HUMAN MONOCYTES AND ALVEOLAR MACROPHAGES

Modulation of phenotype and function
by cytokines and glucocorticoids *in vitro* and in asthma

HUMANE MONOCYTEN EN ALVEOLAIRE MACROFAGEN

Beïnvloeding van fenotype en functie
door cytokinen en glucocorticoïden *in vitro* en bij astmapatiënten

COVER: "Impression of research in the service of patient care"

Lower panel : Patient care (outpatient ward of the Sint-Franciscus Hospital, Schiekade 66, Rotterdam, circa 1910. In this hospital, the author of this thesis was born in 1960, and started his training as a pulmonary physician in January 1992) (by courtesy of Erasmus Publishing, Rotterdam).

Middle panel : Phase contrast microscopy of cultured human alveolar macrophages incubated with and without dexamethasone, respectively (chapter 5.5).

Upper panel : Hypothetical cellular working mechanism of glucocorticoids (chapters 1.3 and 2).

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Aan mijn ouders
Aan Monique

HUMAN MONOCYTES AND ALVEOLAR MACROPHAGES

Modulation of phenotype and function by cytokines and glucocorticoids *in vitro* and in asthma

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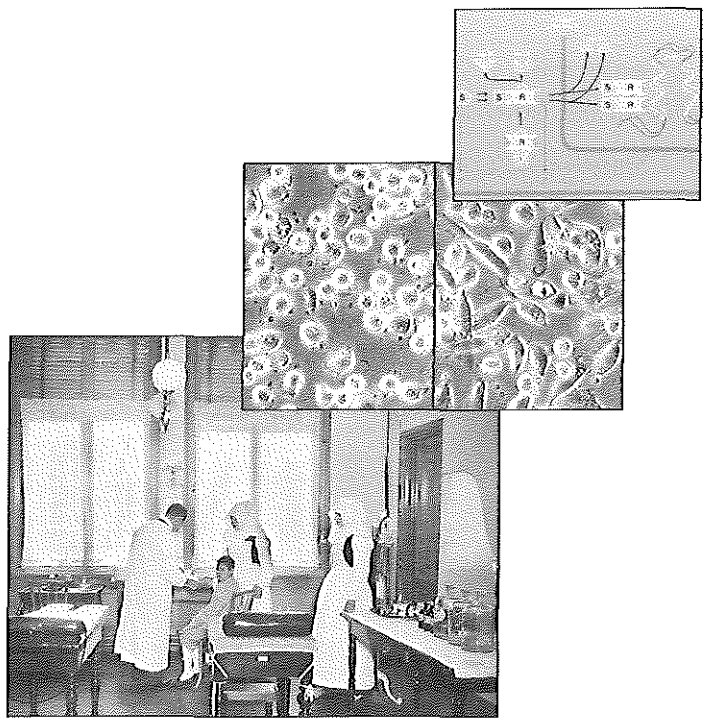

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1.1 MONOCYTES/MACROPHAGES

With emphasis on their role in pulmonary diseases

1.1.1 Historical background

In 1882 Ilya I'litch Metchnikoff, a Russian zoologist and anatomist, described for the first time that a special kind of cell in loose connective tissue could phagocytose foreign material (1,2). Different names were given to this cell, but most people know it nowadays as macrophage (which means "big eater") to underline the morphological difference with the "little eaters" (microphages, which was the early name given to another phagocytosing cell type now known as polymorphonuclear granulocyte). For a long time macrophages were classified together with other cell types (vascular endothelial cells, fibroblasts, reticular cells and histiocytes) as the reticuloendothelial system because of their common ability to concentrate supravital stains injected intravenously (3). However, this concept of the reticuloendothelial system did not recognize differences in function and origin. Therefore, based upon similarities between both morphology, function, kinetics and hemopoietic origin the mononuclear phagocyte system was postulated in 1972 (4,5). Nowadays, the different tissue macrophages, the peripheral blood monocytes (PBM) and their bone marrow precursors are considered to belong to the mononuclear phagocyte system (6,7). Nevertheless, some aspects remain unclear and raise intriguing questions; originally, dendritic cells were excluded from the mononuclear phagocyte system, whereas this status was uncertain for Langerhans cells, veiled cells and lymph node macrophages (4,5). Recently, it was shown that human monocytes could be stimulated *in vitro* to express functional and phenotypic characteristics of dendritic cells (8-11). In these studies, serum (9) and thyroid hormones (11) were used to induce dendritic qualities. Antibodies to GM-CSF, TNF-α and IL-6 inhibited this induction (11). Another study showed that dendritic cells proliferate in cultures of cells from human bone marrow (12). Therefore, evidence is growing that also dendritic cells may be regarded as belonging to the mononuclear phagocyte system (7). However, the origin of follicular dendritic cells present in the germinal centers within the lymph nodes and spleen remains a fascinating field of research where data from different experiments come to different conclusions. A monocytic origin for follicular dendritic cells cannot be excluded, but most results support the view that these cells derive from local cells with a fibroblastic or mesenchymal origin (13).

Mononuclear phagocytes (i.e. cells belonging to the mononuclear phagocyte system) can be found in the bone marrow, circulation and several tissues (6,7,14,15). Blood mononuclear phagocytes are generally called monocytes. Monocytes are able to leave the bloodstream and to enter the tissues or serous body cavities (16,17). These migrated monocytes, tissue mononuclear phagocytes, are generally called macrophages. This process of migration of mononuclear phagocytes from the bone marrow to the bloodstream and eventually to the different tissues represents an important way of maintaining the population of tissue macrophages. However, there are also reports which show that tissue macrophages themselves are able to proliferate, thereby maintaining the population

independently of the bone marrow (16,18-21). Different names were given to the tissue macrophages, which are now all considered to belong to the mononuclear phagocyte system (6). Figure 1 summarizes the different types of tissue mononuclear phagocytes. Other types of macrophages may be found in tissues under inflammatory conditions. Under these conditions monocytes are recruited from the bloodstream generally to a greater extent, and develop into inflammatory macrophage types, usually called exudate macrophages. In chronic inflammatory reactions, which may lead to the formation of granulomas, multinucleated giant cells, which are formed by fusion of cells, and epithelioid cells may be found (22,23). Also these cells arise from monocytes/macrophages and belong to the mononuclear phagocyte system.

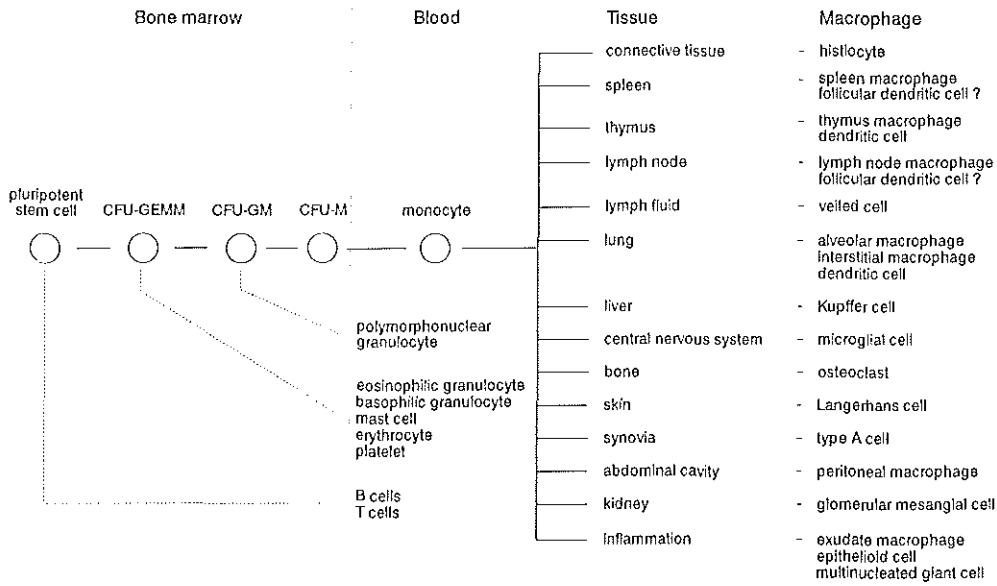

Figure 1. Hypothetical scheme of the developmental stages of human mononuclear phagocytes, leading eventually to the different tissue macrophages. In this scheme, mononuclear phagocyte development is depicted as a linear continuum. Final differentiation into the different tissue macrophages is induced in the specific micro-environment of the different tissues. Other models that may explain mononuclear phagocyte heterogeneity are discussed in the text. Abbreviations used in this figure:
CFU-GEMM : granulocyte erythrocyte macrophage megakaryocyte colony forming unit
CFU-GM : granulocyte macrophage colony forming unit
CFU-M : macrophage colony forming unit

1.1.2 Heterogeneity of monocytes/macrophages

Initially, differences in morphology and anatomical location interfered with the recognition of a distinct cell system, and tissue macrophages were given uncorrelated

names (see Figure 1). Once the concept of a unified mononuclear phagocyte system was established, the obvious question arose how to explain the phenotypic and functional diversity of the cells belonging to this system. On the one hand, mononuclear phagocytes from a particular tissue or anatomical site may differ greatly from mononuclear phagocytes from other sites. The differences in morphology (e.g. cell size, shape of nucleus, ratio of cytoplasm : nucleus, ability to stretch in *in vitro* culture), in expression of cell membrane proteins (e.g. CD14 and RFD9 Ag), and in function (e.g. phagocytosis, cytotoxicity, regulation of inflammatory and immune responses) are best known. On the other hand, the population of mononuclear phagocytes itself from a particular tissue or anatomical site may be heterogeneous, again with regard to the morphology, function and expression of proteins. To facilitate a clear discussion about this subject one has to realize that four different, but interacting, cellular processes (i.e. proliferation, differentiation, maturation, activation) (see Figure 2) determine the development, and the ultimate cellular heterogeneity among mononuclear phagocytes.

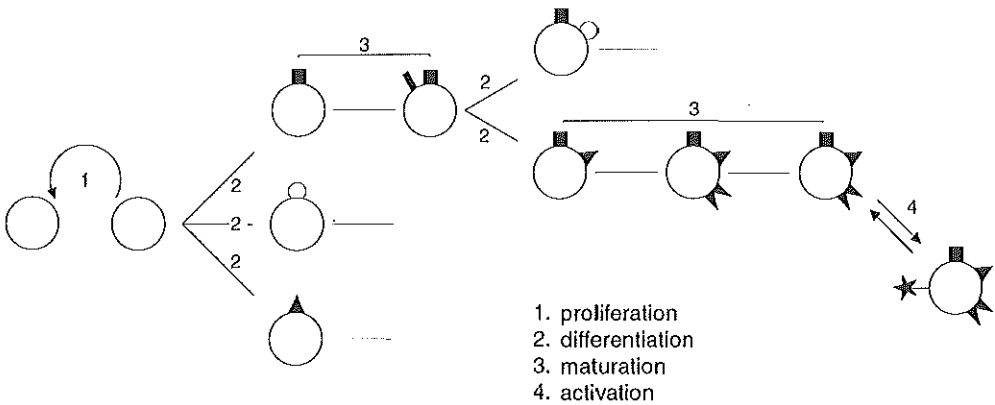

Figure 2. Proliferation, maturation, differentiation and activation in the generation of cellular heterogeneity. In this figure, heterogeneity is represented by differences in the expression of cell membrane antigens (■, ○, ▲, ▮, ▙ and ★).

Proliferation is defined as the replication of cells giving rise to daughter cells which are identical to their parent cell. Proliferation is probably always accompanied by differentiation or maturation, which explains why daughter cells are often different from their parent cell. The only exceptions to this rule concern germ cells and tumor cells.

Differentiation is defined as the development of cellular diversity/heterogeneity by passing through a stage at which a cell is receptive to several stimuli and has the ability to acquire one out of several specific phenotypes with accompanying functions. Under the influence of specific environmental factors only one specific phenotype will be acquired. This new phenotype is different from the parental cell phenotype and the process of differentiation is in principal irreversible. After passing such a point of differentiation and

having acquired a certain differentiated phenotype, a cell will develop to a more mature stage linked with more mature cellular properties.

Maturation concerns the process during which each stage with a certain cellular phenotype will be followed inevitably by the next stage with another, more mature cellular phenotype. Also the process of maturation is considered to be irreversible.

Activation is a reversible process by which cells exhibit a temporary altered phenotype to perform temporarily an enhanced or new function.

Proliferation, differentiation, maturation and activation of mononuclear phagocytes are regulated by environmental factors (24,25), including cell-to-cell interactions (25,26), cell-to-extracellular matrix interactions (27), several soluble regulatory glycoprotein molecules, generally called cytokines (e.g. IL-3, IL-4, M-CSF, GM-CSF) (25,28-32), and other soluble mediators (e.g. 1,25-dihydroxyvitamin D_3, glucocorticoids, substance P, neurotensin) (33-35). It is important to realize that one specific environmental factor may affect a cell type at various stages of development. The outcome of such an effect may, however, depend on the stage of development (30,36,37). For instance, in immature cells TNF-α enhanced IFN-γ-induced MHC class II expression, whereas in more mature cells this additive effect of TNF-α was eliminated (36). Furthermore, it is known now that, in general, a combination of environmental factors is needed to modulate particular aspects of cellular phenotype or function (38,39).

Essentially, all mononuclear phagocytes present in the different tissues derive from precursor cells in the bone marrow. Proliferation and maturation result in more mature cells and are accompanied by changes in morphology and function. At some points in this generation of heterogeneity, differentiation takes place. When and where differentiation leading to distinct and separate cell lineages occurs, is still a matter of argument and heated discussions (7). One view suggests that terminal differentiation of mononunuclear phagocytes is driven by the local microenvironment of the different tissues. The seeding of PBM to different tissues is supposed to be random, and heterogeneity among PBM should be explained as differences in maturation stage (7,42,43).

Another view proposes that already in the bone marrow multiple separate differentiation lineages exist. These different lineages should give rise to separate lineages of PBM. Tissue destination of these lineages of PBM is supposed to be "pre-programmed", and each of the lineages is believed to mature to one particular type of tissue macrophages. A study in the human fetus showed the presence of two accessory cell populations distinguishable by the expression of cell membrane antigens (40). Furthermore, this study demonstrated that this heterogeneity is maintained during fetal development, although both populations seemed to undergo additional phenotypic changes. These results are in concordance with the view that already at an early stage mononuclear phagocytes are committed to one particular phenotype. Recently, data were presented at the eighth "Annual Conference of the European Macrophage Study Group" showing the existence of phenotypically distinct monocyte subsets in humans (41). $CD64^+$ and $CD64^-$ monocytes appeared to be committed to mainly phagocytic and highly efficient antigen-presenting functions, respectively. Further studies are needed to unravel the precise origin of monocyte/macrophage heterogeneity.

1.1.3 Monocytes/macrophages in pulmonary diseases

An important function of macrophages, as the name itself already reveals, is to phagocytose material. These cells are able to phagocytose waste originated in the body itself or from outside the body. For example, worn-out erythrocytes are removed in the spleen, liver and bone marrow by macrophages (44), whereas inhaled carbon particles in cigarette smoke are phagocytosed in the lung by alveolar macrophages (AM) (45,46). Furthermore, they are able to phagocytose and destroy microorganisms (46,47). Phagocytosis is mediated by cell membrane receptors present on monocytes/macrophages, which include Fc receptors (48), complement receptors [CR-1 (CD35) and CR-3 (CD11b/CD18)] (49), and carbohydrate receptors such as the macrophage mannose receptor (50). These properties turn macrophages, as part of innate immunity, into crucial cells in host defense (51). On the other hand, some obligatory or facultative intracellular pathogens use phagocytosis to their advantage, using macrophages to avoid detection and to reach suitable niches within the host (52). *Mycobacterium tuberculosis* represents a notorious example (53). Once inside the macrophage, it prevents phagosome-lysosome fusion and thrives within resident AM eventually causing the death of the host cell. Another microorganism that is able to resist the antibacterial activities of professional phagocytes concerns *Yersinia pestis*. It may cause bubonic or pneumonic plague, and its ability to resist phagocytosis results from the Yop E cytotoxin, a plasmid-encoded protein (54). An example of a viral microorganism which uses macrophages to reach otherwise inaccessible host regions, e.g. the brain, concerns the human immunodeficiency virus (HIV), although its mechanism to "survive" in macrophages differs fundamentally from *M. tuberculosis* or *Y. pestis* (55,56). Some macrophage-tropic HIV strains may integrate in the genomic material of mononuclear phagocytes, turning itself "invisible" for the host defense system. In this way, mononuclear phagocytes may act as major reservoirs of virus infection and the integrated virus may be transported to various organs.

Besides the above-mentioned role as scavenger cells, mononuclear phagocytes are nowadays also known to have important functions in inflammatory and immunological processes (46,57,58). They have been recognized now to initiate and perpetuate or to suppress these processes, acting either as antigen-presenting cells or regulatory cells, respectively (Figure 3). Mononuclear phagocytes, at least some of them in various tissues, are able to take up and process antigens. After endocytosis and processing (59) of a particular antigenic peptide, presentation to $CD4^+$ T cells requires the expression of MHC class II molecules (for a recent review: 60). MHC class II is expressed as an α/β heterodimer on the cell surface of some cell types. A third protein, the invariant chain, is characteristic of newly synthesized MHC class II and may help in optimal processing and presentation of antigens. Most mononuclear phagocytes express MHC class II molecules. It is now known that processed antigenic peptides may already associate with MHC class II molecules intracellularly within lysosomes before surface expression. Processed antigen expressed ("presented") on the cell surface in association with MHC class II may interact with specific T cell receptors (which are associated with the CD3 Ag) leading to T cell activation (61). Antigen presenting capacity can be influenced by environmental factors.

Exposure to T cell derived cytokines such as IFN-γ and IL-4 induces an enhanced expression of MHC class II molecules resulting in increased antigen presenting capacity of macrophages (62). However, although generally regarded as important antigen presenting cells for antigen-primed or allogeneic T cells, most macrophages seem to be unable to stimulate the proliferation of naive syngeneic T cells (63,64). On the other hand, dendritic cells seem to be specialized in stimulation of these T cells (63,64). The latter findings leave us with an apparent paradox of a cell type with, on the one hand, high efficiency of presentation of antigens which require processing, and, on the other hand, poor phagocytic and processing activity.

Table 1. Major secretory products of mononuclear phagocytes.*

Main group of products	Individual products		
cytokines	IL-1α	IL-8	IFN-α
	IL-1β	IL-10	TNF-α
	IL-1RA	IL-12	TNF-β
	IL-6	IL-15	
growth factors	G-CSF	FGF	
	M-CSF	IGF-1	
	GM-CSF	MIP 1+2	
	TGF-β	PDGF	
arachidonic acid metabolites	5-lipoxygenase products		
	cyclooxygenase products		
	PAF		
enzymes and enzyme inhibitors	proteases	phosphatases	α1-anti-proteinase
	lipases	glycosidases	α2-macroglobulin
	DNases	sulphatases	
	RNases	arginases	
clotting factors and complement components	factor VII, IX, X		
	C3a, C3b, C5a		
	C1, C2, C3, C4, C5		
plasma binding proteins	transferrin		
	lactoferrin		
	transcobalamin		
	apolipoprotein E		
reactive oxygen and nitrogen products	superoxide		
	hydrogen peroxide		
	nitric oxide		

* Adapted from references 14, 15, 46, 58, 75 and 183.

Interestingly, in some viral infections macrophages appeared better antigen presenting cells than dendritic cells (65). Whether dendritic cells differentiate *in vivo* from monocytes and acquire this specific antigen presenting capacity within a particular micro-environment or arise from a distinct precursor within the bone marrow is still disputable (60). Evidence from recent *in vitro* studies shows that dendritic cells may be generated from blood monocytes (8-11).

The affinity of MHC class II-bound antigen for T cell receptors is low, and additional protein interactions are required for optimal T cell activation. It is now recognized that, besides MHC class II molecules, also other accessory molecules are of great importance in optimal antigen presentation. The way in which antigen presenting cells and T cells interact is greatly facilitated by adhesion molecules and other accessory molecules. Interactions of MHC class II, LFA-1 (CD11a/CD18), LFA-3 (CD58) and B7 (CD80) with CD4 Ag, ICAM-1 (CD54), CD2 and CD28 Ag, respectively, are best known (66-69; for recent reviews: 70,71). Both the surface expression of these adhesion molecules and the actual results of protein-ligand interaction have been shown to be regulated by cytokines (62,72-74).

In addition to their ability as antigen presenting cells to initiate immunological and inflammatory reactions, mononuclear phagocytes are also potent regulatory cells. This function is created by their ability to secrete over a hundred biologically active molecules and their ability to respond to stimuli through specific receptors for these stimuli (58,75-77). Table 1 shows a summary of substances which can be secreted by mononuclear phagocytes, whether or not after activation or after reaching a certain stage of maturation or differentiation. This potency of mononuclear phagocytes to secrete such a diverse arsenal of biologically active molecules turns these cells into modulators of many physiological processes, such as inflammation, atherosclerosis (78), wound healing (79) and defense against malignancy (80,81) or infection. Such an important role as "process-modulator" can only be performed properly if mononuclear phagocytes are also able to perceive environmental signals. These signals may include direct cellular and matrix interactions or secreted molecules, and are picked up by cell membrane receptors or intracellular receptors (76). Direct cellular and matrix interactions are, from the mononuclear phagocytes' side, mediated by cell membrane receptors. Many of these belong to the integrin superfamily and interact with specific ligand-proteins from the side of the interacting cell or matrix. Soluble molecules secreted by other cells may influence mononuclear phagocytes through specific cell membrane receptors (e.g. receptors for several cytokines) or intracellular receptors (e.g. receptors for various steroids). Table 2 shows a summary of soluble mediators to which mononuclear phagocytes have been shown to be receptive. Beholding the extensiveness of possibilities to influence other cells or to be influenced by other cells *in vitro*, makes us realize that the *in vivo* interactions involving mononuclear phagocytes may be at least as complex and that safe therapeutical intervention still needs further understanding.

Because of their above-mentioned immunological, inflammatory and regulatory properties, and because of their ubiquity in the lung (interstitial monocytes/macrophages, AM, dendritic cells), mononuclear phagocytes are supposed to be pivotal cells in immunological and inflammatory processes underlying many pulmonary diseases.

Table 2. Soluble mediators which have been shown to affect mononuclear phagocytes' function or phenotype.*

Mediator	Action on mononuclear phagocytes
IL-1	chemotaxis; release of IL-1, TNF and CSF
IL-1RA	antagonization of IL-1 effects
IL-2	production of TNF-α
IL-3	maturation/differentiation
IL-4	enhanced MHC class II, CD23 and CD13 Ag expression; maturation/differentiation; decreased production of IL-1 and TNF-α
IL-7	pro-inflammatory actions (increased production of IL-1, IL-6 and TNF-α; increased tumoricidal activity)
IL-10	anti-inflammatory actions (decreased production of IL-1, IL-6, IL-8, TNF-α, GM-CSF and PGE_2; decreased tumoricidal activity)
IL-13	comparable to IL-4 effects
TNF-α	chemotaxis; release of PAF, IL-1 and PGE_2
IFN-α	modulation of activation
IFN-β	modulation of activation
IFN-γ	enhanced MHC class II expression; enhanced bactericidal and tumoricidal activity; enhanced antigen presenting capacity; increased production of IL-1 and TNF-α
TGF-β	chemotaxis; modulation of activation
PDGF	chemotaxis
M-CSF	maturation/differentiation; secretion of IFN-α, TNF-α and G-CSF
GM-CSF	maturation/differentiation
MCP-1	chemotaxis
RANTES	chemotaxis
glucocorticoids	maturation/differentiation; modulation of activation
vitamine D	maturation/differentiation
LPS	induction of immediate early genes (proto-oncogenes, e.g. c-fos, c-myc or c-fms) and cytokine expression (e.g. IL-1 and TNF-α)
eicosanoids	chemotaxis
PAF	chemotaxis
C5a	chemotaxis

* Adapted from references 46, 76, 77 and the relevant references in Table 3.

Their role in antigen-mediated or allergic lung diseases, such as asthma and extrinsic allergic alveolitis, is generally accepted because of their antigen-presenting capacity. The initial event in allergic asthma is thought to be presentation of antigen by dendritic cells/monocytes/macrophages to T lymphocytes as described above. It is still uncertain whether this presentation takes place right in the alveolar compartment where antigens are deposited. Another possible site where antigen presenting cells, after migrating from the alveoli, could interact with lymphocytes may be the draining lymph nodes. Eventually, this cellular interaction results in the production of inflammatory mediators by both antigen presenting cells and T lymphocytes (Figure 3). Furthermore, T lymphocytes will develop into Th2 lymphocytes. This subset of T cells is able to produce certain cytokines (IL-4, IL-5, IL-10 and IL-13) which play a pivotal regulatory role in the pathogenesis of allergic asthma. The development of Th2 cells is influenced by the balance between different cytokines (and probably hormones) in the cellular microenvironment. IL-4, and probably specific factors

produced by antigen-presenting cells, play an important role in the development of these Th2 cells. The initial cellular source of IL-4 is still uncertain, but at present research is focussed on mast cells and a subpopulation of T lymphocytes (Figure 4) (82).

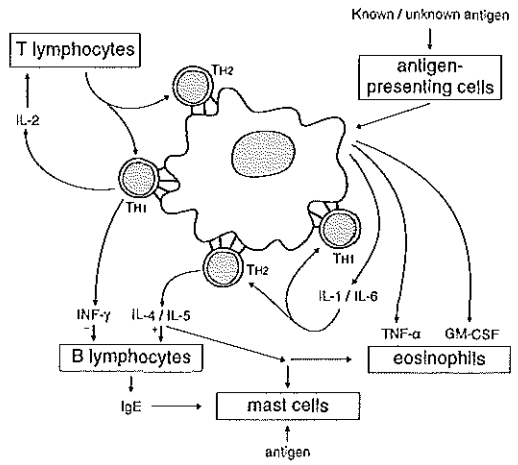

Figure 3. Hypothetical and simplified cellular interactions in the pathogenesis of allergic inflammatory lung diseases. The different cell types are boxed.

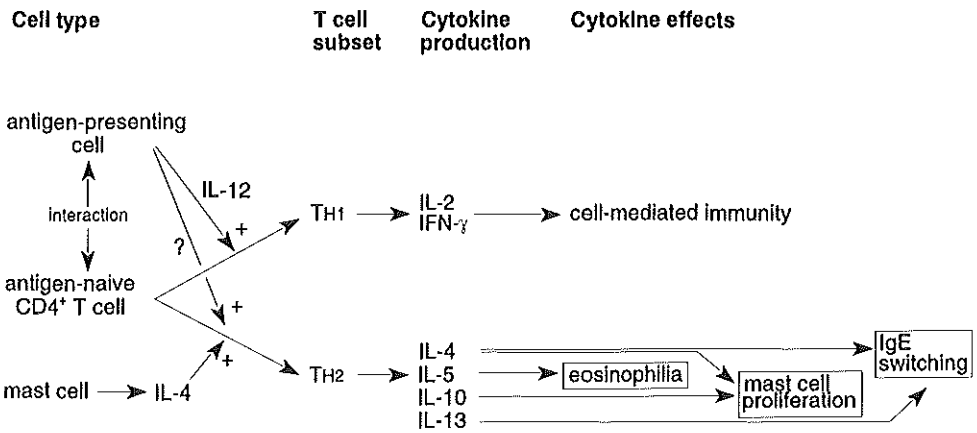

Figure 4. Hypothetical role of cytokines in the development of $CD4^+$ T cell subsets. The effects of cytokines produced by Th2 cells which may play a role in allergic asthma are boxed.

The interaction of T cells with antigen-presenting cells results in the production of a range of cytokines. This will lead to the recruitment and activation of other cell types (e.g. eosinophils and neutrophils, other mononuclear phagocytes and lymphocytes) at the initial

site of antigen deposition in the lung. In allergic asthma, the first contact of T lymphocytes with an antigen presented by mononuclear phagocytes will eventually result in help to B cells to produce specific antibodies. In this way, a person becomes sensitized to the antigen. Every following contact with the antigen will lead to activation of multiple cell types with release of inflammatory mediators, resulting in clinical symptoms. Acute asthmatic symptoms are thought to result from humoral, IgE dependent reactions. B lymphocytes producing these antibodies are at the end of the cellular cascade, but mononuclear phagocytes play the primary and initial role in the generation of these symptoms (Figure 3) (83,84). The late asthmatic symptoms and complaints of bronchial hyperresponsiveness are believed to be caused by (chronic) inflammation of the bronchial wall (85). This inflammation is characterized by infiltrates of T cells and mononuclear phagocytes, stressing again the importance of the latter cell type not only in the initiation, but also in the effector phase of the inflammatory response. The question why not everybody exposed to a particular antigen develops allergic symptoms, is still the base of many research projects.

In addition to their powerful pro-inflammatory functions, a subset of mononuclear phagocytes have also been postulated as important suppressors of T cell-mediated inflammation (86,87). In the healthy lung, pro-inflammatory and immunosuppressive mononuclear phagocytes are balanced in proportion and activity, but in bronchial biopsies of asthmatic patients a reduced proportion of immunosuppressive macrophages was found (86).

Presentation of a hypothesized, but still unidentified antigen by mononuclear phagocytes may underlie the cellular etiology in some lung diseases (88). In sarcoidosis, the limited diversity of the T cell receptor in junctional regions suggests a response from a specific antigenic stimulus (89,90). Furthermore, aberrant cytokine release from AM has been demonstrated in sarcoidosis, suggesting dysregulation of the immune response (91,92).

In granulomatous lung diseases, mononuclear phagocytes are known to be required to generate granulomas. Within a certain microenvironment, these cells mature into epithelioid cells, which, upon fusion, develop into multinucleated giant cells, also called Langhans cells (22,23). A better understanding of the pathogenesis of granulomatous inflammations will come from further insight in the mechanisms underlying maturation, differentiation and functioning of the mononuclear phagocytes involved.

Also in fibrotic lung diseases, either primary or secondary, mononuclear phagocytes are an essential cell type because of the production of factors that cause accumulation, activation and proliferation of structural cells (93). Mononuclear phagocytes are able to release a great variety of such factors. Platelet-derived growth factor (PDGF) is chemotactic and mitogenic for both fibroblasts, neutrophils and monocytes (for a review: 94). The proto-oncogene c-*sis* encodes for the PDGF-B chain, and its expression is increased in AM from patients with idiopathic pulmonary fibrosis (IPF)(95). PDGF secreted by AM may bind to structural cells, e.g. fibroblasts, or smooth muscle cells. Next, these cells become activated to produce an excess of extracellular matrix molecules resulting in fibrotic scarring. Alveolar macrophage-derived growth factor (AMDGF) is constitutively produced

by AM from patients with IPF (96). Together with PDGF and fibronectin, it stimulates proliferation of fibroblasts. This growth factor has been shown to be identical to a high molecular weight form of insulin-like growth factor-I (IGF-I, somatomedin-C) (97). Transforming growth factors (TGF) produced by activated AM are chemotactic for both monocytes and fibroblasts (98-100). They also stimulate monocytes and fibroblasts to produce growth factors and extracellular components, respectively (99,101, for a recent review: 102). The production of tumor necrosis factor (TNF)-α by AM may stimulate migration of neutrophils across the vascular endothelium and growth of fibroblasts (103,104). Therefore, also TNF-α released by AM may be an actual mediator in the pathogenesis of fibrotic lung diseases. Recently, the production of TNF-α by PBM-derived macrophages in cystic fibrosis was shown to be substantially higher than in normal macrophages (105). This appeared to be due to an increased rate of TNF-α gene transcription by cystic fibrosis macrophages, and may underlie the cachexia commonly seen in these patients.

In conclusion, mononuclear phagocytes should be considered as important cells in many diseases, including pulmonary diseases. On the one hand, they protect us, thanks to their phagocytosing and microbicidal capacities, against invading microorganisms. In this way, they may prevent infectious diseases. On the other hand, mononuclear phagocytes are also recognized as central cells in the pathogenesis of lung diseases with either immunological or inflammatory components, because of their ability to present antigens, to secrete a multitude of mediators, and to respond to environmental stimuli. In this way, they are able to control the function of many other cell types. Better insight in the functioning of mononuclear phagocytes will hopefully lead to new possibilities to intervene in the course of pulmonary diseases.

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1.2 CYTOKINES

With emphasis on their production by and influence on monocytes/macrophages

1.2.1. Historical background

Communication between cells, which in concerted action perform immunological or inflammatory tasks, seems obvious at present, and it is known now that cells interact through direct cell-to-cell contacts and through soluble or cell-membrane-bound signalling proteins, called cytokines (106). However, it was only in 1966 that for the first time it was realized that a soluble factor released in cultures of antigen-sensitized lymphocytes could influence the function of phagocytes (reviewed in 39,107). This specific factor was named migration inhibitory factor or MIF. In the following years various biological activities were demonstrated in the culture broth from antigen-stimulated lymphocytes. In 1969, the generic term lymphokines was suggested to be used to describe such biological activities (107). In 1979 the fist attempt was made to devise a nomenclature for lymphokines that clustered certain biological activities and was independent of particular bioassays (108,109). The name interleukin (IL) was chosen describing the communication between white blood cells. At the same time lymphocyte activating factor (LAF), B cell activating factor, B cell differentiation factor and mitogenic protein were classified as IL-1, whereas T cell growth factor, thymocyte mitogenic factor and killer cell helper factor were classified as IL-2. At the sixth "International Congress of Immunology" in 1986 it was agreed that a new interleukin would first be named according to its biologic property or properties, but once the amino acid sequence of the human form was established, it would be assigned an interleukin number (111). Since that time, fifteen interleukins have been described (111-132). Furthermore, the interleukins are nowadays considered as part of a larger group of protein molecules, referred to as cytokines, including the interleukins, interferons, tumor necrosis factors and growth factors (133). This heterogeneous group of cytokines has a number of characteristics:

1. cytokines are low molecular weight (<30 kDa in monomeric form) proteins which are often glycosylated;
2. cytokines regulate a wide spectrum of biologic events relevant to inflammation, immunity, metabolism, cell growth, cell maturation and differentiation, fibrogenesis, and homeostasis;
3. cytokines are usually produced transiently and locally, acting in a paracrine or autocrine, rather than endocrine, manner;
4. cytokines are extremely potent, often being active at nanomolar or picomolar concentrations;
5. interaction of cytokines with high affinity cell surface receptors eventually leads to modulation of RNA and protein synthesis, which results in an altered cell function;
6. individual cytokines have multiple (pleiotropic) activities whilst different cytokines may mediate similar effects (redundancy);
7. cytokines interact in a network by inducing each other, influencing cytokine cell surface receptor expression, and by synergistic, additive or antagonistic effects on cell function;
8. the genes for several cytokines are clustered on chromosomes (IL-1α/β, IL-1RA and IL-1R on chromosome 2; GM-CSF, M-CSF, M-CSFR, IL-3, IL-4, IL-5 and IL-13 on chromosome 5), which may explain why these factors are expressed in a coordinated fashion following cell activation (134,135).

Table 3. Cellular sources and a selection of effects of human cytokines.

Cytokine	Other name	Cellular source	Biological action	Reference
IL-1α/β	BAF, ETAF, LAF	mononuclear phagocytes, endothelial cells, NK cells, B cells, fibroblasts, keratinocytes	maturation and proliferation of B and T cells; stimulation of arachidonic acid metabolism; secretion of collagenase, elastase and plasminogen activator; fever	111
IL-2		T cells (Th1-like)	mitogenic for thymocytes, T and B cells and NK cells; enhanced cytotoxic activity of monocytes	112,113
IL-3	multi-CSF	T cells	hemopoietic growth and differentiation factor	114
IL-4	BSF-I	T cells (Th2-like), mast cells	proliferation of T and B cells; induction of IgE and IgG synthesis; induction of MHC class II, CD13 and CD23 Ag expression on mononuclear phagocytes	28,29,31, 63,115
IL-5	BCGF-II, TRF	T cells (Th2-like)	activation of B cells and eosinophils; chemotactic for eosinophils	116
IL-6	BSF-2, IFN-β2, HSF	mononuclear phagocytes, B cells, T cells, endothelial cells, fibroblasts, keratinocytes	induction of acute phase proteins; synergistic with IL-1; induction of IL-2R on T cells	117,118
IL-7		T cells, fibroblasts	mitogenic for thymocytes and T cells; induction of IL-2R on T cells; induction of IL-1, IL-6, IL-8, TNF-α	119,120, 121
IL-8	NAP-1	mononuclear phagocytes, endothelial cells, epithelial cells, fibroblasts, keratinocytes	chemotactic for and degranulation of PMN	122
IL-9	P40, MEA	T cells	growth factor for some T cell lines; production of IL-6 by IL-3-dependent mast cell lines	123,124
IL-10		mononuclear phagocytes, T cells (Th2-like), B cells	decreased production of IL-1, IL-6, IL-8, TNF-α, GM-CSF, PGE_2	125,126
IL-11		fibroblasts	proliferation and maturation of megakaryocytic and myeloid precursor cells	127
IL-12	NKSF	mononuclear phagocytes, B cells	induction of IFN-γ; augmentation of NK cell-mediated cytotoxicity; initiation of cell-mediated immunity	128,129
IL-13		T cells	many of the effects are similar to IL-4	130
IL-14	HMW-BCGF	T cells, certain malignant B cells	proliferation of B cells; inhibition of immunoglobulin secretion	131
IL-15		adherent mononuclear phagocytes, epithelial and fibroblast cell lines	biological activity resembles that of IL-2	132

Table 3. Cellular sources and a selection of effects of human cytokines (continued).

Cytokine	Other name	Cellular source	Biological action	Reference
IFN-α	viral IFN, type I IFN	mononuclear phagocytes, NK cells, B cells, fibroblasts	induction of antiviral state; fever	139
IFN-β		fibroblasts, epithelial cells	induction of antiviral state; fever	139
IFN-γ		T cells (Th1-like), NK cells	enhanced phagocytosis and tumoricidal activity of mononuclear phagocytes; induction of MHC class II on many cell types; fever	136,139
G-CSF	MGI-2	mononuclear phagocytes, fibroblasts, endothelial cells	maturation/differentiation of granulocytes	140-143
M-CSF	MGF, CSF-1	mononuclear phagocytes, fibroblasts, endothelial cells	maturation/differentiation of mononuclear phagocytes	140,141
GM-CSF	CSF-2, pluripoietin-α	mononuclear phagocytes, T cells, fibroblasts, endothelial cells, mast cells	maturation/differentiation of both granulocytes and mononuclear phagocytes	140,141, 143
IGF	somatomedin, AMDGF	mononuclear phagocytes	growth of tissues	144
FGF		mononuclear phagocytes	induction of neovascularization; mitogenic for endothelial cells	145
MIP-1α/β		mononuclear phagocytes	chemokinetic for mononuclear phagocytes and PMN; fever	146,147
MCP-1	MCAF	keratinocytes, lymphocytes, fibroblasts	chemotactic for mononuclear phagocytes	148,151
RANTES		T cells	migration of blood monocytes and T cells	149-151
MIP-2		mononuclear phagocytes	chemokinetic for PMN	151
PDGF		platelets, endothelial cells, epithelial cells, mononuclear phagocytes	mitogenic for fibroblasts, epithelial cells, capillary endothelial cells and arterial smooth muscle cells; wound repair; chemotactic for mononuclear phagocytes	93,152
TGF-α		epithelial cells, keratinocytes, fibroblasts	proliferation of cells; angiogenesis	153
EGF	β-urogastrone	epithelial cells, keratinocytes, fibroblasts	proliferation of cells; angiogenesis	153
TNF-α/β	cachectin	mononuclear phagocytes, T cells	necrosis of certain tumors; growth of fibroblasts; angiogenesis; fever	154
TGF-β		mononuclear phagocytes, platelets, fibroblasts	inhibition of IL-2 effects and proliferation of certain cells; increased gene expression of collagens	100,101

Abbreviations used in Table 3. Cellular sources and a selection of effects of human cytokines.

AMDGF	: alveolar macrophage-derived growth factor
BAF	: B cell activating factor
BCGF	: B cell growth factor
BSF	: B cell stimulatory factor
CSF	: colony stimulating factor
EGF	: epidermal growth factor
ETAF	: epidermal cell-derived T cell activating factor
FGF	: fibroblast growth factor
G	: granulocyte
HSF	: hepatocyte-stimulating factor
HMW-BCGF	: high molecular weight B cell growth factor
IFN	: interferon
IGF	: insulin-like growth factor
IL	: interleukin
LAF	: lymphocyte-activating factor
M	: macrophage
MCP	: monocyte chemoattractant protein
MCAF	: monocyte chemotactic and activating factor
MEA	: mast cell enhancing activity
MGF	: macrophage/granulocyte factor
MGI	: macrophage/granulocyte inducer
MIP	: macrophage inflammatory protein
NAP	: neutrophil-activating peptide
NKSF	: natural killer cell stimulatory factor
PDGF	: platelet-derived growth factor
PMN	: polymorphonuclear cells
RANTES	: regulated upon activation, normal T expressed, and presumably secreted
R	: receptor
TNF	: tumor necrosis factor
TGF	: transforming growth factor
TRF	: T cell replacing factor

Although once it was thought that a certain cytokine directed its single activity at one particular cell type, it is nowadays appreciated that different cytokines may direct the same change in cell function (136,137), that a single cytokine may effect a multitude of activities, and that these activities may concern different cell types (138). Furthermore, the biologic effect of cytokines are often situation-specific, since they vary depending on the concentration of the cytokine, the cellular stage of activation, maturation and differentiation, and the presence of other cytokines or hormones in the local microenvironment (30, 36,38,39). This context of a particular cytokine determines to a great extent the ultimate effects observed. Table 3 summarizes human regulatory proteins that are at present accepted cytokines, together with their cellular sources and some of their biological effects. It should be emphasized that in this table only the most important effects are selected, and that for the purpose of a complete overview of cytokine effects one has to turn to the references.

1.2.2 Clinical implications

The important role played by cytokines in numerous physiological processes, as mentioned above, constitutes a challenge to clinicians to use these molecules in clinical settings. Moreover, the genetic material of many of these cytokines has been cloned and it is possible to produce huge amounts of pure recombinant cytokines. The cytokines that direct normal hemopoiesis are now being used in a variety of clinical protocols, comparable to the use of erythropoietin (which is, however, considered to be a hormone, rather than a cytokine) to control anemia in chronic renal failure and recently in sickle cell disease (155,156).

G-CSF and GM-CSF are being used in cancer patients with leucopenia induced by chemotherapy and/or irradiation (157,160). At present, these cytokines are administered subcutaneously and adverse effects are mild in case of G-CSF (local rashes, fever, discomfort over bones containing hemopoietic tissue) (161). In some patients, high doses of GM-CSF may have more serious side effects such as capillary leak syndrome, fever, rashes, pericarditis, and pleural effusion (161). Furthermore, the use of these cytokines may entail the risk to convert preleukemic cells to fully neoplastic leukemic cells as also leukemic cells may possess cytokine receptors. Recently, aerosolized GM-CSF was used in nonhuman primates, and was shown to increase the population of macrophages and neutrophils (162). Further studies are needed to determine whether aerosol delivery of GM-CSF will be of use in the treatment of pulmonary diseases.

The gravity of thrombocytopenia induced by treatment with high-dose carboplatin in cancer patients could be diminished by IL-1α (163). The latter, beneficial application of IL-1α contrasts with other more harmful effects of IL-1, as IL-1 is also known to mediate, in part, fever, sleep, anorexia, shock, arthritis, colitis, diabetes and atherosclerosis (164).

Also IL-2 is currently being used in the treatment of various diseases (165-167). It has been shown to be of benefit in cancer and infection with HIV (165,166). Recently, IL-2 was reported to enhance immunity in common variable immunodeficiency which allowed stopping of immunoglobulin infusions (167).

The potential anti-inflammatory effects of IL-4 observed in several *in vitro* experiments are now being tested in *in vivo* studies (168-170). On the contrary, blocking IL-4 effects may also be of therapeutical use, e.g. in the treatment of allergy and asthma. In these diseases, disrupted commitment of T cells to Th2 effector functions may underlie the aberrant IgE production (171). The importance of IL-4 in these processes has been stressed recently, and IL-4 was suggested as a critical drug target (171).

In addition to IL-4, also IL-5 has been suggested as a key cytokine in diseases as allergy and asthma (171). Blocking IL-5 activity has been shown to inhibit eosinophilia and bronchial hyperresponsiveness induced by antigen in sensitized animals (172). Therefore, also IL-5 may be a future target for antagonists in the treatment of allergic diseases.

Recently, the local generation of IL-8 was described as a possible therapeutic target for attempts to control the adult respiratory distress syndrome (ARDS) (173,174). ARDS is characterized by increased capillary permeability and increased numbers of neutrophils within the airspaces of the lungs. It has been suggested that IL-8, a neutrophil chemotaxin,

is an actual candidate for the initiation and progression of neutrophil-mediated events in ARDS (174). Early appearance of IL-8 in BAL-fluid of patients at risk of ARDS may be an important prognostic indicator (174). AM are the principal source of IL-8 within the lungs (175,176).

In summary, single-cytokine therapy is now being evaluated in many clinical trials. As the effect of a certain cytokine is greatly determined by the context, sequential or concurrent combination cytokine therapy may prove to be superior in the future (177-179). On the other hand, blocking harmful cytokine effects may also be of potential clinical use. However, one has to realize that, in addition to their antagonizing activities, soluble cytokine receptors and anticytokine antibodies can also agonize cytokine activity (181). The latter activity may result from an increased half-life time of cytokine-cytokine receptor complexes (182). This "double-edged sword" phenomenon may restrict the therapeutic use of such agents (181). The effects of recombinant IL-1 receptor antagonist is presently studied in clinical trials for treating rheumatoid arthritis, septic shock and chronic myeloid leukemias (180).

1.2.3 Cytokines and monocytes/macrophages

Mononuclear phagocytes are able to "communicate" with many other cell types. As a result, mononuclear phagocytes are important regulators of complex multicellular processes, e.g. inflammation (58,82). Part of this "communication" is mediated by soluble or cellular bound cytokines. On the one hand, mononuclear phagocytes are capable of secreting various cytokines (Table 3) (183,184). On the other hand, these phagocytes are receptive to many cytokines produced by either themselves or other cell types (Table 2) (76).

Effects of cytokines

The influence of cytokines on mononuclear phagocytes has been demonstrated in early events during maturation/differentiation. Generation of CFU-GEMM and CFU-GEM, the earliest precursor cells of mononuclear phagocytes, from the pluripotent stem cells (see figure 1), is under control of both IL-3 and GM-CSF (136). Only more mature/differentiated stages have been shown to be responsive to M-CSF (137). The presence or absence of specific cytokine receptors may underlie the sequential responsiveness for different cytokines during cell maturation/differentiation. M-CSF responsiveness may eventually result from induction of the c-*fms* proto-oncogene, which represents the high affinity receptor for M-CSF, by GM-CSF (137). *In vitro*, it has also been demonstrated that IL-6 and IFN-γ are able to potentiate GM-CSF and M-CSF effects (185). Furthermore, maturation/differentiation of mononuclear phagocytes can be modulated by IL-4 (28).

Besides the effects on maturation/differentiation, some cytokines may induce chemotactic responses in mononuclear phagocytes (Table 4). The β-chemokines (e.g. MIP-1, MCP-1 and RANTES) are chemotactic for mononuclear phagocytes, whereas another

related family of cytokines, the α-chemokines (e.g. IL-8 and NAP-2), is only chemotactic for neutrophils and lymphocytes (186). Also TGF, PDGF and TNF may attract mononuclear phagocytes (98,152,154).

As regulators of complex multicellular processes *in vivo*, many functions of mononuclear phagocytes can be affected by activating and inhibitory signals from their micro-environment. Some cytokines give mononuclear phagocytes properties which may eventually increase the degree of inflammation (Tables 2 and 4). The most important of these cytokines are IL-1, IL-7 and TNF (121,137). In the presence of these cytokines, mononuclear phagocytes enhance the production of other pro-inflammatory mediators (IL-1, IL-6, TNF-α, PAF, PGE_2). Other cytokines may eventually result in a decrease of inflammatory activities (Tables 2 and 4).

Table 4. Functions of mononuclear phagocytes which can be modulated by cytokines.

