and 44/272 = 0.16, respectively, of belonging to either one of the three diagnostic groups. These probabilities, which add up to 1 (as they should), are called 'prior probabilities'. If a set of predefined characteristics (so-called 'explanatory variables') of a patient is known, then these characteristics can be involved in making these probabilities vary among patients. For example, if it is known that a patient is a smoker, then his probabilities may be different from the above mentioned prior probabilities, and so also from the probabilities of a nonsmoker. The latter probabilities, which can be calculated if we know the smoking status of a patient, are the so-called 'posterior probabilities'. A statistical technique which can be used to calculate these posterior probabilities from the prior probabilities and from the patient characteristics is called polychotomous logistic regression analysis.[1,2] If there are only two diagnostic groups, the analysis is called logistic regression analysis, without the adjective 'polychotomous'.

By means of polychotomous logistic regression analysis, an allocation rule can be derived according to which an arbitrary patient is allocated to one and only one of J disjoint diagnostic groups $j = 1,\ldots,J$ on the basis of p explanatory variables $x_1, x_2, \ldots, x_p$ that contain some information about the diagnostic group to which the patient belongs. This information is assembled in J - 1 linear predictor scores y_j, which are defined as linear combinations of the explanatory variables:

$$y_j = \beta_{0j} + \beta_{1j}x_1 + \ldots \beta_{pj}x_p,\ j = 1,\ldots,J\text{-}1.$$

Hence, there are J - 1 sets of unknown coefficients $\{\beta_{0j}, \beta_{1j}, \ldots \beta_{pj}\}$ which are to be estimated. For this purpose a polychotomous logistic regression model is postulated for the probabilities p_j, defined as the

probability that a patient with scores $y_1, \ldots, y_{J-1}$ belongs to diagnostic category j, as follows:

$$p_j = \frac{\exp(y_j)}{1 + \sum_{j=1}^{J-1} \exp(y_j)}, \text{ for } j = 1, \ldots, J - 1,$$

$$p_J = \frac{1}{1 + \sum_{j=1}^{J-1} \exp(y_j)}$$

with $\Sigma_j p_j = 1$.

The coefficients $\{\beta_{0j}, \beta_{1j}, \ldots \beta_{pj}\}$ in the linear predictor scores y_j $(j = 1, \ldots, J\text{-}1)$ can be estimated by means of the maximum likelihood technique, using standard statistical software (*eg,* module polychotomous logistic regression (PR) of the Biomedical Package (BMDP)). After estimating these coefficients, the probabilities p_j can be calculated for each patient with the above formula. The value of j for which p_j is maximum for a patient, denotes the predicted group for that patient.

When the β coefficients are estimated from in total n patients, with n_j patients per diagnostic category j, then an additive part in the estimated β_{0j} coefficients is $\ln(n_j/n_J)$:

$$\hat{\beta}_{0j} = \alpha_j + \ln(n_j/n_J), \quad j = 1, \ldots, J\text{-}1,$$

being the only coefficients influenced by the relative sizes of the diagnostic groups. For predicting group membership of patients in a population with other relative sizes for the diagnostic groups, say N_j/N_J, then the $\hat{\beta}_{0j}$ coefficients have to be adapted as follows:

$$\hat{\beta}_{0j}(\text{new}) = \hat{\beta}_{0j} - \ln(n_j/n_J) + \ln(N_j/N_J).$$

The p explanatory x variables used are the same as those used in chapter 3, except for the percentage of plasma cells, which causes the logistic regression analysis to become degenerate. The reason for this is that this variable is almost a perfect discriminator: a positive value almost certainly implies the presence of the diagnosis EAA.

In our application with three diagnostic groups $(J = 3)$, we have two linear predictors, of which the estimated ß-coefficients and standard errors are presented in table 3.