Function	Cytokine
maturation/differentiation	M-CSF, GM-CSF, IL-3, IL-4, IL-6, IFN-γ
chemotaxis	β-chemokines (MIP-1, MCP-1, RANTES), TNF, TGF, PDGF
pro-inflammatory activities	IL-1, IL-4, IL-7, IFN-γ, TNF-α
anti-inflammatory activities	IL-1RA, IL-4, IL-10, IL-13, TGF, IFN-β

The most important potential terminators of an inflammatory response are IL-1RA (180), IL-4 (168,169) and IL-10 (125,126). They are all able to decrease the production of other mediators (IL-1, IL-6, TNF-α, GM-CSF, PGE_2) in mononuclear phagocytes. Here, it is essential to stress again that the actions of cytokines can only be understood in "context", and can not be categorized as pure stimulators or inhibitors. A particular cytokine may have either pro-inflammatory or anti-inflammatory properties, subject to additional signals. A striking case in point is IL-4, which may induce MHC class II resulting in enhanced antigen presenting capacities (62). However, IL-4 may also decrease the production of IL-1 and TNF-α, enabling a decrease of the acute inflammatory response (168,169).

Production of cytokines

One of the earliest examples of cytokines produced by mononuclear phagocytes are IL-1 (137,187) and TNF-α (137,188,189). The IL-1 family consists of three structurally related polypeptides (164). The first two are IL-1α and IL-1β, each of which has a broad spectrum of both beneficial and harmful biologic actions. The third is IL-1RA, which inhibits the activities of IL-1. IL-1 was originally recognized for its ability to induce proliferation of murine thymocytes (187), but is now also known as an important mediator of coagulation, inflammation and fibrosis (137,164). Functions of a great variety of different cell types may be modulated by IL-1. IL-1RA is a specific inhibitor of IL-1 activity that acts by blocking the binding of IL-1 to its cell surface receptor. It provides some protection against the disease-provoking effects of IL-1 (180). TNF-α was first recognized for its ability to induce hemorrhagic necrosis of certain tumors (190). Besides this cytotoxicity, it has variable

growth activity for fibroblasts, it modulates angiogenesis, induces release of collagenase from structural cells, and produces systemic effects of fever and cachexia (137,154).

Mononuclear phagocytes are the main source of IL-6. It is the cytokine primarily involved in delivering the final signal to hepatocytes for the regulation of protein synthesis during the acute phase protein response (191). Furthermore, IL-6 induces the proliferation of T cells, as well as the expression of the IL-2R on these cells (117).

The chemotaxis and degranulation of neutrophils is regulated by IL-8, which is produced by mononuclear phagocytes and many other cell types (122).

IL-10, IL-12 and IL-15 are also produced by mononuclear phagocytes. IL-10 has been shown to exhibit anti-inflammatory properties. It inhibits the production of many inflammatory cytokines (125,126). Recently, IL-12 was found to induce the differentiation of Th1 cells from uncommitted T cells (129). Consequently, it may play an important role in cell-mediated immunity. The biological activities of IL-15 resemble those of IL-2 (132).

Upon stimulation with LPS or IL-1, mononuclear phagocytes may produce colony stimulating factors, as M-CSF, G-CSF, and GM-CSF (183,192). These factors are important regulators of the maturation/differentiation of myeloid cells.

Mononuclear phagocytes are known to produce IFN-α in response to viruses, bacteria, and tumor cells (193). IFN-α has antiviral and antimitotic effects, and up-regulates MHC class II expression (139).

By means of the production of TGF-β, mononuclear phagocytes are able to modulate many cellular functions, e.g. replication (97). The response of cells to TGF-β is complex, and depends upon the cell type, state of activation, and exposure to other cytokines (101). Cells that respond to TGF-β by undergoing mitosis do so through their ability to secrete and respond in an autocrine fashion to PDGF. Mononuclear phagocytes themselves are also able to produce PDGF (94,194).

Some growth factors produced by mononuclear phagocytes are characterized by a high affinity for heparin. FGF is mitogenic for endothelial cells and represents a potent inducer of neovascularization (145). MIP-1 is produced in response to stimulation with LPS. It is chemotactic for neutrophils, and may induce fever (146,147).

IGF-1 is synthesized by mononuclear phagocytes and other cells in response to growth hormone and mediates its trophic actions (144). It stimulates proliferation of a variety of structural cell types, including fibroblasts, smooth muscle cells and chondrocytes. AM synthesize and secrete a high molecular weight form of IGF-1, which was originally named AMDGF (95,96).

In conclusion, both the production of cytokines by mononuclear phagocytes and the numerous effects of cytokines on their function are likely to play an important role in inflammatory processes and immune reactions. Everyday, interactions between cytokines and these cells seem to become more complex because of new observations (195). Careful interpretation of *in vitro* results will eventually lead to insight into the *in vivo* processes and may open new therapeutic possibilities.

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1.3 GLUCOCORTICOIDS

With emphasis on their influence on monocytes/macrophages

1.3.1 Historical background

Although glucocorticoids are known by almost everybody as a powerful drug feared because of their sometimes impressive adverse effects (196-198), they are still ordinary hormones physiologically present in everyone's body (199). They are synthesized in the adrenal cortex, secreted and circulating at a concentration that fluctuates in a circadian mode (200). In humans the natural glucocorticoid is hydrocortisone (cortisol). It has several vital functions (199,201-202). Life would be impossible without functioning adrenal glands, unless the absent cortisol is substituted for by exogenous glucocorticoids (199,203). Plasma cortisol levels are elevated during periods of stress, probably preventing the body's defense reactions against stress from overshooting and preventing needless tissue damage (201,202). Just these inhibitory and protective effects of glucocorticoids are being used nowadays in the treatment of several diseases. Cortisone, the natural precursor of cortisol, was used first in the treatment of rheumatoid arthritis in 1949 (204). Within a few years glucocorticoids were also used in the treatment of other diseases and in 1950 their beneficial effects in asthma patients were described (205). Cortisone was initially administered intramuscularly, but at present glucocorticoids can be administered also orally, intravenously or via inhalation (206). Furthermore, synthetic glucocorticoids, much more potent than cortisol and without the unwanted mineralocorticoid side effects, have been developed (207-209).

1.3.2 Clinical implications

After their introduction in the treatment of rheumatoid arthritis, glucocorticoids were soon used in the treatment of other diseases with inflammatory or disrupted immunological components (210,211). Since 1950 glucocorticoids are being used in the treatment of various lung diseases (212). In some pulmonary diseases their use is of undisputable value now, whereas in others their use is still controversial (see below and Table 5). In the last decade, insight in the cellular and subcellular mechanisms underlying lung diseases has increased tremendously thanks to new cellular and molecular biological techniques (213,214). With this, also the beneficial effects of glucocorticoids in lung diseases became clear at the cellular and subcellular level (215,216). Hopefully, this knowledge will eventually help to decide whether or not glucocorticoid treatment will be of use in a particular clinical setting. Table 5 gives an overview of pulmonary diseases in which glucocorticoids have become a cornerstone of treatment. With regard to the dosage scheme of oral glucocorticoid treatment it is generally accepted now that alternate-day therapy can prevent or ameliorate the manifestations of Cushing's syndrome, avert or permit recovery from hypothalamus-pituitary-adrenal axis suppression, and is as effective (or nearly as effective) as continuous therapy (197,240,241).

Table 5. Lung diseases in which glucocorticoid treatment is generally accepted or under investigation.

Lung disease	Comment	References
asthma	glucocorticoids generally accepted in reducing BHR; important role of maintenance treatment with inhaled glucocorticoids	217-219
COPD	subgroups of COPD patients may benefit from glucocorticoids	220
tuberculosis: menigitis	use of glucocorticoids controversial	221,222
pericarditis	indications for use of glucocorticoids clear	223
PCP	early adjunctive treatment with glucocorticoids reduces the risks of respiratory failure and death in patients with AIDS and PCP	224,225
sarcoidosis	glucocorticoids used in hypercalcemia or impaired pulmonary function	226-228
EAA	glucocorticoids used to inhibit or delay fibrosis	227-229
fibrosis	glucocorticoids may slow down the progression to end stage fibrosis	227,228,230
PIE syndrome	use of glucocorticoids in chronic or recurrent cases	228
drug induced pneumonitis (e.g. methotrexate, amiodarone, O_2)	glucocorticoids may have some benefit, but results are inconsistent	231,232
ARDS	no benefit from glucocorticoids early in the disease; glucocorticoids being used in a trial 7 to 14 days after the onset of ARDS	233
aspiration pneumonitis	glucocorticoids may prevent ARDS when administered within 12-24 hours after aspiration	234
neonatal respiratory distress syndrome	glucocorticoids given to mother before parturition	235
lung transplantation or lung conditions secondary to general autoimmune diseases (e.g. RA,SLE, scleroderma, Wegener's disease)	glucocorticoids prevent rejection or may be used to treat the primary disease, usually in combination with other immunosuppressive agents (e.g. azathioprine or cyclophosphamide)	236,237
neoplasms	glucocorticoids (in combination with chemotherapy or radiation) used as either cytostatic drug or anti-emetic agent	238,239

Abbreviations used:
ARDS : adult respiratory distress syndrome
BHR : bronchial hyperresponsiveness
COPD : chronic obstructive pulmonary diseases
EAA : extrinsic allergic alveolitis
PIE : pulmonary infiltration with blood eosinophilia
PCP : *Pneumocystis carinii* pneumonia
RA : rheumatoid arthritis
SLE : systemic lupus erythematosus

This has been shown in many non-lung diseases, but also in asthma, sarcoidosis and rheumatoid arthritis associated lung conditions. If alternate-day therapy is unsuccessful because symptoms of the underlying disease occur on the second day, the dosage scheme may be changed to a daily single dose early in the morning. Currently, the use of (intravenous) high-dose pulse therapy is being studied, but up to now its benefits remain controversial (242).

Table 6. The influence of glucocorticoids on the synthesis of proteins with inflammatory effects in human cells or tissue.

Protein	Cell/tissue	Glucocorticoid effect	References
IL-1β	BAL mononuclear phagocytes, monocytic cell line (U937)	*decreased* mRNA expression *in vitro*; blocking post-transcriptional IL-1 synthesis	249,250
IL-2	T lymphocytes	*decreased* gene transcription/mRNA stability	251,252
IL-5	PBM	*decreased* gene expression *in vitro*	253
IL-6	monocytes, endothelial cells, fibroblast cell line	*decreased* mRNA levels and protein production *in vitro*	249,254
IL-8	PBM, AM	*decreased* mRNA levels and protein production *in vitro*	255
TNF-α	lung fragments	*inhibition* of mRNA transcription	256
IFN-γ	lymphocytes	*decreased* mRNA expression	257
collagenase	transfected cell lines (HeLa)	*decreased* gene transcription as a result of GR/AP-1 interaction	258-260
lipocortins	PBM, AM	induction of mRNA expression *in vitro*; influence *in vivo* unclear	261-270
nitric oxide synthase	macrophage cell line (J774), endothelial cells	*inhibition* of mRNA synthesis	271,272
metallothionein IIa	AM	*increased* mRNA expression	265,273
ICAM-1	monocytic and bronchial epithelial cell line, endothelial cells	*decreased* mRNA expression	245,274
ELAM-1	endothelial cells	*decreased* mRNA expression	245
GM-CSF	fibroblasts	*decreased* mRNA stability/ mRNA transcription	275

Although probably all cells in humans possess glucocorticoid receptors, humans are relatively insensitive to the effects of glucocorticoids as compared with rodents (243). This is of great importance when extrapolating glucocorticoid effects in laboratory animals to

humans. One of the first effects of a single dose of glucocorticoids in humans concerns the numbers of circulating leukocytes (244,245). Within 4 to 6 hours a lymphopenia and granulocytosis are the result of redistribution of circulating lymphocytes to other lymphoid compartments and of delayed egress of granulocytes from the intravascular space, respectively. Steroid-altered interactions of lymphocytes and granulocytes with endothelial cells through cell membrane proteins may underlie these effects (246). Furthermore, the number of blood monocytes is decreased (244,245). These effects were the first observed cellular changes induced by glucocorticoids. At present, a lot of subcellular effects of glucocorticoids are known and with this knowledge insight in the working mechanisms of these drugs have increased greatly (213,214). It is known now that glucocorticoids are able to influence cellular protein synthesis (Table 6) (247). These proteins include inflammatory mediators (e.g. cytokines), membrane-bound proteins (e.g. ICAM-1), and enzymes (e.g. collagenase, synthases and phospholipase A_2) which catalyze the production of inflammatory mediators. It should be mentioned that most data are obtained from *in vitro* studies. We understand nowadays that protein mediators (cytokines) and non-protein mediators, which production for their part may be modulated by protein mediators, control to a great extent inflammatory and immunological processes. All this knowledge seems to suggest that the anti-inflammatory working mechanisms of glucocorticoids are clear (see chapter 2 for further detailed information). However, there remain still several unanswered questions. These concern not only virgin research fields but also our recently obtained data. The latter may be exemplified by our current knowledge of lipocortins. In 1986 they were first described to be induced by glucocorticoids and to mediate anti-inflammatory effects (276). Since then, numerous conflicting results were published regarding the inducibility of lipocortins by glucocorticoids *in vivo* and the biological importance of *in vivo* lipocortin-mediated inhibition of phospholipase A_2 (261-270).

Glucocorticoid resistance

Besides the unwanted adverse effects of glucocorticoids in their clinical use, it was also observed that glucocorticoid responsiveness varied among patients (277). Furthermore, syndromes of partial resistance to the physiologically most important glucocorticoid, cortisol, were recognized (for a recent review 278). Hyposensitivity to the glucocorticoid action of cortisol was first described in a family in 1976 (279). This could be explained recently by a defect in the glucocorticoid receptor (280,281). The defect was shown to result from a mutation in the coding sequence. This mutation turned out to be a point mutation with a thymidine substituted for adenine at position 2054 in the ligand-binding domain. This led to an amino acid substitution from aspartate to valine at position 641 of the receptor, resulting in the synthesis of glucocorticoid receptors that are unable to bind their ligand effectively. Furthermore, the molecular basis of two other types of familial glucocorticoid resistance has been elucidated, recently (282-284). In one family, a heterozygous 4 basepair deletion was found, that eventually led to a 50% decrease in the receptor concentration with normal binding affinity (282,283). This reduction was

suggested to result from low glucocorticoid receptor mRNA transcription or rapid degradation of this mRNA. In another family, a point mutation (in position 2317) led to the substitution of valine to isoleucine at amino acid residue 729 again within the ligand-binding domain of the glucocorticoid receptor, resulting in a decreased affinity for its ligand (284; for a recent review 278).

In the reported syndromes of familial, partial resistance to cortisol, patients presented with various symptoms. Most of these symptoms result from compensatory enhanced ACTH levels, which also lead to elevated cortisol concentrations. There are no signs of Cushing's syndrome, but excess ACTH secretion also results in increased levels of both adrenal steroids with salt-retaining activity and adrenal androgens. This may lead to (mild) hypertension, hypokalaemic alkalosis, female masculinization (hirsutism, menstrual irregularities, acne), or abnormalities in spermatogenesis. However, clinical manifestations may be absent, or nonspecific like chronic fatigue as the only symptom (285).

The above-mentioned mutations are rare and the clinical observation of variable clinical response to therapeutically used glucocorticoids may have (an)other explanation(s) (286). The variability can sometimes be explained by variable patient's compliance with taking the prescribed dose of glucocorticoids, mostly as a result of fear of adverse effects. Other causes may be differences in glucocorticoid pharmacokinetics associated with the presence of another disease [e.g. hypo/hyperthyroidism (287,288)], enhanced microsomal activity induced by comedication [e.g. phenobarbital or rifampicin (203,289)], decreased bioconversion of prednisone (290), hypoalbuminemia (291), or the formation of auto-antibodies directed against glucocorticoid-induced proteins such as lipocortins (292). Table 7 shows a summary of theoretical causes of the decreased response to glucocorticoids.

Airway inflammation is believed to be the main cause of bronchial hyperresponsiveness in asthma (84). Therefore, glucocorticoids are frequently used in this disease (217-219). Certain asthma patients, however, fail to respond adequately to glucocorticoid therapy and have been described as suffering from steroid-resistant asthma (277,300,301,304-312). Many mechanisms underlying this apparent resistance have been suggested, but the precise nature remains to be elucidated. On the one hand, a defect in the glucocorticoid responsiveness of blood monocytes has been hypothesized (304-309). Recently, it has been reported that in blood monocytes a defective interaction of the glucocorticoid receptor with transcription factors such as AP-1 may underlie the clinical observation of asthmatics who are less responsive to glucocorticoid treatment (300,301). On the other hand, rapid glucocorticoid clearance (310), abnormal binding of ligand to its receptor (310,311), and reduction in receptor numbers (311) have been described in mononuclear cells. Furthermore, a defective response of T cells to glucocorticoids has been reported in steroid-resistant asthma (302,312). In the patients studied, T cells appeared to be activated persistently, despite steroid therapy (312), whereas IL-2 in combination with IL-4 was shown to reduce the binding affinity of nuclear glucocorticoid receptors for their ligand in mononuclear cells and to reduce the T cell response to glucocorticoids (302). These findings suggest that multiple cellular abnormalities may underlie the clinical presentation of steroid-resistant asthma, and that inflammation in itself may modulate cellular steroid responsiveness via locally produced inflammatory mediators. This mechanism may also

explain the acquired, partial glucocorticoid resistance observed in AIDS-patients (313).

Table 7. Theoretical cellular disturbances underlying decreased glucocorticoid responsiveness.

Disturbance	Comment	References
relatively low levels of ligand	secondary to enhanced steroid metabolism	203,288,289
hampered diffusion through cell membrane	no reports in literature	
decreased number of functional receptors	either decreased number or decreased affinity for ligand	293-297
interactions of (liganded) receptor with other proteins in the cytoplasm	e.g. mutual repression of GR and AP-1 in the regulation of collagenase gene expression	214
hampered translocation of liganded receptor from cytoplasm to nucleus	role of heat shock proteins? no reports in literature (however, interaction of FK 506 with hsp 56 potentiates GR-mediated effects)	(298,299)
altered interactions of liganded receptor with DNA or other transcription factors in the nucleus	1. hypothesized molecular defect in glucocorticoid-resistant asthma	300,301
	2. interaction of inflammation-induced transcription factors (e.g. AP-1) with GR may antagonize glucocorticoid effects	214,302
	3. mutations in the HRR of MMTV affect response to glucocorticoids	303
hampered transcription	no reports in literature	
hampered translation	no reports in literature	
posttranslational interference	auto-antibodies against lipocortins	292

Abbreviations used: AP-1 : activator protein-1; GR : glucocorticoid receptor; HRR : hormone response region; hsp : heat shock protein; MMTV : mouse mammary tumor virus

1.3.3 Glucocorticoids and monocytes/macrophages

Many cell types in various animals, including man, have been shown to possess glucocorticoid receptors. Probably all nucleated cells in man express these receptors and thus are potentially glucocorticoid-responsive (314). In this part of the introduction we shall focus on the influence of glucocorticoids on one particular cell type, mononuclear phagocytes, because of their supposed central role in many physiological and pathological processes as discussed in the first part of the introduction.

The presence of glucocorticoid receptors has been described in PBM, which makes the observed effects of glucocorticoids on these cells understandable (315,316). In other mononuclear phagocytes the presence of specific glucocorticoid receptors has been found to be more difficult to demonstrate (316,317). In human AM, binding assays to determine the numbers of glucocorticoid receptors are hampered by high nonspecific binding. In our

laboratory, we succeeded to by-pass this nonspecific binding and showed conclusively that the effects of glucocorticoids on AM in man are also mediated via glucocorticoid receptors (318).

The effects of glucocorticoids on mononuclear phagocytes are diverse (319-321). Most of the effects have been studied *in vitro*, but extrapolation of such results to the *in vivo* situation may be a tricky operation. Different aspects of mononuclear phagocytes can be influenced by glucocorticoids. Effects on antimicrobial activity, accessory function, maturation/differentiation and activation will be discussed here. Furthermore, interactions of the immune and endocrine systems at the level of mononuclear phagocytes will be mentioned.

Antimicrobial activity of monocytes/macrophages has been described to decrease upon treatment with glucocorticoids, although some reports seem to contradict these findings (319,322-325). *In vitro* treatment of human macrophages impaired the elimination of several bacteria and the fungus Aspergillus (322,323). This effect was glucocorticoid receptor-mediated. However, another study showed that reduced antimicrobial activity of human monocytes *in vitro* could only be achieved with high doses of glucocorticoids exceeding therapeutic levels (324), whereas *in vivo* reduced monocyte killing of *Staphylococcus aureus* was observed after a 3-day course of prednisone therapy (319). Also in animal studies, antimicrobial actions of glucocorticoids show contradictory results. *In vivo*, glucocorticoids suppress resistance to microbial infection in rodents, but the phagocytic and microbicidal activities of macrophages isolated from glucocorticoid-treated animals often remain normal (325). Nowadays, it is possible to understand these puzzling results, as a result of the explosive growth of knowledge of immune processes and cytokines. It has been shown that some cytokines, in particular IFN-γ, could antagonize or enhance effects of glucocorticoids on mononuclear phagocyte functions (323,326-328). The ability of macrophages to inhibit the growth of bacteria has been described to decrease upon treatment with glucocorticoids, but was restored by IFN-γ (323). However, IFN-γ was only capable of restoring antimicrobial activities against some, not all, pathogens (323). These recent findings may help to understand that results of simplified *in vitro* conditions (effect of an isolated cytokine or hormone) cannot be extrapolated to the complicated *in vivo* processes (many interactions between hormones and cytokines) thoughtlessly.

Part of the antimicrobial activities of mononuclear phagocytes results from the expression of membrane receptors that specifically bind the Fc region of immunoglobulins. These FcR greatly enhance the ability of mononuclear phagocytes to recognize and eliminate microorganisms that are coated with specific antibodies. Especially IgG antibodies, which interact with specific FcγR on the cell membrane of mononuclear phagocytes, are able to increase phagocytosis, and are therefore called opsonins (329). Several studies, in man and animals, have shown that glucocorticoids and cytokines, in particular IFN-γ, are able to modulate the expression of FcγR on mononuclear phagocytes (328,330-334). The influence of glucocorticoids may be either a direct effect on FcγR expression (332,333), or an indirect mechanism, such as the inhibition of IFN-γ production (257). These findings enable us to understand the above-mentioned antagonizing effects of IFN-γ on the glucocorticoid-mediated modulation of phagocytosis. However, the effects

of glucocorticoids on FcγR expression are still not fully understood. Some studies showed that glucocorticoids downregulated FcγR expression in the monocytic cell lines U937 and HL60 (332,333), whereas another study showed that in human monocytes FcγR expression was not affected by glucocorticoids alone (334). The model is getting even more complicated, as three distinct FcγR have been demonstrated in man and rodents, which expression is differentially regulated by IFN-γ and glucocorticoids (328,335,336). Murine macrophages constitutively express FcγRII and III. Upon stimulation with IFN-γ, the expression of FcγRI is greatly induced, whereas the expression of FcγR III and II is moderately increased and unaffected, respectively (328). Furthermore, dexamethasone was shown to enhance the effects of IFN-γ on the expression of FcγRI, whilst the effects on FcγRII and III were unaffected by glucocorticoids (328). In human cells, recent *in vitro* findings showed that glucocorticoids inhibited the IFN-γ-induced increase in FcγRI on U937 cells, but that they enhanced the IFN-γ-induced increase on blood monocytes (337). *In vivo* however, glucocorticoids slightly decreased FcγRI-expression on monocytes, but induced a manifest downregulation of FcγRIIIa-expression (Dr. P.M. Guyre, personal communication).

In addition to the antimicrobial activities, also other functions of mononuclear phagocytes are mediated by FcγR (superoxide generation, ADCC, release of cytokines) (336), and may therefore also be influenced by glucocorticoids.

The *accessory function* of mononuclear phagocytes is vitally important in the generation of immune responses. Many aspects of this function are still unknown, but it is realized that the expression of MHC class II and the production of both IL-1 and IL-6 by mononuclear phagocytes are essential (Figure 3) (60). Glucocorticoids are known to interfere with the production of IL-1 and IL-6 both in human monocytes and macrophages *in vitro* (249,254,338,339), and the monocytic cell line U937 (250). However, it has also been shown that (*in vivo*) treatment of patients with rheumatoid arthritis did not influence the ability of isolated synovial macrophages to produce IL-1 or TNF (340). This stresses again the need for reserve in extrapolating *in vitro* results to *in vivo* processes unconditionally. Several reports showed that glucocorticoids decrease MHC class II Ag expression on macrophages in mice (325,341). In mice repression of MHC class II I-A_β gene expression by glucocorticoids has been shown to result from preventing the binding of the X box DNA binding protein to the X box DNA sequence (342). In rats, even the immunostimulatory effects of IFN-γ on antigen expression could be reversed by treatment with methylprednisolone (343). Glucocorticoid treatment of human monocytes decreased their antigen presenting properties (344).

Maturation/differentiation of mononuclear phagocytes has also been shown to be controlled by glucocorticoids (345,346). Different aspects of maturation/differentiation were inhibited by glucocorticoids *in vitro*. Tumoricidal activity, 5' nucleotidase and acid phosphatase activity were found to increase upon maturation, but these increases were inhibited when human monocytes were cultured in the presence of cortisol succinate or dexamethasone (345). In our laboratory, the maturation-associated increases in RFD9 Ag expression and acid phosphatase activity, and decrease in CD14 Ag expression were inhibited by dexamethasone in human monocytes *in vitro* (346). In the monocytic cell line

U937, PMA induced maturation, which was inhibited by cortisol (347). In contrast with these findings, another study in U937 cells showed that TPA-induced maturation was not inhibited by glucocorticoids, whereas TPA-induced activation was (348).

Activation of mononuclear phagocytes may result in an enhanced capacity to present antigens or to kill microbes, both of which have already been described above to be downregulated by glucocorticoids. *In vitro*, activation of mononuclear phagocytes can be studied after stimulation with various compounds, e.g. TPA or LPS. Activation of U937 cells with TPA induced a release of lysozyme and prostanoids, and generated reactive oxygen radicals. This TPA-induced activation with concomitant functions was inhibited by glucocorticoids (348). LPS-stimulated mononuclear phagocytes produce various cytokines, e.g. IL-1β and TNF-α. IFN-γ enhanced the TNF-α production, and even overcame the inhibition of TNF-α by dexamethasone (349). Again, these findings stress that *in vivo*, at the site of inflammation, probably multiple cytokines and hormones determine in concert the eventual effects on the functions of mononuclear phagocytes. Another study showed the influence of glucocorticoids on another aspect of activation with LPS; Upon stimulation of the macrophage cell line J774 with LPS, NO synthase was induced, and this induction could be inhibited by glucocorticoids (271). This resulted in a decreased formation of NO_2^- and NO, which may be part of the anti-inflammatory mechanisms of glucocorticoids.

In addition to the above-mentioned effects of glucocorticoids on (more or less) isolated functions of mononuclear phagocytes, it is important to realize that an additional aspect of the anti-inflammatory effects of glucocorticoids may be a decreased accumulation of mononuclear phagocytes at the site of inflammation. This, in turn, may be caused by a decreased production of myeloid precursors in the bone marrow, inhibition of maturation/differentiation as mentioned in the last paragraph, a decreased production of chemotactic stimuli by other cell types, and a hampered interaction with endothelial cells resulting in decreased diapedesis.

Recent studies have suggested balanced interactions between immunological processes and the endocrine system. Immunological functions of mononuclear phagocytes, in particular the production of a great variety of cytokines (e.g. IL-1, IL-6, IL-8 and TNF-α) (249,254-256,338,339), are regulated by glucocorticoids, adrenal representatives of the hormone system. That part of the mutual influences of the two systems will be discussed in detail in chapter 2. Conversely, cytokines may influence mononuclear phagocytes at the level of glucocorticoid responsiveness. It was shown recently that IFN-γ increased the number of glucocorticoid receptors in the murine macrophage cell line RAW 264.7 (350). It was suggested that this increase in receptor number may be essential for increasing macrophage sensitivity to feedback inhibition by glucocorticoids. Another recent study showed that either IL-1β, IL-6 or TNF-α alone induced a rise in the binding of glucocorticoids in the monocytic cell line U937 (351). Interestingly, antagonistic effects were demonstrated between IL-6 and TNF-α, and between IL-1β and TNF-α when they were applied simultaneously in U937 cells. It was suggested that local imbalance in the ratio of these three cytokines may also influence glucocorticoid responsiveness of mononuclear phagocytes *in vivo*.

1.4 REFERENCES

1. Metchnikoff E. Concerning the relationship between phagocytes and anthrax bacilli. Virch Arch Pathol Anat 1884;97:502-506. Reprinted translation by Magasanik D and Coons AH. Rev Infect Dis 1984;6:761-770.
2. Karnovsky ML. Metchnikoff in Messina: a century of studies on phagocytosis. New Engl J Med 1981;304:1178-1180.
3. Aschoff L. Das reticulo-endotheliale System. Ergbn Inn Med Kinderheilk 1924;26:1-118.
4. Van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull WHO 1972;46:845-852.
5. Van Furth R. Current view on the mononuclear phagocyte system. Immunobiol 1982;161:178-185.
6. Gordon S, Keshav S, Chung LP. Mononuclear phagocytes: tissue distribution and functional heterogeneity. Curr Opin Immunol 1988;1:26-35.
7. Leenen PJM, Campbell PA. Heterogeneity of mononuclear phagocytes; an interpretive review. In: Horton MA, ed. Blood cell biochemistry (Volume 5; Macrophages and related cells). New York: Plenum Press, 1993:29-85.
8. Kabel PJ, De Haan-Meulman M, Voorbij HAM, Kleingeld M, Knol EF, Drexhage HA. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. Immunobiol 1989; 179:395-411.
9. Najar HM, Ruhl S, Bru-Capdeville AC, Peters JH. Adenosine and its derivatives control human monocyte differentiation into highly accessory cells versus macrophages. J Leukocyte Biol 1990;47:429-439.
10. Peters JH, Ruppert J, Gieseler RKH, Najar HM, Xu H. Differentiation of human monocytes into CD14 negative accessory cells: do dendritic cells derive from the monocytic lineage? Pathobiol 1991;59:122-126.
11. Mooij P, Simons PJ, De Haan-Meulman M, De Wit HJ, Drexhage HA. Effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells: role of granulocyte-macrophage colony-stimulating factor, tumour-necrosis factor-α and interleukin-6. J Endocrinol 1994;140:503-512.
12. Reid CDL, Fryer PR, Clifford C, Kirk A, Tikerpae J, Knight SC. Identification of hematopoietic progenitors of macrophages and dendritic Langerhans cells (DL-CFU) in human bone marrow and peripheral blood. Blood 1990;76:1139-1149.
13. Heinen E, Bosseloir A. Follicular dendritic cells: whose children? Immunol Today 1994;15:201-204.
14. Johnston RB. Monocytes and macrophages. New Engl J Med 1988;318:747-752.
15. Gordon S, Fraser I, Nath D, Hughes D, Clarke S. Macrophages in tissues and *in vitro*. Curr Opin Immunol 1992;4:25-32.
16. Ginsel LA. Origin of macrophages. In: Horton MA, ed. Blood cell biochemistry (Volume 5; Macrophages and related cells). New York: Plenum Press, 1993:87-113.
17. Dougherty GJ, McBride WH. Monocyte differentiation *in vitro*. In: Zembala M and Asherson GL, eds. Human Monocytes. London: Academic Press, 1989:49-58.
18. Coggle JE, Tarling JD. The proliferation kinetics of pulmonary alveolar macrophages. J Leukocyte Biol 1984;35:317-327.
19. Sawyer RT. The significance of local resident pulmonary alveolar macrophage proliferation to population renewal. J Leukocyte Biol 1986;39:77-87.
20. Golde DW, Byers LA, Finley TN. Proliferative capacity of human alveolar macrophage. Nature 1974;247:373-375.

21. Kreipe H, Radzun HJ, Heidorn K, Barth J, Kiemle-Kallee J, Petermann W, Gerdes J, Parwaresch MR. Proliferation, macrophage colony-stimulating factor, and macrophage colony-stimulating factor-receptor expression of alveolar macrophages in active sarcoidosis. Lab Invest 1990;62:697-703.
22. Adams DO. The granulomatous inflammatory response. Am J Pathol 1976;84:164-191.
23. Gibbs AR, Williams WJ. The pathology of sarcoidosis. Semin Respir Med 1986;8:10-16.
24. Sachs L. The molecular control of blood cell development. Science 1987;238:1374-1379.
25. Metcalf D. Control of granulocytes and macrophages: molecular, cellular, and clinical aspects. Science 1991;254:529-533.
26. Kierney PC, Dorshkind K. B lymphocyte precursors and myeloid progenitors survive in diffusion chamber cultures but B cell differentiation requires close association with stromal cells. Blood 1987;70:1418-1424.
27. Gordon MY, Riley GP, Watt SM, Greaves MF. Compartmentalization of a haematopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 1987;326:403-405.
28. Te Velde AA, Klomp JPG, Yard BA, De Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
29. Te Velde AA, Rousset F, Peronne C, De Vries JE, Figdor CG. IFN-α and IFN-γ have different regulatory effects on IL-4-induced membrane expression of FcεRIIb and release of soluble FcεRIIb by human monocytes. J Immunol 1990;144:3052-3059.
30. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.
31. Van Hal PThW, Hopstaken-Broos JPM, Prins A, Favaloro EJ, Huijbens RFJ, Hilvering C, Figdor CG, Hoogsteden HC. Potential, indirect anti-inflammatory effects of IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N-activity (CD13; EC 3.4.11.2). J Immunol 1994;153:2718-2728.
32. Groopman JE, Molina J-M, Scadden DT. Hematopoietic growth factors: biology and clinical applications. New Engl J Med 1989;321:1449-1459.
33. Clohisy DR, Bar-Shavit Z, Chappel JC, Teitelbaum SL. 1,25-Dihydroxyvitamin D_3 modulates bone marrow macrophage precursor proliferation and differentiation. Up-regulation of the mannose receptor. J Biol Chem 1987;262:15922-15929.
34. Moore RN, Osmand AP, Dunn JA, Joshi JG, Rouse BT. Substance P augmentation of CSF-1-stimulated *in vitro* myelopoiesis. A two-signal progenitor restricted, tuftsin-like effect. J Immunol 1988;141:2699-2703.
35. Moore RN, Osmand AP, Dunn JA, Joshi JG, Koontz JW, Rouse BT. Neurotensin regulation of macrophage colony-stimulating factor-stimulated *in vitro* myelopoiesis. J Immunol 1989;142:2689-2694.
36. Watanabe Y, Jacob CO. Regulation of MHC class II antigen expression. Opposing effects of tumor necrosis factor-α on IFN-γ-induced HLA-DR and Ia expression depends on the maturation and differentiation stage of the cell. J Immunol 1991;146:899-905.
37. Razak K, Newland AC. Induction of CD13 expression on fresh myeloid leukaemia: correlation of CD13 expression with aminopeptidase-N activity. Leukemia Res 1992;16:625-630.
38. Elias JA, Zitnik RJ. Cytokine-cytokine interactions in the context of cytokine networking. Am J Respir Cell Mol Biol 1992;7:365-367.
39. Nathan C, Sporn M. Cytokines in context. J Cell Biol 1991;113:981-986.
40. Janossy G, Bofill M, Poulter LW, Rawlings E, Burford GD, Navarrete C, Ziegler A, Kelemen E. Separate ontogeny of two macrophage-like accessory cell populations in the human fetus. J Immunol 1986;136:4354-4361.
41. Grage-Griebenow E, Lorenzen D, Fetting R, Flad H-D, Ernst M. Phenotypical and functional characterization of Fcγ receptor I (CD64)-negative monocytes, a minor human monocyte subpopulation with high accessory and antiviral activity. Eur J Immunol 1993;23:3126-3135.

42. Dougherty GJ, Dougherty ST, McBride WH. Monocyte heterogeneity. In: Zembala M and Asherson GL, eds. Human monocytes. London: Academic Press, 1989:71-78.
43. Auger MJ, Ross JA. The biology of the macrophage: macrophage heterogeneity. In: Lewis CE and McGee JOD, eds. The macrophage. Oxford: Oxford University Press, 1992:27-31.
44. Ham AW, Cormack DH. Lymphatic tissue. In: Ham AW and Cormack DH, eds. Histology. Philadelphia: JB Lippincott Company, 1979:323-366.
45. Marcy TW, Merrill WW. Cigarette smoking and respiratory tract infection. Respir Infect 1987;8:381-391.
46. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
47. Speert DP. Macrophages in bacterial infection. In: Lewis CE and McGee JOD, eds. The macrophage. Oxford: Oxford University Press, 1992:215-263.
48. Kinet J-P. Antibody-cell interactions: Fc receptors. Cell 1989;57:351-354.
49. Schlesinger LS, Horwitz MA. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. J Clin Invest 1990;85:1304-1314.
50. Kuhlman M, Joiner K, Ezekowitz RAB. The human mannose-binding protein functions as an opsonin. J Exp Med 1989;169:1733-1745.
51. Brown E, Atkinson JP, Fearon DT. Innate immunity: 50 ways to kill a microbe. Curr Opin Immunol 1994;6:73-74.
52. Reiner NE. Altered cell signaling and mononuclear phagocyte deactivation during intracellular infection. Immunol Today 1994;15:374-381.
53. Chan J, Fan X, Hunter SW, Brennan PJ, Bloom BR. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect Immun 1991;59:1755-1761.
54. Rosqvist R, Forsberg Å, Wolf-Watz H. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. Infect Immun 1991;59:4562-4569.
55. Rosenberg ZF, Fauci AS. Immunology of HIV infection. In: Paul WE, ed. Fundamental Immunology. New York: Raven Press, 1993:1375-1397.
56. Mosier D, Sieburg H. Macrophage-tropic HIV: critical for AIDS pathogenesis? Immunol Today 1994;15:332-339.
57. Herscowitz HB. In defense of the lung: paradoxical role of the pulmonary alveolar macrophage. Ann of Allergy 1985;55:634-648.
58. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
59. Van Noort JM, Boon J, Van der Drift ACM, Wagenaar JPA, Boots AMH, Boog CJP. Antigen processing by endosomal proteases determines which sites of sperm-whale myoglobin are eventually recognized by T cells. Eur J Immunol 1991;21:1989-1996.
60. Chain BM, Levine TP. Antigen processing. In: Horton MA, ed. Blood cell biochemistry (Volume 5; Macrophages and related cells). New York: Plenum Press, 1993:161-182.
61. Yewdell JW, Bennink JR. The binary logic of antigen processing and presentation to T cells. Cell 1990;62:203-206.
62. Zlotnik A, Fischer M, Roehm N, Zipori D. Evidence for effects of interleukin 4 (B cell stimulatory factor 1) on macrophages: enhancement of antigen presenting ability of bone marrow-derived macrophages. J Immunol 1987;138:4275-4279.
63. Inaba K, Steinman RM. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. J Exp Med 1984;160:1717-1735.
64. Macatonia SE, Taylor PM, Knight SC, Askonas BA. Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses *in vitro*. J Exp Med 1989;169:1255-1264.

65. Macatonia SE, Gompels M, Pinching AJ, Patterson S, Knight SC. Antigen presentation by macrophages but not by dendritic cells in human immunodeficiency virus (HIV) infection. Immunology 1992;75:576-581.
66. Nag B, Wada HG, Passmore D, Clark BR, Sharma SD, McConnell HM. Purified β-chain of MHC class II binds to CD4 molecules on transfected HeLa cells. J Immunol 1993;150:1358-1364.
67. Patarroyo M, Prieto J, Rincon J, Timonen T, Lundberg C, Lindbom L, Asjo B, Gahmberg CG. Leukocyte-cell adhesion: a molecular process fundamental in leukocyte physiology. Immunol Rev 1990;114:67-108.
68. Somoza C, Driscoll PC, Cyster JG, Williams AF. Mutational analysis of the CD2/CD58 interaction: the binding site for CD58 lies on one face of the first domain of human CD2. J Exp Med 1993;178:549-558.
69. Walter H, Schepensn S, Van Wauwe J, De Boer M. Ligation of CD28 on resting T cells by its ligand B7 results in the induction of both Th1- and Th2-type cytokines. Eur Cyt Network 1994;5:13-21.
70. Clark EA, Ledbetter JA. How B and T cells talk to each other. Nature 1994;367:425-428.
71. Springer TA. Adhesion receptors of the immune system. Nature 1990;346:425-434.
72. Roberts PJ, Pizzey AR, Khwaja A, Carver JE, Mire-Sluis AR. The effects of interleukin-8 on neutrophil fMetLeuPhe receptors, CD11b expression and metabolic activity, in comparison and combination with other cytokines. Brit J Haematol 1993;84:586-594.
73. Costello R, Brailly H, Mallet F, Mawas C, Olive D. Interleukin-7 is a potent co-stimulus of the adhesion pathway involving CD2 and CD28 molecules. Immunology 1993;80:451-457.
74. Kubin M, Kamoun M, Trinchieri G. Interleukin 12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production of human T cells. J Exp Med 1994;180:211-222.
75. Rappolee DA, Werb Z. Secretory products of phagocytes. Curr Opin Immunol 1988;1:47-55.
76. Auger MJ, Ross JA. The biology of the macrophage: macrophage surface receptors. In: Lewis CE and McGee JOD, eds. The macrophage. Oxford: Oxford University Press, 1992:16-27.
77. Celada A, Nathan C. Macrophage activation revisited. Immunol Today 1994;15:100-102.
78. Parums DV. Macrophages in cardiovascular disease. In: Lewis CE and McGee JOD, eds. The macrophage. Oxford: Oxford University Press, 1992:359-390.
79. Fahey TJ, Sherry B, Tracey KJ, Van Deventer S, Jones WG, Minei JP, Morgello S, Shires GT, Cerami A. Cytokine production in a model of wound healing: the appearance of MIP-1, MIP-2, cachectin/TNF and IL-1. Cytokine 1990;2:92-99.
80. Te Velde AA, Figdor CG. Monocyte mediated cytotoxic activity against melanoma. Melanoma Res 1992;1:303-309.
81. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. Immunol Today 1992;13:265-270.
82. Vries De JE. Atopic allergy and other hypersensitivities. Curr Opin Immunol 1994;6:835-837.
83. Solbach W, Moll H, Röllinghoff M. Lymphocytes play the music but the macrophage calls the tune. Immunol Today 1991;12:4-6.
84. Martin TR, Goodman RB. The role of lung mononuclear cells in asthma. Immunol Allergy Clin North Am 1990;10:295-308.
85. Leff AR, Hamann KJ, Wegner CD. Inflammation and cell-cell interactions in airway hyperresponsiveness. Am J Physiol 1991;260:L189-L206.
86. Poulter LW, Janossy G, Power C, Sreenan S, Burke C. Immunological/physiological relationship in asthma: potential regulation by lung macrophages. Immunol Today 1994;15:258-261.
87. Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. J Exp Med 1993;177:397-407.
88. Rook GA, Stanford JL. Slow bacterial infections or autoimmunity? Immunol Today 1992;13:160-164.

89. Tamura N, Holroyd KJ, Banks T, Kirby M, Okayama H, Crystal RG. Diversity in junctional sequences associated with the common human Vγ9 and Vδ2 gene segments in normal blood and lung compared with the limited diversity in a granulomatous disease. J Exp Med 1990;172:169-181.
90. Forrester JM, Newman LS, Wang Y, King TE, Kotzin BL. Clonal expansion of lung Vδ1$^+$ T cells in pulmonary sarcoidosis. J Clin Invest 1993;91:292-300.
91. Semenzato G. Immunology of interstitial lung diseases: cellular events taking place in the lung of sarcoidosis, hypersensitivity pneumonitis and HIV infection. Eur Respir J 1991;4:94-102.
92. Fireman EM, Topilsky MR. Sarcoidosis: an organized pattern of reaction from immunology to therapy. Immunol Today 1994;15:199-201.
93. Gauldie J, Jordana M, Cox G. Cytokines and pulmonary fibrosis. Thorax 1993;48:931-935.
94. Ross R, Raines EW, Bowen-Pope DF. The biology of platelet-derived growth factor. Cell 1986;46:155-169.
95. Martinet Y, Rom WN, Grotendorst GR, Martin GR, Crystal RG. Exaggerated spontaneous release of platelet-derived growth factor by alveolar macrophages from patients with idiopathic pulmonary fibrosis. New Engl J Med 1987;317:202-209.
96. Bitterman PB, Adelberg S, Crystal RG. Spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. J Clin Invest 1983;72:1801-1813.
97. Rom WN, Basset P, Fells GA, Nukiwa T, Trapnell BC, Crystal RG. Alveolar macrophages release an insulin-like growth factor I-type molecule. J Clin Invest 1988;82:1685-1693.
98. Assoian RK, Fleurdelys BE, Stevenson HC, Miller PJ, Madtes DK, Raines EW, Ross R, Sporn MB. Expression and secretion of type β transforming growth factor by activated human macrophages. Proc Natl Acad Sci USA 1987;84:6020-6024.
99. Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl LM, Roberts AB, Sporn MB. Transforming growth factor type β induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987;84:5788-5792.
100. Postlethwaite AE, Keski-Oja J, Moses HL, Kang AH. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor β. J Exp Med 1987;165:251-256.
101. Ignotz RA, Massagué J. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 1986;261:4337-4345.
102. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. New Engl J Med 1994;331:1286-1292.
103. Mason MJ, Van Epps DE. In vivo neutrophil emigration in response to interleukin-1 and tumor necrosis factor-alpha. J Leukocyte Biol 1989;45:62-68.
104. Austgulen R, Espevik T, Nissen-Meyer J. Fibroblast growth-stimulatory activity released from human monocytes. Scand J Immunol 1987;26:621-629.
105. Pfeffer KD, Huecksteadt TP, Hoidal JR. Expression and regulation of tumor necrosis factor in macrophages from cystic fibrosis patients. Am J Respir Cell Mol Biol 1993;9:511-519.
106. Mantovani A, Dejana E. Cytokines as communication signals between leukocytes and endothelial cells. Immunol Today 1989;10:370-375.
107. Mizel SB. The interleukins. FASEB J 1989;3:2379-2388.
108. Mizel SB, Farrar JJ. Revised nomenclature for antigen-nonspecific T-cell proliferation and helper factors. Cell Immunol 1979;48:433-436.
109. Mizel SB, Farrar JJ. Revised nomenclature for antigen-nonspecific T-cell proliferation and helper factors. J Immunol 1979;123:2928-2929.
110. Dinarello CA, Mier JW. Lymphokines. New Engl J Med 1987;317:940-945.
111. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. There is more than one interleukin 1. Immunol Today 1986;7:45-56.
112. Smith KA. Interleukin-2: inception, impact, and implications. Science 1988;240:1169-1176.
113. Malkovský M, Loveland B, North M, Asherson GL, Gao L, Ward P, Fiers W. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. Nature 1987;325:262-265.

114. Yang Y-C, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC. Human IL-3 (multi-CSF): Indentification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell 1986;47:3-10.
115. Yokota T, Otsuka T, Mosmann T, Banchereau J, DeFrance T, Blanchard D, De Vries JE, Lee F, Arai K-I. Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell- and T-cell-stimulating activities. Proc Natl Acad Sci USA 1986;83:5894-5898.
116. Kinashi T, Harada N, Severinson E, Tanabe T, Sideras P, Konishi M, Azuma C, Tominaga A, Bergstedt-Lindqvist S, Takahashi M, Matsuda F, Yaoita Y, Takatsu K, Honjo T. Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. Nature 1986;324:70-73.
117. Wong GG, Clark SC. Multiple actions of interleukin 6 within a cytokine network. Immunol Today 1988;9:137-139.
118. Kotloff RM, Little J, Elias JA. Human alveolar macrophage and blood monocyte interleukin-6 production. Am J Respir Cell Mol Biol 1990;3:497-505.
119. Namen AE, Schmierer AE, March CJ, Overell RW, Park LS, Urdal DL, Mochizuki DY. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. J Exp Med 1988;167:988-1002.
120. Alderson MR, Tough TW, Ziegler SF, Grabstein KH. Interleukin 7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. J Exp Med 1991;173:923-930.
121. Standiford TJ, Strieter RM, Allen RM, Burdick MD, Kunkel SL. IL-7 up-regulates the expression of IL-8 from resting and stimulated human blood monocytes. J Immunol 1992;149:2035-2039.
122. Baggiolini M, Walz A, Kunkel SL. Neutrophil-activating peptide-1/Interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest 1989;84:1045-1049.
123. Renauld JC, Goethals A, Houssiau F, Merz H, Van Roost E, Van Snick J. Human P40/IL-9. Expression in activated $CD4^+$ T cells, genomic organization, and comparison with the mouse gene. J Immunol 1990;144:4235-4241.
124. Dugas B, Renauld JC, Pène J, Bonnefoy JY, Peti-Frère C, Braquet P, Bousquet J, Van Snick J, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. Eur J Immunol 1993;23:1687-1692.
125. Moore KW, O'Garra A, De Waal-Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165-190.
126. De Waal Malefyt R, Abrams J, Bennett B, Figdor CG, De Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991;174:1209-1220.
127. Quesniaux VF, Mayer P, Liehl E, Turner K, Goldman SJ, Fagg B. Review of a novel hematopoietic cytokine, interleukin-11. Int Rev Exp Pathol 1993;34:205-214.
128. Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, Loudon R, Sherman F, Perussia B, Trinchieri G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 1989;170:827-845.
129. Scott P. IL-12: initiation cytokine for cell-mediated immunity. Science 1993;260:496-497.
130. McKenzie ANJ, Culpepper JA, De Waal Malefyt R, Brière J, Punnonen J, Aversa G, Sato A, Dang W, Cocks BG, Menon S, De Vries JE, Banchereau J, Zurawski G. Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. Proc Natl Acad Sci USA 1993;90:3735-3739.
131. Ambrus JL, Pippin J, Joseph A, Xu C, Blumenthal D, Tamayo A, Claypool K, McCourt D, Srikiatchatochorn A, Ford RJ. Identification of a cDNA for a human high-molecular-weight B-cell growth factor. Proc Natl Acad Sci USA 1993;90:6330-6334.
132. Grabstein KH, Eisenman J, Shanebeck K, Rauch C, Srinivasan S, Fung V, Beers C, Richardson J, Schoenborn MA, Ahdieh M, Johnson L, MR Alderson, Watson JD, Anderson DM, Giri JG. Cloning of a T cell growth factor that interacts with the β chain of the interleukin-2 receptor. Science 1994;264:965-968.