Table 3—*Estimated Coefficients and Standard Errors (SE) of the Polychotomous Logistic Regression Model*

Explanatory variable	F1	SE	F2	SE
Constant	11.68	4.26	16.19	5.51
Age yrs	-0.2072	0.05	-0.1406	0.05
Sex (Female)	0.7863	0.82	0.9682	1.01
Smoking (yes)	2.389	2.65	-0.9572	3.15
Smoking x (age squared)	-0.4481×10^{-3}	0.82×10^{-3}	0.8495×10^{-3}	0.11×10^{-2}
BAL fluid				
Yield(out/in)x100	0.0672	0.04	-0.0310	0.04
Cells $x10^4$/ml	-0.0764	0.02	-0.0120	0.02
AM %; squared	-0.2913×10^{-3}	0.41×10^{-3}	-0.2009×10^{-2}	0.82×10^{-3}
PMN %; squared	0.5536×10^{-3}	0.55×10^{-3}	-0.2613×10^{-2}	0.22×10^{-2}
Lym %; squared	0.1465×10^{-2}	0.13×10^{-2}	0.6466×10^{-3}	0.15×10^{-2}
Eos %; squared	-0.1986	0.0623	-0.0109	0.0116

F1 = Function 1; F2 = Function 2; AM = Alveolar macrophages; PMN = Polymorphonuclear neutrophils; Lym = Lymphocytes; Eos = Eosinophils.

Results

The classification results for all cases used in the analysis are shown in table 4. These results are obtained by applying the allocation rule as described above. The percentage of patients correctly classified in all patients with a given actual diagnosis, called the 'diagnostic effectiveness' is $100((180+31+37)/272) = 91.2$.[3] The diagnostic effectiveness for sarcoidosis is $100(180/190) = 94.7\%$, for EAA $100(31/38) = 81.6\%$ and IPF $100(37/44) = 84.1\%$ (table 4). The predicted value of a classification can be calculated as the probability that a patient actually belongs to the predicted group. For the prediction 'sarcoidosis', the predicted value (PV^+) equals $100(180/192) = 93.8\%$, and for the prediction 'EAA' and 'IPF' these values are $100(31/36) = 86.1\%$ and $100(37/44) = 84.1\%$, respectively.

The three respective prior probabilities that an arbitrary patient (without using any additional information) actually belongs to a diagnostic group,

are for sarcoidosis 100(190/272) = 69.9%, for EAA 100(38/272) = 14.0% and for IPF 100(44/272) = 16.2%.

The specificity, *ie*, the probability of the prediction 'non-sarcoidosis' in the group without sarcoidosis is 100((31 + 1 + 1 + 37)/(38 + 44)) = 85.4%, for 'non-EAA' 100((180 + 6 + 6 + 37)/(190 + 44)) = 97.9% and for 'non-IPF' 100((180 + 4 + 6 + 31)/(190 + 38)) = 94.4%.

The predicted value of the negative result, *ie*, the predicted value of the group with 'non-sarcoidosis' (PV^-) equals 100(31 + 1 + 1 + 37)/(36 + 44) = 87.5%, for 'non-EAA' 100(180 + 6 + 6 + 37)/(192 + 39) = 93.9% and for 'non-IPF' 100 (180 + 4 + 6 + 31)/(192 + 36) = 96.9% (table 4).

Table 4—*Classification Results from the Patients with Interstitial Lung Diseases*

	Predicted Group Membership (n)				Percentage			
Actual Group	Sar	EAA	IPF	Total	SPEC	DE	PV^+	PV^-
Sar	180	4	6	190	85.4	94.7	93.8	85.4
EAA	6	31	1	38	97.9	81.6	86.1	97.9
IPF	6	1	37	44	94.4	84.1	84.1	94.4
Total	192	36	44	272				

n = number of cases; Sar = Sarcoidosis; EAA = Extrinsic allergic alveolitis; IPF = Idiopathic pulmonary fibrosis; SPEC = Specificity; DE = Diagnostic effectiveness (ie, sensitivity); PV^+ = Positive predicted value; PV^- = Negative predicted value.

The polychotomous logistic regression model estimated in table 3 can be used to calculate which diagnosis out of the three above interstitial lung diseases is most likely, given the patient's characteristics. For this purpose a computer program is available, which can be obtained from:

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Comment on the cover illustrations

The use of herbs for therapeutical and culinary purposes probably goes back 60.000 years.[1] In 2800 BC the Chinese herbalist Shen Nung compiled a list of 366 medicinal plants.[1] In the first century AD, Dioscorides wrote his authoritative herbal *'De materia medica libri quinque'*, which would become the basis for our knowledge.[2] In Western Europe, sources going back as far as the seventh century speak of the cultivation of medicinal herbs. Monks, who were spreading the gospel and who considered in their mission to look after the sick, laid out herb gardens at the monasteries in order to have medicinal plants at their disposal.[1]

Plants were the main source of medicines till far into the nineteenth century, but in the course of the twentieth century this whole system of knowledge was put aside to make room for the production of chemical medicines.[1] A number of plants we cannot do without, however, because their active substances cannot be replaced by other herbs or chemicals; they are still being used in any pharmacy. Some examples are: digoxin (from Digitalis lanata or foxglove), colchicine (from Colchicum autumnale or meadow saffron), vincristine and vinblastine (from Vinca rosea), taxol (from Taxus brevifolia) and morphine (from Papaver somniferum or opium poppy).[1,3] Two examples of herbs which are being used in the treatment of lung complaints, will be described below.

Lungwort or *Pulmonaria officinalis* L.

Lungwort is endemic in Central Europe but is also found in other areas. Its generic name is derived from the Latin word *pulmones*, 'lungs', because the white spots on the leaves faintly resemble lungs in form and marking.[1] This refers to the system of 'signaturism' (Paracelsus 1493-1541) which holds that the plant resembles the disease and the organ that is to be treated.[1,4] The flowers are pink at first, then red and violet changing into a violet-blue colour when they start to fade.[5,6] Again, an analogy with the lungs can be recognized.

The leaves contain soluble silicic acid which strengthens the connective tissue, especially of the lungs.[4,7] The tannic acid contained in lungwort has a contracting effect on the mucous and tissue cells decreasing the permeability and preventing alien agents from entering, and stemming

small haemorrhages.[7] A decoction was used for coughs, bronchitis and tuberculosis.[1,5] The leaves contain much potassium nitrate, which has a urine-excreting effect. Lungwort is also used in the distillation of alcoholic drinks, it is a component of vermouth (martini) and the young leaves make a tasty vegetable when boiled.[1]

Marjoram or *Origanum vulgare* L.

Wild marjoram originates from the Mediterranean.[8] The generic name 'origanum' is derived from the Greek words *oros*, 'mountain', and *gamos*, 'joy', and refers to its pretty appearance in the mountains.[1] In Greek mythology its origin is explained as follows: young Amarakos was a servant to the king of Cyprus. When he dropped a valuable jug of perfume, he fainted from fear. Merciful gods carried him away in the form of a herb: marjoram. The god Venus was the first to cultivate the herb.[8] The Greeks believed that marjoram provided joy to the dead and therefore planted it on graves. To the Romans too, it was a symbol of happiness and on Sicily people believed that marjoram could banish sorrow.[1,8]

In the Middle Ages it was used for its strong aromatic smell and its antiseptic properties: it was strewn in the sick room, and used to improve the taste of mixtures and powders and in the composition of scent sachets and bathing oil.[1,8] Nowadays it is often used in the preparation of dishes, in toothpaste and in liquor and perfume production.[8]

Therapeutically, it is known for its powerful, calming effect; it is a good sedative and it also has a soothing effect on the digestive system.[1] It contains essential oil which has a disinfectant and stimulating effect on, especially, the skin and the intestinal and pulmonic mucous membranes.[7] It also contains thymol, a substance with a bactericidal and fungicidal effect.[9,10] When inhaled, marjoram is supposed to make to be suited against coughs, colds, inflammation of the tonsils, and other pulmonary diseases.[1] Marjoram gargles cure infections of the mouth and sore gums. Used in compresses it is a remedy for swollen glands. It also relieves hay fever.[1,5,11]

Toelichting op de omslagillustraties

Het gebruik van kruiden vanwege hun geneeskracht en in de keuken is waarschijnlijk 60.000 jaar geleden al ontstaan.[1] De Chinese kruidkundige Shen Nung heeft in 2800 voor Christus een lijst aangelegd van 366 geneeskrachtige planten.[1] In de eerste eeuw van onze jaartelling schreef Dioscorides zijn gezaghebbende kruidenboek *'De materia medica libri quinque'*, dat de basis van onze kennis zou worden.[2] In West-Europa kent men al uit de 7de eeuw bronnen, die over het kweken van geneeskrachtige kruiden spreken. Monniken, die het Christendom verspreiden en het als hun taak zagen voor de zieken te zorgen, legden bij de kloosters kruidentuinen aan om te kunnen beschikken over medicinale planten.[1]