133. Burke F, Naylor MS, Davies B, Balkwill F. The cytokine wall chart. Immunol Today 1993;14:165-170.
134. Rao A. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. Immunol Today 1994;15:274-281.
135. Ullman KS, Northrop JP, Verweij CL, Crabtree GR. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. Annu Rev Immunol 1990;8:421-452.
136. Becker S, Daniel EG. Antagonistic and additive effects of IL-4 and interferon-γ on human monocytes and macrophages: effects on Fc receptors, HLA-D antigens, and superoxide production. Cell Immunol 1990;129:351-362.
137. Le J, Vilček J. Biology of disease. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 1987;56:234-248.
138. O'Garra A, Umland S, De France T, Christiansen J. 'B-cel factors' are pleiotropic. Immunol Today 1988;9:45-54.
139. Balkwill FR. Interferons. Lancet 1989;i:1060-1063.
140. Metcalf D. Haemopoietic growth factors 1. Lancet 1989;i:825-827.
141. Geissler K, Harrington M, Srivastava C, Leemhuis T, Tricot G, Broxmeyer HE. Effects of recombinant human colony stimulating factors (CSF) (granulocyte-macrophage CSF, granulocyte CSF, and CSF-1) on human monocyte/macrophage differentiation. J Immunol 1989;143:140-146.
142. Welte K, Bonilla MA, Gillio AP, Boone TC, Potter GK, Gabrilove JL, Moore MAS, O'Reilly RJ, Souza LM. Recombinant human granulocyte colony-stimulating factor. Effects on hematopoiesis in normal and cyclophosphamide-treated primates. J Exp Med 1987;165:941-948.
143. Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. New Engl J Med 1992;327:28-35.
144. Han VKM, D'Ercole AJ, Lund PK. Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 1987;236:193-197.
145. Rifkin DB, Moscatelli D. Recent developments in the cell biology of basic fibroblast growth factor. J Cell Biol 1989;109:1-6.
146. Wolpe SD, Davatelis G, Sherry B, Beutler B, Hesse DG, Nguyen HT, Moldawer LL, Nathan CF, Lowry SF, Cerami A. Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. J Exp Med 1988;167:570-581.
147. Davatelis G, Wolpe SD, Sherry B, Dayer J-M, Chicheportiche R, Cerami A. Macrophage inflammatory protein-1: a prostaglandin-independent endogenous pyrogen. Science 1989;243:1066-1068.
148. Leonard EJ, Yoshimura T. Human monocyte chemoattractant protein-1 (MCP-1). Immunol Today 1990;11:97-101.
149. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 1990;347:669-671.
150. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, Krensky AM. A human T cell-specific molecule is a member of a new gene family. J Immunol 1988;141:1018-1025.
151. Oppenheim JJ, Zachariae COC, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene "intercrine" cytokine family. Annu Rev Immunol 1991;9:617-648.
152. Deuel TF, Senior RM, Huang JS, Griffin GL. Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. J Clin Invest 1982;69:1046-1049.
153. Schreiber AB, Winkler ME, Derynck R. Transforming growth factor-α: a more potent angiogenic mediator than epidermal growth factor. Science 1986;232:1250-1253.
154. Tracey KJ, Vlassara H, Cerami A. Cachectin/tumour necrosis factor. Lancet 1989;i:1122-1126.
155. Winearls CG, Oliver DO, Pippard MJ, Reid C, Downing MR, Cotes PM. Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic heamodialysis. Lancet 1986;ii:1175-1178.

156. Rodgers GP, Dover GJ, Uyesaka N, Noguchi CT, Schechter AN, Nienhuis AW. Augmentation by erythropoietin of the fetal-hemoglobin response to hydroxyurea in sickle cell disease. New Engl J Med 1993;328:73-80.
157. Morstyn G, Campbell L, Souza LM, Alton NK, Keech J, Green M, Sheridan W, Metcalf D, Fox R. Effect of granulocyte colony stimulating factor on neutropenia induced by cytotoxic chemotherapy. Lancet 1988;i:667-672.
158. Gabrilove JL, Jakubowski A, Scher H, Sternberg C, Wong G, Grous J, Yagoda A, Fain K, Moore MAS, Clarkson B, Oettgen HF, Alton K, Welte K, Souza L. Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. New Engl J Med 1988;318:1414-1422.
159. Brandt SJ, Peters WP, Atwater SK, Kurtzberg J, Borowitz MJ, Jones RB, Shpall EJ, Bast RC, Gilbert CJ, Oette DH. Effect of recombinant human granulocyte-macrophage colony-stimulating factor on hematopoietic reconstitution after high-dose chemotherapy and autologous bone marrow transplantation. New Engl J Med 1988;318:869-876.
160. Groopman JE, Molina J-M, Scadden DT. Hematopoietic growth factors. Biology and clinical applications. New Engl J Med 1989;321:1449-1459.
161. Metcalf D. Peptide regulatory factors. Haemopoietic growth factors 2: clinical applications. Lancet 1989;i:885-887.
162. Rose RM, Kobzik L, Dushay K, Wolfthal S, Hondalus M, Metzger M, Stoudemire J, Brain JD, Garnick M, O'Donnell C, Donahue RE. The effect of aerosolized recombinant human granulocyte macrophage colony-stimulating factor on lung leukocytes in nonhuman primates. Am Rev Respir Dis 1992;146:1279-1286.
163. Smith II JW, Longo DL, Alvord WG, Janik JE, Sharfman WH, Gause BL, Curti BD, Creekmore SP, Holmlund JT, Fenton RG, Sznol M, Miller LL, Shimizu M, Oppenheim JJ, Fiem SJ, Hursey JC, Powers GC, Urba WJ. The effects of treatment with interleukin-1α on platelet recovery after high-dose carboplatin. New Engl J Med 1993;328:756-761.
164. Dinarello CA, Wolff SM. The role of interleukin-1 in disease. New Engl J Med 1993;328:106-113.
165. Vogelzang NJ, Lipton A, Figlin RA. Subcutaneous interleukin-2 plus interferon alfa-2a in metastatic renal cancer: an outpatient multicenter trial. J Clin Oncol 1993;11:1809-1816.
166. Teppler H, Kaplan G, Smith KA, Montana AL, Meyn P, Cohn ZA. Prolonged immunostimulatory effect of low-dose polyethylene glycol interleukin 2 in patients with human immunodeficiency virus type 1 infection. J Exp Med 1993;177:483-492.
167. Cunningham-Rundles C, Kazbay K, Hassett J, Zhou Z, Mayer L. Enhanced humoral immunity in common variable immunodeficiency after long-term treatment with polyethylene glycol-conjugated interleukin-2. New Engl J Med 1994;331:918-921.
168. Hart PH, Vitti G, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. Proc Natl Acad Sci USA 1989;86:3803-3807.
169. Maher DW, Davis I, Boyd AW, Morstyn G. Human interleukin-4: an immunomodulator with potential therapeutic applications. Prog Growth Fac Res 1991;3:43-56.
170. Wong HL, Lotze MT, Wahl LM, Wahl SM. Administration of recombinant IL-4 to humans regulates gene expression, phenotype, and function in circulating monocytes. J Immunol 1992;148:2118-2125.
171. Anderson GP, Coyle AJ. T_H2 and "T_H2-like" cells in allergy and asthma: pharmacological perspectives. Trends Parmacol Sci 1994;15:324-332.
172. Van Oosterhout AJ, Ladenius AR, Savelkoul HF, Van Ark I, Delsman KC, Nijkamp FP. Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. Am Rev Respir Dis 1993;147:548-552.

173. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, Weiner-Kronish JP, Sticherling M, Christophers E, Matthay MA. Elevated levels of NAP-1/Interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. Am Rev Respir Dis 1992;146:427-432.
174. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, Grant IS, Pollok AJ, Haslett C. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. Lancet 1993;341:643-647.
175. Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8: the major neutrophil chemotactic factor in the lung. Exp Lung Res 1991;17:17-23.
176. Standiford TJ, Kunkel SL, Rolfe MW, Evanoff HL, Allen RM, Strieter RM. Regulation of human alveolar macrophage and blood monocyte-derived interleukin-8 by prostaglandin E_2 and dexamethasone. Am J Respir Cell Mol Biol 1992;6:675-681.
177. Donahue RE, Seehra J, Metzger M, Lefebvre D, Rock B, Carbone S, Nathan DG, Garnick M, Sehgal PK, Laston D, La Vallie E, McCoy J, Schendel PF, Norton C, Turner K, Yang Y-C, Clark SC. Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science 1988;241:1820-1823.
178. Patchen ML, Fischer R, MacVittie TJ. Effects of combined administration of interleukin-6 and granulocyte colony-stimulating factor on recovery from radiation-induced hemopoietic aplasia. Exp Hematol 1993;21:338-344.
179. Van Dijk J, Uemura H, Beniers AJ, Peeten WP, Zegveld ST, Fleuren GJ, Warnaar SO, Oosterwijk E. Therapeutic effects of monoclonal antibody G250, interferons and tumor necrosis factor, in mice with renal-cell carcinoma xenografts. Int J Cancer 1994;56:262-268.
180. Dinarello CA, Thompson RC. Blocking IL-1: interleukin 1 receptor antagonist *in vivo* and *in vitro*. Immunol Today 1991;12:404-410.
181. Debets R, Savelkoul HFJ. Cytokine antagonists and their potential therapeutic use. Immunol Today 1994;15:455-458.
182. Finkelman FD, Madden KB, Morris SC, Holmes JM, Boiani N, Katona IM, Maliszewski CR. Anti-cytokine antibodies as carrier proteins. Prolongation of in vivo effects of exogenous cytokines by injection of cytokine-anti-cytokine antibody complexes. J Immunol 1993;151:1235-1244.
183. Auger MJ, Ross JA. The biology of the macrophage: macrophage functions. In: Lewis CE and McGee JOD, eds. The Macrophage. Oxford: Oxford University Press, 1992:31-49.
184. Kelley J. Cytokines of the lung. Am Rev Respir Dis 1990;141:765-788.
185. Bohbot A, Eischen A, Felden C, Vincent F, Oberling F. U937 cell line: impact of CSFs, IL-6 and IFN-γ on the differentiation and the Leu-CAM proteins expression. Exp Hematol 1993;21:564-572.
186. Nicod LP. Cytokines. Thorax 1993;48:660-667.
187. Gery I, Gershon RK, Waksman BH. Potentiation of cultured mouse thymocyte responses by factors released by peripheral leucocytes. J Immunol 1971;107:1778-1780.
188. Kelker CH, Oppenheim JD, Stone-Wolff D, Henriksen-DeStefano D, Aggarwal BB, Stevenson HC, Vilček J. Characterization of human tumor necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. Int J Cancer 1985;36:69-73.
189. Beutler B, Cerami A. Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature 1986;320:584-588.
190. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Nat Acad Sci USA 1975;72:3666-3670.
191. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon β_2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. Proc Natl Acad Sci USA 1987;84:7251-7255.
192. Crocker PR, Milon G. Macrophages in the control of haematopoiesis: control of haematopoiesis by soluble regulatory factors. In: Lewis CE and McGee JOD, eds. The Macrophage. Oxford: Oxford University Press, 1992:125-135.

193. Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. Annu Rev Biochem 1987;56:727-777.
194. Martinet Y, Bitterman PB, Mornex J-F, Grotendorst GR, Martin GR, Crystal RG. Activated human monocytes express the c-*sis* proto-oncogene and release a mediator showing PDGF-like activity. Nature 1986;319:158-160.
195. Hamilton JA. Colony stimulating factors, cytokines and monocyte-macrophages - some controversies. Immunol Today 1993;14:18-24.
196. David DS, Grieco MH, Cushman P. Adrenal glucocorticoids after twenty years. A review of their clinically relevant consequences. J Chron Dis 1970;22:637-711.
197. Axelrod L. Side effects of glucocorticoid therapy. In: Schleimer RP, Claman HN, Oronsky A, eds. Anti-inflammatory steroid action. Basic and clinical aspects. San Diego: Academic Press, Inc. 1989:377-408.
198. Seale JP, Compton MR. Side-effects of corticosteroid agents. Med J Aust 1986;144:139-142.
199. Tyrrell JB, Aron DC, Forsham PH. Glucocorticoids and adrenal androgens. In: Greenspan FS, ed. Basic and clinical endocrinology. San Francisco: Prentice-Hall International Inc. 1991:323-362.
200. Krieger DT, Allen W, Rizzo F, Krieger HP. Characterization of the normal temporal pattern of plasma corticosteroid levels. J Clin Endocrin 1971;32:266-284.
201. Munck A, Guyre PM. Glucocorticoid physiology and homeostasis in relation to anti-inflammatory actions. In: Schleimer RP, Claman HN, Oronsky A, eds. Anti-inflammatory steroid action. Basic and clinical aspects. San Diego: Academic Press, Inc. 1989:30-47.
202. Munck A, Mendel DB, Smith LI, Orti E. Glucocorticoid receptors and actions. Am Rev Respir Dis 1990;141:S2-S10.
203. Elansary EH, Earis JE. Rifampicin and adrenal crisis. Brit Med J 1983;286:1861-1862.
204. Hench PS, Kendall EC, Slocumb CH, Polley HF. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc Staff Meet, Mayo Clin 1949:24:181-197.
205. Carryer HM, Koelsche GA, Prickman LE, Maytum CK, Lake CF, Williams HL. Effects of cortisone on bronchial asthma and hay fever occurring in subjects sensitive to ragweed pollen. Proc Staff Meet, Mayo Clin 1950;25:482-486.
206. Clark TJH. Effect of beclomethasone dipropionate delivered by aerosol in patients with asthma. Lancet 1972;i:1361-1364.
207. Brown HM. The introduction and early development of inhaled steroid treatment. In: Mygind N, Clark TJH, eds. Topical steroid treatment for asthma and rhinitis. London: Baillière Tindall, 1980:66-76.
208. Szefler SJ. General pharmacology of glucocorticoids. In: Schleimer RP, Claman HN, Oronsky A, eds. Anti-inflammatory steroid action. Basic and clinical aspects. San Diego: Academic Press, Inc. 1989:353-376.
209. Harding SM. The human pharmacology of fluticasone propionate. Resp Med 1990;84(Suppl):25-29.
210. Hench PS, Kendall EC, Slocumb CH, Polley HF. Effects of cortisone acetate and pituitary ACTH on rheumatoid arthritis, rheumatic fever and certain other conditions. Arch Int Med 1950;85:545-666.
211. McGowan JE, Chesney PJ, Crossley KB, LaForce FM. Guidelines for the use of systemic glucocorticosteroids in the management of selected infections. J Infect Dis 1992;165:1-13.
212. Ziment I. Steroids. Clin Chest Med 1986;7:341-354.
213. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
214. De Waal RMW. The anti-inflammatory activity of glucocorticoids. Mol Biol Rep 1994;19:81-88.
215. Schleimer RP. Effects of glucocorticoids on inflammatory cells relevant to their therapeutic applications in asthma. Am Rev Respir Dis 1990;141:S59-S69.
216. Morris HG. Mechanisms of glucocorticoid action in pulmonary disease. Chest 1985;88:133S-141S.
217. Barnes PJ. A new approach to the treatment of asthma. New Engl J Med 1989;321:1517-1527.

218. Juniper EF, Kline PA, Vanzieleghem MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. Am Rev Respir Dis 1990;142:832-836.
219. Costain D, Harrison BDW, Holgate ST, Hopkins AP, Partridge MR, Barnes PJ, Brewis RAL, Bucknall CE, Gribbin HR, Lane DJ, Neville E, Allison SP, Barnett AH, Clark TJH, Connolly CK, Crompton GK, Donaldson J, Evans CC, Ferguson AD, Friend JAR, Hilton SR, Holmes WF, Jones K, Kenwright S, McNicol MW, Page RL, Pantin CFA, Pearson MG, Rudolf M, Smith A, Stark JE, Thomas GO, Turner-Warwick ME, Waine C. Guidelines for management of asthma in adults: I-chronic persistent asthma. Brit Med J 1990;301:651-653.
220. Wedzicha JA. Inhaled corticosteroids in COPD: awaiting controlled trials. Thorax 1993;48:305-307.
221. Allen MB, Cooke NJ. Corticosteroids and tuberculosis. For systemic symptoms and local pressure effects. Brit Med J 1991;303:871-872.
222. Humphries M. The management of tuberculous meningitis. Thorax 1992;47:577-581.
223. Strang JIG, Kakaza HHS, Gibson DG, Allen BW, Mitchison DA, Evans DJ, Girling DJ, Nunn AJ, Fox W. Controlled clinical trial of complete open surgical drainage and of prednisolone in treatment of tuberculous pericardial effusion in Transkei. Lancet 1988;ii:759-764.
224. Gagnon S, Boota AM, Fischl MA, Baier H, Kirksey OW, La Voie L. Corticosteroids as adjunctive therapy for severe *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. New Engl J Med 1990;323:1444-1450.
225. Masur H, and other members of the panel for corticosteroids as adjunctive therapy for pneumocystis pneumonia. Consensus statement on the use of corticosteroids as adjunctive therapy for pneumocystis pneumonia in the acquired immunodeficiency syndrome. New Engl J Med 1990;323:1500-1504.
226. Lawrence EC, Teague RB, Gottlieb MS, Jhingran SG, Liebermann J. Serial changes in markers of disease activity with corticosteroid treatment in sarcoidosis. Am J Med 1983;74:747-756.
227. Turner-Warwick M. Interstitial lung disease: approaches to therapy. Semin Respir Med 1984;6:92-102.
228. Schwarz MI, King Jr TE, eds. Interstitial lung disease. Burlington and Philadelphia: BC Decker Inc. 1988.
229. Pickering CAC, Newman Taylor AJ. Extrinsic allergic bronchioloalveolitis (hypersensitivity pneumonia). In: Parkes WR, ed. Occupational lung disorders. Oxford: Butterworth-Heineman Ltd. 1994:667-709.
230. Venuta F, Rendina EA, Ciriaco P, De Giacomo T, Pompeo E, Bachetoni A, Ricci C. Efficacy of cyclosporine to reduce steroids in patients with idiopathic pulmonary fibrosis before lung transplantation. J Heart Lung Transplant 1993;12:909-914.
231. Hargreaves MR, Mowat AG, Benson MK. Acute pneumonitis associated with low dose methotrexate treatment for rheumatoid arthritis: report of five cases and review of published reports. Thorax 1992;47:628-633.
232. Pitcher WD. Amiodarone pulmonary toxicity. Am J Med Sci 1992;303:206-212.
233. Kollef MH, Schuster DP. The acute respiratory distress syndrome. New Engl J Med 1995;332:27-37.
234. Hupp JR. Use of corticosteroids for aspiration pneumonitis. J Oral Maxillofac Surg 1993;51:103-104. Comment on: Vaughan GG, Grycko RJ, Montgomery MT. The prevention and treatment of aspiration of vomitus during pharmacosedation and general anesthesia. J Oral Maxillofac Surg 1992;50:874-879.
235. Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 1972;50:515-520.
236. Fauci AS, Haynes BF, Katz P, Wolff SM. Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years. Ann Intern Med 1983;98:76-85.
237. Turner-Warwick M, Turner-Stokes L. Thoracic manifestations of multisystem diseases. In: Baum GL, Wolinsky E, eds. Textbook of pulmonary diseases. Boston-Toronto: Little, Brown and Company, 1989:689-714.
238. Walsh D, Avashia J. Glucocorticoids in clinical oncology. Clev Clin J Med 1992;59:505-515.

239. Roila F, and the Italian group for antiemetic research. Dexamethasone, granisetron, or both for the prevention of nausea and vomitting during chemotherapy for cancer. New Engl J Med 1995;332:1-5.
240. Harter JG, Reddy WJ, Thorn GW. Studies on an intermittent corticosteroid dosage regimen. New Engl J Med 1963;269:591-596.
241. Axelrod L. Glucocorticoids. In: Kelley WN, Harris ED, Ruddy S, Sledge CB, eds. Textbook of Rheumatology. Philadelphia: WB Saunders Company, 1993:779-796.
242. Keogh BA, Bernardo J, Hunninghake GW, Line BR, Price DL, Crystal RG. Effect of intermittent high dose parenteral corticosteroids on the alveolitis of idiopathic pulmonary fibrosis. Am Rev Respir Dis 1983;127:18-22.
243. Claman HN, Moorhead JW, Benner WH. Corticosteroids and lymphoid cells in vitro. I. Hydrocortisone lysis of human, guinea pig, and mouse thymus cells. J Lab Clin Med 1971;78:499-507.
244. Fauci AS, Dale DC. The effect of in vivo hydrocortisone on subpopulations of human lymphocytes. J Clin Invest 1974;53:240-246.
245. Kehrl JH, Fauci AS. The clinical use of glucocorticoids. Ann Allerg 1983;50:2-8.
246. Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc Natl Acad Sci USA 1992;89:9991-9995.
247. Guyre PM, Girard MT, Morganelli PM, Manganiello PD. Glucocorticoid effects on the production and actions of immune cytokines. J Steroid Biochem 1988;30:89-93.
248. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray MA, Anderson WH, eds. Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:593-617.
249. Amano Y, Lee SW, Allison AC. Inhibition by glucocorticoids of the formation of interleukin-1α, interleukin-1β, and interleukin-6: mediation by decreased mRNA stability. Mol Pharmacol 1993;43:176-182.
250. Knudsen PJ, Dinarello CA, Strom TB. Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin 1 in U937 cells. J Immunol 1987;139:4129-4134.
251. Boumpas DT, Anastassiou ED, Older SA, Tsokos GC, Nelson DL, Balow JE. Dexamethasone inhibits human interleukin 2 but not interleukin 2 receptor gene expression in vitro at the level of nuclear transcription. J Clin Invest 1991;87:1739-1747.
252. Paliogianni F, Raptis A, Ahuja SS, Najjar SM, Boumpas DT. Negative transcriptional regulation of human interleukin 2 (IL-2) gene by glucocorticoids through interference with nuclear transcription factors AP-1 and NF-AT. J Clin Invest 1993;91:1481-1489.
253. Fushimi T, Okayama H, Shimura S, Sasaki H, Takishima T, Shirato K. Dexamethasone suppresses both IL-5 production and its gene expression by human PBMC. Am Rev Respir Dis 1993;147:A980.
254. Waage A, Slupphaug G, Shalaby R. Glucocorticoids inhibit the production of IL-6 from monocytes, endothelial cells and fibroblasts. Eur J Immunol 1990;20:2439-2443.
255. Standiford TJ, Kunkel SL, Rolfe MW, Evanoff HL, Allen RM, Strieter RM. Regulation of human alveolar macrophage- and blood monocyte-derived interleukin-8 by prostaglandin E_2 and dexamethasone. Am J Respir Cell Mol Biol 1992;6:75-81.
256. Natrajan S, Schulman ES, Rozans M, Glaum M, Raible D, Gillespie D. Dexamethasone suppresses the generation of TNF-α in human lung at a transcriptional level. Am Rev Respir Dis 1993;147:A981.
257. Arya SK, Wong-Staal F, Gallo RC. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. J Immunol 1984;133:273-276.
258. Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 1990;62:1189-1204.
259. Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J, Karin T. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 1990;62:1205-1215.

260. Schüle R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell 1990;62:1217-1226.
261. Flower RJ. Background and discovery of lipocortins. Agents Actions 1985;17:225-262.
262. Northup JK, Valentine-Braun KA, Johnson LK, Severson DL, Hollenberg MD. Evaluation of the antiinflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J Clin Invest 1988;82:1347-1352.
263. Brönnegård M, Andersson O, Edwall D, Lund J, Norstedt G, Carlstedt-Duke J. Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol Endocrinol 1988;2:732-739.
264. Goulding NJ, Godolphin JL, Sharland PR, Peers SH, Sampson M, Maddison PJ, Flower RJ. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. Lancet 1990; 335:1416-1418.
265. Ambrose MP, Hunninghake GW. Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. J Appl Physiol 1990;68:1668-1671.
266. Solito E, Raugei G, Melli M, Parente L. Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated, but not undifferentiated U-937 cells. FEBS Lett 1991;291:138-244.
267. Goulding NJ, Guyre PM. Regulation of inflammation by lipocortin 1. Immunol Today 1992;13:295-297.
268. Ambrose MP, Bahns C-LC, Hunninghake GW. Lipocortin I production by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;6:17-21.
269. Goulding NJ, Guyre PM. Glucocorticoids, lipocortins and the immune response. Curr Opin Immunol 1993;5:108-113.
270. Flower RJ, Rothwell NJ. Lipocortin-1: cellular mechanisms and clinical relevance. Trends Parmacol Sci 1994;15:71-76.
271. Di Rosa M, Radomski M, Carnuccio R, Moncada S. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem Biophys Res Commun 1990;172:1246-1252.
272. Radomski MW, Palmer RMJ, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. Proc Natl Acad Sci USA 1990;87:10043-10047.
273. Mayo KE, Palmiter RD. Glucocorticoid regulation of metallothionein-I mRNA synthesis in cultured mouse cells. J Biol Chem 1981;256:2621-2624.
274. Van de Stolpe A, Caldenhoven E, Raaijmakers JAM, Van der Saag PT, Koenderman L. Glucocorticoid-mediated repression of intercellular adhesion molecule-1 expression in human monocytic and bronchial epithelial cell lines. Am J Respir Cell Mol Biol 1993;8:340-347.
275. Tobler A, Meier R, Seitz M, Dewald B, Baggiolini M, Fey MF. Glucocorticoids downregulate gene expression of GM-CSF, NAP-1/IL-8, and IL-6, but not of M-CSF in human fibroblasts. Blood 1992;79:45-51.
276. Wallner BP, Mattaliano RJ, Hession C, Cate RL, Tizard R, Sinclair LK, Foeller C, Pingchang Chow E, Browning JL, Ramachandran KL, Pepinsky RB. Cloning and expression of human lipocortin, a phospholipase A2 inhibitor with potential anti-inflammatory activity. Nature 1986;320:70-81.
277. Carmichael J, Paterson IC, Diaz P, Crompton GK, Kay AB, Grant IWB, Corticosteroid resistance in chronic asthma. Brit Med J 1981;282:1419-1422.
278. Arai K, Chrousos GP. Glucocorticoid resistance. Ballière's Clin Endocrinol Metab 1994;8:317-331.
279. Vingerhoeds ACM, Thijssen JHH, Schwarz F. Spontaneous hypercortisolism without Cushing's syndrome. J Clin Endocrinol Metab 1976;43:1128-1133.
280. Linder MJ, Thompson EB. Abnormal glucocorticoid receptor gene and mRNA in primary cortisol resistance. J steroid Biochem 1989;32:243-249.

281. Hurly DM, Accili D, Stratakis CA, Karl M, Vamvakopoulos N, Rorer E, Constantine K, Taylor SI, Chrousos GP. Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 1991;87:680-686.
282. Lamberts SWJ, Poldermans D, Zweens M, De Jong FH. Familial cortisol resistance: differential diagnostic and therapeutic aspects. J Clin Endocrinol Metab 1986;63:1328-1333.
283. Karl M, Lamberts SWJ, Detera-Wadleigh SD, Encio IJ, Stratakis CA, Hurley DM, Accili D, Chrousos GP. Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. J Clin Endocrinol Metab 1993;76:683-689.
284. Malchoff DM, Brufsky A, Reardon G, McDermott P, Javier EC, Bergh C-H, Rowe D, Malchoff CD. A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 1993;91:1918-1925
285. Brönnegård M, Werner S, Gustafsson J-Å. Primary cortisol resistance associated with a thermolabile glucocorticoid receptor in a patient with fatigue as the only symptom. J Clin Invest 1986;78:1270-1278.
286. Morris HG. Factors that influence clinical responses to administered corticosteroids. J Allergy Clin Immunol 1980;66:343-346.
287. Parfitt AM. Cushing's syndrome with "normal" replacement dose of cortisone in pituitary hypothyroidism. J Clin Endocrinol Metab 1964;24:560-562.
288. Lipsett MB. Factors influencing the rate of metabolism of steroid hormones in man. Ann NY Acad Sci 1971;179:442-449.
289. Brooks SM, Sholiton LJ, Werk EE, Altenau P. The effects of ephedrine and theophylline on dexamethasone metabolism in bronchial asthma. J Clin Pharmacol 1977;17:308-318.
290. Powell LW, Axelsen E. Corticosteroids in liver disease: studies on the biological conversion of prednisone to prednisolone and plasma protein binding. Gut 1972;13:690-696.
291. Lewis GP, Jusko WJ, Burke CW, Graves L. Prednisone side-effects and serum-protein levels. Lancet 1971;ii:778-781.
292. Chung KF, Podgorski MR, Goulding NJ, Godolphin JL, Sharland PR, O'Connor B, Flower RJ, Barnes PJ. Circulating autoantibodies to recombinant lipocortin-1 in asthma. Resp Med 1991;85:121-124.
293. Chrousos GP, Vingerhoeds A, Brandon D, Eil C, Pugeat M, DeVroede M, Loriaux L, Lipsett MB. Primary cortisol resistance in man. A glucocorticoid receptor-mediated disease. J Clin Invest 1982;69:1261-1269.
294. Homo-Delarche F. Glucocorticoid receptors and steroid sensitivity in normal and neoplastic human lymphoid tissues: a review. Cancer Res 1984;44:431-437.
295. Gomi M, Iida S, Tsugawa M, Itoh Y, Moriwaki K, Yamashita S, Tarui S. Decreased glucocorticoid receptors in cultured skin fibroblasts from a patient with primary cortisol resistance. New Engl J Med 1986;314:1194.
296. Lamberts SWJ, Koper JW, Biemond P, Den Holder FH, De Jong FH. Cortisol receptor resistance: the variability of its clinical presentation and response to treatment. J Clin Endocrinol 1992;74:313-321.
297. Kato GJ, Quddus FF, Shuster JJ, Boyett J, Pullen JD, Borowitz MJ, Whitehead VM, Crist WM, Leventhal BG. High glucocorticoid receptor content of leukemic blasts is a favorable prognostic factor in childhood acute lymphoblastic leukemia. Blood 1993;82:2304-2309.
298. Yem AW, Tomasselli AG, Heinrikson RL, Zurcher-Neely H, Ruff VA, Johnson RA, Deibel MR. The Hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP13. J Biol Chem 1992;267:2868-2871.
299. Ning Y-M, Sanchez ER. Potention of glucocorticoid receptor-mediated gene expression by the immunophilin ligands FK506 and rapamycin. J Biol Chem 1993;268:6073-6076.
300. Adcock IM, Brown CR, Peters MJ, Lane S, Gelder CM, Virdee H, Lee T, Barnes PJ. DNA binding of glucocorticoid receptor from peripheral blood monocytes of steroid sensitive and resistant patients. Am Rev Respir Dis 1993;147:A244.

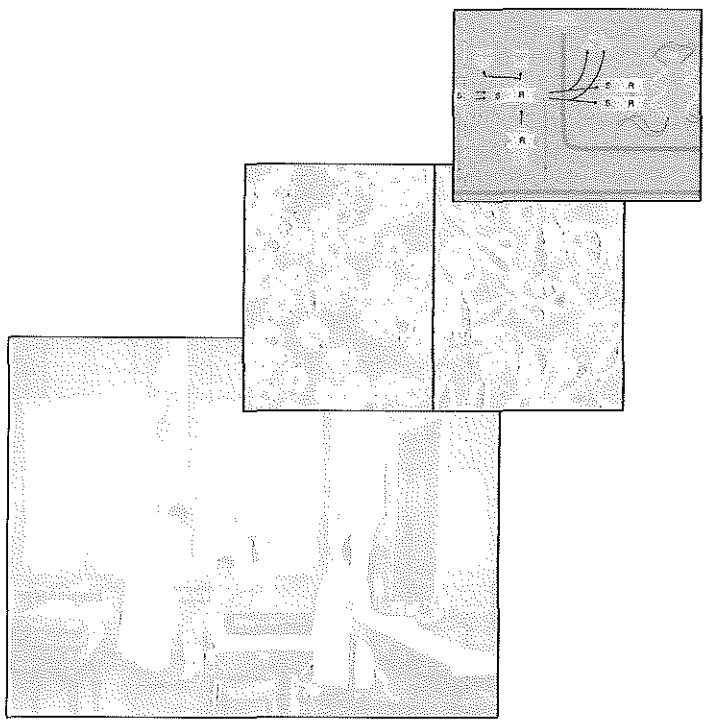
301. Adcock IM, Brown CR, Virdee H, Peters MJ, Gelder CM, Lane S, Lee T, Barnes PJ. Transcription factor interactions in peripheral blood monocytes of steroid sensitive and resistant asthmatic patients. Am Rev Respir Dis 1993;147:A292.
302. Kam JC, Szefler SJ, Surs W, Sher ER, Leung DYM. Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. J Immunol 1993;151:3460-3466.
303. Gowland PL, Buettl E. Mutations in the hormone regulatory element of mouse mammary tumor virus differentially affect the response to progestins, androgens, and glucocorticoids. Mol Cell Biol 1989;9:3999-4008.
304. Kay AB, Diaz P, Carmichael J, Grant IWB. Corticosteroid resistant chronic asthma and monocyte complement receptors. Clin Exp Immunol 1981;44:576-580.
305. Poznansky MC, Gordon ACH, Douglas JG, Krajewski S, Wyllie AH, Grant IWB. Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients. Clin Sci 1984;67:639-645.
306. Poznansky MC, Gordon ACH, Grant IWB, Wyllie AH. A cellular abnormality in glucocorticoid resistant asthma. Clin Exp Immunol 1985;61:135-142.
307. Corrigan CJ, Brown PH, Barnes NC, Szefler SJ, Tsai JJ, Frew AJ, Kay AB. Glucocorticoid resistance in chronic asthma. Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids *in vitro*. Am Rev Respir Dis 1991;144:1016-1025.
308. Wilkinson JRW, Crea AEG, Clark TJH, Lee TH. Identification and characterization of a monocyte-derived neutrophil-activating factor in corticosteroid-resistant bronchial asthma. J Clin Invest 1989;84:1930-1940.
309. Lane SJ, Lee TH. Glucocorticoid receptor characteristics in monocytes of patients with corticosteroid-resistant bronchial asthma. Am Rev Respir Dis 1991;143:1020-1024.
310. Kamada AK, Spahn JD, Surs W, Brown E, Leung DYM, Szefler SJ. Coexistence of glucocorticoid receptor and pharmacokinetic abnormalities: factors that contribute to a poor response to treatment with glucocorticoids in children with asthma. J Pediatr 1994;124:984-986.
311. Sher ER, Leung DYM, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Cellular mechanism contributing to inadequate response to glucocorticoid therapy. J Clin Invest 1994;93:33-39.
312. Corrigan CJ, Brown PH, Barnes NC, Tsai J-J, Frew AJ, Kay AB. Glucocorticoid resistance in chronic asthma: peripheral blood T lymphocyte activation and comparison of the T lymphocyte inhibitory effects of glucocorticoids and cyclosporin A. Am Rev Respir Dis 1991;144:1026-1032.
313. Norbiato G, Bevilacqua M, Vago T, Baldi G, Chebat E, Bertora P, Moroni M, Galli M, Oldenburg N. Cortisol resistance in acquired immunodeficiency syndrome. J Clin Endocrinol Metab 1992;74:608-613.
314. Ballard PL, Baxter JD, Higgins SJ, Rousseau GG, Tomkins GM. General presence of glucocorticoid receptors in mammalian tissues. Endocrinology 1974;94:998-1002.
315. Lippman M, Barr R. Glucocorticoid receptors in purified subpopulations of human peripheral blood lymphocytes. J Immunol 1977;118:1977-1981.
316. Lacronique JG, Rennard SI, Bitterman PB, Ozaki T, Crystal RG. Alveolar macrophages in idiopathic pulmonary fibrosis have glucocorticoid receptors, but glucocorticoid therapy does not suppress alveolar macrophage release of fibronectin and alveolar macrophage derived growth factor. Am Rev Respir Dis 1984;130:450-456.
317. Van Hal PThW, Mulder E, Hoogsteden HC, Hilvering C, Benner R. Glucocorticoid receptors in alveolar macrophages: methodological aspects of the determination of the number of glucocorticoid receptors per cell. Agents Actions 1989;26:128-131.
318. Van Hal PThW, Wijkhuijs JM, Hoogsteden HC, Van der Kwast ThH, Zegers ND, Mulder E. Functional glucocorticoid receptors in human alveolar macrophages. Submitted for publication.
319. Rinehart JJ, Sagone AL, Balcerzak SP, Ackerman GA, LoBuglio AF. Effects of corticosteroid therapy on human monocyte function. New Engl J Med 1975;292:236-241.

320. Guyre PM, Munck A. Glucocorticoid actions on monocytes and macrophages. In:Schleimer RP, Claman HN, Oronsky A, eds. Anti-inflammatory steroid action. Basic and clinical aspects. San Diego: Academic Press, Inc. 1989:199-225.
321. Russo-Marie F. Macrophages and the glucocorticoids. J Neuroimmunol 1992;40:281-286.
322. Schaffner A, Douglas H, Braude A. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. J Clin Invest 1982;69:617-631.
323. Schaffner A. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. J Clin Invest 1985;76:1755-1764.
324. Rinehart JJ, Balcerzak SP, Sagone AL, LoBuglio AF. Effects of corticosteroids on human monocyte function. J Clin Invest 1974;54:1337-1343.
325. Nugent KM, Pesanti EL. Chronic glucocorticosteroid therapy impairs staphylococcal clearance from murine lungs. Infect Immun 1982;38:1033-1036.
326. Warren MK, Vogel SN. Opposing effects of glucocorticoids on interferon-γ-induced murine macrophage Fc receptor and Ia antigen expression. J Immunol 1985;134:2462-2469.
327. Hogan MM, Vogel SN. Inhibition of macrophage tumoricidal activity by glucocorticoids. J Immunol 1988;140:513-519.
328. Sivo J, Politis AD, Vogel SN. Differential effects of interferon-γ and glucocorticoids on FcγR gene expression in murine macrophages. J Leukocyte Biol;1993:54:451-457.
329. Roitt I. Extracellular bacteria susceptible to killing by phagocytosis and complement. In: Essential Immunology. Oxford: Blackwell Scientific Publications, 1994:247-255.
330. Guyre PM, Morganelli PM, Miller R. Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. J Clin Invest 1983;72:393-397.
331. Perussia B, Dayton ET, Lazarus R, Fanning V, Trinchieri G. Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. J Exp Med 1983;158:1092-1113.
332. Crabtree GR, Munck A, Smith KA. Glucocorticoids inhibit expression of Fc receptors on the human granulocytic cell line HL-60. Nature 1979;279:338-339.
333. Náray-Fejes-Tóth A, Cornwell GG, Guyre PM. Glucocorticoids inhibit IgE receptor expression on the human monocyte cell line U937. Immunology 1985;56:359-366.
334. Girard MT, Hjaltadottir S, Náray-Fejes-Tóth A, Guyre PM. Glucocorticoids enhance the γ-interferon augmentation of human monocyte immunoglobulin G Fc receptor expression. J Immunol 1987;138:3235-3241.
335. Anderson CL, Looney RJ. Human leukocyte IgG Fc receptors. Immunol Today 1986;7:264-266.
336. Van de Winkel JGJ, Anderson CL. Biology of human immunoglobulin G Fc receptors. J Leukocyte Biol 1991;49:511-524.
337. Pan L, Mendel DB, Zurlo J, Guyre PM. Regulation of the steady state level of FcγRI mRNA by IFN-γ and dexamethasone in human monocytes, neutrophils, and U-937 cells. J Immunol 1990;145:267-275.
338. Van Hal PThW, Prins A, Hoogsteden HC. IL-1β gene expression in cultured bronchoalveolar macrophages from smokers and nonsmokers, and modulation by IL-4 and dexamethasone. Submitted for publication.
339. Zanker B, Walz G, Wieder KJ, Sirom TB. Evidence that glucocorticosteroids block expression of the human interleukin-6 gene by accessory cells. Transplantation 1990;49:183-185.
340. Weinberg JB, Wortham TS, Misukonis MA, Patton KL, Chitneni SR. Synovial mononuclear phagocytes in rheumatoid arthritis and osteoarthritis: quantitative and functional aspects. Immunol Invest 1993;22:365-374.
341. Snyder DS, Unanue ER. Corticosteroids inhibit murine macrophage Ia expression and interleukin 1 production. J Immunol 1982;129:1803-1805.

342. Celada A, McKercher S, Maki RA. Repression of major histocompatibility complex Ia expression by glucocorticoids: the glucocorticoid receptor inhibits the DNA binding of the X box DNA binding protein. J Exp Med 1993;177:691-698.
343. Leszczynski D, Ferry B, Schellekens H, Van de Melde PH, Häyry P. Antagonistic effects of γ interferon and steroids on tissue antigenicity. J Exp Med 1986;164:1470-1477.
344. Hirschberg H, Hirschberg T, Nousiainen H, Braathen LR, Jaffe E. The effects of corticosteroids on the antigen presenting properties of human monocytes and endothelial cells. Clin Immunol Immunop 1982;23:577-585.
345. Rinehart JJ, Wuest D, Ackerman GA. Corticosteroid alteration of human monocyte to macrophage differentation. J Immunol 1982;129:1436-1440.
346. Van Hal PThW, Hoogsteden HC, Te Velde AA, Wijkhuijs JM, Figdor CG, Hilvering C. IL-4-induced maturation of monocytes/macrophages is inhibited by glucocorticoids. Submitted for publication.
347. Baybutt HN, Holsboer F. Inhibition of macrophage differentiation and function by cortisol. Endocrinology 1990;127:476-480.
348. Hoff T, Spencker T, Emmendoerffer A, Goppelt-Struebe M. Effects of glucocorticoids on the TPA-induced monocytic differentiation. J Leukocyte Biol 1992;52:173-182.
349. Luedke CE, Cerami A. Interferon-γ overcomes glucocorticoid suppression of cachectin/tumor necrosis factor biosynthesis by murine macrophages. J Clin Invest 1990;86:1234-1240.
350. Salkowski CA, Vogel SN. IFN-γ mediates increased glucocorticoid receptor expression in murine macrophages. J Immunol 1992;148:2770-2777.
351. Rakasz E, Gal A, Biró J, Balas G, Falus A. Modulation of glucocorticosteroid binding in human lymphoid, monocytoid and hepatoma cell lines by inflammatory cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α. Scand J Immunol 1993;37:684-689.

CHAPTER 2

ANTI-INFLAMMATORY MECHANISMS OF GLUCOCORTICOIDS



CHAPTER 2

ANTI-INFLAMMATORY MECHANISMS OF GLUCOCORTICOIDS*

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CHAPTER 2

2.1 INTRODUCTION

2.2 HISTORICAL BACKGROUND

2.3 NUCLEAR MECHANISM

2.3.1. Introduction

2.3.2. Glucocorticoid receptor

2.3.3. Glucocorticoid-regulated mediators
- *Phospholipase A_2 inhibitory proteins*
- *Interleukin-1*
- *Interleukin-2*
- *Interleukin-3*
- *Interleukin-5*
- *Interleukin-6*
- *Interleukin-8*
- *Tumor necrosis factor-α*
- *Interferon-γ*

2.3.4. Other glucocorticoid-regulated proteins
- *Collagen*
- *Collagenase*
- *Nuclease*
- *ICAM-1 (CD54)*
- *Neutral endopeptidase (CD10)*

2.4 NONNUCLEAR MECHANISM

2.5 OTHER ANTI-INFLAMMATORY ACTIONS

2.6 CONCLUSION AND FUTURE RESEARCH

2.7 REFERENCES

2.1 INTRODUCTION

Controlling inflammation is still one of the major aims in medicine. Today, many anti-inflammatory drugs are available (Table 1), but new agents are still being searched for.

Table 1. Overview of anti-inflammatory drugs.

Main groups of anti-inflammatory drugs	Examples/comments
1. Derivatives of salicylic acid	- Aspirin
2. Derivatives of acetic acid	- Indomethacin, diclofenac
Derivatives of propionic acid	- Ibuprofen, naproxen
Derivatives of oxicam	- Piroxicam
Derivatives of pyrazolone	- Phenylbutazone
3. Disease modulating/slow acting agents	- Gold, antimalarial compounds (chloroquine), penicillamine
4. Anti-neoplastic drugs	- Cyclophosphamide, azathioprine, methotrexate
5. Glucocorticoids (derivates of cortisol)	- Prednisone, dexamethasone
6. Cytokines, cytokine receptors and their antagonists	- Clinical trials recently started (e.g. IL-1R antagonist and IL-4)

Glucocorticoids are powerful anti-inflammatory agents. In humans there is a natural production of mainly hydrocortisone by the adrenal cortex, and this steroid hormone at physiological levels has several vital effects (1). An excessive production, however, leads to a disturbance of a hormonal equilibrium resulting in a salient constellation of symptoms and physical features. During periods of stress, plasma hydrocortisone concentration is elevated, but paradoxically glucocorticoids are able to inhibit the body's defense systems against stress factors. It is proposed that this stress-induced increase in hydrocortisone level prevents the body's defense reactions from overshooting and needless tissue damage (2). Since 1948 these inhibitory effects of glucocorticoids have been used in the treatment of several inflammatory diseases, and up until now no equally potent agent has been found.

This chapter deals with some recently unraveled molecular mechanisms of glucocorticoid actions at the level of synthesis and release of mediators in inflammatory processes. With the help of these mechanisms it is possible to understand more clearly why some inflammatory lung diseases can be treated with glucocorticoids. On the other hand, knowledge of the importance and role of certain mediators in inflammatory lung diseases combined with understanding of the basic glucocorticoid influences in inflammation will lead to predictions as to whether or not such diseases can be combatted with these steroids. Hopefully, it will also create possibilities to eliminate the more serious side effects.

2.2 HISTORICAL BACKGROUND

Today it is impossible to imagine life without glucocorticoids in the treatment of a large variety of diseases (3), although not so long ago Philip S. Hench wrote that "... no supplies of compound E are expected for treatment or additional research until sometime in 1950 at the earliest at which time supplies still will be exceedingly small" (4). In his paper he mentioned the use of cortisone, originally termed compound E and first isolated 16 years earlier (5), in the treatment of rheumatoid arthritis. Following the footsteps of Hench, glucocorticoids were soon introduced in the treatment of other inflammatory diseases including some lung diseases, in particular asthma (6). Unfortunately this apparent panacea turned out also to have undesirable side effects, just as impressive as the intended therapeutic effects (7).

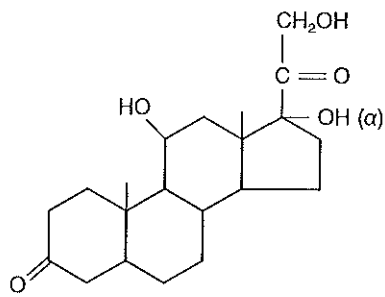

Figure 1. Structure of hydrocortisone.

In the beginning, cortisone was used and administered intramuscularly, but because of the side effects a search for a more ideal anti-inflammatory therapeutic with fewer side effects was initiated. In principal two potentialities were investigated. First, it proved to be possible to reduce the mineralocorticoid action, which is one of the causes of the unwanted side effects. At the same time, the anti-inflammatory effects could be potentiated. This was achieved by the production of synthetic glucocorticoid derivatives instead of isolation and separation of steroids from adrenal extracts. All these derivatives have, as does the very first administered compound E, the same basic structure as hydrocortisone (cortisol) (Figure 1). In humans, hydrocortisone is the most important glucocorticoid, while cortisone happens to be its natural precursor. Table 2 shows some glucocorticoids with their relative potencies. Second, attempts were made to reduce the systemic side effects by delivering the glucocorticoids locally to the place of disease. This search led in 1972 to the introduction of beclomethasone dipropionate (BDP) as a topical aerosol glucocorticoid in the treatment of asthma (8). The absence of significant systemic side effects can be explained by the fact that the small part of BDP that is absorbed systemically will be converted directly in the liver to the much less potent beclomethasone. Since this success, other topical glucocorticoids were introduced in the treatment of some lung diseases and several other inflammatory disorders.

In the 46 years since the first scientific paper on the clinical application of glucocorticoids, much clinical and fundamental research has unraveled some of the basic mechanisms through which glucocorticoids influence inflammatory and immunological processes. Especially the deeper understanding of influences on the production or secretion of mediators enables us to explain clinical (side) effects in a more satisfying way. Nevertheless, many riddles remain, and scientists are aware of the great diversity of still uncomprehended mechanisms, which together define the outcome of glucocorticoid treatment (9).

Table 2. Relative properties of some glucocorticoids.

	Relative mineralocorticoid effects	Relative anti-inflammatory effects	Equivalent dose (mg) when systemically administered	General comments
Cortisone (compound E)	+ +	0.8	25	Natural precursor of hydrocortisone
Hydrocortisone	+ +	1.0	20	Natural glucocorticoid in humans
Prednisone	+	4.0	5	Conversion to prednisolone in liver
Prednisolone	+	4.0	5	
Methylprednisolone	0	5.0	4	
Triamcinolone	0	5.0	4	
Betamethasone	0	20 to 30	0.6	Long biologic half-life with prolonged duration of effects and side effects
Dexamethasone	0	20 to 30	0.75	
Budesonide	0	Developed for inhalation therapy. Higher affinity for glucocorticoid receptors than dexamethasone and betamethasone. No systemic effects because of an efficient hepatic first-pass metabolism		
Fluticasone propionate	0			

2.3 NUCLEAR MECHANISM

2.3.1. Introduction

Most investigators are convinced that a so-called "nuclear mechanism" is responsible for most, if not all, of the glucocorticoid effects (10). In this mechanism the leading role is played by glucocorticoid receptors. These receptors are not membrane receptors, but are believed to be situated in the cytoplasm at least in the absence of glucocorticoids. Addition of glucocorticoids leads to a shift from the cytoplasm to the nucleus (11). These observations were made in experiments with a monoclonal antibody against the glucocorticoid receptor on cell cultures. *In vivo* there probably exists an equilibrium between cytoplasmic- and nuclear-situated receptors.

The initial events in the nuclear pathway that prelude the glucocorticoid effects concern the passive diffusion of glucocorticoids through the cell membrane and the specific binding to the glucocorticoid receptor, which is associated with at least three types of heat shock proteins (hsp) (Figure 2). The unliganded glucocorticoid receptor exists in the cytoplasm as a hetero-oligomer and interacts with two molecules of hsp 90 and one molecule of hsp 70 (12,13). Hsp 56 presumably does not interact with the receptor itself, but interacts with and regulates the function of hsp 90 (14,15). As a result of the binding of glucocorticoids to their receptors, the receptor molecule undergoes some changes, probably expressed in the steric conformation, and acquires a high affinity for binding to specific DNA sequences in the nucleus. The original receptor has now turned into an activated (transformed) receptor-ligand complex. Moreover, this activation is accompanied by the dissociation of two types of the heat shock proteins (hsp 90 and hsp 56) from the receptor (Figure 2). Dissociation of hsp70 from the receptor is thought to take place not in the cytoplasm, but at a later stage in the nucleus (16). The precise functions of these hsp are not yet clear. Probably, hsp 90 plays a role in transport of the glucocorticoid receptor towards the nucleus through interactions with the cytoskeleton (17,18). The same

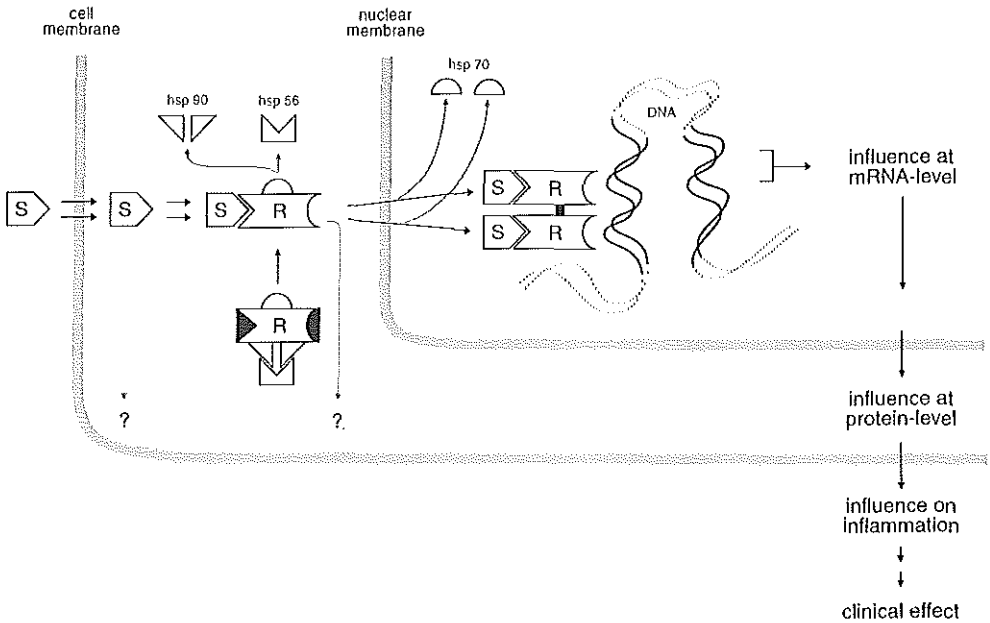

Figure 2. Hypothetical cellular events after glucocorticoid administration. Glucocorticoids (S), steroid hormones, pass the cell membrane and bind to their receptors (R). The unliganded glucocorticoid receptor is part of a multiprotein complex, that also consists of several heat shock proteins (hsp). Upon binding of glucocorticoids to their receptors, some hsp (hsp 56 and hsp 90) dissociate from the receptor in the cytoplasm. Others (hsp 70) probably dissociate from the receptor in the nucleus. Furthermore, in the nucleus liganded glucocorticoid receptors form homodimers and interact with specific DNA sequences. Eventually, this interaction results in modulation of transcription.

function has been suggested for hsp 70, whereas hsp 56 may play a role in the regulation of hsp 90 function (15). Furthermore, hsp 56 may interact with the immunosuppressive FK 506, leading to an increased translocation of the glucocorticoid receptor to the nucleus (19,20).

Next, the activated receptor-glucocorticoid complex passes the nuclear membrane, "recognizes" a specific DNA sequence, and binds to it (for recent reviews: 21,22). These DNA sequences are now recognized as enhancers of DNA transcription (23) and are called glucocorticoid response elements (GRE). It is known now that there exist two classes of steroid hormone responsive elements in general. One group mediates response to glucocorticoids, progestins, mineralocorticoids, and androgens. This group is known as the group of glucocorticoid/progesterone response elements (GRE/PRE), and has the consensus nucleotide sequence GGTACAnnnTGTYCY (24). The other group mediates response to estrogens, thyroid hormones, vitamin D_3 and retinoic acid, and has the consensus sequence GGTCAnnnTGACC (24). The prototype of this group is the estrogen response element (ERE). In common with the other response elements, GRE are either palindromic or half-palindromic sequences. This probably accounts for the fact that in general not one activated receptor-ligand complex binds to DNA, but a homo- or hetero-dimer of complexes (25,26). Through this binding, the transcription of DNA to mRNA can be influenced at places of the genome, mostly far removed from the GRE (27). Steroid hormone response elements found in hormonally responsive genes are usually clustered as hormone response regions (HRR). Examples of such clusters of binding sites for the same class of steroid hormone receptor are found in the HRR of the MMTV (21; Figure 3), the rabbit uteroglobuline gene (28), and the chicken lysozyme gene (29).

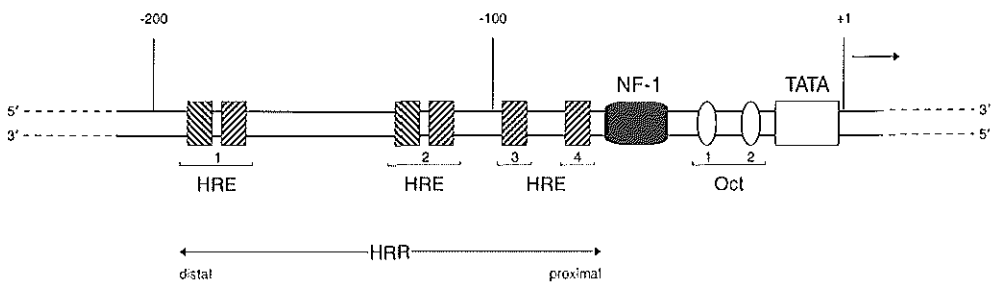

Figure 3. Schematic representation of the mouse mammary tumor virus (MMTV) promoter. The hormone response region (HRR) consists of four separate hormone response elements (HRE). HRE 1 and 2 are palindromic structures, whereas HRE 3 and 4 are half-palindromes. Furthermore, the binding site for NF-1, the two octamer motifs (Oct), and the TATA box are shown.

Furthermore, HRR may be integrated in the context of a complex array of additional sites for other transcription factors (21; Figure 3). The interactions among receptors bound to these clusters of response elements and the interactions between these receptors and other transcription factors play an important role in steroid hormone response (21,30,31). Influence of the GRE-bound receptor-ligand complex on transcription is mediated partially

by transcription-activation domains on the receptor. The glucocorticoid receptor contains at least three transcription-activation domains, one in the steroid-binding, one in the DNA-binding, and one in the N-terminal domain (22,32-34). It is generally thought that these transcription-activation domains contact transcription factors, whereupon the transcription machinery is either activated or deactivated (21,22,35). It is possible to get either an increased transcription or a decreased transcription. This regulation at the level of mRNA finally results in induction or inhibition of the translation of mRNA to protein. The induced or inhibited proteins probably account for many of the clinical glucocorticoid effects, although as yet only a few glucocorticoid-regulated proteins have been identified (Table 3). The intermediate between administration of glucocorticoids and the clinical effects caused by influenced protein synthesis usually takes a minimum of several hours.

Table 3. Influence of glucocorticoids on the synthesis of some proteins involved in inflammation.

Protein	Inflammatory function of protein	Glucocorticoid effect on gene transcription	References
Lipocortins	Regulate the production of arachidonic acid metabolites by phospholipase A_2 inhibition	Increase	77-100
Interleukin-1	Early role in inflammation	Decrease	103,104
Interleukin-2	Induces T cell proliferation	Decrease	106-108
Interleukin-3	Regulates hematopoiesis	Decrease	110
Interleukin-5	Induces eosinophilia	Decrease	113
Interleukin-6	Early role in inflammation	Decrease	116
Interleukin-8	Chemotactic for neutrophils	Decrease	119
TNF-α	Role in shock, capillary leak	Decrease	120
Interferon-γ	Activation of mononuclear phagocytes	Decrease	107
Collagens	Enhanced deposition in fibrosis	Decrease	122-124,126
Nuclease	Degrades DNA leading to lymphocytolysis in mice	Increase	130

2.3.2. Glucocorticoid receptor

With the nuclear mechanism in mind, it is possible to imagine the glucocorticoid receptor (GR) as a protein with different functional domains: one domain with the function to bind steroid and another domain with the function to bind DNA (Figure 4). The existence of such functional domains has been confirmed by limited proteolysis of crude and purified GR (36). Mild digestion with different proteases resulted in different proteolytic fragments, some still able to bind glucocorticoids or DNA. At that time, these findings strengthened the hypothesis that separate DNA- and steroid-binding domains were present within the GR. Apart from the steroid-binding capacity, the steroid-binding domain is nowadays also believed to possess a transcription activation function (37). The DNA-binding region contains two subregions which are both able to form a "zinc finger" (38,39). The first, N-terminally located, "zinc finger" is believed to recognize one half of the palindromic GRE and to interact with the major groove of the DNA double helix. More precisely, three amino

acids at the C-terminal site of this finger are believed to determine DNA binding specificity (for a recent review: 38). The second "zinc finger" plays a role in the dimerization of two glucocorticoid receptor molecules upon binding to GRE (38,40). The DNA-binding region is also known to have a transcription-activation function (33,34). Furthermore, a third domain has been recognized by making antibodies against the receptor (immunogenic domain in Figure 4) (41). The precise role of this third domain is still uncertain, but it is required in transcriptional regulation. A portion of the receptor molecule between the DNA-binding and steroid-binding domain has been termed "hinge region", because of supposed flexibility in determining the three-dimensional structure (Figure 4).

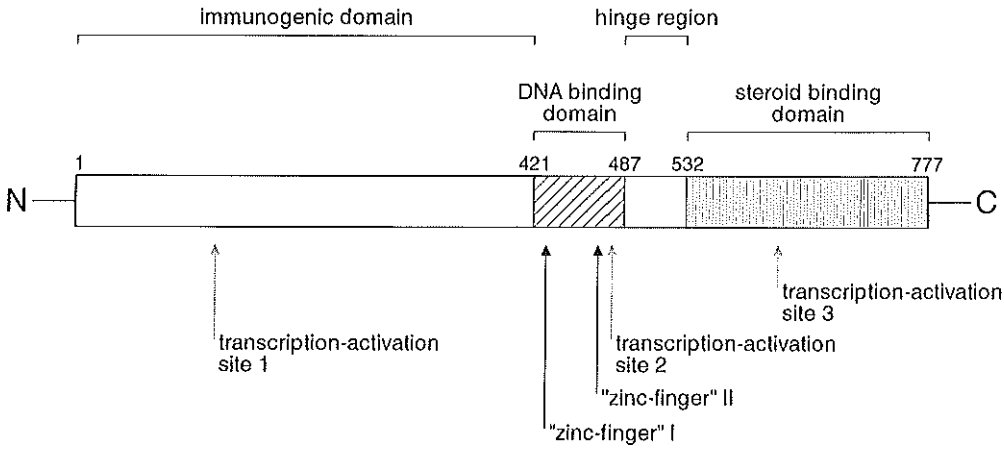

Figure 4. Linear alignment of the human glucocorticoid receptor. N and C represent the N-terminal and C-terminal end of the protein, respectively.

This subdivision of the GR into three main domains is also visible at the level of DNA. This observation was enabled by the cloning of the human GR cDNA (42). Thanks to the cloning of other hormone receptors with a transcriptional regulatory function, it became possible to compare these receptors at the DNA and protein level (estrogen receptor (43), mineralocorticoid receptor (44), progesteron receptor (45), androgen receptor (46). In particular the cloning of the mineralocorticoid receptor needs to be mentioned because it also has, added to its aldosteron-binding capacity, a high cortisol-binding capacity, but a low dexamethasone-binding capacity. Therefore, this receptor is often referred to as a type I corticosteroid binding site, whereas the glucocorticoid receptor, having a high affinity for dexamethasone and a low one for aldosteron, is often referred to as a type II binding site (47).

Comparisons at the level of DNA led to the discovery that there were important homologies between the different steroid receptors. The regions of highest homology at the DNA level appeared to code for two of the three above-mentioned functional domains at the protein level. Because of these regions of similarities, it is thought that steroid receptors

in general are built up as variations on a shared theme (48). At the protein level the above-mentioned homology among the steroid receptors can be expressed as the percentage of identical amino acids. The DNA-binding domains have the highest homology, the steroid-binding domains possess a lower homology, whereas the immunogenic domain expresses the lowest homology (Table 4). These homologies can be explained functionally: to bind cortisol the GR has to possess at least the structure to bind a steroid in general. Amino acids stabilizing this structure have to be present also in other steroid receptors, and these amino acids represent the homology. Specificity for cortisol is expressed by those amino acids that are not homologous. The same thinking concerns the DNA-binding domains: to bind DNA a certain protein structure is demanded (the homology). Binding to specific GRE in DNA is determined by some of the remaining amino acids, which differ from those in other steroid receptors. It is known now which amino acids determine specificity for either a GRE or a ERE. Combining the results from three laboratories it became clear that three amino acids play a key role in distinguishing between these two response elements (38,49-51). These amino acids are located at the C-terminal site of the first zinc finger, at positions 458, 459, and 462. It was demonstrated that a three amino acid change of Glu-Gly-Ala (EGA) in the ER to Gly-Ser-Val (GSV) completely switched receptor specificity from an ERE to a GRE (50).

Table 4. Comparison of the human glucocorticoid receptor with other human receptors with transcriptional regulatory functions concerning the DNA-binding and steroid-binding domains.

	Percentage homology with the glucocorticoid receptor	
	DNA-binding domain	steroid binding domain
Mineralocorticoid receptor	94	57
Progesterone receptor	83	54
Androgen receptor	76	50
Estrogen receptor	62	30
Thyroid hormone receptors	47	17
Retinoic acid receptor	45	<15
Vitamin D_3 receptor	42	<15

On the other hand, specificity is also determined by the structure of the response elements: the response elements for estrogens differ from the GRE. However, the GRE appears to be similar to response elements for progesterone, mineralocorticoids and androgens, indicating that hormone-specific gene regulation is not determined by DNA binding alone. Additional receptor-protein interactions, which are not fully understood, play an important role (21,52). Specific interaction of transcription factors [e.g. nuclear factor I (NF I), octamer transcription factor-1 (OTF-1), activator protein-1 (AP-1)] with steroid hormone receptors and/or promoters may determine further hormone-specific gene regulation not shared with other steroids. Furthermore, the relative levels of expression of the different steroid receptors in a particular cell may also determine hormone-specific actions.

As steroid hormone receptors are phosphoproteins, phosphorylation may also be a way to modulate receptor function (53). Receptors are basally phosphorylated in the absence of ligand, but a certain degree of hyperphosphorylation is observed in the presence of hormone. Phosphorylation may affect intracellular traffic of receptors, but also reutilization, transcriptional regulation and interaction of receptors with other molecules are probably influenced by the degree of phosphorylation (21,53).

Steroid receptors belong to a superfamily of DNA-binding proteins able to modulate gene expression (54). On one hand, this family concerns proteins (e.g., GR) which need the binding of a ligand (e.g., cortisol) before being able to bind to DNA. On the other hand, there are supposed to be proteins (products of oncogenes) able to bind to DNA without the need for a ligand. Therefore, these proteins can influence the transcription of genes continuously in the absence of a ligand. In case these genes concern the production of a growth factor, the continuous stimulation of transcription may lead to an overproduction of growth factors. This mechanism attractively suggests a relation between oncogene products and hormone-independent neoplasms (55). At the moment this superfamily concerns the different steroid receptors, the c-erb-A proto-oncogen (identical to the thyroid hormone receptor) (56), another thyroid hormone receptor (present in the central nervous system) (57), the retinoic acid receptor families (58,59), the vitamin D receptor (60), and numerous orphan receptors, e.g. hERR 1 and 2 (61), COUP-TF's and EAR's (for a recent review: 62,63).

The search for the putative ligands of the orphan receptors has recently led to new insights into activation of the steroid hormone receptors. It was found that the neurotransmitter dopamine stimulated COUP-TF-dependent activation of a target gene (62,64). This effect did not appear to be mediated through direct binding of dopamine to COUP-TF, but was suggested to result from the interaction of dopamine with its membrane-bound receptor. This "cross-talk" between a cell membrane signal transduction pathway and the modulation of gene expression by steroid hormones may be mediated by phosphorylation of the steroid hormone receptor. These findings shed a new light on the ligand-independent activation of steroid hormone receptors and stress the above-mentioned significance of phosphorylation for receptor function (53). On the other hand, not all steroid receptor systems may be activated by such neurotransmitters. This may apply to the glucocorticoid receptor system, as CV1 cells co-transfected with a human glucocorticoid expression vector and a target gene were transcriptionally responsive to dexamethasone, but totally unresponsive to dopamine (62). Other evidence for "cross-talk" between the steroid hormone signalling pathway and signal transduction via cell membrane receptors comes from the fields of cytokines (for a recent review: 65) and oncogenes (66-69). Recently, IFN-α was shown to modulate phosphorylation of transcription factors in the cytoplasm, which then translocate into the nucleus and regulate transcription (66; Figure 5). The Ras-dependent signalling pathway was shown to run from the cell membrane to the nucleus (67). Growth factors may affect Ras activity, which eventually leads to translocation of activated protein kinases into the nucleus, where they phosphorylate transcription factors (67; Figure 5). Final regulation of gene transcription is thought to depend on the interactions of steroid hormone receptors with transcription factors in the

nucleus (69). In addition to these interactions in the nucleus, interactions of the glucocorticoid receptor with transcription factors in the cytoplasm have also been suggested (70-72). The transcription factor AP-1, a protein complex of the products of the c-jun and c-fos proto-oncogenes, was shown to interact with the liganded glucocorticoid receptor (73). This interaction prevents translocation of the receptor from the cytoplasm into the nucleus (Figure 5). In this way, AP-1 is able to counteract glucocorticoid actions. On the other hand, glucocorticoids may antagonize the actions of AP-1. The latter represents an important mechanism of the anti-inflammatory effects of glucocorticoids, and explains the repressive effects of glucocorticoids on IL-1-induced expression of the collagenase gene (74). Recently, direct interactions of glucocorticoid receptors with other transcription factors resulting in altered gene expression have been reported (75,76). A functional antagonism between the transcription factor NF-κB and the glucocorticoid receptor has been demonstrated in the regulation of the expression of the IL-6 gene (75). This antagonism resulted from physical association between the two proteins. In epithelial cells, it was shown that a direct interaction between the transcription factor CREB and the glucocorticoid receptor reduced binding of the latter protein to GRE (76).

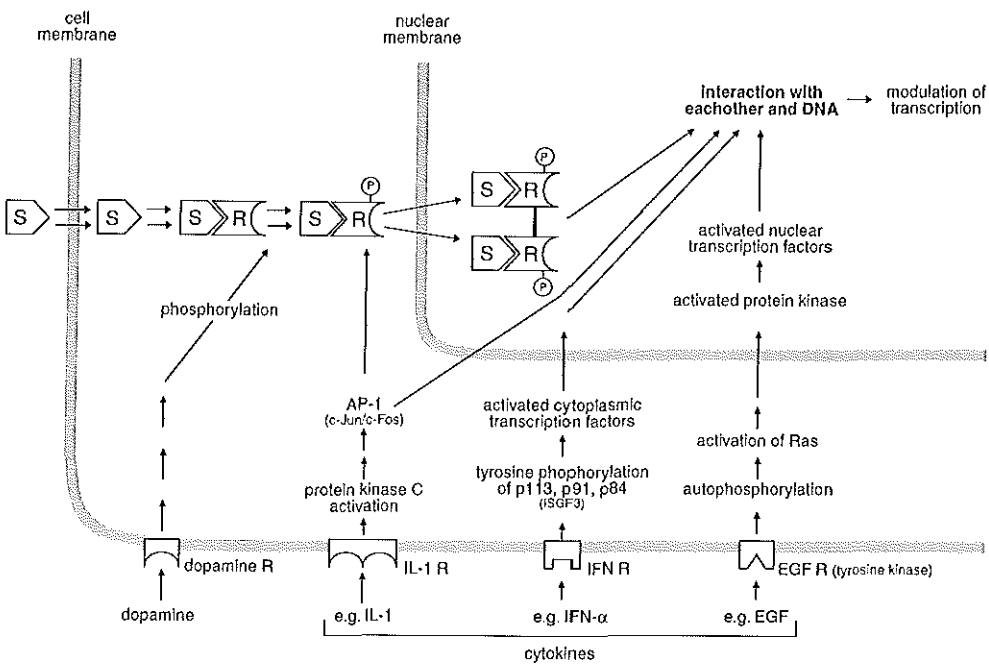

Figure 5. Hypothetical "cross talk" between different cellular signalling pathways.
Abbreviations used in this Figure: activator protein (AP); deoxyribonucleic acid (DNA); epidermal growth factor (EGF); interferon (IFN); interleukin (IL); interferon-stimulated gene factor-3 (ISGF3); protein (p); phosphate group (Ⓟ); receptor (R); steroid, in casu glucocorticoid (S).

2.3.3. Glucocorticoid-regulated mediators

Phospholipase A_2 inhibitory proteins

One group of proteins of which synthesis is thought to be inducible by glucocorticoids involves lipocortins (77,78). Lipocortin is the proposed unified name of a family of phospholipase A_2 inhibiting proteins. The first identified inhibitor was isolated from guinea pig lungs and peritoneal macrophages, and was named macrocortin (79). In other experiments, proteins with phospholipase A_2 inhibitory properties were also demonstrable and christened as renocortin and lipomodulin (80). Also, the release of stored lipocortins can be induced by glucocorticoids (81). Recently, human lipocortin complementary DNA has been cloned, and the gene has been expressed in E. *coli* (82).

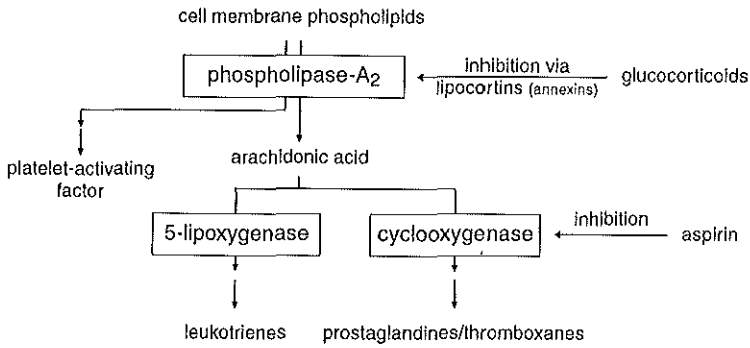

Figure 6. Interaction sites of steroidal (glucocorticoids) and nonsteroidal (e.g. aspirin) anti-inflammatory drugs.

In vitro these proteins inhibit phospholipase A_2 activity leading to a reduced production of arachidonic acid and consequently to a reduced production of arachidonic acid metabolites (Figure 6). How inhibition of phospholipase A_2 by lipocortins is achieved is still a matter of debate. In the beginning it was suggested that lipocortin inactivated the enzyme by direct binding. However, a research line involving substrates of viral and growth factor receptor tyrosine kinases suggested another possibility (83). This line of research led to the discovery of calpactins: proteins with *cal*cium-dependent *p*hospholipid- and *actin*-binding capacity. Eventually, calpactins were found to be equivalent to lipocortins: lipocortin I equals calpactin II, and lipocortin II is comparable to calpactin I. Furthermore, recent data show that calpactin-mediated inhibition of phospholipase A_2 is most probably due to Ca^{2+}-dependent binding of calpactin to phospholipids, the substrate of phospholipase A_2 (84). The discovery of these calpactins expanded the number of potential inhibitors of phospholipase A_2 (83). Recently, it has been suggested to rename this family of proteins "annexins" (85; Table 5). A novel annexin (annexin XI) has been identified recently (86). Some members of this group of proteins are suggested to be a secretory form (annexin I is secreted by macrophages), whereas others are intracellular proteins (annexin II). These different forms might be regulated in different ways by glucocorticoids (87).

No matter what the exact mechanism of lipocortin action, inhibition of phospholipase A_2 leads indirectly to an inhibition of both the cyclooxygenase and the lipoxygenase pathway (Figure 6). As a result, the synthesis of all arachidonic acid metabolites (prostaglandins, thromboxanes, and leukotrienes) is reduced. In addition, by inhibition of phospholipase A_2 activity, the production of platelet-activating factor (PAF) will also be impaired (Figure 6) (88). In contrast, nonsteroidal anti-inflammatory drugs like aspirin are only able to inhibit the cyclooxygenase pathway without influencing the production of leukotrienes and PAF (89).

Table 5. Revised nomenclature of lipocortins, calpactins and blood coagulation inhibitors.

Annexin	I	II	III	IV
Previous terminology	Lipocortin I p35 Calpactin II Chromobindin 9 GIF	Calpactin I Lipocortin II p36 Chromobindin 8 Protein I PAP-IV	Lipocortin III PAP-III 35-α Calcimedin	Endonexin I Protein II 32.5K Calelectrin Lipocortin IV Chromobindin 4 PAP-II PP4-X

Annexin	V	VI	VII	VIII
Previous terminology	PAP-1 Lipocortin V 35K Calelectrin Endonexin II PP4 VAC-α 35-γ Calcimedin Calphobindin I Anchorin CII	p68, p70, 73K 67K Calelectrin Lipocortin VI Protein III Chromobindin 20 67K Calcimedin	Synexin	VAC-β

Abbreviations used: GIF : glycosylation-inhibiting factor
PAP : placental anticoagulant protein
PP : placental protein
VAC : vascular anticoagulant (protein)

All these phospholipase A_2-dependent metabolites are important mediators in inflammatory processes (90,91). Therefore, on the basis of *in vitro* experiments, regulation of the expression and activity of lipocortins has been postulated to be at least one of the keystones by which glucocorticoids exert their anti-inflammatory actions *in vivo*. It has been shown that monoclonal antibodies against lipocortins block the suppressive effects of glucocorticoids on the release of arachidonic acid (92). In some patients suffering from autoimmune disease, autoantibodies against lipocortins have been found (92). Probably the effective serum level of lipocortin in these patients is inversely related to the severity of their disease. Research is focused now on the direct application of lipocortins instead of glucocorticoids as a therapeutic in inflammatory diseases. Notwithstanding the overwhelming data on the action of lipocortins *in vitro* leading to an attractive general model to explain the inhibition of the mediator production by glucocorticoids, there still remain

questions about differences between *in vivo* and *in vitro* experiments (82,93). After *in vivo* treatment of rats with dexamethasone, rat peritoneal macrophages showed a sixfold increase of lipocortin I mRNA (82), whereas *in vitro* treatment of human cells did not lead to a detectable increase of lipocortin I mRNA (93). In both experiments the increase is puzzlingly not accompanied by an increase of the protein level. Analyzing these experiments, one has to bear in mind that glucocorticoids can affect posttranslational processing and compartmentalization of proteins without changing the total protein level (94). Moreover, in *in vivo* experiments with rabbits and healthy human volunteers, dexamethasone treatment does not lead to a decrease in the level of eicosanoid metabolites in urine (95,96). In contrast, dexamethasone is indeed able to block increased LTB_4 production during endotoxin-induced lung injury in pigs *in vivo* (97).

Recently, another aspect of the biological activity of lipocortin I was published. It was shown that lipocortin I could modulate cell functions by binding to specific cell surface receptors on blood monocytes and neutrophils (98).

In conclusion, it can be said that there is compelling evidence that glucocorticoid-induced phospholipase A_2 inhibitors play an important role in regulating the production of inflammatory mediators, although their exact mechanisms of inhibition remain to be elucidated. Under certain conditions glucocorticoids can influence the production of lipocortins *in vitro* and *in vivo*. The precise role of these glucocorticoid-inducible proteins as physiological regulators of inflammatory reactions in humans has to be further evaluated (for recent reviews: 99,100).

Interleukin-1

Another protein with an important immunological and inflammatory role and transcription of which is believed to be influenced by glucocorticoids is interleukin-1 (101). Interleukin-1 (IL-1) can be produced by a variety of cell types, but the most important source in view of inflammatory processes concerns antigen-presenting cells. The lungs harbor a large number of antigen-presenting cells, represented mostly by dendritic cells and alveolar monocytes/macrophages. Therefore, a large source of IL-1 is potentially present in the lung, although alveolar monocytes/macrophages differ in their ability to produce IL-1, probably related to differences in cell maturation (102). IL-1 is able to influence many different target cells, among which are cells with important inflammatory properties (101). In this respect, the influence of IL-1 on cytokine production and proliferation of T lymphocytes is important, whilst polymorphonuclear leukocytes and macrophages have been reported to be chemotactically attracted to IL-1 and to become activated.

Since many years, it is known that glucocorticoids inhibit IL-1 release from murine peritoneal macrophages (103). Recently, it was reported that pharmacological doses of dexamethasone could block transcription of the IL-1 gene in a bacterial toxin-stimulated human monocytic cell line, U937 (104). It was also shown in this study that higher doses of dexamethasone could block IL-1 release. This was not due to an enhanced degradation of IL-1 mRNA, but most likely to an elevation of cAMP levels. It is therefore conceivable

that *in vivo* anti-inflammatory actions of glucocorticoids may also, at least partly, be caused by suppression of IL-1 expression.

Interleukin-2

Interleukin-2 (IL-2), also termed T cell growth factor, is a protein of which the most important immunological function is to enable the proliferation of activated T cells. Activated T cells possess the dimeric, high affinity IL-2 receptor and show clonal expansion upon stimulation with IL-2 (105). In addition, IL-2 is able to enhance the cytolytic activity of natural killer cells.

Glucocorticoids have been shown to suppress directly the production of this lymphokine *in vitro*, and at methylprednisolone concentrations over 10 μg/ml the induction of the IL-2 receptor was also inhibited *in vitro* (106). Glucocorticoids are now known to inhibit the IL-2 production at the level of mRNA (107). These data strongly suggest that *in vivo* regulation of IL-2-driven immunological processes can be a mechanism through which glucocorticoids exert their anti-inflammatory and immunosuppressive actions.

Unfortunately, one has also to bear in mind that the effects of glucocorticoids *in vitro* can be dependent upon the choice of cell type. It has been shown that glucocorticoids exert different actions on the proliferation of a murine helper and a cytolytic T cell clone after addition of IL-2 or IL-4 (108). It was shown that the IL-2-driven proliferation of a cytolytic clone did not respond to glucocorticoids, while the proliferative response to IL-4 could be blocked by glucocorticoids. In contrast, addition of glucocorticoids to a (Th2-type) helper clone blocked the IL-2-driven proliferation, while the IL-4-driven proliferation was not affected. In this view, the *in vivo* actions of glucocorticoids on concerted lymphocyte functions are probably even more complex.

Interleukin-3

Interleukin-3 (IL-3) has been described to be produced by human activated T cell clones (109). Probably IL-3 plays a significant role in the rapid expansion of hemopoietic cells in response to immunological stress. Recently, IL-3 expression was also shown to be under control of glucocorticoids, at least in cloned murine T lymphocytes (110).

Because experiments were done with a murine helper T cell clone incapable of producing IL-2, the possibility that IL-3 mRNA is regulated by glucocorticoid-regulated IL-2 concentrations could be ruled out.

Interleukin-5

Interleukin-5 (IL-5) has been shown to be a potent and specific growth factor for eosinophilic granulocytes (111). Because of the eosinophilia seen in bronchial asthma, IL-5

has been suggested to play a key role in the pathogenesis of asthma. It was also shown that anti-IL-5 monoclonal antibodies inhibited BAL eosinophilia and airway hyperresponsiveness in an animal model (112). In human mononuclear cells, using the PCR-technique, it was shown that dexamethasone (10^{-8}-10^{-6}M) suppressed IL-5 mRNA expression *in vitro* (113). These effects may be a mechanism of the beneficial effects of glucocorticoids in asthma.

Interleukin-6

Another cytokine believed to contribute to the process of inflammatory responses is interleukin-6 (IL-6). Different inflammatory stimuli like LPS, IL-1 and IFN-γ induce IL-6 transcription in monocytes (114). IL-6 promotes growth of B cells, plasma cells, T cells, and induces acute phase proteins in hepatocytes (115). Dexamethasone (10^{-9}-10^{-6}M) was shown to inhibit IL-6 production at the transcriptional level in human monocytes, endothelial cells and fibroblasts (116).

Interleukin-8

The cytokine interleukin-8 (IL-8) has been implicated as a major chemotactic and activating factor for neutrophils. Mononuclear phagocytes are the predominant source of IL-8 in various inflammatory diseases. It was shown that dexamethasone decreased IL-8 mRNA levels in both LPS-stimulated human peripheral blood monocytes and alveolar macrophages (119). It is not known whether glucocorticoids decrease IL-8 mRNA synthesis or accelerate its decay (119).

Tumor Necrosis Factor-α

Tumor necrosis factor-α (TNF-α) has been described to be a central cytokine in inflammation (117). Many of its effects are caused by the induction of other mediators, and it plays a role in leucocyte chemotaxis and activation, shock, capillary leak syndrome, necrosis and many other aspects of inflammatory diseases (118). It was shown in mice and human lung fragments that dexamethasone (10^{-6}M) inhibited the transcription of TNF-α, providing an additional mechanism for the anti-inflammatory and immunosuppressive effects of glucocorticoids (120).

Interferon-γ

Interferon-γ (IFN-γ) belongs to a family of proteins first recognized by their antiviral properties. It is produced by activated T lymphocytes and is able to increase the numbers

of monocyte and macrophage Fc receptors, thereby augmenting some immunological functions of monocytes and macrophages. Furthermore, it is capable of inducing class I and class II histocompatibility antigens (121). Dexamethasone inhibits *in vitro* the accumulation of IFN-γ mRNA in human, phytohemagglutinin-stimulated peripheral blood lymphocytes (107). It is not yet known whether this inhibited accumulation is mediated through a direct effect on the IFN-γ gene transcription or whether it is regulated by another glucocorticoid-induced mediator.

2.3.4. Other glucocorticoid-regulated proteins

Besides the above-mentioned regulation of lipocortin and cytokines, the genes of other proteins with important functions in inflammatory processes have been proven to be under regulation of glucocorticoids. These regulations take place at the level of DNA transcription and can be explained by the nuclear working mechanism of glucocorticoids.

Collagen

Glucocorticoids have been shown to inhibit the synthesis of collagen in many *in vitro* models (122,123). The exact mechanism by which this inhibition takes place has been a matter of debate. Some studies suggest that transcriptional regulation by glucocorticoids causes the decrease in mRNA (122), while other studies propose a change in the half-life of mRNA (123). Recently these two views were united when it was shown that dexamethasone both decreases the transcription of the collagen gene *in vitro* and appears to lead to a posttranslational effect on the procollagen mRNA content (124). Analyzing the above-mentioned studies one has to keep in mind the possible influence of the used glucocorticoid and its concentration on the ultimate results. In one study, dexamethasone was used at the concentration of 1×10^{-7} M, while in another 250×10^{-7} M was used. Cortisol at 100×10^{-7} M was also used in some experiments. The mechanism of collagen inhibition may depend on the glucocorticoid concentration. Although in some pulmonary diseases the use of glucocorticoids is debatable (125), in pulmonary diseases with active collagen secretion leading to restriction of lung volume, the use of glucocorticoids to diminish the production of collagen seems tenable, especially in view of the above-mentioned *in vitro* results. In late stage adult respiratory distress syndrome (ARDS), lung edema is no longer present. Instead, extensive collagen formation with the development of fibrosis takes place. Even early stages of some forms of ARDS are characterized by active collagen synthesis in the pulmonary interstitium (126). Administration of dexamethasone especially during the late stage of ARDS may thus be useful. This is demonstrated in Figure 7 showing the results of dexamethasone administration in a patient who developed ARDS after a pneumococcal pneumonia. Dexamethasone application resulted in a downregulation of collagen formation with an increase in lung compliance allowing a successful weaning of the ventilator.

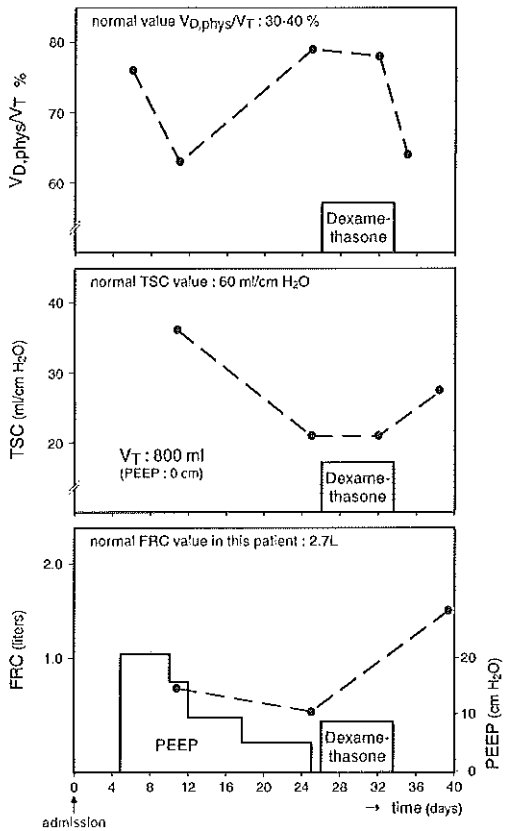

Figure 7. Illustration of the influence of dexamethasone on the late (proliferative) phase with newly formed collagen and fibrosis in a patient with adult respiratory distress syndrome (ARDS). A 40-year-old female admitted with a pneumonia developed ARDS. Because of deteriorating lung function parameters, dexamethasone (16 mg/day) was given on day 26. This resulted in a down regulation of fibrosis and clinical improvement. The dead-space ventilation decreased resulting in a decreased physiological dead space/tidal volume ($V_{D,phys}/V_T$) ratio. The compliance of the lung increased as indicated by the increase in total static compliance (TSC), whereas the lung volume increased as indicated by the increase in functional residual capacity (FRC).

Collagenase

The collagenase family, a group of metalloprotease enzymes, is involved in the degradation of collagen and other basal membrane constituents. In inflammatory diseases, tissue damage may be caused by the action of metalloproteases (e.g. damage to cartilage in rheumatoid arthritis) (127). Inflammatory cytokines as IL-1 and TNF-α induce the expression of some metalloprotease genes (128,129). This effect is mediated via the transcription factor AP-1. Glucocorticoids bound to their receptor are known to interact with AP-1 in the cytoplasm, thus preventing the gene expression of collagenase (70-72). This anti-inflammatory action of glucocorticoids results from modulation of transcription without binding of the receptor-steroid complex to DNA.

Nuclease

In man, cells are relatively corticosteroid resistant, compared with rodents such as mice and rats (9). In these rodents, glucocorticoids cause lymphocyte depletion resulting from cell death and shrinkage of lymphoid tissue, while in man this kind of cell death can only be observed in glucocorticoid treatment of certain lymphatic leukemias. The central event in this cytolytic process concerns degradation of the genome, and it was hypothesized that steroid-induced cell death was dependent upon the induction of a protein. In rats this protein has now been identified as a nuclease (130). Whether the induction of such a protein also plays a role in man in the treatment of glucocorticoid-responsive diseases, in particular the treatment of inflammatory lung diseases, has still to be evaluated.

ICAM-1 (CD54)

The intercellular adhesion molecule-1 (ICAM-1; CD54) represents a member of the immunoglobulin gene-like superfamily and plays a role in cellular interactions. It interacts with integrin receptors on other cell types and mediates diapedesis and infiltration of tissue by inflammatory cells. Regulation of the expression of these interaction-regulating proteins may modulate inflammation (131). Recently, it was shown that glucocorticoids are able to downregulate ICAM-1 expression in both a human monocytic and bronchial epithelial cell line (132). The mechanism of this effect is not clear yet. It has been suggested that ICAM-1 mRNA expression may be regulated by transcription factors. A direct interaction of the steroid-glucocorticoid receptor complex with these transcription factors, in the absence of GRE, may eventually downregulate ICAM-1 mRNA expression.

Neutral endopeptidase (CD10)

Neutral endopeptidase [NEP, EC 3.4.24.11, common acute lymphoblastic leukemia Ag (CALLA), CD10] belongs to the group of ectoenzymes, so-called because of their extracellularly located catalytic sites. The physiological function of these enzymes is being unraveled nowadays. NEP has been reported to process a number of biologically active peptides (133), including neuropeptides (134). It has been suggested that NEP may modulate neurogenic inflammation (e.g. in asthmatic lungs) by enzymatic degradation of neuropeptides (e.g. substance P) (134). Glucocorticoids have been shown to increase NEP expression in airway epithelial cells (135), although a recent study could not reproduce these findings (136). This increase in expression may represent an increased activity to terminate inflammation, and may be an actual working mechanism of glucocorticoids.

2.4 NONNUCLEAR MECHANISM

Because some glucocorticoid actions appear faster than could be expected through the above-mentioned influence on mRNA production, some investigators have postulated that glucocorticoids are also able to influence cellular events without interfering with nuclear processes (137,138). One of the actions concerns the inhibition of ACTH release that begins minutes after administration of glucocorticoids. Probably such effects are attributable to an interference of steroid (whether or not bound to the glucocorticoid receptor; see Figure 2) with protein phosphorylation or to an interaction with the cell membrane leading to a change in intracellular Ca^{2+}. An important role of intracellular Ca^{2+} concerns the activation of a cAMP-dependent protein kinase which is able to phosphorylate lipocortin. Upon phosphorylation lipocortin is inactivated, leading to an increase in phospholipase A_2 activity and an increase in arachidonic acid products. Therefore, besides their regulation of lipocortin synthesis at mRNA level, it also seems possible that glucocorticoids exert action on arachidonic acid metabolism via a nonnuclear mechanism mediated by interference with Ca^{2+} entry into the cell and lipocortin phosphorylation.

2.5 OTHER ANTI-INFLAMMATORY ACTIONS

Besides the already mentioned basic effects of glucocorticoids on the production of some inflammatory mediators (arachidonic acid metabolites, IL-1, IL-2, IL-3, IL-5, IL-6, TNF-α, IFN-γ) and two specific proteins (collagen and nuclease), there exist numerous other effects influencing the inflammatory process, which are not yet fully clarified (9). Glucocorticoids exert an effect on the cellular distribution in peripheral blood. A lymphocytopenia occurs in man 4-6 hours after glucocorticoid administration (139). This results from a redistribution of the peripheral blood lymphocytes (mostly T lymphocytes) into other body compartments, especially the bone marrow. Probably in this way fewer lymphocytes are available to leave the circulation at a site of inflammation.

There are also glucocorticoid effects on the other cellular components of peripheral blood. Like the induced lymphocytopenia, the monocytopenia probably results from a redistribution of monocytes, although in animal models a decreased release from the bone marrow has also been described (140). Another important contribution to the anti-inflammatory action of glucocorticoids concerns the decreased influx of neutrophilic granulocytes to the site of inflammation leading to a neutrophilia (141). The precise underlying mechanisms of this hampered accumulation are unknown, but it partially depends on decreased margination of the neutrophils and is probably the most important anti-inflammatory factor at pharmacological glucocorticoid doses. Probably, glucocorticoids alter cellular surfaces leading to a disturbance of neutrophil-endothelium interactions and preventing regression from the microvasculature to the site of inflammation. Actually, it was shown recently that glucocorticoids (at nanomolar concentrations) inhibited the expression of adhesion molecules by endothelial cells (142). Glucocorticoids may thereby interfere with the traffic of leukocytes into inflamed areas. Effects on cellular distribution

in blood can therefore also be explained as mediated via the nuclear mechanism of glucocorticoids and their influence on transcription of adhesion proteins. Furthermore, it was shown in hamsters that dexamethasone inhibited leukocyte diapedesis, but not leukocyte rolling or adhesion. This dexamethasone-induced inhibition of leukocyte transmigration appeared to be mediated by lipocortin I (143).

Stabilization of lysosomal membranes, thereby leading to an inhibition of the release of lysosomal contents, has been proposed to be an important anti-inflammatory factor of glucocorticoid treatment (137). However, this inhibition was achieved at very high doses on isolated rabbit liver lysosomes and was not reproducible on isolated lysosomes from human neutrophils (144). Lysosomal release from intact human cells proved to be inhibitable (145). Whether this mechanism plays a role at glucocorticoid concentrations during treatment is still a matter of debate.

Vasoconstriction can be achieved by glucocorticoids. In this way the vascular response to inflammation can be compensated for as some inflammatory mediators are capable of producing vasodilatation (146). It is possible that inhibition of the production of arachidonic acid metabolites is responsible for this phenomenon, but a still controversial antagonism of the kinin system has also been proposed (147).

Many inflammatory mediators cause an increase in vascular permeability (148) leading to edema. Glucocorticoids reduce formation of edema by a still unknown mechanism. Because formation of some edema-causing mediators requires the presence of neutrophils, it has been suggested that inhibition of the interaction between neutrophils and vascular endothelium can be a mechanism through which glucocorticoid-induced edema inhibition takes place (149).

Alveolar macrophages are believed to secrete a mediator responsible for an increase of mucus secretion (150). This mediator is newly synthesized after cell surface activation of macrophages. It was called macrophage-derived mucus secretagogue (MMS), and it was shown not to be a derivative of arachidonic acid. *In vitro* studies on human airways have described the inhibition of mucus secretion by glucocorticoids (151). It is not yet known whether these two findings may be combined, leading to the hypothesis that glucocorticoids are able to reduce the macrophage production of the above-mentioned secretagogue by inhibition of gene transcription.