Tot in de 19de eeuw waren planten de belangrijkste bron van geneesmiddelen, maar in de loop van de 20ste eeuw werd dat hele stelsel van kennis terzijde geschoven om ruimte te maken voor de produktie van chemische medicamenten. Een aantal gewassen zijn onmisbaar, omdat hun werking niet door andere kruiden of chemicaliën te vervangen is en worden nog in de apotheek gebruikt.[1] Enkele voorbeelden zijn: digoxine (uit Digitalis lanata of wollig vingerhoedskruid), colchicine (uit Colchicum autumnale of herfststijlloos), vincristine en vinblastine (uit Vinca rosea of maagdenpalm), paditaxel ofwel taxol (uit Taxus brevifolia) en morfine (uit Papaver somniferum of slaapbol).[1,3] Twee voorbeelden van kruiden, die vroeger werden gebruikt bij de behandeling van longaandoeningen, worden nu beschreven.

Breed longkruid of *Pulmonaria officinalis* L.

Longkruid is inheems in Midden-Europa, maar ook ingeburgerd in andere gebieden. De geslachtsnaam is afgeleid van het Latijnse woord *pulmones*, 'longen', doordat de witte vlekken op de bladen in vorm en tekening iets op longen lijken.[1] Dit verwijst naar de 'signatuurleer' (Paracelcus 1493-1541), deze houdt in dat de plant lijkt op de te behandelen ziekte en het orgaan.[1,4] De bloemen van het longkruid zijn eerst roze, dan roodpaars en worden tijdens de bloei violetachtig blauw.[5,6] Ook hierin is de analogie te herkennen met de longen.

De bladeren bevatten oplosbaar kiezelzuur, de werkzaamheid hiervan is dat het bindweefsel versterkt wordt, vooral dat van de longen.[4,7] Het looizuur dat zich in longkruid bevindt, heeft een samentrekkende invloed

op de slijm- en weefselcellen, waardoor de doorlaatbaarheid verminderd wordt en het vreemde agentia belemmert binnen te treden en kleine bloedingen stelpt.[7] Een afkooksel werd gebruikt tegen hoest, bronchitis en tuberculose.[1,5] De bladeren bevatten tevens veel kaliumnitraat, wat een urine-uitdrijvende werking heeft. Longkruid wordt ook gebruikt bij de distillatie van dranken, het is een bestanddeel van vermouth en de jonge blaadjes zijn een smakelijk gekookte groente.[1]

Wilde marjolein of *Origanum vulgare* L.

Wilde marjolein stamt uit het gebied rondom de Middellandse zee.[8] De geslachtsnaam origanum is afgeleid van de Griekse woorden *oros*, 'een berg' en *gamos*, 'vreugde' en verwijst naar de fraaie verschijning in de bergen.[1] In de Griekse mythologie wordt het ontstaan als volgt verklaard. De jonge Amarakos was in dienst van de koning van Cyprus. Toen hij een kostbare kruik parfum liet vallen, viel hij bewusteloos neer van angst. Genadige goden voerden hem weg in de vorm van een kruid: Marjolein. De god Venus heeft het kruid het eerst gekweekt.[8] De Grieken geloofden dat marjolein de doden vreugde verschafte en plantten dit kruid daarom op de graven, voor de Romeinen was marjolein een symbool van geluk en op Sicilië geloofde men dat marjolein verdriet kon verdrijven.[1,8]

Vanwege haar aromatische geur en anti-septische kwaliteiten werd marjolein in de middeleeuwen gebruikt als strooikruid in de ziekenkamer, om drankjes en poeders lekkerder te maken en in geurzakjes en badolie.[1,8] Tegenwoordig wordt marjolein in tandpasta verwerkt en gebruikt bij de bereiding van gerechten en in de dranken- en parfumindustrie.[8]

In geneeskrachtig opzicht heeft marjolein een sterk kalmerende werking, is een goed slaapmiddel en kalmeert tevens de spijsvertering.[1] Het bevat etherische olie, wat desinfecterend en prikkelend werkt op vooral de huid en slijmvliezen van darm en longen.[7] Tevens bevat het thymol; een stof met een bactericide en fungicide werking.[9-10] Dit zou marjolein bij inademing geschikt maken tegen hoest, verkoudheid, ontsteking van de keelamandelen en andere problemen met de luchtwegen.[1] Gorgeldranken van marjolein kunnen mondinfecties genezen; marjolein verlicht hooikoorts en kompressen van marjolein kunnen opgezette lymfeklieren verhelpen.[1,5,11]

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