2.6 CONCLUSION AND FUTURE RESEARCH

Since the first clinical application of glucocorticoids in the treatment of rheumatoid arthritis, the use of these drugs has been extended to the fight against a large variety of other inflammatory diseases, including several lung diseases. During the past 46 years both basic and clinical investigations have expanded our insight into their working mechanisms, although many questions remain. Nowadays, glucocorticoids are known to exert many of their effects by influencing the transcription of genes, and many proteins encoded by these genes play a role at different levels in inflammatory processes. On the other hand, one realizes that inflammatory processes usually represent a complex of interactions between

many different cell types, inflammatory mediators, and other secretory products. Because of this involvement of many different cell types and cell products an anti-inflammatory agent intervening at the level of one specific cell type or cell product would be of little value. This explains, in part, why glucocorticoids, with their wide range of effects on cellular events, have proved to be such successful anti-inflammatory drugs. Unfortunately, their broad range of effects has also proved not to be restricted to beneficial ones, given their impressive side effects, while glucocorticoid responsiveness seems to vary among individuals. Future investigations face the arduous task of adapting the working mechanisms of these hormones in order to diminish side effects, while leaving enough of their many influences to maintain their strong anti-inflammatory action. This task would be made easier by a better understanding of the molecular working mechanisms of glucocorticoids. Furthermore, the recently discovered "cross-talk" between membrane-associated protein receptors and intracellular steroid hormone receptors may also have therapeutical implications.

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2.7 REFERENCES

1. Tyrrell JB, Aron DC, Forsham PH. Glucocorticoids and adrenal androgens. In: Greenspan FS, ed. Basic and clinical endocrinology. San Francisco: Prentice-Hall International Inc. 1991:323-362.
2. Munck A, Guyre PM, Holbrook NJ. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Rev 1984;5:25-44.
3. Kehrl JH, Fauci AS. The clinical use of glucocorticoids. Ann Allergy 1983;50:2-8.
4. Hench PS, Kendall EC, Slocumb CH, Polley HF. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc Staff Meet, Mayo Clin 1949;24:181-197.
5. Mason HL, Myers CS, Kendall EC. The chemistry of crystalline substances isolated from the suprarenal gland. J Biol Chem 1936;114:613-631.
6. Carryer HM, Koelsche GA, Prickman LE, Maytum CK, Lake CF, Williams HL. Effects of cortisone on bronchial asthma and hay fever occurring in subjects sensitive to ragweed pollen. Proc Staff Meet, Mayo Clin 1950;25:482-486.
7. Seale JP, Compton MR. Side-effects of corticosteroid agents. Med J Austral 1986;144:139-142.
8. Clark TJH. Effect of beclomethasone dipropionate delivered by aerosol in patients with asthma. Lancet 1972;i:1361-1364.
9. Fauci AS. Immunosuppressive and anti-inflammatory effects of glucocorticoids. In: Baxter JD, Rousseau G, eds. Monographs in endocrinology: glucocorticoid hormone action. Berlin, Heidelberg, New York: Springer-Verlag. 1979;12:449-465.
10. Munck A, Mendel DB, Smith LI, Orti E. Glucocorticoid receptors and actions. Am Rev Respir Dis 1990;141:2-10.

11. Gustafsson J-A, Carlstedt-Duke J, Poellinger L, Okret S, Wikström A-C, Brönnegård M, Gillner M, Dong Y, Fuxe K, Cintra A, Härfstrand A, Agnati L. Biochemistry, molecular biology, and physiology of the glucocorticoid receptor. Endocrine Rev 1987;8:185-234.
12. Mendel DB, Orti E. Isoform composition and stoichiometry of the ~90-kDa heat shock protein associated with glucocorticoid receptors. J Biol Chem 1988;263:6695-6702.
13. Smith DF, Stensgard BA, Welch WJ, Toft DO. Assembly of progesterone receptor with heat shock proteins and receptor activation are ATP mediated events. J Biol Chem 1992;267:1350-1356.
14. Sanchez ER. Hsp56: a novel heat shock protein associated with untransformed steroid receptor complexes. J Biol Chem 1990;265;22067-22070.
15. Lebeau M-C, Massol N, Herrick J, Faber LE, Renoir J-M, Radanyi C, Baulieu E-E. P59, an hsp 90-binding protein. Cloning and sequencing of its cDNA and preparation of a peptide-directed polyclonal antibody. J Biol Chem 1992;267:4281-4284.
16. Sanchez ER, Hirst M, Scherrer LC, Tang H-Y, Welsh MJ, Harmon JM, Simons SS Jr., Ringold GM, Pratt WB. Hormone-free mouse glucocorticoid receptors overexpressed in chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90. J Biol Chem 1990;265:20123-20130.
17. Akner G, Mossberg K, Wikström A-C, Sundqvist K-G, Gustafsson J-Å. Evidence for colocalization of glucocorticoid receptor with cytoplasmic microtubules in human gingival fibroblasts, using two different monoclonal anti-GR antibodies, confocal laser scanning microscopy and image analysis. J Steroid Biochem Molec Biol 1991;39:419-432.
18. Miyata Y, Yahara I. Cytoplasmic 8 S glucocorticoid receptor binds to actin filaments through the 90-kDa heat shock protein moiety. J Biol Chem 1991;266:8779-8783.
19. Yem AW, Tomasselli AG, Heinrikson RL, Zurcher-Neely H, Ruff VA, Johnson RA, Deibel MR Jr. The Hsp56 component of steroid receptor complexes binds to immobilized FK506 and shows homology to FKBP-12 and FKBP-13. J Biol Chem 1992;267:2868-2871.
20. Ning Y-M, Sanchez ER. Potentiation of glucocorticoid receptor-mediated gene expression by the immunophilin ligands FK506 and rapamycin. J Biol Chem 1993;268:6073-6076.
21. Truss M, Beato M. Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 1993;14:459-479.
22. Beato M. Gene regulation by steroid hormones. Cell 1989;56:335-344.
23. Parker M. Enhancer elements activated by steroid hormones? Nature 1983;304:687-688.
24. Beato M, Chalepakis G, Schauer M, Slater EP. DNA regulatory elements for steroid hormones. J Steroid Biochem 1989;32:737-748.
25. Kumar V, Chambon P. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 1988;55:145-156.
26. Glass CK. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. Endocr Rev 1994;15:391-407.
27. Miesfeld RL. Molecular genetics of corticosteroid action. Am Rev Respir Dis 1990;141:11-17.
28. Jantzen C, Fritton HP, Igo-Kemenes T, Espel E, Janich S, Cato ACB, Mugele K, Beato M. Partial overlapping of binding sequences for steroid hormone receptors and DNaseI hypersensitive sites in the rabbit uteroglobin gene region. Nucleic Acids Res 1987;15:4535-4552.
29. Hecht A, Berkenstam A, Strömstedt PE, Gustafsson J-Å, Sippel AE. A progesterone responsive element maps to the far upstream steroid dependent DNase hypersensitive site of chicken lysozyme chromatin. EMBO J 1988;7:2063-2073.
30. Schüle R, Muller M, Kaltschmidt C, Renkawitz R. Many transcription factors interact synergistically with steroid receptors. Science 1988;242:1418-1420.
31. Strähle U, Schmid W, Schütz G. Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J 1988;7:3389-3395.
32. Giguère V, Hollenberg SM, Rosenfeld MG, Evans RM. Functional domains of the human glucocorticoid receptor. Cell 1986;46:645-652.

33. Hollenberg SM, Giguère V, Segui P, Evans RM. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 1987;49:39-46.
34. Schena M, Freedman LP, Yamamoto KR. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev 1989;3:1590-1601.
35. Ptashne M, Gann AAF. Activators and targets. Nature 1990;346:329-331.
36. Wrange O, Gustafsson J-Å. Separation of the hormone- and DNA-binding sites of the hepatic glucocorticoid receptor by means of proteolysis. J Biol Chem 1978;253:856-865.
37. Webster NJG, Green S, Jin JR, Chambon P. The hormone-binding domains of the estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell 1988;54: 199-207.
38. Freedman LP. Anatomy of the steroid receptor zinc finger region. Endocr Rev 1992;13:129-145.
39. Green S, Kumar V, Theulaz J, Wahli W, Chambon P. The N-terminal DNA-binding "zinc finger" of the oestrogen and glucocorticoid receptors determines target gene specificity. EMBO J 1988;7:3037-3044.
40. Luisi BF, Xu WX, Otwinowski Z, Freedman LP, Yamamoto KR, Sigler PB. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature 1991;352:497-505.
41. Okret S, Wikström A-C, Wrange O, Andersson B, Gustafsson J-A. Monoclonal antibodies against the rat liver glucocorticoid receptor. Proc Natl Acad Sci USA 1984;81:1609-1613.
42. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM. Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985;318:635-641.
43. Green S, Walter P, Kumar V, Krust A, Bornert J-M, Argos P, Chambon P. Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. Nature 1986;320:134-139.
44. Arriza JL, Weinberger C, Cerelli G, Claser TM, Handelin BL, Housman DE, Evans RM. Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor. Science 1987;237:268-275.
45. Misrahi M, Atger M, d'Auriol L, Loosfelt H, Meriel C, Fridlansky F, Guoichon-Mantel A, Galibert F, Milgrom E. Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem Biophys Res Commun 1987;143:740-748.
46. Trapman J, Klaassen P, Kuiper GGJM, van der Korput JAGM, Faber PW, van Rooij HCJ, Geurts van Kessel A, Voorhorst MM, Mulder E, Brinkmann AO. Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Comm 1988;153:241-248.
47. Funder JW. Adrenal steroids: New answers, new questions. Science 1987;237:236-237.
48. Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988;240:889-895.
49. Danielsen M, Hinck L, Ringold GM. Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. Cell 1989;57:1131-1138.
50. Mader S, Kumar V, De Verneuil H, Chambon P. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature 1989;338:271-274.
51. Umesono K, Evans RM. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell 1989;57:1139-1146.
52. Nordheim A. CREB takes CBP to tango. Nature 1994;370:177-178.
53. Orti E, Bodwell JE, Munck A. Phosphorylation of steroid hormone receptors. Endocr Rev 1992;13:105-128.
54. Green S, Chambon P. A superfamily of potentially oncogenic hormone receptors. Nature 1986;324:615-617.
55. Sluyser M, Mester J. Oncogenes homologous to steroid receptors? Nature 1985;315:546.
56. Weinberger C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM. The c-erb-A gene encodes a thyroid hormone receptor. Nature 1986;324:641-646.

57. Thompson CC, Weinberger C, Lebo R, Evans RM. Identification of a novel thyroid hormone receptor expressed in the mammalian central nervous system. Science 1987;237:1610-1613.
58. Rowe A, Brickell PM. The nuclear retinoid receptors. Int J Exp Path 1993;74:117-126.
59. Becker-André M, André E, DeLamarter JF. Identification of nuclear receptor mRNAs by RT-PCR amplification of conserved zinc-finger motif sequences. Biochem Biophys Res Com 1993;194:1371-1379.
60. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW. Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc Natl Acad Sci USA 1988;85:3294-3298.
61. Giguère V, Yang N, Segui P, Evans RM. Identification of a new class of steroid hormone receptors. Nature 1988;331:91-94.
62. Power RF, Conneely OM, O'Malley BW. New insights into activation of the steroid hormone receptor superfamily. Trends Pharmacol Sci 1992;13:318-323.
63. Laudet V, Hänni C, Coll J, Catzeflis F, Stéhelin D. Evolution of the nuclear receptor gene superfamily. EMBO J 1992;11:1003-1013.
64. Power RF, Lydon JP, Conneely OM, O'Malley BW. Dopamine activation of an orphan of the steroid receptor superfamily. Science 1991;252:1546-1548.
65. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253-262.
66. Hunter T. Cytokine connections. Nature 1993;366:114-116.
67. Egan SE, Weinberg RA. The pathway to signal achievement. Nature 1993;365:781-783.
68. Ponta H, Cato ACB, Herrlich P. Interference of pathway specific transcription factors. Biochim Biophys Acta 1992;1129:255-261.
69. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
70. Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 1990;62:1189-1204.
71. Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J, Karin T. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 1990;62:1205-1215.
72. Schüle R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell 1990;62:1217-1226.
73. Schüle R, Evans RM. Cross-coupling of signal transduction pathways: zinc finger meets leucine zipper. Trends in Genetics 1991;7:377-381.
74. De Waal RMW. The anti-inflammatory activity of glucocorticoids. Mol Biol Rep 1994;19:81-88.
75. Ray A, Prefontaine KE. Physical association and functional antagonism between the p65 subunit of transcription factor NK-κB and the glucocorticoid receptor. Proc Natl Acad Sci USA 1994;91:752-756.
76. Adcock IM, Peters MJ, Kwon OJ, Barnes PJ. Interaction of glucocorticoid receptors and cAMP responsive element binding protein (CREB) in human epithelial cells: effects of antisense oligonucleotides. Am J Respir Crit Care Med 1994;149:A1026.
77. Hirata F, Notsu Y, Yamada R, Ishihara Y, Wano Y, Kunos I, Kunos G. Isolation and characterization of lipocortin (lipomodulin). Agents Actions 1985;17:263-266.
78. Peers SH, Flower RJ. The role of lipocortin in corticosteroid action. Am Rev Respir Dis 1990;141:18-21.
79. Blackwell GJ, Carnuccio R, Di Rosa M, Flower RJ, Parente L, Persico P. Macrocortin: A polypeptide causing the antiphospholipase effect of glucocorticoids. Nature 1980;287:147-149.
80. Flower RJ. Background and discovery of lipocortins. Agents Actions 1985;17:225-262.
81. Blackwell GJ, Flower RJ. Inhibition of phospholipase. Brit Med Bull 1983;39:260-264.

82. Wallner BP, Mattaliano RJ, Hession C, Cate RL, Tizard R, Sinclair LK, Foeller C, Pingchang Chow E, Browning JL, Ramachandran KL, Pepinsky RB. Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature 1986;320:70-81.
83. Saris CJM, Tack BF, Kristensen T, Glenney JR, Hunter T. The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell 1986;46:201-212.
84. Davidson FF, Dennis EA, Powell M, Glenney JR. Inhibition of phospholipase A_2 by lipocortins and calpactins. J Biol Chem 1987;262:1698-1705.
85. Crumpton MJ, Dedman JR. Protein terminology tangle. Nature 1990;345:212.
86. Towle CA, Treadwell BV. Identification of a novel mammalian annexin. cDNA cloning, sequence analysis, and ubiquitous expression of the annexin XI gene. J Biol Chem 1992;267:5416-5423.
87. Huang K-S, Wallner BP, Mattaliano RJ, Tizard R, Burne C, Frey A, Hession C, McGray P, Sinclair LK, Chow EP, Browning JL, Ramachandran KL, Tang J, Smart JE, Pepinsky RB. Two human 35 kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. Cell 1986;46:191-199.
88. Parente L, Flower RJ. Hydrocortisone and "macrocortin" inhibit the zymosan-induced release of lyso-paf from rat peritoneal leucocytes. Life Sci 1985;36:1225-1231.
89. Vane J, Botting R. Inflammation and the mechanism of action of anti-inflammatory drugs. FASEB J 1987;1:89-96.
90. Malmsten CL. Prostaglandins, thromboxanes, and leukotrienes in inflammation. Am J Med 1986;80:11-17.
91. Vane JR. Antiinflammatory drugs and the many mediators of inflammation. Int J Tiss Reac 1987;9:1-14.
92. Hirata F, Carmine del R, Nelson CA, Axelrod J, Schiffmann E, Warabi A, De Blas AL, Nirenberg M, Manganiello V, Vaughan M, Kumagai S, Green I, Decker JL, Steinberg AD. Presence of autoantibody for phospholipase inhibitory protein, lipomodulin, in patients with rheumatic diseases. Proc Natl Acad Sci USA 1981;78:3190-3194.
93. Brönnegård M, Andersson O, Edwall D, Lund J, Norstedt G, Carlstedt-Duke J. Human calpactin II (lipocortin II) messenger ribonucleic acid is not induced by glucocorticoids. Mol Endocrin 1988;2:732-739.
94. Firestone GL, Payvar F, Yamamoto KR. Glucocorticoid regulation of protein processing and compartmentalization. Nature 1982;300:221-225.
95. Náray-Fejes-Tóth A, Fejes-Tóth G, Fischer C, Frölich JC. Effect of dexamethasone on *in vivo* prostanoid production in the rabbit. J Clin Invest 1984;74:120-123.
96. Rosenkrantz B, Náray-Fejes-Tóth A, Fejes-Tóth G, Fischer C, Sawada M, Frölich JC. Dexamethasone effect on prostanoid formation in healthy man. Clin Sci 1985;68:681-685.
97. Olson NC, Dobrowsky RT, Fleisher LN. Dexamethasone blocks increased leukotriene B4 production during endotoxin-induced lung injury. J Appl Physiol 1988;64:2100-2107.
98. Goulding NJ, Luying P, Guyre PM. Characteristics of lipocortin 1 binding to the surface of human peripheral blood leukocytes. Biochem Soc Trans 1990;18:1237-1238.
99. Goulding NJ, Guyre PM. Regulation of inflammation by lipocortin 1. Immunol Today 1992;13:295-297.
100. Goulding NJ, Guyre PM. Glucocorticoids, lipocortins and the immune response. Curr Opin Immunol 1993;5:108-113.
101. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. There is more than one interleukin-1. Immunol Today 1986;7:45-56.
102. Elias JA, Schreiber AD, Gustilo K, Chien P, Rossman MD, Lammie PJ, Daniele RP. Differential interleukin 1 elaboration by unfractionated and density fractionated human alveolar macrophages and blood monocytes: relationship to cell maturity. J Immunol 1985;135:3198-3204.

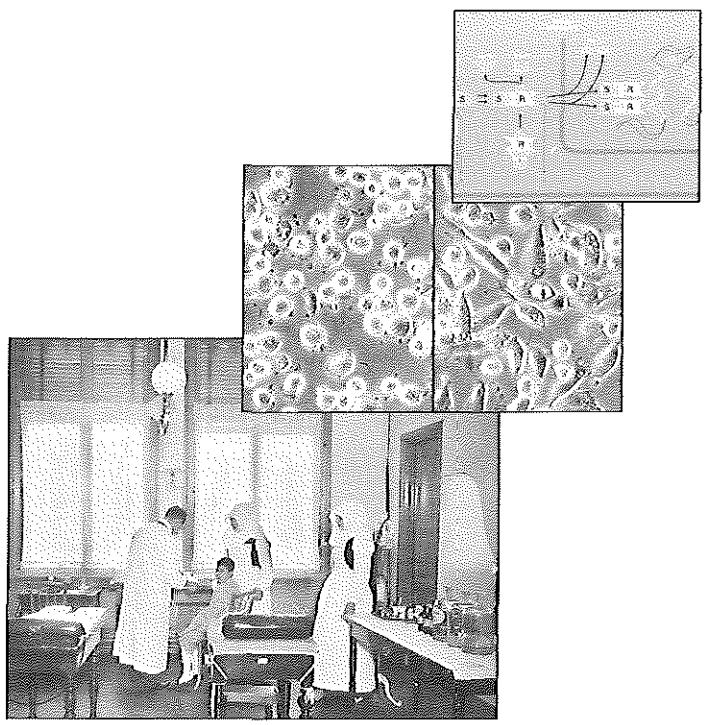
103. Snyder DS, Unanue ER. Corticosteroids inhibit murine macrophage Ia expression and interleukin-1 production. J Immunol 1982;129:1803-1805.
104. Knudsen J, Dinarello CA, Strom TB. Glucocorticoids inhibit transcriptional and posttranscriptional expression of interleukin-1 in U937 cells. J Immunol 1987;139:4129-4134.
105. Tsudo M, Kozak RW, Goldman CK, Waldmann TA. Demonstration of a non-Tac peptide that binds interleukin-2: A potential participant in a multichain interleukin-2 receptor complex. Proc Natl Acad Sci USA 1986;83:9694-9698.
106. Randazzo B, Hirschberg T, Hirschberg H. Inhibition of the antigen activated T cell response by methylprednisolone is caused by inhibition of interleukin-2 (IL-2) production. J Immunopharmac 1984;6:419-423.
107. Arya SK, Wong-Staal F, Gallo RC. Dexamethasone-mediated inhibition of human T cell growth factor and γ-interferon messenger RNA. J Immunol 1984;133:273-276.
108. Bertoglio JH, Leroux E. Differential effects of glucocorticoids on the proliferation of a murine helper and a cytolytic T cell clone in response to IL-2 and IL-4. J Immunol 1988;141:1191-1196.
109. Otsuka T, Miyajima A, Brown N, Otsu K, Abrams J, Saeland S, Caux C, De Waal Malefijt R, Vries J de, Meyerson P, Yokota K, Gemmel L, Rennick D, Lee F, Arai N, Arai K-I, Yokota T. Isolation and characterization of an expressible cDNA encoding human IL-3. Induction of IL-3 mRNA in human T cell clones. J Immunol 1988;140:2288-2295.
110. Culpepper JA, Lee F. Regulation of IL-3 expression by glucocorticoids in cloned murine T lymphocytes. J Immunol 1985;135: 3191-3197.
111. Sanderson CJ, Campbell HD, Young IG. Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. Immunol Rev 1988;102:29-50.
112. Van Oosterhout AJM, Ladenius ARC, Savelkoul HFJ, Van Ark I, Delsman KC, Nijkamp FP. Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. Am Rev Respir Dis 1993;147:548-552.
113. Fushimi T, Okayama H, Shimura S, Sasaki H, Takishima T, Shirato K. Dexamethasone suppresses both IL-5 production and its gene expression by human PBMC. Am Rev Respir Dis 1993;147:A980.
114. Navarro S, Debili N, Bernaudin J-F, Vainchenker W, Doly J. Regulation of the expression of IL-6 in human monocytes. J Immunol 1989;142:4339-4345.
115. Wong GG, Clark SC. Multiple actions of interleukin 6 within a cytokine network. Immunol Today 1988;9:137-139.
116. Waage A, Slupphaug G, Shalaby R. Glucocorticoids inhibit the production of IL 6 from monocytes, endothelial cells and fibroblasts. Eur J Immunol 1990;20:2439-2443.
117. Talmadge JE, Phillips H, Schneider M, Rowe T, Pennington R, Bowersox O, Lenz B. Immunomodulatory properties of recombinant murine and human tumor necrosis factor. Cancer Res 1988;48:544-550.
118. Tracey KJ, Vlassara H, Cerami A. Cachectin/tumor necrosis factor. Lancet 1989;i:1122-1126.
119. Standiford TJ, Kunkel SL, Rolfe MW, Evanoff HL, Allen RM, Strieter RM. Regulation of human alveolar macrophage- and blood monocyte-derived interleukin-8 by prostaglandin E_2 and dexamethasone. Am J Respir Cell Mol Biol 1992;6:75-81.
120. Natrajan S, Schulman ES, Rozans M, Glaum M, Raible D, Gillespie D. Dexamethasone suppresses the generation of TNF-α in human lung at a transcriptional level. Am Rev Respir Dis 1993;147:A981.
121. Dinarello CA, Mier JW. Lymphokines. New Engl J Med 1987;317:940-945 .
122. Cockayne D, Sterling KM, Shull S, Mintz KP, Illeyne S, Cutroneo KR. Glucocorticoids decrease the synthesis of type I procollagen mRNAs. J Biochem 1986;25:3202-3209.
123. Hämäläinen L, Oikarinen J, Kivirikko K. 1. Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and the effect of cortisol. J Biol Chem 1985;260:720-725.
124. Weiner FR, Czaja MJ, Jefferson DM, Giambrone M-A, Kaspa R-T, Reid LM, Zern MA. The effects of dexamethasone on *in vitro* collagen gene expression. J Biol Chem 1987;262:6955-6958.
125. Ziment I. Steroids. Clin Chest Med 1986;7:341-354.

126. Auler JOC, Fermandes Calheiros D, Brentani MM, Santello JL, Lemos PCP, Saldiva PHN. Adult respiratory distress syndrome: Evidence of early fibrinogenesis and absence of glucocorticoid receptors. Eur J Respir Dis 1986;69:261-269.
127. Janusz MJ, Hare M. Cartilage degradation by cocultures of transformed macrophage and fibroblast cell lines. J Immunol 1993;150:1922-1931.
128. Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M. Prolonged activation of *jun* and collagenase genes by tumour necrosis factor-α. Nature 1989;337:661-663.
129. Conca W, Kaplan PB, Krane SM. Increases in levels of procollagenase messenger RNA in cultured fibroblasts induced by human recombinant interleukin 1β or serum follow c-*jun* expression and are dependent on new protein synthesis. J Clin Invest 1989;83:1753-1757.
130. Compton MM, Cidlowski JA. Identification of a glucocorticoid-induced nuclease in thymocytes. J Biol Chem 1987;262:8288-8292.
131. Wegner CD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. Science 1990;247:456-459.
132. Van de Stolpe A, Caldenhoven E, Raaijmakers JAM, Van der Saag PT, Koenderman L. Glucocorticoid-mediated repression of intercellular adhesion molecule-1 expression in human monocytic and bronchial epithelial cell lines. Am J Respir Cell Mol Biol 1993;8:340-347.
133. Erdös EG, Skidgel RA. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB J 1989;3:145-151.
134. Matsas R, Kenny AJ, Turner AJ. The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase 24.11. Biochem J 1984;223:433-440.
135. Lang Z, Murlas CG. Dexamethasone increases airway epithelial cell neutral endopeptidase by enhancing transcription and new protein synthesis. Lung 1993;171:161-172.
136. Proud D, Subauste MC, Ward PE. Glucocorticoids do not alter peptidase expression on a human bronchial epithelial cell line. Am J Respir Cell Mol Biol 1994;11:57-65.
137. Duval D, Durant S, Homo-Delarche F. Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. Biochim Biophys Acta 1983;737:409-442.
138. Johnson LK, Longenecker JP, Baxter JD, Dallman MF, Widmaier EP, Eberhardt NL. Glucocorticoid action: A mechanism involving nuclear and non-nuclear pathways. Br J Dermatol 1982;107:6-23.
139. Fauci AS, Dale DC. The effect of *in vivo* hydrocortisone on subpopulations of human lymphocytes. J Clin Invest 1974;53:240-246.
140. Thompson J, Van Furth R. The effect of glucocorticosteroids on the proliferation and kinetics of promonocytes and monocytes of the bone marrow. J Exp Med 1973;137:10-20.
141. Boggs DR, Athens JW, Cartwright GE, Wintrobe MM. The effect of adrenal glucocorticosteroids upon the cellular composition of inflammatory exudates. Am J Path 1964;44:763-773.
142. Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc Natl Acad Sci USA 1992;89:9991-9995.
143. Mancuso F, Flower RJ, Perretti M. Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule: involvement of endogenous lipocortin 1. J Immunol 1995;155:377-386.
144. Persellin RH, Ku LC. Effects of steroid hormones on human polymorphonuclear leukocyte lysosomes. J Clin Invest 1974;54:919-925.
145. Goldstein IM, Roos D, Weissmann G, Kaplan HB. Influence of corticosteroids on human polymorphonuclear leukocyte function *in vitro*. Inflamm 1976;1:305-315.
146. Chang S-W, Voelkel NF. Inflammatory mediator effects on pulmonary blood flow, edema, and the vascular endothelium. In: Bray MA, Anderson WH, eds. Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:403-453.

147. Lefer AM, Inge TF. Lack of interaction between glucocorticoids and the kallikrein-kinin systems. Proc Soc Exp Biol Med 1974;145:658-662.
148. Hoogsteden HC, Van Hal PThW. Mediators of the induction of non-allergic pulmonary inflammation. In: Bray MA, Anderson WH, eds. Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:185-277.
149. Schleimer RP. Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic applications in asthma. Am Rev Respir Dis 1990;141:59-69.
150. Marom Z, Shelhamer JH, Kaliner M. Human pulmonary macrophage-derived mucus secretagogue. J Exp Med 1984;159:844-860.
151. Marom Z, Shelhamer J, Alling D, Kaliner M. The effects of corticosteroids on mucous glycoprotein secretion from human airways *in vitro*. Am Rev Respir Dis 1984;129:62-65.

CHAPTER 3

INTRODUCTION TO THE EXPERIMENTAL WORK



Since the idea that organisms are made up of cells, doctors have been aimed at understanding diseases by searching for disturbances of cellular processes. Concurrently, the realization dawned upon doctors that recovery of derailed cellular processes was accompanied by recovery of the patient. Furthermore, it became clear that medicines were able to influence this process of recovery of "diseased cells". This search for relations between cell function and disease has led to many victories in the history of medicine and have realized new and powerful methods of treatment, stressing the importance of basic sciences and knowledge. Although it is almost unavoidable for our understanding to simplify the complex reality of cellular disturbances underlying diseases, one has to take care not to overestimate the role of one single cell type in a particular disease. It is realized now that different cell types are able to interact with each other. Therefore, medical intervention has to concentrate preferably on influencing cellular interactions, rather than on only one cell type.

Nowadays, great efforts are made and a lot of money is spent to find new ways to influence specifically cellular functions or interactions. Monocytes/macrophages are being studied all over the world because of their supposed crucial role in several diseases, partly for their own specific properties and partly for their ability to interact with and influence other cell types. The great variety of cellular functions of mononuclear phagocytes described in chapter 1.1 support the idea that these cells are essential in many physiological and pathological processes. Because of these cellular properties and their abundance in the lung, mononuclear phagocytes should be considered as pivotal cells in many lung diseases, including asthma. In chapters 1.2, 1.3 and 2 it is shown that glucocorticoids and cytokines can modulate functions of mononuclear phagocytes. Consequently, the course of the diseases in which mononuclear phagocytes are central cells may be influenced by the clinical application of glucocorticoids or cytokines. Since 1949, glucocorticoids are being used successfully in the treatment of many inflammatory diseases, including asthma, but the clinical use of cytokines is still in its infancy.

The base of the investigations presented in this thesis was the central role of mononuclear phagocytes in the chronic airway inflammation, which underlies many of the clinical symptoms in asthma. The purpose of the investigations was to get more insight into the influence of glucocorticoids and cytokines on the phenotype and function of these cells, since glucocorticoid- or cytokine-induced modulation of monocyte/macrophage functions may be relevant to the treatment of asthmatic patients. *In vitro* experiments concerning these aspects are described in chapters 4 and 5. In chapter 6, studies are described in which it was analyzed whether *in vivo* treatment of asthma patients with inhaled glucocorticoids was associated with effects on peripheral blood monocytes and alveolar macrophages similar to the effects described in our *in vitro* studies.

The precise working mechanisms of glucocorticoids in the inhibition of inflammation are still incompletely understood. Therefore, the investigations presented here were started to gain more insight into their cellular working mechanisms. With regard to cytokines, their clinical application as anti-inflammatory drugs is less clear compared to glucocorticoids, and therefore, the *in vitro* studies presented here were done to search for effects which may be of use to modulate inflammation *in vivo*. In most experiments, we focussed on the

effects of IL-4, as this cytokine has been described previously to have potential anti-inflammatory effects.

Chapter 4 describes the effects of cytokines *in vitro* on the expression of cell membrane proteins, the function and the proliferative activity of monocytes/macrophages. Especially the influence of IL-4 on aminopeptidase-N is described, and it is shown that IL-4-mediated effects are determined partly by the maturation stage of monocytes/macrophages. Furthermore, it is shown that *in vivo* the lungs create an environment, probably through local production of cytokines, in which monocytes/macrophages re-acquire proliferative activity.

Chapter 5 is a compilation of four *in vitro* studies concerning the effects of glucocorticoids on some functional aspects of monocytes/macrophages. An earlier described effect of IL-4 on the maturation of monocytes/macrophages is confirmed in our studies. We investigated the influence of glucocorticoids on this IL-4 effect, and based on our findings we hypothesized a balanced *in vivo* regulation of the phenotype and function of monocytic cells by cytokines and steroid hormones. Furthermore, we focussed on the gene expression of two proteins with pro- and anti-inflammatory properties, respectively, and its regulation by IL-4 and glucocorticoids. Additional, direct evidence for specific effects of glucocorticoids on monocytes/macrophages comes from the development of an assay to detect glucocorticoid receptors in these cells conclusively.

Chapter 6 describes some cellular effects during glucocorticoid therapy. The *in vitro* influences of glucocorticoids on monocytes/ macrophages, as described in chapters 4 and 5, were reason to investigate whether *in vivo* treatment with glucocorticoids resulted in cellular effects comparable with the *in vitro* findings.

Chapter 7 discusses the significance of the presented experimental data in the context of the literature. In addition, the clinical use of cytokines, in particular the applicability of the described potential anti-inflammatory properties of IL-4, are discussed in the context of recent clinical studies. Special attention is paid to the understanding that the anti-inflammatory properties of both glucocorticoids and cytokines are based on the modulation of cell functions via modulation of gene expression. New openings for improvement of the treatment of inflammatory lung diseases including asthma, may be the specific modulation of the expression of only the genes relevant to inflammation.

CHAPTER 4

MONOCYTES/MACROPHAGES AND CYTOKINES

4.1 Introduction

4.2 Potential, indirect anti-inflammatory effects of IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N (CD13; EC 3.4.11.2) activity

4.3 Regulation of aminopeptidase-N (CD13) and $F_c\varepsilon$RIIb (CD23) expression by interleukin-4 depends on the stage of maturation of monocytes/macrophages

4.4 Proliferation of mature and immature subpopulations of bronchoalveolar monocytes/macrophages and peripheral blood monocytes

INTRODUCTION: DIFFERENCES IN EXPRESSION OF MONOCYTE/MACROPHAGE SURFACE ANTIGENS IN PERIPHERAL BLOOD AND BRONCHOALVEOLAR LAVAGE CELLS*

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SUMMARY

Peripheral blood monocytes (PBM) are generally considered as precursors of various populations of tissue macrophages, e.g. alveolar macrophages (AM). These macrophages differ from their precursors in morphology and function. Monoclonal antibodies can be used to study differences in the expression of cell membrane antigens. In this study, differences in the expression of cell surface antigens between PBM and AM (isolated from bronchoalveolar lavage fluids) from healthy volunteers were evaluated.

In blood monocytes, the percentages of cells expressing the antigens recognized by CD14, CD33 and Max 3, respectively, were higher than in AM. On the other hand, the percentages of AM expressing RFD9 antigen were higher than blood monocytes expressing this antigen.

These data suggest that upon migration of PBM to the alveoli, the expression of some cell membrane antigens is reduced, whereas the expression of other antigens is upregulated. These changes in expression eventually determine the phenotype of AM and may be considered as maturation-associated. Our findings may be used to study and influence maturation of mononuclear phagocytes *in vitro*.

INTRODUCTION

AM represent a heterogeneous population of mononuclear phagocytes (1-3). This heterogeneity is believed to represent differences in maturation and activation. A continuous influx of PBM maintains, probably in addition to local proliferation, the local population of AM (4-6). PBM leave the circulation, pass the lung interstitium and mature into AM. At each level of this migratory process, PBM are thought to pass through different stages of maturation. The presence of different maturation stages may be visualized by studying the expression of cell surface antigens. Different stages of the life cycle of mononuclear phagocytes are characterized by the expression of different cell surface antigens or combinations of different antigens. Expression of these different antigens is related to different cellular functions of the respective stages of the life cycle. Monoclonal antibodies (mAb) are useful to detect these differences in expression of surface antigens (7). Other cellular qualities to study the existence of different maturation stages or, in a more general sense, different subpopulations among AM may be their morphology, density, motile capacity and immunological functions (1).

Activation at all stages of maturation may also contribute to the cellular heterogeneity of mononuclear phagocytes. Activation may influence the expression of cell surface antigens, cell function and cell morphology. Whether differences in differentiation stages also determine the heterogeneity of AM remains controversial. Some studies appear to demonstrate that subpopulations of AM arise from distinct bone marrow precursors. After this differentiation process in the bone marrow, these different lineages should mature independently. Dendritic cells, which are also found in bronchoalveolar lavage (BAL) fluid, may represent such a distinct lineage (8). However, recent studies in humans showed that

also dendritic cells may originate from PBM (9,10). Therefore, differentiation does not appear to contribute to the cellular heterogeneity of BAL monocytes/macrophages in humans.

Activation is obviously a cellular response to extracellular stimuli. These environmental stimuli may be mediated via direct intercellular contact, interactions between cells and extracellular matrix, or soluble factors, e.g. cytokines. With regard to maturation, the importance of environmental stimuli is less self-evident. Maturation may be considered as an autonomous cellular process which takes place independently of extracellular influences. However, recent studies have shown conclusively that maturation of monocytes/macrophages can be modulated by environmental factors *in vitro*, suggesting that also maturation *in vivo* may be under the influence of extracellular stimuli (11,12). Furthermore, differences in phenotype between monocytes/macrophages from different tissues are thought to result from environmental differences created by the tissue where these cells reside (13).

This study was partly designed to investigate differences in the expression of monocytic cell surface antigens on PBM and AM in healthy volunteers. Differences were suspected because of the above-mentioned migration of PBM to the lung, which is associated with maturation of PBM into AM. Differences were studied with mAb.

MATERIALS AND METHODS

Subjects and bronchoalveolar lavage

BAL fluid (BALF) was obtained from 10 healthy volunteers. This group consisted of non-smoking individuals without chest abnormalities and with normal chest X-ray films and lung functions. Approval for all lavage studies was obtained from the Medical Ethics Committee of the Erasmus University/University Hospital Rotterdam.

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

Immunologic characterization of BAL cells

The BAL cells were immunologically characterized by use of the following mAb: CD3(Leu-4), CD4(Leu-3a), CD8(Leu-2a) (Becton Dickinson, San Jose, CA), CD13(Q20) (Dr. C.E. van der Schoot, Amsterdam, The Netherlands), CD14(My3) (Coulter Clone, Hialeah, FL), U26 (Dr. R. Winchester, NY), CD68(Y2/131) (Dr. D.Y. Mason, Oxford, UK), Max3 and Max24 (Dr. F. Emmrich, Erlangen, FRG), RFD9 (Dr. L. Poulter, London, UK), CD68(Ki-M6), CD68(Ki-M7) and Ki-M8 (Behring, Marburg, FRG).

For immunologic staining, 50 µl of the cell suspensions (8 x 10^6 cells per ml) were incubated for 30 min at 4°C with 50 µl of the relevant, optimally-titrated antibody. As a control, cells were incubated with normal mouse serum (NMS). After this incubation the cells were washed twice and subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat-anti-mouse immunoglobulin antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands). Subsequently the cells were washed twice and the

cell pellet was mounted in glycerol/PBS (9:1), containing 1 mg *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of FITC. Coverslips were sealed to the slide with paraffin wax mixed with ceresin (BDH Chemicals). The fluorescence was evaluated using Zeiss fluorescence microscopes equipped with phase contrast facilities. The phase contrast facilities were used to evaluate the morphology of the cells (lymphocytes, small monocyte-like macrophages and mature macrophages) and for discrimination between labeled cells and contaminating fluorescent particles. In this way it was possible to determine which proportion of the monocytes and macrophages expressed the various immunologic markers. For each immunologic marker at least 300 cells were evaluated.

Immunologic characterization of PB cells

Mononuclear cells (MNC) from PB were isolated by Ficoll density centrifugation (Ficoll Paque, density: 1.077 g/cm^3, Pharmacia, Uppsala, Sweden) for 15 min at room temperature at a centrifugal force of 1,000 g. All standard washings and immunologic stainings of MNC from PB were done as described above for BAL cells. Forward and sideward light scatter signals were used to differentiate between lymphocytes and monocytes and made it possible to analyze expression of the relevant Ag on PBM by FACScan analysis.

Statistical analysis

Comparisons of cellular parameters from BALF and PB were done with Wilcoxon's test. Five percent was considered as the limit of statistical significance.

RESULTS

Some characteristics of the BALF from healthy volunteers are shown in Table 1. The majority of BAL cells was found to be monocytes/macrophages as determined by May-Grünwald Giemsa staining. A small percentage (1%) of this population resembled blood monocytes, and was thought to represent immature macrophages (Figure 1). Around 6% of the total cell population concerned lymphocytes, whereas less than 2% concerned granulocytes.

Table 1. Total and differential cell counts in BALF from healthy volunteers.

Recovered BALF	Healthy volunteers (n = 10)
Recovered volume in ml	122.0 (23.9)[a]
Total cell recovery x 10^6	8.9 (5.2)
Differential cell count (%):	
Macrophages	93.1 (7.7)
Granulocytes	1.9 (2.9)
Lymphocytes	5.9 (7.7)
Monocyte-like cells	1.0 (0.1)

a. Mean with standard deviation in parentheses.

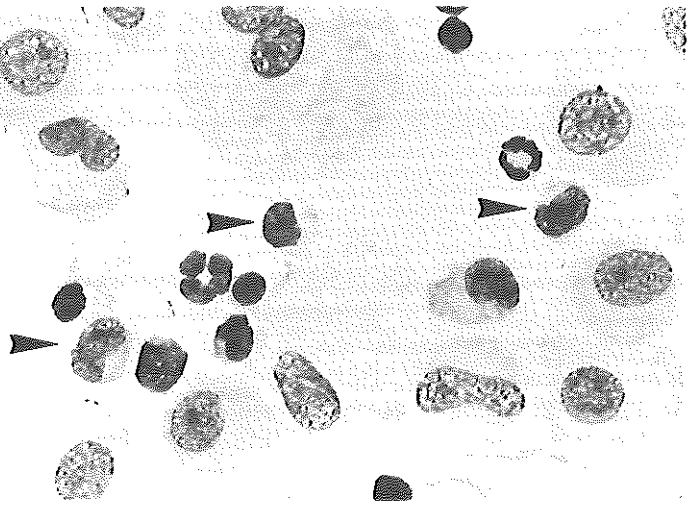

Figure 1. May-Grünwald Giemsa staining of cells present in the BAL fluid from a healthy volunteer. The majority of the cells is macrophage (mature mononuclear phagocyte). A small percentage of the cell population resembles blood monocytes (arrows), and is thought to represent immature macrophages.

Table 2. Immunologic characterization of PBM and BAL monocytes/macrophages from healthy volunteers.

Monoclonal antibody	PBM	BAL monocytes/macrophages	
	%	%	x 10^4/ml
CD13(Q20)	78.3 (29.1)[a]	68.4 (16.6)	5.2 (1.3)[b]
CD14(My3)	89.0 (10.1)	49.3 (23.3)[c]	3.5 (0.9)
CD33(My9)	89.3 (8.7)	42.9 (34.4)[c]	3.1 (1.2)
CD68(Ki-M6)	0.2 (0.3)	2.2 (2.6)	0.2 (0.2)
CD68(Ki-M7)	52.4 (29.5)	50.6 (6.0)	4.8 (5.6)
CD68(Y2/131)	27.4 (16.8)	45.0 (19.6)	4.5 (1.7)
U26	93.7 (6.5)	91.7 (7.6)	7.0 (1.4)
Max3	96.6 (1.8)	81.6 (17.0)[d]	6.4 (1.5)
Max24	92.0 (4.2)	93.0 (5.9)	7.2 (1.6)
Ki-M8	79.4 (6.4)	82.7 (8.6)	7.6 (2.1)
RFD9	0.3 (0.6)	84.9 (23.3)[c]	5.8 (0.9)

a. Mean with standard deviation in parentheses.
b. The absolute numbers of cells are given as positive macrophages x 10^4/ml recovered BAL fluid.
c. Different from PBM ($p < 0.01$).
d. Different from PBM ($p < 0.05$).

The immunologic characterization of monocytes/macrophages in BAL fluid and PBM is shown in Table 2. An important difference between AM and PBM was the higher percentage of PBM positive for some mAb used: CD14, CD33 and Max 3. Statistical analysis indicated that these differences were significant. The percentages of PBM positive

for two of the mAb used, RFD9 and CD68, appeared to be lower than the percentages of AM positive for these markers. Both the percentages of RFD9$^+$ and CD68 (Ki-M6)$^+$ PBM were less than 0.5%, whereas RFD9$^+$ and CD68 (Ki-M6)$^+$ AM represented 85% and 2.2% of the total cell population in BALF, respectively. However, only the difference in expression of RFD9 Ag between PBM and AM was statistically significant.

DISCUSSION

In this study the expression of a panel of monocytic antigens was analyzed on PBM and AM. The background of this study is the hypothesis that a continuous influx of PBM into the lungs maintains, at least partially, the local population of AM. This migration is associated with phenotypic and functional changes, which are considered to represent maturation of PBM (12). Some of the mAb used in this study recognize cell surface antigens with known functions, whereas others react with proteins with still unknown functions.

CD13 antibodies recognize a cell membrane-bound glycoprotein, aminopeptidase-N (EC 3.4.11.2), involved in the metabolism of regulatory peptides (14). The expression and the functional enzyme-activity of CD13 Ag can be modulated by cytokines *in vitro*. As part of these findings, IL-4 was shown recently to increase CD13 Ag expression (15) in addition to its effect on PBM maturation (11, chapter 5.2). However, no differences in the expression of CD13 Ag were observed between PBM and AM in this study. One explanation may be that IL-4 has only a transient effect on CD13 Ag expression (15), whereas the effects on PBM maturation (visualized by changes in the expression of CD14 and RFD9 Ag) are long-lasting (chapter 5.2).

CD14 antibodies recognize three or four distinct epitopes on a 55 kDa cell membrane glycoprotein (16). This glycoprotein may function as a receptor for LPS. The binding of LPS to the CD14 glycoprotein can be enhanced markedly in the presence of LPS-binding protein (LBP). Only a minor population of AM, i.e. the monocyte-like cells (Figure 1), expressed CD14 Ag, in contrast to the majority of PBM. This small population of AM was thought to represent newly arrived monocytes, which had finished their migration from the vasculature to the alveolar compartment. It may be hypothesized that upon maturation of PBM to AM, CD14 Ag expression decreases. This decrease is also seen during *in vitro* maturation, and can be accelerated by IL-4 (chapter 5.2).

CD33, U26, Max 3, Max 24 and Ki-M8 antibodies are known to recognize cell membrane antigens on monocytes/macrophages, but the functions of these antigens have to be elucidated still (17,18). The expression of CD33 and Max 3 Ag may be related to the maturation stage of monocytes/macrophages, as in this study the expression in PBM was found to be higher than in AM.

RFD9 Ag were nearly absent from PBM, whereas the majority of AM expressed this Ag. Further studies are necessary to determine whether only the small RFD9$^+$ subpopulation of PBM migrates to the lungs, or RFD9 Ag becomes progressively expressed upon migration of PBM to the lungs.

The three mAb CD68(Ki-M6), CD68(Ki-M7) and CD68(Y2/131) are panmacrophage antibodies (19-21). The markers recognized by these mAb are restricted to cells of the monocyte/macrophage system, and these antigens are mainly confined to lysosome and phagosome structures. For CD68(Ki-M6) and CD68(Ki-M7), a stepwise increase in cytoplasmic expression has been described during maturation of monocytes into macrophages, *in vitro* as well as *in vivo* (19-21). In our study we did not perform immunoperoxidase stainings on cytocentrifuge preparations (in which cytoplasmic expression of antigens can easily be detected), but instead incubated cells in suspension in order to analyze differences in cell surface expression between the different CD68 mAb. In this way, we demonstrated that these markers are not strictly cytoplasmic. Surface expression was also found by Cordell et al. (22). Our findings are also in line with the findings of Parwaresch et al. (19) and Kreipe et al. (21) that the expression of CD68 is increased in more mature cells. We found lower percentages of $CD68^+$ macrophages than in their studies because with our technique we detect cell-surface markers, whereas in the other studies an alkaline phosphatase technique was used that detects cell-surface as well as intracytoplasmatically located antigens.

In conclusion, this study shows that PBM and AM differ in the expression of cell surface Ag as demonstrated with mAb. These findings help us to understand maturation of mononuclear phagocytes. Further studies are needed to evaluate whether influencing maturation and Ag-related cellular functions may be useful in the treatment of pulmonary diseases.

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REFERENCES

1. Spiteri MA, Clarke SW, Poulter LW. Isolation of phenotypically and functionally distinct macrophage subpopulations from human bronchoalveolar lavage. Eur Respir J 1992;5:717-726.
2. Biondi A, Rossing TH, Bennett J, Todd RF III. Surface membrane heterogeneity among human mononuclear phagocytes. J Immunol 1984;132:1237-1243.
3. Gant VA, Hamblin AS. Human bronchoalveolar macrophage heterogeneity demonstrated by histochemistry, surface markers and phagocytosis. Clin Exp Immunol 1985;60:539-545.
4. Johnston RB. Monocytes and macrophages. New Engl J Med 1988;318:747-752.
5. Radzun H, Parwaresch MR, Kreipe H. Monocytic origin of human alveolar macrophages. J Histochem Cytochem 1983;31:318-324.
6. Bitterman PB, Saltzman LE, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication: one mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. J Clin Invest 1984;74:460-469.

7. Mechtersheimer G, Möller P. Phenotyping of human epithelioid cells, alveolar macrophages, and foamy cells by means of mAb of the myeloid section. In: Knapp W, Dörken B, Gilkes WR, Rieber EP, Schmidt RE, Stein H, Von dem Borne AEGKr, eds. Leucocyte Typing IV. Oxford: Oxford University Press. 1989:918-921.
8. Van Furth R. Current view on the mononuclear phagocyte system. Immunobiol 1982;161:178-185.
9. Kabel PJ, De Haan-Meulman M, Voorbij HAM, Kleingeld M, Knol EF, Drexhage HA. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. Immunobiol 1989;179:395-411.
10. Najar HM, Ruhl S, Bru-Capdeville AC, Peters JH. Adenosine and its derivatives control human monocyte differentiation into highly accessory cells versus macrophages. J Leukocyte Biol 1990;47:429-439.
11. Te Velde AA, Klomp JPG, Yard BA, De Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
12. Andreesen R, Brugger W, Scheibenbogen C, Kreutz M, Leser H-G, Rehm A, Löhr GW. Surface phenotype analysis of human monocyte to macrophage maturation. J Leukocyte Biol 1990;47:490-497.
13. Gordon S, Keshav S, Chung LP. Mononuclear phagocytes: tissue distribution and functional heterogeneity. Curr Opin Immunol 1988;1:26-35.
14. Kenny AJ, O'Hare MJ, Gusterson BA. Cell-surface peptidases as modulators of growth and differentiation. Lancet 1989;ii:785-787.
15. Van Hal PThW, Hopstaken-Broos JPM, Prins A, Favaloro EJ, Huijbens RJF, Hilvering C, Figdor CG, Hoogsteden HC. Potential, indirect anti-inflammatory effects of IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase activity (CD13; EC 3.4.11.2). J Immunol 1994;153:2718-2728.
16. Ziegler-Heitbrock HWL, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today 1993;14:121-125.
17. Peiper SC, LeBoeuf RD, Prasthofer EF, Borowitz MJ, Walker WS, Ashmun RA, Look AT. Biochemical and genetic characterization of gp67, the CD33 myeloid antigen. Tissue Antigens 1989;33:229.
18. Emmrich H, Andreesen R. Monoclonal antibodies against differentiation antigens on human macrophages. Immunol Lett 1985;9:321-324.
19. Parwaresch MR, Radzun HJ, Kreipe H, Hansmann M-L, Barth J. Monocyte/macrophage-reactive monoclonal antibody Ki-M6 recognizes an intracytoplasmic antigen. Am J Pathol 1986;124:141-151.
20. Micklem K, Rigney E, Pulford K, Turley H, Mason DY. Four pan-macrophage antibodies from the third workshop recognise the same antigen. Tissue Antigens 1989;33:206.
21. Kreipe H, Radzun HJ, Parwaresch MR, Haislip A, Hansmann M-L. Ki-M7 monoclonal antibody specific for myelomonocytic cell lineage and macrophages in human. J Histochem Cytochem 1987;35:1117-1126.
22. Cordell J, Simmons D, Micklem K, Stross P, Mason DY. The expression of pan-macrophage antigens in transfected cells. Tissue Antigens 1989;33:204.

POTENTIAL, INDIRECT ANTI-INFLAMMATORY EFFECTS OF IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N-activity (CD13; EC 3.4.11.2)*

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SUMMARY

IL-4 up-regulates various monocytic properties, which are associated with pro-inflammatory functions. Paradoxically, IL-4 may also act as an anti-inflammatory agent by down-regulating the production of several inflammatory mediators. As the activity of some mediators has recently been shown to be regulated by peptidases, we examined whether IL-4 was able to modulate the expression of a cell membrane associated peptidase, aminopeptidase-N (CD13). IL-4 caused a dose-dependent increase in the expression of CD13 Ag on highly purified human blood monocytes. Maximal expression was observed around 48 h of culture. This IL-4-induced increase was completely blocked by anti-IL-4 antiserum. Furthermore, the increase in surface expression was preceded by increased mRNA levels of CD13, which was maximal around 24 h of culture. Additionally, we observed that also CD13-mediated leucine-aminopeptidase activity of monocytes was induced by IL-4. Also other CD13-expressing cells were sensitive to IL-4, since CD13 Ag expression and CD13 mRNA levels were up-regulated in human alveolar macrophages and endothelial cells upon IL-4 treatment. The increased expression of cell membrane aminopeptidase-N represents a potentially increased cellular ability to inactivate inflammatory mediators. Therefore, these findings represent further evidence of IL-4-mediated anti-inflammatory actions. We postulate that up-regulation of aminopeptidase-N expression may be an indirect working-mechanism of IL-4 to modulate the action of bioactive peptides. This mechanism as such may underlie, at least partially, the anti-inflammatory effects of IL-4 *in vivo*.

INTRODUCTION

IL-4 was originally characterized by its ability to effect differentiation of resting B lymphocytes (1). More recently, it has been shown that IL-4 may also display a wide range of effects on several cell types (2). It can act as a growth factor for activated T lymphocytes, thymocytes, NK cells (3-5), and mast cells (5,6). In addition, monocytic cells can also be influenced by IL-4, which is reported to induce monocyte maturation (7). Furthermore, some of the actions of IL-4 on monocytic cells are considered to be stimulatory. IL-4 increases expression of MHC class II Ag (7), increases antigen-presenting capacity (8), and induces FcεRIIb expression (9). On the other hand, IL-4 also appears to inhibit some parameters of monocyte activation. Thus, IL-4 inhibits superoxide production (10), inhibits the release of PGE2 (11), IL-1β (11-14), IL-6 (12,15,16), IL-8 (17), TNF-α (11-13), and up-regulates the production of IL-1R antagonist (14,18). Based upon the latter findings, it was suggested that IL-4 may have anti-inflammatory properties (11-19).

During our studies on the influence of cytokines on monocytes/macrophages, we observed that IL-4 was also able to modulate the expression of aminopeptidase-N. Initially, this enzyme (EC 3.4.11.2, gp150, CD13) was designated as a marker for subpopulations of hemopoietic cells (20-23). Currently, it is generally accepted that many other cell types (including fibroblasts, the renal tubular and intestinal epithelium (24,25), endothelial cells

(26,27), and synaptic membranes of cells of the central nervous system (28)) express this enzyme. Comparable to the role of neutral endopeptidase (EC 3.4.24.11), it is hypothesized that aminopeptidase-N also plays an important role in modulating the activity of bioactive oligopeptides (29-32). Accordingly, aminopeptidase-N may play a role in regulation of inflammatory and immunological responses. Modulation of the expression of aminopeptidase-N may therefore influence these oligopeptide-mediated responses. In this report, we demonstrate that IL-4 is able to up-regulate the expression of CD13 Ag on monocytic cells. Up-regulation is also seen in non-monocytic cells (i.c. endothelial cells), although this was less pronounced. This up-regulation is accompanied by an increase in aminopeptidase-activity, indicating that cells activated by IL-4 may acquire potential anti-inflammatory properties.

MATERIALS AND METHODS

Isolation of PBM

Human PBM were isolated from 500 ml blood from healthy volunteers as described elsewhere (33). Briefly, mononuclear cells were separated by density centrifugation with a blood component separator. Next, the mononuclear cells were fractionated into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was over 95% pure as judged by May-Grünwald Giemsa staining and contained more than 98% viable cells as judged by trypan blue exclusion. Lymphocytes and granulocytes constituted less than 2% and 3% of the monocyte preparation, respectively. Before analysis of the surface membrane determinants the cells were washed twice with a PBS solution (300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated BSA (Organon Teknika, Oss, The Netherlands). Before isolation of total RNA or detection of LAP activity the cells were washed twice with a PBS solution.

Isolation of AM

All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. BAL was performed in healthy, nonsmoking volunteers after premedication with thiazinamium and local anesthesia using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection, the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles, whereafter the BAL cells were isolated by centrifugation at 4°C at a force of 400 × g. More than 90% of the BAL cells appeared to be macrophages as judged by May-Grünwald Giemsa staining. Before analysis of the surface membrane determinants the cells were washed twice with PBS/0.5% BSA. Before isolation of total RNA or detection of LAP activity the cells were washed twice with a PBS solution.

Isolation of EC

Human EC were isolated from umbilical cord vein by collagenase digestion as described elsewhere (34).

Cell lines

THP-1 cells (36) were used as control cells because of their CD13 mRNA and CD13 Ag expression. They were obtained from The American Type Culture Collection (Rockville, MD) and maintained according to their instructions.

Recombinant IL-4, rIL-2, rIFN-γ, GM-CSF, LPS and anti-IL-4 antiserum

Human rIL-4 was a generous gift from Dr. H.F.J. Savelkoul from our department and Dr. K. Arai (DNAX Research Institute, Palo Alto, CA). Human rIL-2 was obtained from Eurocetus (Amsterdam, The Netherlands). Human rIFN-γ (specific activity 10^7 U/mg) and human rGM-CSF (specific activity 10^7 U/mg) were kindly provided by Drs. P. Trotta and S. Naghabhushan (Schering-Plongh Corp, Bloomfield, NJ). LPS (E. coli 0127:B8) was purchased from Difco Laboratories (Detroit, MI). A polyclonal rabbit anti-IL-4 antiserum was used to block the biologic activity of IL-4, as has been described elsewhere (36).

Culture of cells

PBM and AM were cultured in a modified Iscove's medium (GIBCO, Paisley, U.K.) as described elsewhere (37), in which BSA was replaced by human serum albumin. In some experiments this medium was supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin free (defined as less than 1.0 ng endotoxin/ml as quantified by the Limulus amoebocyte lysate assay). PBM ($2\text{-}4 \times 10^6$ /ml) and AM ($1\text{-}2 \times 10^6$ /ml) were cultured at 37°C, 5% CO2, and 100% humidity in Teflon bags (Janssen's MNL, St-Niklaas, Belgium) for 1 to 7 days. For detection of surface membrane determinants, isolation of total RNA, or detection of LAP activity, cells were harvested from the Teflon bags and separated from the culture supernatant by centrifugation. Subsequently, the cells were washed twice with either PBS/0.5% BSA/0.5% sodium azide when cells were used for detection of surface membrane determinants, or PBS when cells were used for isolation of total RNA or detection of LAP activity. The viability as determined by propidium iodide or trypan blue exclusion exceeded 85%. The numbers of cells recovered after culture in the presence of rIL-4, rIL-2, or anti-IL-4 antiserum did not differ significantly.

EC were cultured in flat bottom 6-well plates as described elsewhere (38). For detection of CD13 Ag expression, plates were washed twice with PBS (37°C) to remove nonviable, nonadherent EC. Thereafter, EC were incubated (5 min, 37°C) with a collagenase solution (Worthington, Biochemical Corporation, NJ) to harvest adherent cells. From this point, EC were handled as PBM and AM. For isolation of total RNA, plates were washed twice with PBS (37°C). Thereafter, adherent cells were homogenized with 0.5 ml of solution D and transferred to 1.5-ml Eppendorf tubes. From this point, EC were handled as PBM and AM.

Detection of surface membrane determinants

For immunofluorescence stainings of PBM, AM and EC the following mAb were used: CD13 (Q20(IgG2a), Dr. C. E. van der Schoot, CLB, Amsterdam, The Netherlands; My7 (IgG1), Coulter Clone, Hialeah, FL); CD23 (Tü1(IgG1), Biotest, Dreieich, Germany; Leu-20 (IgG1), Becton Dickinson, San Jose, CA). Isotype matched control antibodies were directed against idiotypic determinants on a B cell lymphoma cell line and did not react with monocytes, macrophages or endothelium. Fifty µl of the EC, PBM, or AM cell suspension (2×10^6 cells/ml) were incubated for 30 min at 4°C with 50 µl of one of the, optimally titrated, mAb. Incubation was performed in U-bottom 96-well microtiter plates. After three washings the cells were incubated with FITC-labeled GAM $F(ab')_2$ (De Beer Medicals B.V., Hilvarenbeek, The Netherlands) for 30 min at 4°C. After another three washings the cell pellets were resuspended for analysis of the fluorescence

intensity by means of a FACScan (Becton Dickinson). The fluorescence intensities of Ag expression are expressed as a ratio relative to the background fluorescence intensity of cells stained with an isotype matched control antibody. A value of 1.0 reflects fluorescence equivalent to background. When fluorescence intensity was determined on cultured cells, background fluorescence was determined on similarly cultured cells.

All standard washings were performed with PBS/0.5% BSA/0.5% sodium azide.

Preparation of total cellular RNA and Northern blot analysis

Total cellular RNA was isolated from THP-1 cells, freshly isolated or cultured PBM and AM, and cultured EC largely according to Chomczynski and Sacchi (39). Briefly, cells (5-10 x 10^6) were homogenized with 0.5 ml of solution D in a 1.5-ml Eppendorf tube. Sequentially, 50 µl of 2 M sodium acetate (pH 4.0), 0.5 ml of water-saturated phenol, and 0.1 ml of chloroform were added to the homogenate, with thorough mixing after the addition of each reagent. The final suspension was cooled on ice for 15 min. Samples were centrifuged at 10,000 × g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube containing 0.6 ml of isopropanol, and then placed at 4°C for at least 6 h to precipitate RNA. RNA was recovered by centrifugation (10,000 × g, 15 min, 4°C), dissolved in 0.3 to 0.5 ml solution D, and precipitated again with 1 volume of isopropanol. After centrifugation (10,000 × g, 15 min, 4°C), the RNA-pellet was washed in 0.6 to 1.0 ml 75% ethanol and eventually dissolved in 25 to 50 µl RNase-free water. Total RNA (5-20 µg) was separated by electrophoresis in a 1%-agarose gel (40), subsequently vacuum transferred (41) onto a nylon membrane (NY-13 N; Schleicher and Schuell, Dassel, Germany), fixed to the membrane with a 254 nm UV crosslinker (Stratalinker, Stratagene, La Jolla, CA) and hybridized with a CD13 probe, which was labeled according to the Klenow-oligonucleotide method (42). The CD13 cDNA probe was prepared as described elsewhere (43). Prehybridization and hybridization of RNA blots were performed as described elsewhere (40). After hybridization the blots were washed and exposed to Fuji NIF-RX films (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens. The quality and the amounts of RNA applied were controlled by rehybridization of the blots with a GAPDH probe. This probe was a 0.7 kb *Eco*RI - *Pst*I fragment (44).

Semiquantification of CD13 mRNA levels

The relative density for bands on autoradiograms was estimated by laser scanning densitometry (video densitometer model 620, Bio-Rad Laboratories, Richmond, CA). Each gel containing RNA from kinetic IL-4 experiments with PBM, AM or EC also contained a series of 7 1:2 dilutions (0.5-32 µg) of total RNA isolated from CD13 mRNA$^+$ THP-1 cells. As the linear relation between band density on autoradiograms and the quantity of CD13 mRNA is only limited, we related the density of a particular CD13 band from PBM, AM or EC to bands of comparable density in the dilution series of CD13 bands from THP-1 cells. In this way, it was possible to quantitate, indirectly but in a linear way, CD13 bands of different density from PBM, AM or EC, whether or not exposed to IL-4 during different periods of time. CD13 mRNA levels are expressed in units, where a value of X represents the level of CD13 mRNA (present in the quantity electrophorized total RNA) which is identical to the level of CD13 mRNA in X µg of THP-1 total RNA. If X is greater than the quantity of electrophorized total RNA, the particular cell sample contained relatively more CD13 mRNA than THP-1 cells. The same procedure was applied to estimation of GAPDH band densities.

For final interpretation of CD13 mRNA levels, i.e. to compensate for differences in RNA loading per lane or culture-induced influences on general mRNA levels, CD13 mRNA levels were related to GAPDH mRNA levels.

Functional assay for LAP-activity

The presence of aminopeptidase-N activity on PBM was detected largely as described elsewhere (45). Briefly, the assay was performed in triplicate in flat bottom wells of 96-well microtiter plates. Fifty µl of a cell suspension (0.5 - 1.0 x 10^6 cells/ml) were incubated with 50 µl of L-leucine-p-nitroanilide (Sigma) (8.36 mM) for 30 min at 37°C. After this incubation enzymatic activity was blocked by addition of 35 µl of a 30% (v/v) acetic acid solution. The increase in specific absorbance at 405 nm (as a result of accumulation of free p-nitroanilide) was determined by using a Titertek Multiskan MCC plate reader (I.C.N. Biomedicals B.V., Amsterdam, The Netherlands). Aminopeptidase-N activity was expressed as the production of p-nitroanilide in 30 min by 10^6 cells (nmol/30 min x 10^6 cells).

In some experiments cells were pre-incubated with the CD13 mAb WM15 (IgG1, Dr. E. J. Favaloro, Westmead, Australia) for 30 min at 4°C to block CD13-mediated LAP-activity. As a control procedure, cells were pre-incubated with an isotype-matched control antibody. After this pre-incubation, cells were directly assayed for their LAP-activity as described above.

RESULTS

IL-4 up-regulates CD13 Ag expression on human monocytes

Freshly isolated PBM expressed CD13 Ag. However, even after one day of culture, expression of CD13 Ag was found to increase significantly upon IL-4 treatment (Figure 1, closed circles). This increase was consistently seen in all experiments (n = 9). Maximal increase was observed around day 2 of culture. To a limited extent, CD13 Ag expression was also enhanced in control cultures of PBM (Figure 1, open circles). These findings could

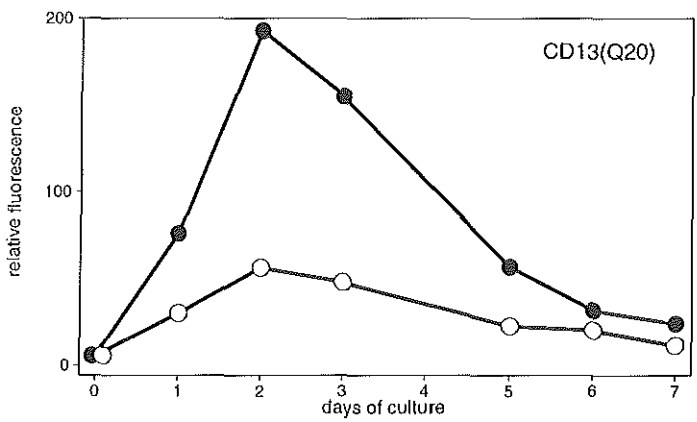

Figure 1. Kinetics of the effect of IL-4 on the cell membrane expression of CD13 Ag on human PBM. Monocytes were cultured without (O) or with (●) 100 U IL-4/ml. On the ordinate is given the relative fluorescence, i.e. the ratio between the mean linear fluorescence intensity of cells labeled with CD13 mAb and the mean linear fluorescence intensity of cells labeled with an IgG2a control antibody. Data of a representative experiment out of five is shown.

not be explained by an increase in cell-size as there was only a limited rise in forward light scatter signal after 2 days, and CD13 Ag expression decreased upon further culture while cell-size increased gradually. After more than 2 days of culture, expression of CD13 Ag diminished, but even after 7 days of culture expression was higher than the expression on freshly isolated PBM or PBM cultured in medium without IL-4. This IL-4-induced increase was dependent on the concentration of IL-4 and could be blocked by anti-IL-4 antiserum (Table 1). We also studied whether other cytokines were able to affect CD13 Ag expression comparable to IL-4. Therefore, we cultured PBM for 2 or 3 days with either IL-2, IFN-γ, GM-CSF or LPS. Neither LPS nor these cytokines were able to induce any additional increase in CD13 Ag expression as compared with the spontaneous increase observed when cells were cultured in mock-medium (Table 2). Taken together, these results suggest that the transient increase in CD13 Ag expression upon culture with IL-4 is specific for IL-4.

Table 1. Influence of IL-4 on the expression of the CD13 Ag on blood monocytes can be blocked by anti-IL-4 antiserum.[a]

Days of Culture	Medium	Medium + IL-4 (100 U/ml)	Medium + IL-4 (100 U/ml) + anti-IL-4
0	17.3[b]	17.3	17.3
3	35.2	94.8	34.3

a. Freshly isolated monocytes or monocytes cultured either in mock medium or in the presence of IL-4 alone or both IL-4 and anti-IL-4 were labeled with Q20(CD13) and FITC-conjugated GAM $F(ab')_2$. A representative experiment out of seven is shown.

b. Data are expressed as relative fluorescence, i.e. the ratio between the mean linear fluorescence intensity of cells labeled with the relevant antibody and the mean fluorescence intensity of cells labeled with the isotype-matched control antibody.

IL-4 increases CD13 mRNA levels in human monocytes

To investigate whether increased CD13 Ag expression required enhanced mRNA levels, total RNA was prepared from freshly isolated PBM, and PBM after culture without or with IL-4. Total RNA was size-separated by electrophoresis and hybridized with the ^{32}P-labeled CD13 cDNA probe and, at a later point of time, the ^{32}P-labeled GAPDH cDNA probe. Analysis of the CD13 mRNA levels showed that culture of PBM with IL-4 caused a consistent and marked additional increase in CD13 mRNA level as compared with the spontaneous increase in control cultures (Figure 2). Maximal induction of CD13 mRNA was seen around day 1 of culture. Semiquantification of the mRNA levels revealed that the level of CD13 mRNA after 1 day of culture in medium without IL-4 was more than 10 times as high as the level in freshly isolated PBM. After 1 day of culture in medium with IL-4, the level of CD13 mRNA was more than 30 times as high (Table 3). Culture of PBM for a

longer period of time revealed that the level of CD13 mRNA gradually diminished after day 1 of culture. Without IL-4, the level of CD13 mRNA decreased to the level of freshly isolated PBM after 5 days of culture. With IL-4, the level of CD13 mRNA also decreased, but was still markedly increased even after 5 days of culture (Figure 2 and Table 1). These results indicate that IL-4 specifically increased the level of CD13 mRNA, and that maximal induction of CD13 mRNA preceded maximal IL-4-induced expression of CD13 Ag on the cell surface by one day.

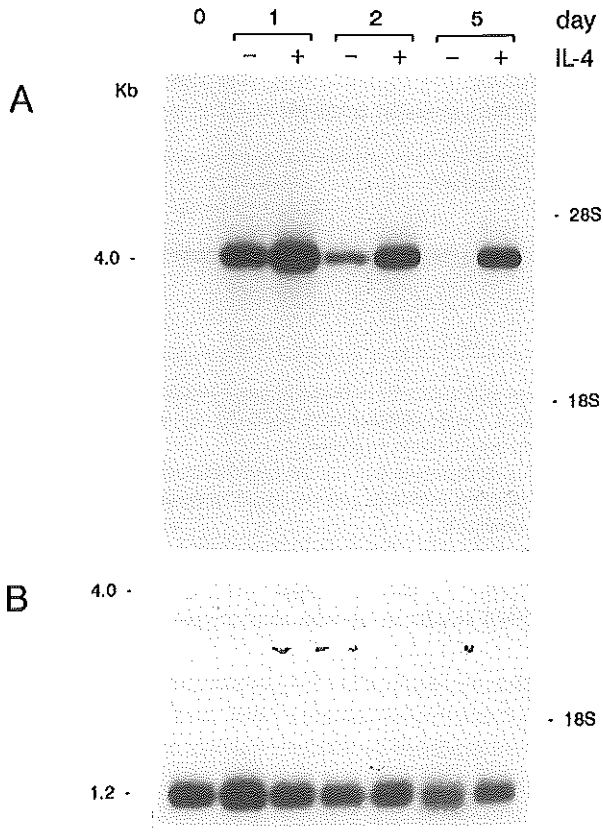

Figure 2. Kinetics of the IL-4-induced up-regulation of CD13 mRNA expression in human PBM. Total RNA was prepared from freshly isolated monocytes (lane 1), and from cells cultured in the absence (lanes 2, 4 and 6) or presence (lanes 3, 5 and 7) of 100 U IL-4/ml. Each lane contains 5 µg of total RNA. RNA was size-separated by electrophoresis, transferred onto a nylon membrane, hybridized with the ^{32}P-labeled CD13 cDNA probe, and exposed for 72 hours (upper panel, A). The lower panel (B) shows the same membrane rehybridized with the GAPDH cDNA probe, which was used as the internal standard for RNA content.

Influence of IL-4 on LAP activity of cultured human monocytes

To determine whether the IL-4-induced up-regulation of CD13 mRNA levels and CD13 Ag expression in PBM was accompanied by a functional increase in aminopeptidase activity, we studied whether PBM cultured with IL-4 would degrade a chromogenic substrate more efficiently than PBM cultured without IL-4. Initially, freshly isolated and shortly cultured PBM were assayed for their capacity to degrade L-leucine-p-nitroanilide. These experiments revealed a decrease in LAP activity after 1 or 2 days of culture whether or not in the presence of IL-4, although in all experiments LAP activity was significantly higher in the IL-4-treated PBM than the nontreated PBM (data not shown). As also other membrane enzymes may display LAP-like activity comparable to CD13 Ag (Dr. E. Favaloro, unpublished data), we tried to make our assay more specific for LAP activity due to CD13 Ag. Therefore, we pre-incubated cultured PBM with the mAb WM-15, which has been described to block LAP activity due to CD13 Ag (23) or, as a control procedure, with an isotype-matched control antibody. Furthermore, we studied the kinetics of the effect of IL-4 on LAP activity in more detail. After culture of PBM without IL-4 we observed an unchanged or slightly decreased total (i.e. after pre-incubation with a control antibody) LAP activity despite the invariably observed increases in CD13 mRNA level and CD13 Ag expression (Figure 3, open circles of panels A and C). After 1 day of culture with IL-4, PBM showed only a slight increase or a slight decrease in total LAP activity (Figure 3, closed circles of panels A and C). Upon further culture, however, a gradual increase in total LAP activity was seen. Maximal total LAP activity was seen around 60 hours of culture with IL-4. Culture with IL-4 for more than 60 hours resulted in a decrease of total LAP activity. During the whole culture period, total LAP activity of PBM cultured with IL-4 was higher than total LAP activity of PBM cultured without IL-4. This was observed in all our experiments ($n = 4$).

CD13-specific LAP activity of PBM cultured without IL-4 (i.e. the decrease of LAP activity after pre-incubation with the mAb WM-15) also decreased upon culture (Figure 3, open circles of panels B and D), while this enzyme activity of PBM cultured with IL-4 showed a transient increase which was maximal around 60 hours of culture (Figure 3, closed circles of panels B and D). The apparent discrepancy between the increased CD13 Ag expression already seen after 1 day of culture with IL-4 and the initially unchanged LAP activity was also observed. Since mAb WM-15 has been reported to block CD13-mediated LAP activity completely, but was unable to block all LAP activity of PBM in our experiments, we concluded that in our experiments also other enzymes displayed LAP-like activity. How expression and function of these other enzymes are influenced by IL-4 is unclear.

Table 2. Influence of IL-2, IFN-γ, GM-CSF and LPS on the expression of the CD13 Ag on blood monocytes as compared with the influence of IL-4.[a]

Days of Culture	Medium	Medium + IL-2	Medium + IFN-γ	Medium + GM-CSF	Medium + LPS	Medium + IL-4
0	39.3[b]	39.3	39.3	39.3	39.3	39.3
3	59.1	61.6	24.9	48.9	55.4	143.6

a. Freshly isolated monocytes or monocytes cultured either in mock medium or in the presence of one of the indicated cytokines (IL-2, IFN-γ, GM-CSF, IL-4) or LPS were labeled with Q20 (CD13) and FITC-conjugated GAM $F(ab')_2$. A representative experiment out of three is shown.

b. Data are expressed as relative fluorescence, calculated as described in the footnote to Table 1.

Table 3. Time course of the influence of IL-4 on the level of CD13 mRNA in human PBM.[a]

	Medium			Medium + IL-4		
Days of Culture	CD13 mRNA level	GAPDH mRNA level	CD13 mRNA relative to GAPDH mRNA	CD13 mRNA level	GAPDH mRNA level	CD13 mRNA relative to GAPDH mRNA
0	<2[b]	5.2[c]	<0.4[d]	<2	5.2	< 0.4
1	34.2	6.8	5.0	>70	4.4	>15.9
2	7.2	3.1	2.3	37.6	4.1	9.2
5	<2	4.7	<0.4	34.5	2.5	13.8

a. Total RNA was prepared from freshly isolated PBM and PBM cultured in medium either without or with 100 U IL-4/ml. After fractionation of 5 µg RNA in an agarose gel, transfer to a nylon membrane, hybridization with the CD13 or GAPDH cDNA probe, and exposure to a film, the relative radioactivity for bands on the autoradiogram was estimated by laser scanning densitometry. Results in this table are derived from the autoradiograms shown in Figure 2. A representative experiment out of three is shown.

b. Data represent expression of CD13 mRNA in PBM relative to the expression in THP-1 cells. A value of X represents the level of CD13 mRNA present in the quantity of total RNA used for electrophoresis (i.c. 5 µg), which is identical to the level of CD13 mRNA present in X µg of total RNA isolated from THP-1 cells (see Materials and Methods for further explanation).

c. Data represent expression of GAPDH mRNA in PBM relative to the expression in THP-1 cells. Interpretation of the data is identical to the interpretation as described in footnote b.

d. Data represent the ratio between the CD13 mRNA (footnote b) and GAPDH mRNA (footnote c) levels.

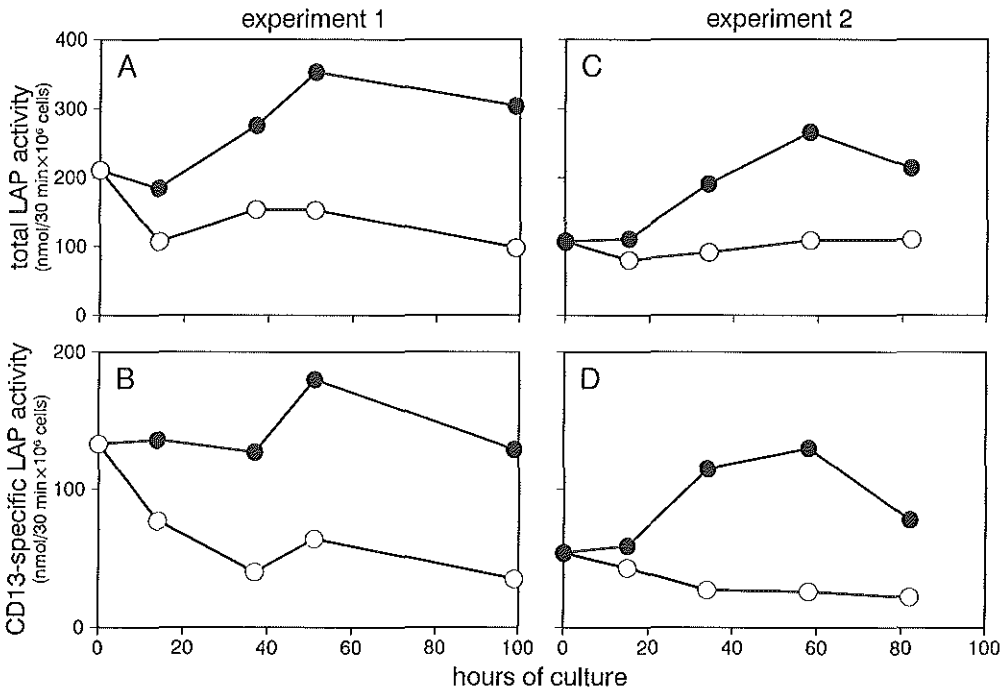

Figure 3. Kinetics of the influence of IL-4 on the LAP activity of human PBM. Monocytes were assayed for their LAP activity directly after isolation (0 hours of culture) and after culture without (○) or with (●) 100 U IL-4/ml. Total LAP activity (panels A and C) represents enzyme activity of PBM which were pre-incubated with an IgG1 control antibody. CD13-specific LAP activity (panels B and D) is calculated by subtracting the remaining LAP activity of PBM which were pre-incubated with the mAb WM-15 from the total LAP activity, and assumes that after pre-incubation with WM-15 the only aminopeptidase activity detected is that which is not mediated by CD13 Ag.

Influence of IL-4 on the expression of CD13 Ag and CD13 mRNA levels in cultured human AM

We have demonstrated earlier that freshly isolated AM express CD13 Ag (43). Upon culture, a spontaneous and transient increase in CD13 Ag expression was observed, comparable with PBM. Likewise, IL-4 induced a more substantial increase in CD13 Ag expression with a maximum around day 2 of culture (Figure 4, right-hand panel). We studied also the kinetics of the effect of IL-4 on CD13 mRNA levels in AM. As in PBM, we observed an IL-4-induced rise in the level of CD13 mRNA. This increase was significantly larger than the spontaneous increase, which was seen upon culture without IL-4 (Figure 5, lanes 2 and 3). Furthermore, this increase was already seen after 5 h of culture and was

maximal after 1 day of culture. The level of CD13 mRNA decreased upon further culture. After 4 days of culture, the level of CD13 mRNA in AM cultured with IL-4 did not differ anymore from the level in AM cultured without IL-4 (Figure 5, lanes 8 and 9).

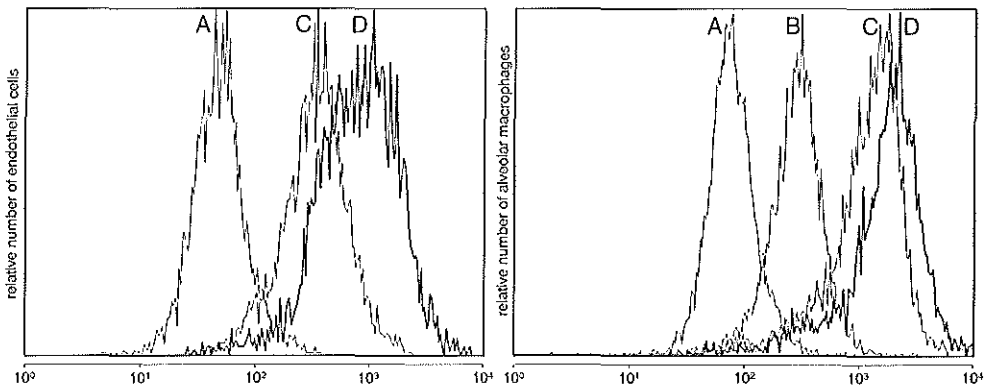

Figure 4. Influence of IL-4 on the expression of CD13 Ag on human EC and AM. The left-hand panel represents EC, which were stained with CD13 mAb and FITC-labeled GAM $F(ab')_2$, either after a culture period of 2 days in medium without (C) or with (D; bold histogram) 100 U IL-4/ml. Histogram A represents EC stained with an IgG2a control antibody and FITC-labeled GAM $F(ab')_2$. One representative experiment out of two is shown. The right-hand panel represents AM, which were also stained after a culture period of 2 days in medium without (C) or with (D; bold histogram) 100 U IL-4/ml. Histogram A represents AM stained with an IgG2a control antibody and FITC-labeled GAM $F(ab')_2$, whereas histogram B represents staining of freshly isolated AM with CD13 mAb and FITC-labeled GAM $F(ab')_2$. One representative experiment out of seven is shown. In both the left-hand and right-hand panel, fluorescence intensity of 7500 cells was determined on a log scale with a FACScan.

Influence of IL-4 on the expression of CD13 Ag and CD13 mRNA levels in cultured human EC

In our experiments we could confirm, as has been demonstrated elsewhere (26,27), that human endothelial cells in *in vitro* culture expressed CD13 Ag. Already after 13 h of culture in the presence of IL-4 (100 U/ml) we found a slight increase in the expression of CD13 Ag (relative fluorescence increased from 100 ± 11% to 122 ± 7% (n = 2)). Maximal increase of CD13 Ag expression was observed between 24 and 48 h of culture. The left-hand panel of Figure 4 shows the maximal IL-4-induced expression of CD13 Ag of one representative experiment (relative fluorescence increased from 7.9 (100%) to 18.8 (238%)). Incubation for more than 48 h resulted in a decrease of the expression of CD13 Ag. Therefore, the transient induction of CD13 Ag expression in human EC resembled the kinetics observed in our experiments with PBM and AM. Moreover, IL-4 also increased CD13 mRNA levels in EC comparable with the increase seen in PBM and AM (Figure 6).

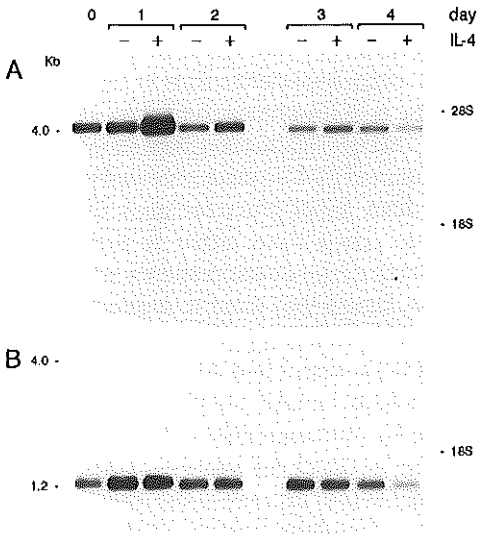

Figure 5. Kinetics of the IL-4-induced up-regulation of CD13 mRNA expression in human AM. Total RNA was prepared from freshly isolated macrophages (lane 1), and from macrophages cultured either in the absence (lanes 2, 4, 6 and 8) or presence (lanes 3, 5, 7 and 9) of 100 U IL-4/ml. Each lane contains 5 µg of total RNA. RNA was electrophorized, transferred onto a nylon membrane, hybridized with the ^{32}P-labeled CD13 cDNA probe, and exposed for 26 hours (upper panel, A). The lower panel (B) shows the same blot rehybridized with the GAPDH cDNA probe, which was used as the internal standard for RNA content.

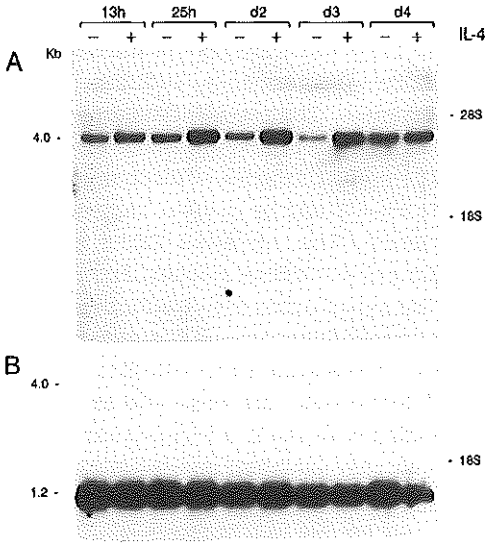

Figure 6. Kinetics of the IL-4-induced up-regulation of CD13 mRNA expression in human EC. Total RNA was prepared from EC cultured either in the absence of IL-4 (lanes 1, 3, 5, 7 and 9) or for 13h, 25h, 2, 3, and 4 days in the presence (lanes 2, 4, 6, 8, and 10) of 100 U IL-4/ml. Each lane contains 5 µg of total RNA. RNA was electrophorized, transferred onto a nylon membrane, hybridized with the ^{32}P-labeled CD13 cDNA probe, and exposed for 3 days (upper panel A). The lower panel B shows the same blot rehybridized with the GAPDH cDNA probe, which was used as the internal standard for RNA content.

DISCUSSION

Although IL-4 was initially described as a B cell stimulatory factor, it is now also known to have regulatory activity on human monocyte/macrophage functions. IL-4 may activate PBM to express FcεRIIb and MHC class II Ag (7-9), and may thus contribute to inflammatory processes. Paradoxically, IL-4 has also been reported recently to display anti-inflammatory functions. The monocyte production of several cytokines and reactive oxygen species is suppressed by IL-4 (10-18), and the capacity to lyse tumor cells and microorganisms is decreased (10,46,47). Furthermore, IL-4 may control the accumulation of monocytes in areas of inflammation by enhancing apoptosis in stimulated monocytes (19). Evidence that IL-4 may exhibit anti-inflammatory properties is accumulating and the

first results of the use of IL-4 *in vivo* have been published recently (48,49).

In the present study, it is shown that IL-4 up-regulates the expression of CD13 Ag on monocytes/macrophages, and thus may be a newly described mechanism in modulating inflammation. The transient induction of CD13 Ag by IL-4 resembles the earlier reported IL-4-induced up-regulation of FcεRIIb on PBM (9,50). Maximal induction was seen after 2 days of culture. Kinetics of the IL-4-induced up-regulation of CD13 mRNA revealed maximal levels after 1 day of culture, indicating that peak mRNA levels preceded peak CD13 Ag expression. The increased CD13 mRNA levels may result from either IL-4-increased transcription or mRNA stability. Future nuclear run-off transcription experiments and mRNA half-life experiments may clarify this point. Indirect evidence that most IL-4 effects occur at the transcriptional level comes from other studies which described influences of IL-4 on mRNA transcription of IL-1Ra (51), IL-2 (52), IL-6, CD23, type I and III procollagen.

Functionally, we observed an IL-4-induced increase in LAP activity more or less coinciding with the IL-4-induced expression of CD13 Ag. Although PBM cultured in mock-medium also exhibited a slight, spontaneous increase in CD13 Ag expression and CD13 mRNA level, this was not accompanied by an increase in LAP activity in our experiments. This may be due to a negative influence on the optimal functional enzyme structure when cells are isolated from the *in vivo* environment and transferred to the artificial *in vitro* culture conditions. Nevertheless, IL-4-treated PBM exhibited a higher LAP activity than untreated PBM, indicating that IL-4 is able to increase the capacity of PBM to degrade aminopeptidase substrates. Not all LAP activity appeared to be due to the presence of CD13 Ag, as the mAb WM-15 was unable to block LAP activity totally. This was demonstrated earlier (53) and may indicate that also other cell membrane enzymes display aminopeptidase-N-like activity. A more specific assay for measuring peptidase activity due to aminopeptidase-N-activity (which is not available yet) would address this issue appropriately.

PBM cultured in mock-medium exhibited a slight, spontaneous increase in CD13 mRNA and CD13 Ag. These increases in expression are unlikely to be caused by contaminating amounts of IL-4, as culture of PBM in the presence of both IL-4 and anti-IL-4 antiserum reduced CD13 Ag expression to the levels of PBM cultured in mock-medium, and not to the levels of freshly isolated PBM. Furthermore, this spontaneous increase could not be explained by either serum components or LPS or cellular adherence, as culture of PBM under serum-free conditions gave similar results, culture in the presence of LPS did not result in a more pronounced increase and PBM were cultured under nonadherent conditions in Teflon bags.

Recently, it was described that activity of cell membrane peptidases could be modulated by cellular environmental factors. Werfel et al. demonstrated that the cell membrane expression of CD13 Ag was rapidly increased on PBM upon stimulation with C5a (54). This increase was observed within minutes, indicating translocation of an intracellular pool of CD13 Ag to the cell surface. Together with our results presented here, this suggests that different mechanisms induce increased cell membrane expression of CD13 Ag. Rohrbach and Conrad reported that LAP activity in cultured PBM was enhanced by the presence of T lymphocytes (55). Although they did not speculate on the mechanism

by which LAP activity was increased, we think that their results can be explained by the production of IL-4 by activated T lymphocytes, which is consistent with our own results. Kondepudi and Johnson studied another cell type and another cell membrane peptidase (56). They reported that several cytokines increased neutral endopeptidase activity in lung fibroblasts, and they suggested that up-regulation of this peptidase may limit inflammation in the pulmonary interstitium. In their study several cytokines were shown to increase neutral endopeptidase activity in human lung fibroblasts, but our results show that IL-4 uniquely upregulates CD13 Ag expression/aminopeptidase-N-activity in human monocytes/macrophages. Our conclusion is supported by others who have shown that IFN-γ and IL-10 were without effect on CD13 Ag expression in human monocytes (57).

Originally, the cell membrane glycoprotein (gp 150) defined by CD13 mAb has been regarded as a marker for subpopulations of hemopoietic cells (20-23). Nowadays, it is known to be expressed by a number of different cell types, including fibroblasts, the renal tubular and intestinal epithelium (24,25), endothelial cells (26,27), and synaptic membranes of cells of the central nervous system (28). Recently, the gene for this protein has been cloned, leading to the recognition of this protein as aminopeptidase-N (EC 3.4.11.2) (58). Aminopeptidase-N belongs to the group of ectoenzymes, so-called because of their extracellularly located catalytic sites (25,29,30). It has been speculated that the actual, physiological substrate(s) of these ectoenzymes may be either membrane-bound, soluble or part of the extracellular matrix. Another member of this group, neutral endopeptidase (EC 3.4.24.11, common acute lymphoblastic leukemia Ag, CD10), has been studied extensively. This enzyme has been reported to process a number of biologically active peptides (59), including IL-1β (60), substance P (29,61), enkephalin (62), C5a (63), and FMLP (64). Less is known about the physiological substrate(s) of aminopeptidase-N, but substrates comparable to those of neutral endopeptidase have been suggested (31). This is strengthened by the findings that different peptidases may be able to process the same peptide, or that inactivation of biologically active peptides may require sequential hydrolysis by a battery of distinct ectoenzymes in a multistep fashion (29,30). In the central nervous system the role of aminopeptidase-N, together with other cell surface peptidases, is believed to inactivate neuropeptides (28). Comparatively, the action of neuropeptides at places of neurogenic inflammation (e.g. neurogenic inflammation in asthmatic lungs) is thought to be terminated, at least partially, by enzymatic degradation (65,66). It has been reported that neutral endopeptidase is able to inactivate the neuropeptide substance P, which plays an important role in the pathogenesis of asthma (61). As hydrolysis of a particular peptide may result from the single action of different peptidases, and inactivation of biologically active peptides may need sequential hydrolysis by both endo- and exopeptidases (29,30), aminopeptidase-N may, in concert with neutral endopeptidase, play a role in modulating inflammation in inflammatory diseases like asthma.

As IL-4 has also been described in PBM to down-regulate cell membrane proteins such as CD14 Ag[3] and the three Fcγ R (47), and the physiological substrate of ectoenzymes may be membrane-bound, IL-4-induced up-regulation of aminopeptidase-N may, in the same cell, play a decisive role in processing other cell membrane-bound Ag. Transfection experiments in $CD14^+$ or $Fc\gamma R^+$ cells using cDNA coding for enzymatically active aminopeptidase-N

may clarify this issue.

The role of monocytes and macrophages in initiating and amplifying immunological and inflammatory responses has been described extensively (67,68). Both cell types may generate a variety of pro-inflammatory mediators and cytokines (68). On the other hand, it has been reported that macrophages may also be able to down-regulate inflammation (69). This down-regulation may be (partially) mediated by cell membrane peptidases. The IL-4-induced increase in LAP activity, as described here, may be a new, additional anti-inflammatory mechanism of IL-4.

Partly comparable to the role of monocytes and macrophages, EC are nowadays recognized as cells which actively participate in a variety of physiological processes, such as inflammation and hemostasis (27,70). Inflammation is characterized by perturbation of EC integrity, leading to increased vascular permeability. Several inflammatory peptides may be responsible for the increase in vascular permeability. A balanced regulation of production and degradation of these peptides determines the final development of localized edema. Recently, it was shown that neutral endopeptidase plays an important role in modulating the edema-forming effects of bradykinin (70). In view of the effects on aminopeptidase-N activity, IL-4 may therefore suppress indirectly the inflammatory response by increasing the capacity of EC to degrade pro-inflammatory peptides.

In conclusion, our results show that IL-4 is able to increase the expression of CD13 Ag on monocytes, macrophages and endothelial cells. We postulate that up-regulation of CD13 Ag expression may be an indirect working-mechanism of IL-4 to modulate the action of bioactive peptides.

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REFERENCES

1. Oliver K, Noelle RJ, Uhr JW, Krammer PH, Vitetta ES. B-cell growth factor (B-cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proc Natl Acad Sci USA 1985;82:2465-2467.
2. O'Garra A, Umland S, De France T, Christiansen J. 'B-cell factors' are pleiotropic. Immunol Today 1988;9:45-54.
3. Spits H, Yssel H, Takebe Y, Arai N, Yokota T, Lee F, Arai K-I, Banchereau J, De Vries JE. Recombinant interleukin 4 promotes the growth of human T cells. J Immunol 1987;139:1142-1142.
4. Defrance T, Vanbervliet B, Aubry J-P, Takebe Y, Arai N, Miyajima A, Yokota T, Lee F, Arai K-I, De Vries JE, Banchereau J. B cell growth-promoting activity of recombinant human interleukin 4. J Immunol 1987;139:1135-1141.
5. Mosmann TR, Bond MW, Coffman RL, Ohara J, Paul WE. T-cell and mast cell lines respond to B-cell stimulatory factor 1. Proc Natl Acad Sci USA 1986;83:5654-5658.

6. Lee F, Yokota T, Otsuka T, Meyerson P, Villaret D, Coffman R, Mosmann T, Rennick D, Roehm N, Smith C, Zlotnik A, Arai K-I. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. Proc Natl Acad Sci USA 1986;83:2061-2065.
7. Te Velde AA, Klomp JPG, Yard BA, De Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
8. Zlotnik A, Fischer M, Roehm N, Zipori D. Evidence for effects of interleukin 4 (B cell stimulatory factor 1) on macrophages: enhancement of antigen presenting ability of bone marrow-derived macrophages. J Immunol 1987;138:4275-4279.
9. Vercelli D, Jabara HH, Lee B-W, Woodland N, Geha RS, Leung DYM. Human recombinant interleukin 4 induces FcεR2/CD23 on normal human monocytes. J Exp Med 1988;167:1406-1416.
10. Abramson SL, Gallin JI. IL-4 inhibits superoxide production by human mononuclear phagocytes. J Immunol 1990;144:625-630.
11. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. Proc Natl Acad Sci USA 1989;86:3803-3807.
12. Te Velde AA, Huijbens RJF, Heije K, De Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990;76:1392-1397.
13. Essner R, Rhoades K, McBride WH, Morton DL, Economou JS. IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J Immunol 1989;142:3857-3861.
14. Vannier E, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4: Interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc Natl Acad Sci USA 1992;89:4076-4080.
15. Yanagawa H, Sone S, Sugihara K, Tanaka K, Ogura T. Interleukin-4 downregulates interleukin-6 production by human alveolar macrophages at protein and mRNA levels. Microbiol Immunol 1991;35:879-893.
16. Szabo G, Kodys K, Miller-Graziano CL. Elevated monocyte interleukin-6 (IL-6) production in immunosuppressed trauma patients. II. Downregulation by IL-4. J Clin Immunol 1991;11:336-344.
17. Standiford TJ, Strieter RM, Chensue SW, Westwick J, Kasahara K, Kunkel SL. IL-4 inhibits the expression of IL-8 from stimulated human moncytes. J Immunol 1990;145:1435-1439.
18. Orino E, Sone S, Nii A, Ogura T. IL-4 up-regulates IL-1 receptor antagonist gene expression and its production in human blood monocytes. J Immunol 1992;149:925-931.
19. Mangan DF, Robertson B, Wahl SM. IL-4 enhances programmed cell death (apoptosis) in stimulated human monocytes. J Immunol 1992;148:1812-1816.
20. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 1981;68:932-941.
21. Look AT, Ashmun A, Shapiro LH, O'Connell PJ, Gerkis V, D'Apice AJ, Sagawa K, Peiper SC. Report on the CD13 (aminopeptidase N) cluster Workshop. In: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, Von Dem Borne AEGKr, eds. Leucocyte typing IV. White cell differentiation antigens. New York: Oxford University Press, 1989:784-787.
22. Sobol RE, Mick R, Royston I, Davey FR, Ellison RR, Newman R, Cuttner J, Griffin JD, Collins H, Nelson DA, Bloomfield CD. Clinical importance of myeloid antigen expression in adult acute lymphoblastic leukemia. New Engl J Med 1987;316:1111-1117.
23. Ashmun RA, Look AT. Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells. Blood 1990;75:462-469.
24. Hogg N, Horton MA. Myeloid antigens: new and previously defined clusters. In: McMichael AJ, ed. Leucocyte typing III. White cell differentiation antigens. Oxford, Oxford University Press, 1987:576-602.
25. Semenza G. Anchoring and biosynthesis of stalked brush border membrane proteins: Glycosidases and peptidases of enterocytes and renal tubuli. Annu Rev Cell Biol 1986;2:255-313.

26. Favaloro EJ, Bradstock KF, Kabral A, Grimsley P, Zowtyj H, Zola H. Further characterization of human myeloid antigens (gp160,95;gp150;gp67): investigation of epitopic heterogeneity and non-haemopoietic distribution using panels of monoclonal antibodies belonging to CD-11b, CD-13 and CD-33. Br J Haematol 1988;69:163-171.
27. Favaloro EJ. CD-13 ('gp150'; aminopeptidase-*M*): Co-expression on endothelial and haemopoietic cells with conservation of functional activity. Immunol Cell Biol 1991;69:253-260.
28. Matsas R, Stephenson SL, Hryszko J, Kenny AJ, Turner AJ. The metabolism of neuropeptides. Phase separation of synaptic membrane preparations with Triton X-114 reveals the presence of aminopeptidase N. Biochem J 1985;231:445-449.
29. Turner AJ, Matsas R, Kenny AJ. Are there neuropeptide-specific peptidases? Biochem Pharmacol 1985;34:1347-1356.
30. Kenny J. Cell surface peptidases are neither peptide- nor organ-specific. Trends Biochem Sci 1986;11:40-42.
31. Kenny AJ, O'Hare MJ, Gusterson BA. Cell-surface peptidases as modulators of growth and differentiation. Lancet 1989;ii:785-787.
32. Bauvois B, Sancéau J, Wietzerbin J. Human U937 cell surface peptidase activities: characterization and degradative effect on tumor necrosis factor-α. Eur J Immunol 1992;22:923-930.
33. Figdor CG, Bont WS, Touw I, De Roos J, Roosnek EE, De Vries JE. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. Blood 1982;60:46-53.
34. Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973;52:2745-2756.
35. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 1980;26:171-176.
36. Chrétien I, Van Kimmenade A, Pearce MK, Banchereau J, Abrams JS. Development of polyclonal and monoclonal antibodies for immunoassay and neutralization of human interleukin-4. J Immunol Meth 1989;117:67-81.
37. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Meth 1984;72:219-227.
38. Te Velde AA, Keizer GD, Figdor CG. Differential function of LFA-1 family molecules (CD11 and CD18) in adhesion of human monocytes to melanoma and endothelial cells. Immunology 1987;61:261-267.
39. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
40. Sambrook J, Fritsch EF, Maniatis T. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989: 7.3-7.87.
41. Olszewska E, Jones K. Vacuum blotting enhances nucleic acid transfer. Trends Genet 1988;4:92-94.
42. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clinica Chimica Acta 1991;198:1-92.
43. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.
44. Benham FJ, Hodgkinson S, Davies KE. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X chromosomes defines a multigene family. EMBO J. 1984;3:2635-2640.
45. Ruseler-van Embden JGH, Van Lieshout LMC. Increased proteolysis and leucine aminopeptidase activity in faeces of patients with Crohn's disease. Digestion 1988;40:33-40.

46. Nishioka Y, Sone S, Orino E, Nii A, Ogura T. Down-regulation by interleukin 4 of activation of human alveolar macrophages to the tumoricidal state. Cancer Res 1991;51:5526-5531.
47. Te Velde AA, Huijbens RJF, De Vries JE, Figdor CG. IL-4 decreases FcγR membrane expression and FcγR-mediated cytotoxic activity of human monocytes. J Immunol 1990;144:3046-3051.
48. Maher DW, Davis I, Boyd AW, Morstyn G. Human interleukin-4: an immunomodulator with potential therapeutic applications. Prog Growth Factor Res 1991;3:43-56.
49. Wong HL, Lotze MT, Wahl LM, Wahl SM. Administration of recombinant IL-4 to humans regulates gene expression, phenotype, and function in circulating monocytes. J Immunol 1992;148:2118-2125.
50. Te Velde AA, Rousset F, Peronne C, De Vries JE, Figdor CG. IFN-α and IFN-γ have different regulatory effects on IL-4-induced membrane expression of FcεRIIb and release of soluble FcεRIIb by human monocytes. J Immunol 1990;144:3052-3059.
51. Werfel T, Sonntag G, Weber MH, Götze O. Rapid increases in the membrane expression of neutral endopeptidase (CD10), aminopeptidase N (CD13), tyrosine phosphatase (CD45), and Fcγ-RIII (CD16) upon stimulation of human peripheral leukocytes with human C5a. J Immunol 1991;147:3909-3914.
52. Rohrbach MS, Conrad AK. Comparison of the T lymphocyte-dependent induction of angiotensin-converting enzyme and leucine aminopeptidase in cultured human monocytes. Clin Exp Immunol 1991;83:510-515.
53. Kondepudi A, Johnson A. Cytokines increase neutral endopeptidase activity in lung fibroblasts. Am J Respir Cell Mol Biol 1993;8:43-49.
54. Look AT, Ashmun RA, Shapiro LH, Peiper SC. Human myeloid plasma membrane glycoprotein CD13 (gp150) is identical to aminopeptidase N. J Clin Invest 1989;83:1299-1307.
55. Erdös EG, Skidgel RA. Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones. FASEB J 1989;3:145-151.
56. Pierart ME, Najdovski T, Appelboom TE, Deschodt-Lanckman MM. Effect of human endopeptidase 24.11 ("enkephalinase") on IL-1-induced thymocyte proliferation activity. J Immunol 1988;140:3808-3811.
57. Matsas R, Kenny AJ, Turner AJ. The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues, by endopeptidase-24.11. Biochem J 1984;223:433-440.
58. Shipp MA, Stefano GB, D'Adamio L, Switzer SN, Howard FD, Sinisterra J, Scharrer B, Reinherz EL. Downregulation of enkephalin-mediated inflammatory responses by CD10/neutral endopeptidase 24.11. Nature 1990;347:394-396.
59. McCormack RT, Nelson RD, Chenoweth DE, LeBien TW. Identification and characterization of a unique subpopulation (CALLA/CD10-Negative) of human neutrophils manifesting a heightened chemotactic response to activated complement. Blood 1987;70:1624-1629.
60. Painter RG, Dukes R, Sullivan J, Carter R, Erdös EG, Johnson AR. Function of neutral endopeptidase on the cell membrane of human neutrophils. J Biol Chem 1988;263:9456-9461.
61. Barnes PJ. Neurogenic inflammation in airways. Int Arch Allergy Appl Immunol 1991;94:303-309.
62. Barnes PJ, Baraniuk JN, Belvisi MG. Neuropeptides in the respiratory tract. Part II. Am Rev Respir Dis 1991;144:1391-1399.
63. Lee TH, Lane SJ. The role of macrophages in the mechanisms of airway inflammation in asthma. Am Rev Respir Dis 1992;145:S27-S30.
64. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
65. Holt PG. Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. Clin Exp Immunol 1986;63:261-270.
66. Yong T, Gao XP, Koizumi S, Conlon JM, Rennard SI, Mayhan WG, Rubinstein I. Role of peptidases in bradykinin-induced increase in vascular permeability in vivo. Circ Res 1992;70:952-959.

REGULATION OF AMINOPEPTIDASE-N (CD13) AND FcεRIIb (CD23) EXPRESSION BY IL-4 DEPENDS ON THE STAGE OF MATURATION OF MONOCYTES/MACROPHAGES*

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SUMMARY

IL-4 has multiple biologic activities and it has been shown to have effects on B and T lymphocytes, mast cells, NK cells and monocytes. We studied the influence of IL-4 on the expression of cell membrane determinants, in particular aminopeptidase-N (CD13) and FcεRIIb (CD23), on human peripheral blood monocytes. We compared the response of monocytes with the response of human alveolar macrophages and monocytic cell lines (U937 and THP1), as mature and more immature representatives of the mononuclear phagocyte system, respectively. A dose-dependent increase of the expression of CD13 Ag was observed when monocytes were cultured with IL-4. Kinetic analyses revealed that this induction was maximal after 2-3 days of culture and resembled the kinetics of IL-4-induced expression of FcεRIIb on monocytes. This IL-4-induced increase was absent when monocytes were cultured with IL-4 and an anti-IL-4 antiserum. Concomitantly, an IL-4-induced increase in leucine-aminopeptidase activity could be observed. Northern blot analysis showed that incubation of monocytes with IL-4 induced a marked increase in CD13 mRNA. Alveolar macrophages also exhibited an increase in CD13 Ag expression when exposed to IL-4. Surprisingly, IL-4 was unable to induce expression of FcεRIIb on alveolar macrophages. U937 and THP1 cells did not show an induction of CD13 Ag when cultured in the presence of IL-4. However, IL-4 did induce the expression of FcεRIIb on both cell lines, suggesting the presence of functional IL-4 R.

Our data demonstrate that IL-4 increases the expression of CD13 Ag on monocytes. This IL-4-induced increase can also be observed in more mature monocytic cells such as alveolar macrophages, but is absent in immature cells such as U937 or THP1 cells. This is functionally accompanied by an increase in leucine-aminopeptidase activity, and may be part of the general activation of monocytes/ macrophages by IL-4. In conclusion, the data suggest that IL-4 responsiveness, in particular the induction of CD13 Ag and FcεRIIb expression, may be dependent on the stage of maturation of monocytes/macrophages.

INTRODUCTION

Initially, IL-4 was described to act as a differentiation factor for resting B lymphocytes (1). Nowadays, IL-4 has been shown to have a variety of effects on different cell types. It may act as a growth factor for activated T lymphocytes, thymocytes, NK cells (2-4), and mast cells (4,5). It may modulate the expression of cell membrane determinants, as FcεRIIb (CD23) and class II MHC antigen (Ag) on B lymphocytes (6,7) and monocytes (8,9), and IL-2R (CD25) on B lymphocytes (10). Furthermore, it has been shown that IL-4 is able to stimulate monocyte maturation accompanied by modulation of maturation markers as CD14 and RFD9 (see chapter 5.2 of this thesis). In the course of studying the influence of IL-4 on monocytes/macrophages we observed that IL-4 is also able to regulate the expression of another myeloid antigen, CD13 Ag.

Originally, the cell membrane glycoprotein (gp150) defined by CD13 mAb was seen as a marker for subpopulations of hemopoietic cells (11-14). Currently, it is known to be

present on many nonhemopoietic tissues, including fibroblasts, the intestinal and renal tubular epithelium (15,16), and on synaptic membranes of the central nervous system (17). As the glycoprotein has been identified as aminopeptidase-N (EC 3.4.11.2) (14,18), it has been suggested that this cell membrane enzyme may play a role in modulating the activity of bioactive oligopeptides (19). In the knowledge that the effects of cytokines on a certain cell type may depend on the maturation or differentiation stage of the cell (20,21), we studied the influence of IL-4 on the expression of the CD13 Ag on PBM as well as AM and monocytic cell lines (U937 and THP1), which can be regarded as more mature and immature representatives of the mononuclear phagocyte system, respectively. In this report, we demonstrate that IL-4 is able to induce a strong increase in expression of the CD13 Ag. This induction can be demonstrated on PBM and AM, but not on U937 and THP1 cells.

MATERIALS AND METHODS

Isolation of PBM

Human PBM were isolated from 500 ml blood from healthy volunteers as described elsewhere (22). Briefly, mononuclear cells were separated by density centrifugation with a blood component separator. Next, the mononuclear cells were fractionated into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was over 95% pure and contained more than 98% viable cells as judged by Trypan blue exclusion. Lymphocytes and granulocytes constituted less than 2% and 3% of the monocyte preparation, respectively. Before analysis of the surface membrane determinants the cells were washed twice with a PBS solution (300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated BSA (Organon Teknika, Oss, The Netherlands).

Isolation of AM

All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. BAL was performed in healthy, nonsmoking volunteers after premedication with thiazinamium and local anesthesia using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles, whereafter the BAL cells were isolated by centrifugation at 4°C at a force of 400 x g. More than 90% of the BAL cells appeared to be macrophages as judged by May-Grünwald Giemsa staining. Before analysis of the surface membrane determinants the cells were washed twice with PBS/0.5% BSA.

Cell lines

U937 cells, originally described by Sundström et al. (23) were maintained at concentrations of 0.25-1.0 x 10^6/ml in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with FCS (GIBCO, Paisley, UK) (10%), penicillin G sodium (10^2 U/ml; Gist-Brocades, Delft, The Netherlands), and streptomycin-sulfate (0.1 mg/ml; Biochrom KG, Berlin, Germany). THP1 cells (24) were obtained from the American Type Culture

Collection (Rockville, MD) and maintained according to their instructions. In some experiments, to culture U937 and THP1 cells under the same conditions as PBM or AM, cells were maintained in a modified Iscove's medium (see below).

Culture of cells

Generally, U937 and THP1 cells were cultured as described above. In some experiments they were cultured as PBM and AM. PBM and AM were cultured in a modified Iscove's medium (GIBCO, Paisley, UK) as described previously (25), in which BSA is replaced by human serum albumin supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin-free (defined as less than 1.0 ng/ml of endotoxin as quantified by the *Limulus* amoebocyte lysate assay). PBM (4×10^6/ml) and AM ($1\text{-}2 \times 10^6$/ml) were cultured at 37°C, 5% CO_2 and 100% humidity in Teflon bags (Janssen's MNL, St-Niklaas, Belgium) for 1 to 7 days. For detection of surface membrane determinants, cells were harvested from the Teflon bags and culture supernatant was removed after centrifugation. Subsequently, the cells were washed twice with PBS/0.5% BSA/0.5% sodium azide. The viability as determined by propidium iodide exclusion exceeded 85%. No significant differences in the numbers of cells recovered after culture in the presence of rIL-4 or anti-IL-4 antiserum could be observed.

rIL-4, rIL-2 and anti-IL-4 antiserum

Human rIL-4 was a generous gift from Dr. H.F.J. Savelkoul (Erasmus University, Rotterdam, The Netherlands) and Dr. K. Arai (DNAX Research Institute, Palo Alto, CA). A polyclonal rabbit anti-IL-4 antiserum was used. This antiserum blocks the biologic activity of IL-4, as has been described earlier (26). Human rIL-2 was obtained from Eurocetus (Amsterdam, The Netherlands).

Detection of surface membrane determinants

For immunofluorescence staining of PBM and AM the following monoclonal antibodies (mAb) were used: CD13 (Q20(IgG2a), Dr. C. E. van der Schoot, Amsterdam, The Netherlands; My7 (IgG1) Coulter Clone, Hialeah, FL); CD23 (Tü1(IgG1), Biotest, Dreieich, Germany; Leu-20 (IgG1), Becton Dickinson, San Jose, CA). Isotype matched antibodies which were directed against idiotypic determinants on a B cell lymphoma cell line and did not react with monocytes were used as control antibodies. Fifty µl of the PBM or AM cell suspension (2×10^6 cells/ml) were incubated for 30 min at 4° C with 50 µl of one of the, optimally titrated, mAb. Incubation was performed in 96-well microtiter plates. After three washings the cells were incubated with FITC-labeled GAM $F(ab')_2$ (De Beer Medicals B.V., Hilvarenbeek, The Netherlands) for 30 min at 4°C. After another three washings the cell pellets were resuspended for analysis of the fluorescence intensity by means of a FACScan (Becton Dickinson). The fluorescence intensities of Ag expression are expressed as a ratio relative to the background fluorescence intensity of cultured cells stained with an isotype matched antibody, where a value of 1.0 reflects fluorescence equivalent to background.

For immunological staining of U937 and THP1 cells, the cell suspension (2×10^6/ml) was preincubated for 10 min at 4°C with a solution of normal horse serum (final concentration 5%, v/v). Without this preincubation, interpretation of the immunological stainings with the relevant mAb was troublesome because of high aspecific binding. After this preincubation, the cells were stained with the relevant mAb as described above for PBM and AM.

All standard washings were performed with PBS/0.5% BSA/0.5% sodium azide.

Preparation of total cellular RNA and Northern blot analysis

Total cellular RNA was isolated from U937 and THP1 cells, and freshly isolated or cultured PBM and AM largely according to Chomczynski and Sacchi (27). Briefly, cells were washed twice in PBS. Cells (5-10 x 10^6) were homogenized with 0.5 ml of solution D in a 1.5-ml Eppendorf tube. Sequentially, 50 µl of 2M sodium acetate (pH 4.0), 0.5 ml of water-saturated phenol, and 0.1 ml of chloroform were added to the homogenate, with thorough mixing after the addition of each reagent. The final suspension was cooled on ice for 15 min. Samples were centrifuged at 10,000 x g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube containing 0.5 ml of isopropanol, and then placed at 4°C for at least 6 h to precipitate RNA. RNA was recovered by centrifugation (10,000 x g, 15 min, 4°C), dissolved in 0.3 to 0.5 ml solution D, and precipitated again with 1 volume of isopropanol. After centrifugation (10,000 x g, 15 min, 4°C), the RNA-pellet was washed in 0.6 to 1.0 ml 75% ethanol and eventually dissolved in 25 to 50 µl RNase-free water. Total RNA (10-20 µg) was separated by electrophoresis in a 1%-agarose gel (28), subsequently vacuum transferred (29) onto a nylon membrane (NY-13 N; Schleicher and Schuell, Dassel, Germany), fixed to the membrane with a 254 nm UV crosslinker (Stratalinker, Stratagene, La Jolla, CA) and hybridized with the CD13 probe, which was labeled according to the Klenow-oligonucleotide method (30). Prehybridization and hybridization of RNA blots were performed as described elsewhere (28). After hybridization the blots were washed and exposed to Fuji NIF-RX films (Fuji Photo Film Co., Japan) with intensifying screens.

Preparation of CD13 cDNA probe

Total RNA was extracted from AM, which were obtained from a healthy individual. The cDNA was synthesized with reverse transcriptase (31) and subsequently amplified by using the PCR method as described elsewhere (32). For the PCR-mediated amplification, synthetic oligonucleotide primers were designed according to the published CD13 cDNA sequence (18). The CD13 upstream primer, aacggaa TTCCAGAGTGTGAGGAGATGGTC, including an aspecific 5′ tail (underlined) with an Eco*R1* restriction site, and the CD13 downstream primer, tgtgtctaga CTTCACCCACTTGATGTTGGCTT, including an aspecific 5′ tail (underlined) with a Xba*I* restriction site, were used in the PCR-reaction. The obtained 562 base pairs PCR-product was electrophorized in a 0.7%-agarose gel, and the band containing the probe was cut out of the gel. The probe was electrophoretically recovered from the agarose gel fragment by using a separation chamber (Biotrap; Schleicher and Schuell) and cloned in the pUC19 vector with Eco*RI* and Xba*I* restriction enzymes. Sequence analysis of the cloned PCR-fragment confirmed that the isolated probe represented the expected CD13 sequence.

Functional assay for aminopeptidase-N activity

The presence of aminopeptidase-N activity on PBM, U937 or THP1 cells was detected largely as described earlier (33). Briefly, the assay was performed in wells (in triplicate) of 96-well microtiter plates. Cells were washed twice in PBS. Fifty µl of a cell suspension (1-3 x 10^6 cells/ml) were incubated with 50 µl of L-leucine-p-nitroanilide (Sigma) (8.36 mM) for 30 min at 37°C. After this incubation enzymatic activity was blocked by addition of 35 µl of a 30% (v/v) acetic acid solution and the increase in specific absorbance at 405 nm (as a result of accumulation of free p-nitroanilide) was determined by using a Titertek Multiskan MCC plate reader (I.C.N. Biomedicals B.V., Amsterdam, The Netherlands). Aminopeptidase-N activity was expressed as the production of p-nitroanilide in 30 min by 10^6 cells (nmol/30 min x 10^6 cells).

RESULTS

CD13 and CD23 surface expression on U937 cells, THP1 cells, PBM and AM

U937 cells cultured in medium containing RPMI 1640 and 10% FCS do not express the CD13 Ag. On the other hand, THP1 cells and freshly isolated PBM or AM clearly express this Ag (Figure 1). The CD23 Ag could not be demonstrated on either one of these cell sources (data not shown).

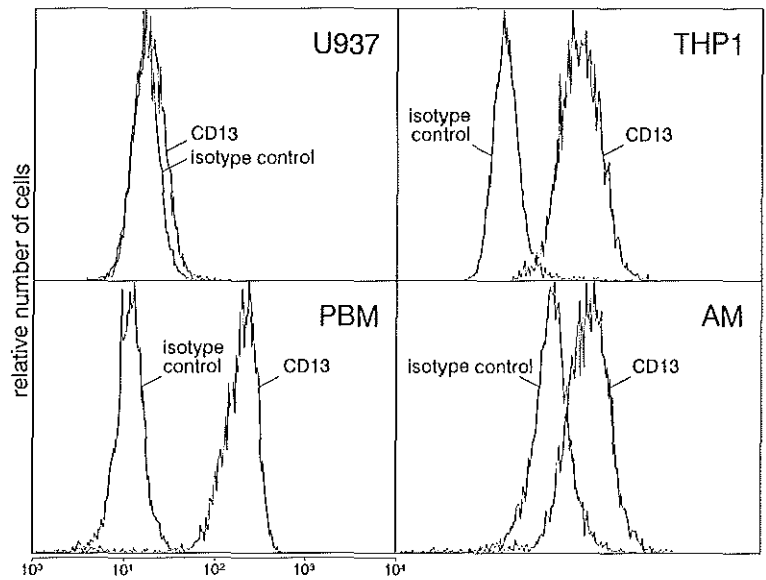

Figure 1. Expression of the CD13 Ag on human monocytic cell lines (U937 and THP1), freshly isolated PBM and AM. The fluorescence intensity of 7500 cells was determined on a log scale with a FACScan. The left-hand histogram in each of the four panels represents cells stained with an IgG2a control antibody and FITC-labeled GAM $F(ab')_2$, whereas the right-hand histogram represents cells stained with Q20 (CD13) and FITC-labeled GAM $F(ab')_2$.

Influence of IL-4 on the expression of the CD13 Ag on human monocytes

Highly purified PBM were cultured for 3 days. As compared with the expression directly after isolation, the expression of the CD13 Ag was enhanced after culture (Figure 2). This increase was consistently seen in all our experiments ($n = 7$). It could only partially be explained by an increase in cell-size as there was only a limited rise in forward light scatter signal. Besides this spontaneous increase in CD13 Ag expression upon culture, IL-4 caused a considerable further increase (Figure 2). Compared with the control cultures, IL-4

induced a 2- to 3-fold further increase in CD13 Ag expression. This further increase was dependent on the concentration of IL-4 (maximal increase was found at a concentration of 50 U IL-4/ml) (data not shown) and could be blocked by the anti-IL-4 antiserum, adding proof to the specificity of the induction (Table 1). PBM cultured in the presence of both IL-4 and the anti-IL-4 antiserum exhibited the same spontaneous but small up-regulation of CD13 Ag as PBM cultured in mock-medium (Table 1). In addition, IL-2 did not induce a further increase in CD13 Ag expression (Table 1). Because IL-4 is known to have longlasting (MHC class II Ag (9), CD14 Ag, RFD9 Ag) and transient (CD23 Ag (8)) effects on the expression of cell membrane determinants, we studied the kinetics of the influence of IL-4 on CD13 Ag expression.

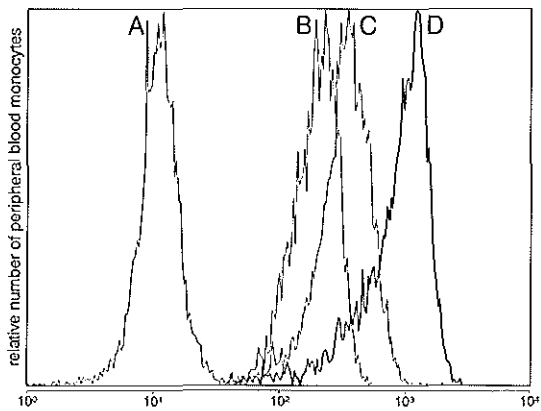

Figure 2. Effects of IL-4 on the expression of the CD13 Ag on blood monocytes. Cells were stained with Q20 (CD13) and FITC-labeled GAM F(ab')$_2$ either directly after isolation (B), or after a culture period of 3 days in modified Iscove's medium without (C) or with (D; bold histogram) 100 U IL-4/ml. Histogram A represents monocytes stained with an IgG2a control antibody and FITC-labeled GAM F(ab')$_2$. Fluorescence intensity of 7500 cells was determined on a log scale with a FACScan. A representative experiment out of seven is shown.

Table 1. Influence of IL-4 on the expression of the CD13 Ag on blood monocytes can be blocked by anti-IL-4 antiserum.[a]

Days of Culture	Medium	Medium + IL-2 (1000 U/ml)	Medium + IL-4 (100 U/ml)	Medium + IL-4 (100 U/ml) + anti-IL-4
0	17.3[b]	17.3	17.3	17.3
3	35.2	36.7	94.8	34.3

a. Freshly isolated monocytes or monocytes cultured either in mock medium or in the presence of IL-2, IL-4 alone or both IL-4 and anti-IL-4 were labeled with Q20(CD13) and FITC-conjugated GAM F(ab')$_2$. A representative experiment out of seven is shown.

b. Data are expressed as relative fluorescence, i.e. the ratio between the mean linear fluorescence intensity of cells labeled with the relevant antibody and the mean fluorescence intensity of cells labeled with the isotype-matched control antibody.

Influence of IL-4 on the expression of the CD13 Ag on human monocytes during a culture period of 7 days

An up-regulation of the determinant recognized by CD13 was already observed after 1 day of culture (Figure 3 and Table 2). This increase was observed in medium without IL-4, but was much more substantial when PBM were cultured with IL-4. Maximal induction of CD13 Ag was seen after 2-3 days of culture. Thereafter, expression of CD13 Ag diminished. However, even after 7 days of culture expression of CD13 Ag was higher than the expression on freshly isolated PBM. During the whole culture period, PBM cultured in the presence of IL-4 exhibited a higher expression of CD13 Ag than PBM cultured in medium without IL-4.

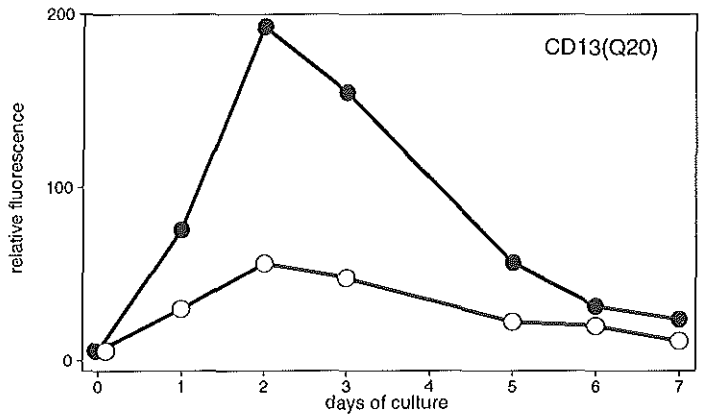

Figure 3. Kinetics of the effect of IL-4 on the membrane expression of CD13 Ag on human monocytes. Monocytes were cultured without (O) or with (●) IL-4 (100 U/ml). Fluorescence intensity of 7500 cells was determined with a FACScan. On the ordinate is given the relative fluorescence, calculated as described in the footnote to Table 1. Data of a representative experiment out of three are shown.

We compared the transient induction of CD13 Ag by IL-4 with the known transient induction of FcεRIIb (CD23) and the IL-4-induced downregulation of CD14 Ag (Table 2). Also in our experiments we observed a transient induction of FcεRIIb by IL-4. Maximal expression was seen after 2 to 3 days of culture, coinciding with the maximal induction of CD13 Ag expression. In contrast, downregulation of CD14 Ag by IL-4 seemed to be a long-lasting phenomenon. Expression of CD14 Ag was almost completely lost after 2 days of culture in the presence of IL-4 and reexpression was not observed even after 7 days of culture. This indicates that IL-4 has transient and sustained effects. The transient IL-4 effects probably reflect a temporary change in state of activation, while the long-lasting effects may regard monocyte maturation (see chapter 5.2 of this thesis).

As there are indications that the maturation/differentiation state of the cell may determine its susceptibility to influences of IFN-γ or TNF-α (21), we investigated whether more mature and immature representatives of the mononuclear phagocyte system respond to IL-4 in the same way as PBM.

Table 2. Time course of the influence of IL-4 on the expression of the CD13, CD14, and CD23 Ag on human blood monocytes.[a]

Days of Culture	Medium			Medium + IL-4 (100 U/ml)		
	CD13	CD14	CD23	CD13	CD14	CD23
0	5.8[b]	9.4	1.1	5.8	9.4	1.1
1	30.0	11.0	1.1	75.7	7.6	3.6
2	56.2	–[c]	1.0	192.8	1.8	6.6
3	47.8	–	–	155.1	–	–
5	22.5	10.4	1.0	56.7	1.0	2.1
6	20.2	7.0	1.0	31.6	1.1	1.2
7	11.4	5.1	0.8	23.9	1.3	1.1

a. Monocytes were labeled with either Q20 (CD13), or UCHM1 (CD14), or Tü1 (CD23), followed by labeling with FITC-conjugated GAM $F(ab')_2$ either directly after isolation or after 1, 2, 5, 6, or 7 days of culture with or without IL-4. A representative experiment out of three is shown.

b. Data are expressed as relative fluorescence, calculated as described in the footnote of Table 1.

c. Not determined.

Influence of IL-4 on the expression of the CD13 Ag on U937 cells, THP1 cells and human AM

U937 cells are known to express FcεRIIb upon culture in the presence with IL-4 (34). In our studies, we could also demonstrate induction of a weak expression of FcεRIIb by IL-4. This induction appeared to be transient and was maximal after 3 days of culture. On the other hand, culture with IL-4 did not result in induction of CD13 Ag (Figure 4). Neither culture in RPMI 1640 supplemented with 10% FCS and rIL-4 nor culture in modified Iscove's medium supplemented with rIL-4 revealed induction of CD13 Ag expression on U937 cells. Although on PBM CD13 Ag expression increased after only 1 day of culture with 30 U IL-4/ml, induction of CD13 Ag expression on U937 cells could not be detected even after 2-3 days of culture with 100 U IL-4/ml.

Human AM were found to express CD13 Ag constitutively (Figure 1). Upon culture, this expression increased and this increase appeared to be maximal after 2 to 3 days of culture (Figure 4). With respect to the kinetics of increase in CD13 Ag expression during culture, PBM and AM appeared to be comparable. Moreover, IL-4 caused a further increase in CD13 Ag expression similar to that observed in PBM (Figure 4 and 5). Maximal expression was observed after 2 to 3 days of incubation. After 4-5 days, expression of CD13 Ag on AM cultured with IL-4 was almost identical to the expression on AM cultured without IL-4.

However, expression after 4-5 days of culture was still higher than expression on freshly isolated AM. These data indicate that, besides the basal culture-induced increase in CD13 Ag expression, IL-4 induced an additional transient up-regulation of CD13 Ag on AM. On the other hand, IL-4 was unable to induce FcεRIIb expression on AM (Figure 4). Because an IL-4-induced increase of CD13 Ag expression was observed only in constitutively CD13-positive cells (PBM and AM) and not in constitutively CD13-negative cells (U937), we examined whether IL-4 was able to modulate expression on THP1 cells, which are, like U937 cells, immature monocytic cells but constitutively express CD13 Ag. In THP1 cells, expression of CD13 Ag was not changed, neither when these cells were cultured in RPMI 1640 supplemented with 10% FCS and rIL-4 nor when they were cultured in modified Iscove's medium supplemented with rIL-4 (data not shown). On the other hand, THP1 cells were able to respond to IL-4, just as expression of FcεRIIb was induced on U937 cells (data not shown).

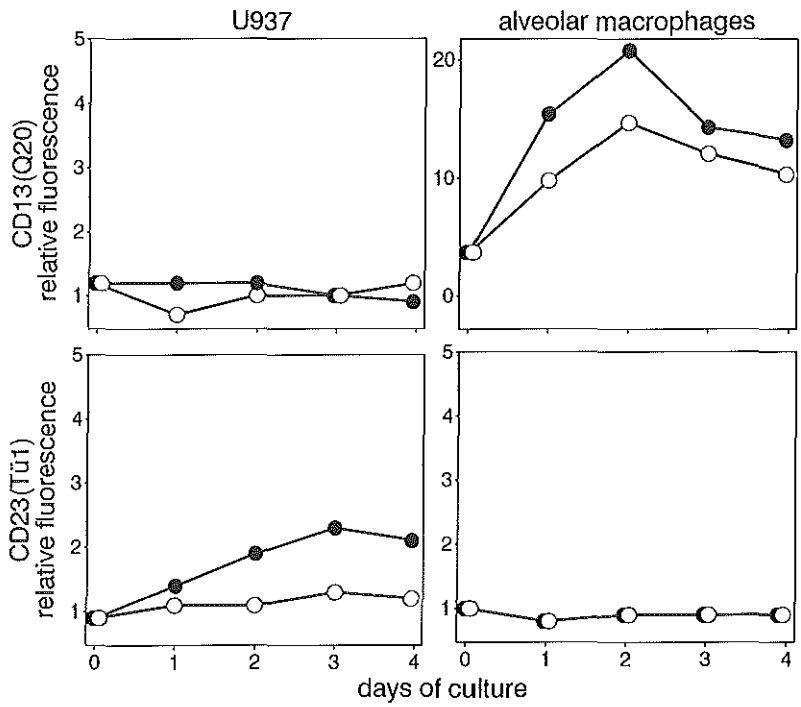

Figure 4. Time course of the influence of IL-4 on the membrane expression of the CD13 and CD23 Ag on the monocytic cell line U937 and human alveolar macrophages. Cells were cultured either without (○) or with (●) 30 U IL-4/ml. Fluorescence intensity of 7500 cells was determined with a FACScan. On the ordinate is given the relative fluorescence, calculated as described in the footnote to Table 1. Both data of U937 cells and macrophages represent one representative experiment of four.

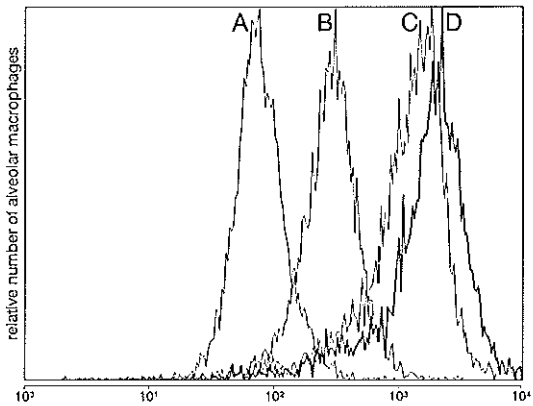

Figure 5. Effects of IL-4 on the expression of the CD13 Ag on alveolar macrophages. Cells were stained with Q20 (CD13) and FITC-labeled GAM F(ab')$_2$ either directly after isolation (B), or after a culture period of 2 days in modified Iscove's medium without (C) or with (D; bold histogram) 30 U IL-4/ml. Histogram A represents macrophages stained with an IgG2a control antibody and FITC-labeled GAM F(ab')$_2$. Fluorescence intensity of 7500 cells was determined on a log scale with a FACScan. A representative experiment out of three is shown.

IL-4 increases LAP activity of human monocytes

To determine whether the IL-4-induced up-regulation of CD13 Ag reflects a functional increase in aminopeptidase activity of PBM, we studied whether IL-4 enhanced the capacity of PBM to degrade L-leucine-p-nitroanilide. PBM cultured for 2 days showed a decrease in LAP activity compared with freshly isolated PBM, despite an increase in CD13 Ag membrane expression (Table 3). These apparent contradicting data may be explained by preliminary observations that also other membrane enzymes (which may be down-regulated upon culture) can display peptidase-activity comparable to CD13 Ag (Dr. E. Favaloro, personal communication). On the other hand, PBM cultured for 2 days with IL-4 exhibited a higher activity to degrade L-leucine-p-nitroanilide than PBM cultured without IL-4 (Table 3). These data add proof that IL-4 is able to increase the functional activity of PBM to degrade L-leucine-p-nitroanilide. U937 cells, which do not express CD13 Ag, exhibited a 20-fold lower capacity to degrade L-leucine-p-nitroanilide, while THP1 cells, which do express CD13 Ag, had a capacity comparable with freshly isolated PBM (data not shown). To obtain more direct evidence for the influence of IL-4 on the expression of CD13 Ag and because IL-4 has been described to induce CD23 mRNA (35), we investigated the effect of IL-4 on CD13 mRNA.

Table 3. Influence of IL-4 on the LAP activity of human monocytes, related to the expression of the CD13 Ag.[a]

Days of Culture	Experiment 1				Experiment 2			
	Medium		Medium + IL-4		Medium		Medium + IL-4	
	LAP	CD13	LAP	CD13	LAP	CD13	LAP	CD13
0	231[b]	3.8[c]	231	3.8	171	5.1	171	5.1
2	108	17.0	229	158.2	108	14.1	199	189.4

a. Monocytes were assayed for their LAP activity directly after isolation and after 2 days of culture with or without 100 U of IL-4/ml. Monocytes of the same donor were also labeled with Q20 and FITC-conjugated GAM $F(ab')_2$ directly after isolation and after two days of culture with or without 100 U IL-4/ml.
b. Data represent enzymatic activity and are expressed as the rate of accumulation of *p*-nitroanilide, nmol/30 min x 10^6 cells.
c. Data are expressed as relative fluorescence, calculated as described in the footnote of Table 1.

IL-4 increases the expression of CD13 mRNA

Total RNA was isolated from freshly isolated human PBM, and PBM cultured for 2 days with or without IL-4. Total RNA was separated by electrophoresis and hybridized with a ^{32}P-labeled CD13 cDNA probe. The results in Figure 6 show that IL-4 induced a strong increase in the level of CD13 mRNA in PBM (Figure 6, lane 2). PBM cultured for 2 days in mock-medium exhibited a small, spontaneous increase in the level of CD13 mRNA (Figure 6, lane 3). CD13 mRNA was not detectable in U937 cells, while the level of CD13 mRNA in THP1 cells resembled the level in freshly isolated PBM and AM.

DISCUSSION

Initially, it was thought that a certain cytokine directed its single activity at one particular cell type. Nowadays, it is generally accepted that different cytokines can regulate the same immune activity, that a single cytokine may effect a multitude of activities and that these activities may be directed at multiple cell types (36-39). Moreover, recent reports describe that the responsiveness of a certain cell type to a particular cytokine may depend on the stage of maturation/differentiation of that cell type (21). IL-4 was originally described as a B cell growth factor, but is now also known to influence T lymphocytes, NK cells, and monocytes as well. Some of the many effects of IL-4 can also be brought about by other cytokines (38,39). The data presented here widen the range of activities of IL-4 and show that IL-4 responsiveness of monocytic cells depends on their stage of maturation.

It has been reported by different investigators that IL-4 is able to modulate the expression of several cell surface determinants. IL-4 is able to induce a transient expression of FεRIIb (CD23) on PBM (8) and monocytic cell lines (35). Furthermore, IL-4 enhances the expression of class II MHC Ag on PBM (9) and AM (P.Th.W. van Hal, unpublished

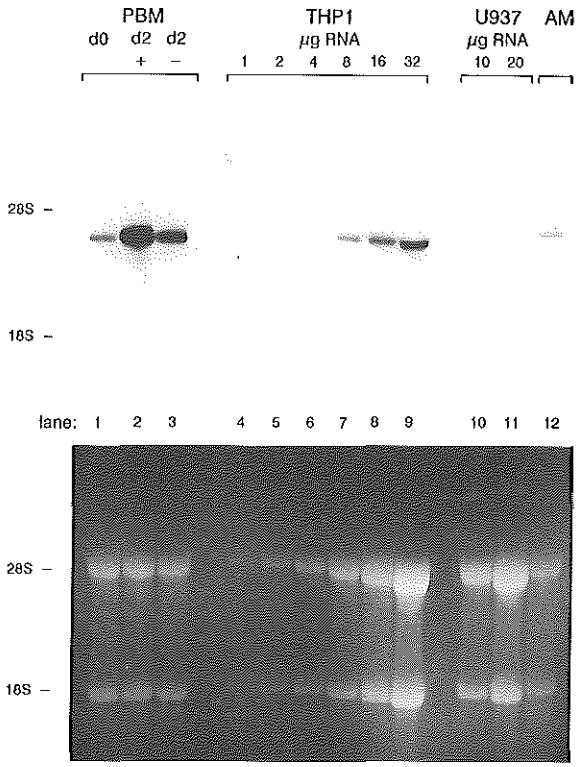

Figure 6. Induction of CD13 mRNA by IL-4 in human peripheral blood monocytes. Total RNA was isolated from freshly isolated monocytes (lane 1), and from cells cultured for 2 days in the presence (lane 2, +) or absence (lane 3,-) of IL-4. Each of these three lanes contains 10 μg of total RNA. Lanes 4 to 9 contain 1, 2, 4, 8, 16 and 32 μg of RNA from the $CD13^+$ cell line THP1, respectively. Lanes 10 and 11 contain 10 and 20 μg of RNA from the $CD13^-$ cell line U937, respectively. Lane 12 contains 5 μg of total RNA from freshly isolated alveolar macrophages. RNA was separated by electrophoresis, transferred onto a nylon membrane and hybridized with the ^{32}P-labeled CD13 cDNA probe (upper panel). The lower panel shows ethidiumbromide staining of the gel after electrophoresis to visualize loading per lane.

observations), downregulates the expression of FcγR on PBM (40), and modulates maturation markers on PBM (see chapter 5.2 of this thesis). Our data indicate that IL-4 is also able to increase the expression of the CD13 Ag. This increase appears to be transient and resembles the kinetics of IL-4-induced FcεRIIb expression. Furthermore, IL-4 increases the level of CD13 mRNA and raises the capacity to degrade L-leucine-p-nitroanilide. Besides these IL-4-induced increases, a small and spontaneous up-regulation of CD13 Ag was observed following culture of monocytes in mock-medium. It is unlikely that this spontaneous up-regulation is due to contaminating amounts of IL-4, as culture of PBM in the presence of both IL-4 and the anti-IL-4 antiserum reduced CD13 Ag expression to the levels of PBM cultured in mock-medium, and not to the levels of freshly isolated PBM. Furthermore, this spontaneous increase could not be explained by serum components or adherence, as culture of PBM in serum-free medium gave similar results (data not shown) and PBM were cultured under nonadherent conditions in Teflon bags, respectively. As culture of PBM in mock-medium induces also a spontaneous increase in MHC class II expression (9), it appears that in our system activation takes place, most likely because of medium components, e.g., low levels of LPS. The spontaneous increase in CD13 Ag expression may be part of this activation.

To our knowledge this is the first report on IL-4-induced expression of CD13 Ag,

although recently Rohrbach et al. (41) reported that leucine-aminopeptidase (EC 3.4.11.2 and identical to CD13 Ag) activity in cultured PBM was enhanced by the presence of T lymphocytes. They found that the majority of increase occurred during the first day of culture, while they saw a further slight increase up until day 6 of culture. Although they did not confirm their data with immunofluorescence staining for CD13 Ag and did not speculate on the mechanism by which leucine-aminopeptidase activity increased, we think that their results can be explained by the production of IL-4 by activated T lymphocytes, thus being consistent with our own results.

Surprisingly, induction of CD13 Ag by IL-4 could not be achieved on immature monocytic cell lines, such as U937 and THP1. We confirmed the earlier observations that IL-4 is able to induce FcεRIIb expression on these cell lines. Therefore, unresponsiveness to IL-4 concerning the induction of CD13 Ag can not be explained as a result of absence of functional IL-4 R. On the other hand, AM, just like PBM, increased their expression of CD13 Ag upon culture with IL-4. AM and PBM constitutively express CD13 Ag, while U937 cells lack this Ag. Therefore, one could speculate that IL-4 is only able to enhance actual expression of CD13 Ag, while it is unable to induce expression on a CD13-negative cell type. If so, one would expect an increase in expression of CD13 Ag on THP1 cells, which are constitutively positive for this marker, upon culture with IL-4. This, however, could not be demonstrated in our experiments. We therefore propose, that immature monocytic cells are unable to enhance CD13 Ag expression upon culture with IL-4 despite the presence of functional IL-4 R, and that the ability to enhance CD13 Ag expression after culture with IL-4 is only acquired upon maturation. On the other hand, it may also be possible that the cell lines in question are unable to upregulate CD13 Ag because of cell line-related properties other than immaturity, e.g. altered state of (de)activation.

An analysis of the literature showed that others also indicated that cytokine effects are variable depending on the cell type and/or the stages of differentiation. Kawabe et al. (34) mentioned that CD23 Ag could be upregulated by IL-4 on normal B lymphocytes, but not on EBV-transformed B lymphoblastoid cell lines. Littman et al. (39) found that IL-4 did not stimulate undifferentiated HL-60 cells to produce C2. However, when these cells were first pre-incubated with vitamine D3 to induce monocytic differentiation, they did produce C2 in response to IL-4. Recently, Watanabe et al. (21) demonstrated that TNF-α enhanced IFN-γ-induced MHC class II Ag expression in immature cells, such as U937 cells, while TNF-α had no effect on IFN-γ-induced MHC class II Ag expression in TPA-differentiated U937 cells. They also proposed that cytokine effects depend on the maturation stage of cells. As IL-4 was able to induce FcεRIIb expression on U937 cells and PBM, we were surprised to see that it was unable to induce expression on AM. Although there are some reports showing expression of FcεRIIb on AM (42,43), it seems unlikely from our study that IL-4 plays a decisive role in this expression, at least as an isolated factor. Bieber et al. (44) were able to induce FcεRIIb on a subpopulation of human epidermal Langerhans cells, but they used IL-4 in a high dose (1000 U/ml) and in combination with IFN-γ (1000 U/ml). However, it should be emphasized that we cannot exclude the possibility that IL-4 in combination with other cytokines may be able to induce FcεRIIb on AM, or that IL-4 is able to induce FcεRIIb on a small (immature) subpopulation of AM, as FACScan analysis is inappropriate

for detecting positive subpopulations constituting less then 1-2% of the total cell population. Nevertheless, virtually the whole population of U937 cells and PBM express FcεRIIb after culture with IL-4, whereas virtually the whole population of AM does not respond. These findings suggest that upon maturation the ability to express FcεRIIb during culture with IL-4 is lost. This notion is supported by the findings of Te Velde et al. (45), who demonstrated that IL-4-matured PBM do not re-express FcεRIIb upon renewed addition of IL-4 on day 4 of culture.

In conclusion, our findings reveal a new property of IL-4, namely an up-regulation of the expression of CD13 Ag on monocytic cells. As the CD13 Ag represents aminopeptidase-N activity, this up-regulation may be part of an IL-4-induced activation of monocytic cells. Furthermore, our findings provide a model to explain the paradoxical effects of cytokines on a certain cell type. Positive or negative effects of a cytokine on a single cell type may be related to differences in stage of maturation, differentiation or activation.

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REFERENCES

1. Oliver K, Noelle RJ, Uhr JW, Krammer PH, Vitetta ES. B-cell growth factor (B-cell growth factor I or B-cell-stimulating factor, provisional 1) is a differentiation factor for resting B cells and may not induce cell growth. Proc Natl Acad Sci USA 1985;82:2465-2467.
2. Spits H, Yssel H, Takebe Y, Arai N, Yokota T, Lee F, Arai K-I, Banchereau J, De Vries JE. Recombinant interleukin 4 promotes the growth of human T cells. J Immunol 1987;139:1142-1147.
3. Defrance T, Vanbervliet B, Aubry J-P, Takebe Y, Arai N, Miyajima A, Yokota T, Lee F, Arai K-I, De Vries JE, Banchereau J. B cell growth-promoting activity of recombinant human interleukin 4. J Immunol 1987;139:1135-1141.
4. Mosmann TR, Bond MW, Coffman RL, Ohara J, Paul WE. T-cell and mast cell lines respond to B-cell stimulatory factor 1. Proc Natl Acad Sci USA 1986;83:5654-5658.
5. Lee F, Yokota T, Otsuka T, Meyerson P, Villaret D, Coffman R, Mosmann T, Rennick D, Roehm N, Smith C, Zlotnik A, Arai K-I. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. Proc Natl Acad Sci USA 1986;83:2061-2065.
6. Defrance T, Aubry JP, Rousset F, Vanbervliet B, Bonnefoy JY, Arai N, Takebe Y, Yokota T, Lee F, Arai K, De Vries J, Banchereau J. Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. J Exp Med 1987;165:1459-1467.
7. Noelle R, Krammer PH, Ohara J, Uhr JW, Vitetta ES. Increased expression of Ia antigens on resting B cells: An additional role for B-cell growth factor. Proc Natl Acad Sci USA 1984;81:6149-6153.
8. Vercelli D, Jabara HH, Lee B-W, Woodland N, Geha RS, Leung DYM. Human recombinant interleukin 4 induces FcεR2/CD23 on normal human monocytes. J Exp Med 1988;167:1406-1416.

9. Te Velde AA, Klomp JPG, Yard BA, De Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
10. Tomizawa K, Ishizaka A, Kojima K, Nakanishi M, Sakiyama Y, Matsumoto S. Interleukin-4 regulates the interleukin-2 receptors on human peripheral blood B lymphocytes. Clin Exp Immunol 1991;83:492-496.
11. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 1981;68:932-941.
12. Look AT, Ashmun A, Shapiro LH, O'Connell PJ, Gerkis V, D'Apice AJ, Sagawa K, Pelper SC. Report on the CD13 (aminopeptidase N) cluster workshop. In: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, Von dem Borne AEGKr, eds. Leucocyte Typing IV. White Cell Differentiation Antigens. New York: Oxford University Press, 1989:784-787.
13. Sobol RE, Mick R, Royston I, Davey FR, Ellison RR, Newman R, Cuttner J, Griffin JD, Collins H, Nelson DA, Bloomfield CD. Clinical importance of myeloid antigen expression in adult acute lymphoblastic leukemia. New Engl J Med 1987;316:1111-1117.
14. Ashmun RA, Look AT. Metalloprotease activity of CD13/aminopeptidase N on the surface of human myeloid cells. Blood 1990;75:462-469.
15. Hogg N, Horton MA. Myeloid antigens: new and previously defined clusters. In: McMichael AJ, ed. Leucocyte typing III. White Cell Differentiation Antigens. Oxford: Oxford University Press, 1987:576-602.
16. Semenza G. Anchoring and biosynthesis of stalked brush border membrane proteins: Glycosidases and peptidases of enterocytes and renal tubuli. Annu Rev Cell Biol 1986;2:255-313.
17. Matsas R, Stephenson SL, Hryszko J, Kenny AJ, Turner AJ. The metabolism of neuropeptides. Phase separation of synaptic membrane preparations with Triton X-114 reveals the presence of aminopeptidase N. Biochem J 1985;231:445-449.
18. Look AT, Ashmun RA, Shapiro LH, Peiper SC. Human myeloid plasma membrane glycoprotein CD13(gp150) is identical to aminopeptidase N. J Clin Invest 1989;83:1299-1307.
19. Kenny AJ, O'Hare MJ, Gusterson BA. Cell-surface peptidases as modulators of growth and differentiation. Lancet 1989;ii:785-787.
20. Cao H, Wolff RG, Meltzer MS, Crawford RM. Differential regulation of class II MHC determinants on macrophages by IFN-γ and IL-4. J Immunol 1989;143:3524-3531.
21. Watanabe Y, Jacob CO. Regulation of MHC class II antigen expression. Opposing effects of tumor necrosis factor-α on IFN-γ-induced HLA-DR and Ia expression depends on the maturation and differentiation stage of the cell. J Immunol 1991;146:899-905.
22. Figdor CG, Bont WS, Touw I, De Roos J, Roosnek EE, De Vries JE. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. Blood 1982;60:46-53.
23. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565-577.
24. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 1980;26:171-176.
25. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Meth 1984;72:219-227.
26. Chrétien I, Van Kimmenade A, Pearce MK, Banchereau J, Abrams JS. Development of polyclonal and monoclonal antibodies for immunoassay and neutralization of human interleukin-4. J Immunol Meth 1989;117:67-81.
27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.

28. Sambrook J, Fritsch EF, Maniatis T. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. In: Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989:7.3-7.87.
29. Olszewska E, Jones K. Vacuum blotting enhances nucleic acid transfer. Trends Genet 1988;4:92-94.
30. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clinica Chimica Acta 1991;198:1-92.
31. Krug MS, Berger SL. First-strand cDNA synthesis primed with oligo(dT). Methods Enzymol 1987;152:316-325.
32. Breit TM, Wolvers-Tettero ILM, Hählen K, Van Wering ER, Van Dongen JJM. Extensive junctional diversity of γδ T-cell receptors expressed by T-cell acute lymphoblastic leukemias. Implications for the detection of minimal residual disease. Leukemia 1992;6:169-170.
33. Ruseler-van Embden JGH, Van Lieshout LMC. Increased proteolysis and leucine aminopeptidase activity in faeces of patients with Crohn's disease. Digestion 1988;40:33-40.
34. Kawabe T, Takami M, Hosoda M, Maeda Y, Sato S, Mayumi M, Mikawa H, Arai K-I, Yodoi J. Regulation of FcεR2/CD23 gene expression by cytokines and specific ligands (IgE and anti-FcεR2 monoclonal antibody). Variable regulation depending on the cell types. J Immunol 1988;141:1376-1382.
35. Yokota A, Kikutani H, Tanaka T, Sato R, Barsumian EL, Suemura M, Kishimoto T. Two species of human Fcε receptor II (FcεII/CD23): tissue-specific and IL-4-specific regulation of gene expression. Cell 1988;55:611-618.
36. O'Garra A, Umland S, De France T, Christiansen J. "B-cell factors" are pleiotropic. Immunol Today 1988;9:45-54.
37. Le J, Vilček J. Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest 1987;56:234-248.
38. Becker S, Daniel EG. Antagonistic and additive effects of IL-4 and interferon-γ on human monocytes and macrophages: effects on Fc receptors, HLA-D antigens, and superoxide production. Cell Immunol 1990;129:351-362.
39. Littman BH, Dastvan FF, Carlson PL, Sanders KM. Regulation of monocyte/macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFN-γ. J Immunol 1989;142:520-525.
40. Te Velde AA, Huijbens RJF, De Vries JE, Figdor CG. IL-4 decreases FcγR membrane expression and FcγR-mediated cytotoxic activity of human monocytes. J Immunol 1990;144:3046-3051.
41. Rohrbach MS, Conrad AK. Comparison of the T lymphocyte-dependent induction of angiotensin-converting enzyme and leucine aminopeptidase in cultured human monocytes. Clin Exp Immunol 1991;83:510-515.
42. Joseph M, Tonnel A-B, Torpier G, Capron A, Arnoux B, Benveniste J. Involvement of immunoglobulin E in the secretory processes of alveolar macrophages from asthmatic patients. J Clin Invest 1983;71:221-230.
43. Pforte A, Breyer G, Prinz JC, Gais P, Burger G, Häussinger K, Rieber EP, Held E, Ziegler-Heitbrock HWL. Expression of the Fc-receptor for IgE (FcεRII, CD23) on alveolar macrophages in extrinsic allergic alveolitis. J Exp Med 1990;171:1163-1169.
44. Bieber T, Rieger A, Neuchrist C, Prinz JC, Rieber EP, Boltz-Nitulescu G, Scheiner O, Kraft D, Ring J, Stingl G. Induction of FcεR2/CD23 on human epidermal Langerhans cells by human recombinant interleukin 4 and γ interferon. J Exp Med 1989;170:309-314.
45. Te Velde AA, Rousset F, Peronne C, De Vries JE, Figdor CG. IFN-α and IFN-γ have different regulatory effects on IL-4-induced membrane expression of FcεRIIb and release of soluble FcεRIIb by human monocytes. J Immunol 1990;144:3052-3059.

PROLIFERATION OF MATURE AND IMMATURE SUBPOPULATIONS OF BRONCHOALVEOLAR MONOCYTES/MACROPHAGES AND PERIPHERAL BLOOD MONOCYTES*

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SUMMARY

A continuous influx of peripheral blood monocytes (PBM) to the lung is thought to maintain the local population of alveolar macrophages (AM). However, local proliferation of a small subpopulation of AM has been demonstrated in animal studies and in humans. AM exhibit a great heterogeneity with regard to their morphology (cell size, shape of nucleus), immunophenotype (expression of CD14 Ag and RFD9 Ag), and function. Part of this heterogeneity may be explained by the presence of different maturation stages of AM, ranging from small immature, $CD14^+$ $RFD9^-$ PBM-like cells to large, $CD14^-$ $RFD9^+$ mature AM. These findings prompted us to study whether proliferation of PBM and AM is related to their stage of maturation.

The expression of the proliferation marker Ki-67 was studied in AM from both healthy volunteers and patients suffering from sarcoidosis. Using double immunofluorescence staining, we studied proliferation of immature, $CD14^+$ AM, and mature, $RFD9^+$ AM in sarcoidosis, and we compared this with PBM. A significantly larger percentage of AM in general expressed Ki-67 Ag in sarcoidosis [3.0 (median); range 1.1 – 5.5] as compared with healthy volunteers (0.8; 0.2 – 1.3). In sarcoidosis, proliferation was observed in both the immature and the mature subpopulation of AM. Proliferating PBM were rarely observed [less than 0.2% of the $CD14^+$ mononuclear cells (MNC)] both in healthy volunteers and sarcoidosis patients. A small subpopulation of PBM showed a weak expression of RFD9 Ag (less than 1% of MNC). Interestingly, proliferation of PBM was concentrated in this subpopulation (15% of the $RFD9^+$ MNC).

These data show that even mature AM, which are generally thought to be terminally differentiated cells with little capacity to replicate, are able to proliferate, whereas a relatively very low percentage of their precursors in the blood circulation proliferates. Furthermore, the findings suggest that lung tissue in sarcoidosis creates an environment which promotes proliferation of monocytic cells.

INTRODUCTION

Pulmonary alveolar macrophages (AM) were originally recognized as phagocytosing scavenger cells (1), but nowadays they are also known to initiate and regulate inflammatory and immunological processes in several lung diseases (2-4). AM are thought to represent more mature cells of the mononuclear phagocyte system, and to be derived from peripheral blood monocytes (PBM)(5,6). As AM are continuously lost (mainly through a transport from the peripheral airways, via the trachea to the pharynx), the local AM population must be constantly replenished. This may be enabled either through a continuous influx of PBM from the vasculature, or through local proliferation of AM, or a combination of these processes. Historically, the former vision was described first (7,8), whereas the second and latter ideas evolved more recently (9,10). In mice, it was shown that AM renewal may be accounted for by local proliferation alone, and that the influx of PBM was not needed to sustain AM (10). Also in human studies, local proliferation of a small subpopulation of AM

has been demonstrated (11). Moreover, in some inflammatory lung diseases and in the lung of smokers an increase in the number of proliferating AM has been reported (12,13). These latter findings may indicate that inflammation, or in general the local environment of AM, is able to modulate the capacity of AM to proliferate. The local environment may be determined by local cytokines, direct cell contact of AM with other inflammatory cells, or contact with extracellular matrix molecules (14,15). Recent studies on rat and murine AM suggested the significance of cytokines as M-CSF and GM-CSF in *in vitro* proliferation of AM (16-18). In contrast to murine AM, human AM were reported not to respond to M-CSF, but GM-CSF was shown to be a likely candidate as a promotor of local proliferation of AM in the lungs (19).

Human AM represent a heterogenous population of cells with regard to their morphology (cell size, shape of nucleus, ratio of cytoplasm : nucleus)(15,20), the expression of cell membrane proteins (CD14 and RFD9 Ag)(21) and their function (expression of acid phosphatase, Ag presenting capacity, response to cytokines)(15,21,22). The majority of this population is generally considered as more mature mononuclear phagocytes as compared with PBM. As mitotic activity is believed to diminish upon maturation, it is expected that proliferation would be more abundant among the immature, monocyte-like AM. However, a recent study suggested that proliferating AM were not monocyte-like but appeared mature (19).

The purpose of this study was to evaluate as to whether or not proliferation of human AM is related to their stage of maturation and is restricted to the more immature AM. Our findings show that both immature and mature AM may be able to proliferate, whereas a relatively low percentage of PBM proliferate. Furthermore, it is suggested that lung tissue in sarcoidosis creates an environment which induces proliferation of monocytic cells.

MATERIALS AND METHODS

Isolation of mononuclear cells

Peripheral blood samples were obtained from non-smoking healthy volunteers and sarcoidosis patients. Mononuclear cells (MNC) were isolated by Ficoll-Paque (density 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation for 15 min at room temperature with a centrifugal force of 1,000 g. All standard washings of cells were performed with phosphate-buffered saline (PBS; 300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated bovine serum albumin (BSA; Organon Teknika, Oss, The Netherlands). Washing centrifugations were performed with a force of 400 g at 4°C for 5 min.

Isolation of AM

All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. Bronchoalveolar lavage (BAL) was performed in healthy, non-smoking volunteers (n = 12) or patients suffering from sarcoidosis (n = 12) after premedication with thiazinamium and local anesthesia using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a

siliconized specimen trap placed on melting ice. Immediately after collection, the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles, whereafter the BAL cells were isolated by centrifugation at 4°C at a force of 400 g. In the healthy volunteers, more than 90% of the BAL cells appeared to be macrophages as judged by May-Grünwald Giemsa staining. Before analysis of the surface membrane determinants or expression of the proliferation marker Ki-67, the cells were washed twice with PBS/0.5% BSA.

Double immunofluorescence staining

Double immunofluorescence stainings were performed for either the mature monocyte/macrophage marker RFD9 [(not clustered), Dr. L.W. Poulter, London, UK] or the immature monocyte/macrophage marker CD14 (UCHM1, Dr. P.C.L. Beverly, London, UK; My4, Coulter Clone, Hialeah, FL) and the proliferation marker Ki-67 (Dakopatts, Danmark). In some staining experiments also the T lymphocyte marker CD3 (Leu-4, Becton Dickinson, San Jose, CA) was used. Both RFD9, UCHM1, My4, and Leu-4 recognize cell membrane determinants, whereas Ki-67 reacts with a nuclear antigen present in proliferating cells (23). Ki-67 recognizes an antigen that is associated with chromosomes (24). This antigen is expressed in the G_1, S, G_2 and M phase of continuously cycling cells, but is absent in G_0 cells (23). Fifty µl of the MNC or AM cell suspension (10×10^6 and 8×10^6 cells/ml, respectively) were incubated for 30 min at 4°C with 50 µl of the optimally titrated biotinylated monoclonal antibody against one of the cell membrane markers (or NMS as a control). After two washings, cells were incubated (30 min, 4°C) with tetramethylrhodamine isothiocyanate (TRITC)-labeled streptavidin. The labeled cells were washed twice and cytocentrifuge preparations were made. These preparations were fixed in methanol (30 min, 4°C) and subjected to indirect immunofluorescence staining with Ki-67 (or IgG1 as a control) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands). Subsequently, the slides were washed twice and mounted in glycerol/PBS (9:1) containing phenylenediamine (BDH Chemicals, Poole, UK) (1 mg/ml) to prevent fading of the fluorescence. The fluorescence was evaluated using Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) equipped with phase contrast facilities and filter combinations for the evaluation of both FITC and TRITC. At least 500 cells positive for one of the cell membrane markers (either CD3, CD14, or RFD9) were evaluated for the expression of Ki-67 Ag. In some experiments, the expression of cell membrane Ag was determined in Ki-67$^+$ cells. There, at least 200 Ki-67$^+$ cells were screened, which implied that $1\text{-}2 \times 10^4$ cells were evaluated.

Statistical analysis

Differences in the expression of Ki-67 Ag or the various cell membrane Ag between subject groups were tested with the Mann-Whitney U test. Within group differences in the Ag expression between different cell types were tested with the Wilcoxon matched-pairs signed-ranks test. A p value less than 0.05 was considered statistically significant.

Subject population

BAL fluid was obtained from 14 patients with sarcoidosis. The diagnosis was based on the clinical history, physical examination, radiologic findings and the presence of non-caseating granulomas on histologic examination of biopsy specimens. Only those patients were included in this study when cellular analysis of BAL fluid revealed specific findings of alveolitis, i.e. at least 15% T lymphocytes (CD3-positive cells) and a CD4/CD8-ratio equal to or higher than 4.0. The CD4/CD8-ratio of blood T lymphocytes did not exceed 2.5. The patients did not receive glucocorticoids or other medication and did not smoke. For our studies on BAL cells, the control group consisted of 12 healthy, nonsmoking volunteers without chest abnormalities and with

normal chest X-ray films and lung functions. Another comparable control group (n=8) was used in our studies on blood MNC.

RESULTS

Ki-67 Ag expression by AM

It was demonstrated earlier that the number of AM expressing Ki-67 Ag was higher in smokers or patients suffering from sarcoidosis than in healthy, nonsmoking volunteers (12,13). In those studies, expression of Ki-67 Ag was demonstrated by immuno-enzyme staining. In the present study an immunofluorescence staining technique was used and a similar increase in Ki-67$^+$ AM was found in sarcoidosis patients (Table 1). In healthy volunteers, only 0.8% of the AM expressed the proliferation marker, whereas in sarcoidosis this percentage was almost four times higher (3.0%). This difference was statistically significant ($p < 0.0005$). Upon evaluation of AM for expression of the Ki-67 Ag, we found that expression could be observed in both small, monocyte-like AM (with a low cytoplasm : nucleus ratio) and large AM (with a high cytoplasm : nucleus ratio). This, against expectations, suggested that proliferation was not restricted to the more immature, small AM.

Table 1. Expression of Ki-67 Ag in AM from healthy volunteers and patients suffering from sarcoidosis.[a]

	healthy volunteers (n=12)	sarcoidosis (n=14)
Ki-67	0.8 (0.2-1.3)[b]	3.0 (1.1-5.5)[c]

a. Expression of Ki-67 Ag was determined by evaluating at least 1000 AM. Lymphocytes, granulocytes and epithelial cells were excluded on morphogical criteria.
b. Data are expressed as median percentage (range).
c. Statistically different from healthy volunteers ($p < 0.0005$).

Ki-67 Ag expression by immature and mature AM

Heterogeneity among AM can be explained partly by differences (Tables 2 and 3) in maturation stage. Maturation of monocytes/macrophages is accompanied by changes in the expression of cell membrane determinants. Mature AM are positive for RFD9, whereas virtually all immature AM, comparable to PBM, do not express the RFD9 Ag (21). Only a small subpopulation of PBM (0,4%) has been reported to express RFD9 Ag with a low intensity (21). In contrast, immature AM and PBM express the CD14 Ag, whereas mature AM do not (21). The combined application of a maturation-associated marker and Ki-67 revealed that both CD14$^+$ and RFD9$^+$ AM could express the proliferation-associated marker

(Tables 2 and 3). (This could only be demonstrated in patients suffering from sarcoidosis, because the low expression of Ki-67 Ag in AM from healthy volunteers hampered the combined evaluation of Ki-67 Ag with cell membrane markers). These findings confirmed the above-mentioned conclusion that also the more mature, RFD9$^+$ AM may express the Ki-67 Ag (Figure 1). The expression of Ki-67 Ag in mature RFD9$^+$ AM was in the same range as in immature CD14$^+$ AM (Table 2). Therefore, we were also interested to find out to what extent PBM, the precursors of both mature and immature AM, expressed the Ki-67 Ag.

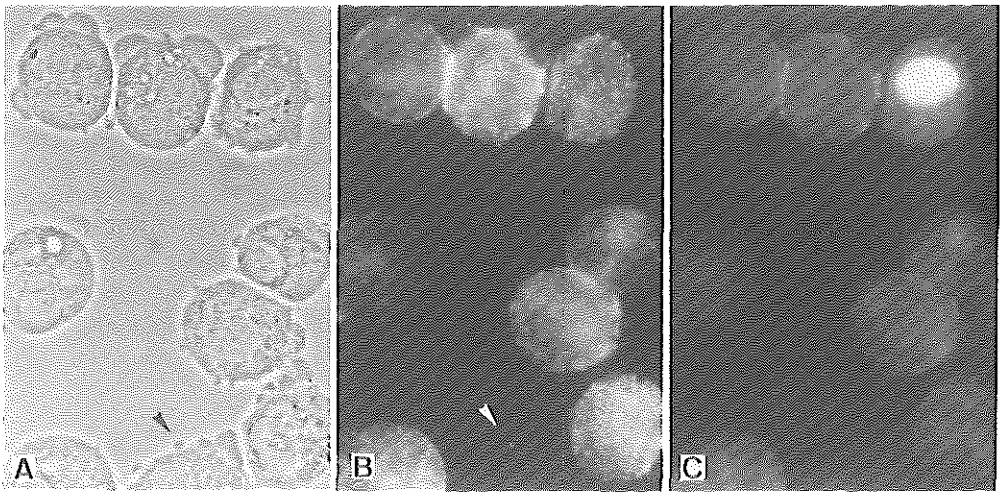

Figure 1. Double immunofluorescence staining of BAL cells for RFD9 and Ki-67 Ag.
A: Phase contrast picture, which enables morphological differentiation between monocytes/macrophages, granulocytes and lymphocytes. In this picture only monocytes/macrophages are seen.
B: Immunofluorescence staining of the cell membrane marker RFD9 (TRITC labeled). Most monocytes/macrophages strongly express this marker; some cells exhibit a weak signal, whereas one cell does not express RFD9 Ag (arrow).
C: Immunofluorescence staining of the proliferation marker Ki-67 (FITC labeled). Antigen recognized by this monoclonal antibody is located in the nucleus. One cell, which also expresses RFD9 Ag (see B), expresses Ki-67 Ag.

Ki-67 Ag expression by PBM

MNC were isolated from healthy volunteers and patients suffering from sarcoidosis, and tested for expression of Ki-67 Ag in PBM. In the MNC samples derived from control subjects, expression of Ki-67 Ag could not be detected in the CD14 (UCHM1)$^+$ cells (Table 4). At least 500 CD14 (UCHM1)$^+$ cells were screened in all control subjects ($n = 8$). Use of another monoclonal antibody also clustered within CD14 (My4) revealed that a very low percentage (0,2%) of My4$^+$ cells expressed Ki-67 Ag (Table 4). When T lymphocytes, as determined by the expression of CD3 Ag, were evaluated for the expression of Ki-67 Ag,

Table 2. Expression of Ki-67 Ag in blood MNC versus mature and immature subpopulations of AM in sarcoidosis (n = 5).

subpopulation of cells[a]	Ki-67
CD14$^+$ MNC (23 ± 7)[b]	0.0 (0.0 - 0.0)[c]
RFD9$^+$ MNC (< 1)	14.5 (2.0 - 46.0)
CD14$^+$ AM (9 ± 6)[d]	1.8 (1.0 - 2.2)
RFD9$^+$ AM (40 ± 16)	2.3 (0.7 - 4.3)

a. At least 500 cells positive for either CD14 (UCHM1) or RFD9 were evaluated for expression of Ki-67 Ag.
b. In parentheses is shown the mean percentage ± S.D. of CD14 (UCHM1)$^+$ or RFD9$^+$ cells in the total population of MNC.
c. Data are expressed as median percentage (range).
d. In parentheses is shown the mean percentage ± S.D. of CD14 (UCHM1)$^+$ or RFD9$^+$ monocytes/macrophages in the total population of BAL cells.

Table 3. Expression of different cell membrane Ag in the Ki-67$^+$ subpopulation of blood MNC and AM in sarcoidosis (n = 5).[a]

	blood MNC		AM	
	CD14 (UCHM1)	RFD9	CD14 (UCHM1)	RFD9
Ki-67$^+$ cells	0.0 (0.0 - 1.0)[b]	10.5 (3.0 - 38.0)	24.4 (12 - 48.9)	81.6 (55.2 - 84.0)

a. Expression of the relevant cell membrane marker was determined by evaluating at least 200 Ki-67$^+$ blood MNC or AM.
b. Data are expressed as median percentage (range).

a higher percentage of Ki-67$^+$ cells was found as compared with CD14$^+$ myeloid cells (Tables 4 and 5).

Conversely, MNC expressing Ki-67 Ag were screened with regard to their cell membrane marker (Table 5). At least 200 Ki-67$^+$ cells were evaluated in each subject. As only a small percentage (1-2%) of the MNC expressed Ki-67 Ag, the screening of 200 positive cells implied that 1-2 x 10^4 cells were evaluated. Only a minor part of the Ki-67$^+$ population expressed CD14 Ag. In the healthy volunteers, less than 1% (median 0.3%) was positive for CD14 (UCHM1), whereas 1,5% of the Ki-67$^+$ cells was also positive for CD14 (My4). Most of the Ki-67$^+$ cells turned out to be CD3$^+$, i.e. T lymphocytes (Table 5).

In the subpopulations of MNC derived from sarcoidosis patients, the expression of Ki-67 Ag was not significantly different from the expression in healthy volunteers (Table 4). When the expression of Ki-67 Ag in CD14 (UCHM1)$^+$ MNC was compared with CD14 (UCHM1)$^+$ AM in sarcoidosis, expression of this proliferation marker was clearly higher in AM (Tables 2 and 3). This difference could not be tested statistically, as the number of subjects was too small for the Wilcoxon matched-pairs signed-ranks test becoming

significant. In sarcoidosis patients, we also evaluated the expression of Ki-67 in RFD9$^+$ MNC. Less than 1% of MNC expressed the RFD9 Ag with low intensity (Table 2). Interestingly, within this cell population there was a distinct subpopulation positive for Ki-67 (Table 2 and 3).

Table 4. Expression of Ki-67 Ag in blood MNC and different subpopulations of MNC in sarcoidosis (n = 5) and healthy volunteers (n = 8).

	percentage of Ki-67$^+$ cells	
(sub)population of blood MNC	sarcoidosis	healthy volunteers
total population[a]	1.0 (0.4 - 1.7)[b,c]	1.6 (0.2 - 1.7)
CD14 (UCHM1)$^+$ cells[d]	0.0 (0.0-0.0)[c]	0.0 (0.0-0.0)
CD14 (My4)$^+$ cells	0.0 (0.0-0.0)[c]	0.2 (0.0 - 0.2)
CD3 (Leu-4)$^+$ cells	0.5 (0.2 - 4.2)[c]	0.6 (0.0 - 1.8)

a. At least 1000 MNC (not further characterized with cell membrane markers) were evaluated for expression of Ki-67 Ag.
b. Data are expressed as median percentage (range).
c. Not significantly different from healthy volunteers.
d. At least 500 cells positive for the different cell membrane markers were evaluated for expression of Ki-67 Ag.

Table 5. Expression of different cell membrane Ag in the Ki-67$^+$ subpopulation of blood MNC in sarcoidosis (n = 5) and healthy volunteers (n = 8).[a]

	sarcoidosis			healthy volunteers		
	CD14 (UCHM1)	CD14) (My4	CD3	CD14 (UCHM1)	CD14 (My4)	CD3
Ki-67$^+$ cells	0.0 (0.0 - 1.0)[b,c]	0.5 (0.0 - 2.0)[c]	77.0 (50.6 - 87.0)[c]	0.3 (0.0 - 3.0)	1.5 (1.0 - 4.0)	58.5 (28.5 - 84.5)

a. Expression of the relevant cell membrane marker was determined by evaluating at least 200 Ki-67$^+$ blood MNC.
b. Data are expressed as median percentage (range).
c. Not significantly different from healthy volunteers.

DISCUSSION

In this study, we have demonstrated that in the lung proliferation of AM is not restricted to the immature, monocyte-like and CD14$^+$ subpopulation, and that a substantial number of the larger, more mature and RFD9$^+$ AM also expressed the proliferation marker Ki-67. Moreover, we showed that proliferation is clearly more abundant among CD14$^+$ AM

than $CD14^+$ PBM. These findings suggest that (part of the) PBM, upon entering the lung tissue maintain or re-acquire their proliferative activity in spite of the changes in their phenotype which suggest maturation/differentiation. We also showed that a small subpopulation of PBM (less than 1% of MNC) expressed the RFD9 Ag. Just within this $RFD9^+$ subpopulation of PBM, proliferation appeared to be concentrated. Proliferation, i.e. expression of Ki-67 Ag, was beyond the detection limit in the CD14 $(UCHM1)^+$ MNC, whereas 15% of the $RFD9^+$ MNC exhibited proliferative activity. These findings may suggest that RFD9 Ag play a role in the proliferation of monocytes/macrophages, although its precise function is still unknown (25).

Current concepts on the origin of tissue macrophages suggest that these cells derive from PBM (1,5,6), although also local proliferation of tissue macrophages has been demonstrated (9,10-13). Accordingly, AM are generally thought to represent terminally differentiated cells with no or very little capacity to replicate. The general idea implies that upon leaving the circulation, PBM mature or differentiate to AM via interstitial monocytes and loose their ability to proliferate. Therefore, one would expect that the proliferating subpopulation of AM, if any, is more immature, and resembles PBM. Surprisingly, we found in sarcoidosis that both the mature ($RFD9^+$) and immature ($CD14^+$) subpopulation of AM exhibited proliferative activity, whilst $CD14^+$ AM had a much higher proliferative activity than $CD14^+$ PBM. These findings indicate that lung tissue creates an environment which appears to induce proliferation of migrated PBM. Prior to their appearance in the alveolar lumen, PBM migrating from the circulation have to pass the interstitial compartment. It would be interesting to compare the proliferative activity of these interstitial monocytes/macrophages with PBM and AM, and to relate proliferation to the expression of cell surface markers.

Here, we also confirmed the findings of others (12,13) that the proliferative activity of AM is higher in sarcoidosis than in healthy volunteers. An enhanced number of proliferating AM could be due to the local presence of a growth factor which is normally absent, or to a changed cellular responsiveness to local growth factors which are constitutively present. Recently, Kreipe et al. showed that the increased proliferation of AM in sarcoidosis may be explained by the increased presence of younger and higher c-fms-expressing AM (12). It is known that PBM express c-fms transcripts (26), whereas AM from non-inflamed lungs show only reduced levels of c-fms RNA (27). Kreipe et al. suggested that the combination of constitutive production of M-CSF by AM and the increased numbers of younger and higher c-fms-expressing AM may result in an enhanced mitotic activity of AM (12). In contrast, our findings indicate that both immature, i.e. younger, and more mature AM show mitotic activity. Furthermore, we showed that PBM, which are known to express the c-fms protein, exhibit only very low mitotic activity as compared with AM. Therefore, it must be concluded that either there is no M-CSF in the environment of PBM, or c-fms expression is not related to the proliferation of monocytes/macrophages in the lung environment. With our findings of proliferation in the $RFD9^+$ subpopulation of PBM, it would be worthwhile to study the expression of c-fms transcripts and the response to growth factors in the various subpopulations of PBM. Nakata et al. studied the proliferation of human AM *in vitro* in the presence of either M-CSF

or GM-CSF (19). They found that GM-CSF increased the proportion of proliferating AM, in contrast to M-CSF. This GM-CSF effect could be abrogated by an antibody against GM-CSF. Furthermore, they showed that human lung conditioned medium contained GM-CSF. Itoh et al. found high levels of GM-CSF mRNA in BAL cells from sarcoidosis patients compared with subjects suffering from extrinsic allergic alveolitis (28). It may therefore by hypothesized that in sarcoidosis increased expression of GM-CSF results in an increased population of proliferating AM, although the trigger of GM-CSF production is still unknown.

In addition to the contribution of local proliferation to the accumulation of monocytes/macrophages in pulmonary inflammation, prolonged survival may also play a substantial role. Recently, it was shown that GM-CSF, and to a lesser degree M-CSF, enhanced survival of human monocytes *in vitro* (29). Both epithelial cell- and fibroblast-conditioned medium contained GM-CSF and M-CSF activity, suggesting that the lung environment *in vivo* may be a potential source of GM-CSF and M-CSF.

Summarizing the findings of different studies in humans, it is suggested that in the lung both GM-CSF and M-CSF may enhance survival of newly arrived PBM, whereas GM-CSF (and not M-CSF) may induce the proliferation of more mature AM. Differential responses of PBM and AM on GM-CSF therapy were also observed *in vivo*, recently, although the influence on cell proliferation was not studied (30).

The data presented here show only a limited expression of Ki-67 Ag in human PBM, suggesting that PBM have only a minute proliferative activity. However, RFD9$^+$ PBM represent a small subpopulation with a much higher proliferative activity than the majority of PBM which are CD14$^+$. Furthermore, a higher percentage of AM expressed Ki-67 Ag as compared with PBM. Even the CD14$^+$ AM, which resemble PBM most, have a higher mitotic activity than PBM. It may therefore be concluded that upon leaving the circulation and entering lung tissue, monocytes enter an environment which seems to induce proliferation. This environment may be determined by local production of GM-CSF, whether or not in combination with other cytokines or tissue factors. This idea is in line with the conclusions of Nakata et al. and Itoh et al. (19,28). Future studies, which should unravel differences in local environmental factors of different organs, will lead to a better understanding of cellular processes as proliferation, maturation, differentiation and activation, and may eventually enable clinical intervention where these cellular processes are disturbed.

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REFERENCES

1. Ham AW, Cormack DH. The origins, morphologies, and functions (including immunological functions) of the cells of loose connective tissue. In: Ham AW, Cormack DH. Histology. Philadelphia: JB Lippincott Company, 1979:225-259.
2. Herscowitz HB. In defence of the lung: paradoxical role of the pulmonary alveolar macrophage. Ann Allergy 1985;55:634-648.
3. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
4. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
5. Van Furth R. Current view on the mononuclear phagocyte system. Immunobiol 1982;161:178-185.
6. Ginsel LA. Origin of macrophages. In: Horton MA, ed. Blood cell biochemistry (Volume 5; Macrophages and related cells). New York: Plenum Press, 1993:87-113.
7. Van Furth R. Cells of the mononuclear phagocyte system. Nomenclature in terms of sites and conditions. In: Van Furth R, ed. Mononuclear phagocytes. Functional aspects. The Hague: Martinus Nijhoff Publishers, 1980:1-30.
8. Blussé Van Oud Alblas A, Van Furth R. The origin, kinetics and characteristics of pulmonary macrophages in the normal steady state. J Exp Med 1979;149:1504-1518.
9. Coggle JE, Tarling JD. The proliferation kinetics of pulmonary alveolar macrophages. J Leukocyte Biol 1984;35:317-327.
10. Sawyer RT. The significance of local resident pulmonary alveolar macrophage proliferation to population renewal. J Leukocyte Biol 1986;39:77-87.
11. Golde DW, Byers LA, Finley TN. Proliferative capacity of human alveolar macrophage. Nature 1974;247:373-375.
12. Kreipe H, Radzun HJ, Heidorn K, Barth J, Kiemle-Kallee J, Petermann W, Gerdes J, Parwaresch MR. Proliferation, macrophage colony-stimulating factor, and macrophage colony-stimulating factor-receptor expression of alveolar macrophages in active sarcoidosis. Lab Invest 1990;62:697-703.
13. Bitterman PB, Saltzman LE, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication. One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. J Clin Invest 1984;74:460-469.
14. Donaldson K, Slight J, Brown DM. Effects of products from inflammatory pulmonary neutrophils on alveolar macrophage chemotaxis, spreading, and thymidine incorporation. Inflam 1989;13:443-453.
15. Gordon S, Keshav S, Chung LP. Mononuclear phagocytes: tissue distribution and functional heterogeneity. Current Opinion Immunol 1988;1:26-35.
16. Lehnert BE, Valdez YE, Lehnert NM, Park MS, Englen MD. Stimulation of rat and murine alveolar macrophage proliferation by lung fibroblasts. Am J Respir Cell Mol Biol 1994;11:375-385.
17. Chen BDM, Clark CR, Chou TH. Granulocyte/macrophage colony-stimulating factor stimulates monocyte and tissue macrophage proliferation and enhances their responsiveness to macrophage colony-stimulating factor. Blood 1988;71:997-1002.
18. Akagawa KS, Kamoshita K, Tokunaga T. Effects of granulocyte-macrophage colony-stimulating factor and colony-stimulating factor-1 on the proliferation and differentiation of murine alveolar macrophages. J Immunol 1988;141:3383-3390.
19. Nakata K, Akagawa KS, Fukayama M, Hayashi Y, Kadokura M, Tokunaga T. Granulocyte-macrophage colony-stimulating factor promotes the proliferation of human alveolar macrophages in vitro. J Immunol 1991;147:1266-1272.
20. Leenen PJM, Campbell PA. Heterogeneity of mononuclear phagocytes; an interpretive review. In: Horton MA, ed. Blood cell biochemistry (Volume 5; Macrophages and related cells). New York: Plenum Press, 1993:29-85.

21. Hoogsteden HC, Van Hal PThW, Wijkhuijs JM, Hop W, Hilvering C. Differences in expression of monocyte/macrophage surface antigens in peripheral blood and bronchoalveolar lavage cells in interstitial lung diseases. Lung 1993;171:149-160.
22. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by IL-4 depends of the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.
23. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710-1715.
24. Verheijen R, Kuijpers HJH, Van Driel R, Beck JLM, Van Dierendonck JH, Brakenhoff GJ, Ramaekers FCS. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. J Cell Sci 1989;92:531-540.
25. Munro CS, Campbell DA, Collings LA, Poulter LW. Monoclonal antibodies distinguish macrophages and epitheloid cells in sarcoidosis and leprosy. Clin Exp Immunol 1987;68:282-287.
26. Kreipe H, Radzun HJ, Heidorn K, Parwaresch MR, Verrier B, Müller R. Lineage-specific expression of c-*fos* and c-*fms* in human hematopoietic cells: discrepancies with the in vitro differentiation of leukemia cells. Differentiation 1986;33:56-60.
27. Radzun HJ, Kreipe H, Heidorn K, Parwaresch MR. Modulation of c-fms proto-oncogene expression in human blood monocytes and macrophages. J Leukocyte Biol 1988;44:198-204.
28. Itoh A, Yamaguchi E, Furuya K, Hizawa N, Ohnuma N, Kawakami Y, Kuzumaki N. Correlation of GM-CSF mRNA in bronchoalveolar fluid with indices of clinical activity in sarcoidosis. Thorax 1993;48:1230-1234.
29. Xing Z, Ohtoshi T, Ralph P, Gauldie J, Jordana M. Human upper airway structural cell-derived cytokines support human peripheral blood monocyte survival: a potential mechanism for monocyte/macrophage accumulation in the tissue. Am J Respir Cell Mol Biol 1992;6:212-218.
30. Thomassen MJ, Ahmad M, Barna BP, Antal J, Wiedemann HP, Meeker DP, Klein J, Bauer L, Gibson V, Andresen S, Bukowski RM. Induction of cytokine messenger RNA and secretion in alveolar macrophages and blood monocytes from patients with lung cancer receiving granulocyte-macrophage colony-stimulating factor therapy. Cancer Res 1991;51:857-862.

CHAPTER 5

MONOCYTES/MACROPHAGES AND GLUCOCORTICOIDS

5.1 Introduction

5.2 IL-4-induced maturation of monocytes/macrophages is inhibited by glucocorticoids

5.3 IL-1β gene expression in cultured human bronchoalveolar monocytes/macrophages from smokers and non-smokers, and modulation by IL-4 and dexamethasone

5.4 Inverse modulation of lipocortin-1 (annexin-1) and IL-1β gene expression by dexamethasone in cultured human bronchoalveolar macrophages

5.5 Functional glucocorticoid receptors in human alveolar macrophages

CHAPTER 5.1

INTRODUCTION

Nowadays, we know that modulation of cellular functions underlies the clinical and therapeutical effects of glucocorticoid treatment in inflammatory diseases. The beneficial effects of glucocorticoids in asthma result from the inhibition of the chronic airway inflammation, which is considered as a major base of the clinical symptoms, especially bronchial hyperresponsiveness. Furthermore, we understand that inflammation in asthma results from the concerted actions of a variety of different cell types including mononuclear phagocytes, lymphocytes, eosinophils, neutrophils, mast cells, epithelial cells, and others. The response of these cells to glucocorticoids requires the presence of functional and specific glucocorticoid receptors. A still increasing number of inflammatory cell functions is being shown to be affected by glucocorticoids.

In this chapter, we shall focus on cellular effects of glucocorticoids in two representatives of the mononuclear phagocyte system, i.e. peripheral blood monocytes and alveolar macrophages, obtained from healthy volunteers. These cells are believed to play a central role in different aspects, i.e. initiation, perpetuation and inhibition, of inflammatory processes. Therefore, mononuclear phagocytes represent an important target cell for glucocorticoids. A better understanding of the cellular working mechanisms of glucocorticoids may enable us to find new ways to by-pass their unwanted "side" effects, or to recognize glucocorticoid-resistent individuals.

In this chapter, we aim

1. to study the effects of glucocorticoids on the expression of cell surface antigens in blood monocytes *in vitro*,
2. to study the effects of glucocorticoids on the gene expression of IL-1β (a pro-inflammatory cytokine) and lipocortin-1 (a potential anti-inflammatory protein) in blood monocytes and alveolar macrophages *in vitro*,
3. to compare IL-4 effects with the anti-inflammatory effects of glucocorticoids, because also IL-4 has been resorted to exhibit various potential anti-inflammatory effects,
4. to demonstrate the presence of specific glucocorticoid receptors in human alveolar macrophages.

We studied the effects of the synthetic glucocorticoid dexamethasone on the expression of cell membrane antigens in peripheral blood monocytes. Upon culture of these cells, changes in the expression of cell membrane antigens appeared to correlate with cell maturation. In some maturation experiments, we shall show the combined effects of both glucocorticoids and IL-4, because this cytokine was found to accelerate monocytic maturation. The results of these combined effects will be discussed in the light of the recently described evidence of cross-talk between the cytokine and the steroid signalling pathways. Furthermore, we will show that human alveolar macrophages may be an actual cellular target of glucocorticoids. Both the application of the glucocorticoid antagonist RU 38486 and the development of a specific receptor assay provided conclusive evidence of receptor-mediated effects of glucocorticoids in human alveolar macrophages. Both IL-1β and lipocortin-1 gene expression were modulated by glucocorticoids. Since some of the pleiotropic IL-4 effects appear to be anti-inflammatory, we compared those effects with the anti-inflammatory effects of glucocorticoids.

IL-4-INDUCED MATURATION OF MONOCYTES/MACROPHAGES IS INHIBITED BY GLUCOCORTICOIDS*

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*This chapter is submitted for publication.

SUMMARY

In vitro culture of peripheral blood monocytes (PBM) induces changes in the morphology and functions of these cells. The nature of these changes suggests that cultured PBM progressively exhibit characteristics of more mature mononuclear phagocytes. In this study, the expression of cell surface antigens (Ag) and the intracellular enzyme acid phosphatase was studied in various maturation stages of mononuclear phagocytes, i.e. in both freshly isolated and cultured human PBM, and freshly isolated alveolar macrophages (AM). Furthermore, we tried to modulate the maturation-associated changes with IL-2, IL-4, and dexamethasone.

Almost all freshly isolated PBM expressed the CD14 surface membrane determinant, but were negative for RFD9. In contrast, almost all AM were positive for RFD9, whereas only a minority was positive for CD14. Both cell types were negative for CD23. Furthermore, AM were strongly positive for endogenous acid phosphatase, whilst PBM were virtually negative. Upon culturing in control medium, PBM became progressively positive for RFD9 and part of the PBM lost the CD14 Ag. In addition, PBM became positive for endogenous acid phosphatase. These data strongly suggested *in vitro* maturation of PBM towards a cell type resembling AM. IL-4 induced both an accelerated maturation of PBM and a transient expression of CD23 Ag, whereas IL-2 had no effects. Maturation was partly inhibited by dexamethasone. Dexamethasone inhibited, in a dose-dependent way, also the IL-4-accelerated maturation and IL-4-induced expression of CD23 Ag, and these effects were counteracted by the glucocorticoid antagonist RU 38486. This suggests that glucocorticoid receptors are involved in mediating the antagonizing influence of dexamethasone on the IL-4 effects.

In conclusion, our findings support the conclusions that maturation of PBM can be achieved *in vitro*, that IL-4 accelerates and dexamethasone slows down this maturation process, whilst dexamethasone is also able to counteract the IL-4 effects. Maturation of monocytic cells *in vivo* may therefore represent a fine-tuned process, regulated by multiple factors including IL-4 in concert with steroid hormones. It is hypothesized that interactions between the signalling pathways of IL-4 and glucocorticoids may occur at the level of transcription factors. One of the working mechanisms of therapeutical glucocorticoids in inflammatory diseases, in which mononuclear phagocytes play a role as immune effector cells, may be modulation of the maturation of monocytic cells.

INTRODUCTION

Both peripheral blood monocytes (PBM) and tissue macrophages belong to the mononuclear phagocyte system, which is widely distributed throughout many tissues [1,2]. Current concepts on the origin of tissue macrophages suggest that these cells derive from PBM [1-4]. PBM are able to leave the circulation and enter almost every tissue [4]. Upon leaving the vasculature, PBM mature into tissue macrophages, and acquire, depending on the tissue in which they reside, a site-specific morphological and immunological phenotype

[5,6]. Alveolar macrophages (AM) are considered to be more mature representatives of the mononuclear phagocyte system and are mainly derived from PBM [3,7,8]. Both the morphological and immunological phenotype and the functions of AM differ from PBM [6,9-11]. It is believed that during their migration from blood to alveoli PBM undergo a maturation process resulting in a change of their phenotype. The precise nature of the *in vivo* stimuli leading to these changes is still unknown.

In vitro studies have suggested that cytokines, e.g. IL-4, GM-CSF and IFN-γ, may be among the central factors that direct the changes in phenotype and function which accompany maturation [12-16]. Comparatively, local *in vivo* production of these cytokines may play an important part in the physiological maturation of PBM in tissues, e.g. the lung. Furthermore, at sites of inflammation or immune responses, inflammatory cytokines and mediators may also alter the phenotype, and, concomitantly, the function of monocytic cells [6]. In addition, *in vitro* studies have shown that maturation of PBM can be influenced by glucocorticoids [17-20]. Glucocorticoids may inhibit the production of inflammatory mediators and cytokines, as a result of which an indirect effect of these hormones on PBM is imaginable [17]. However, also a direct influence of glucocorticoids on the maturation of PBM has been suggested [18-20]. Further studies are needed to understand the various mechanisms underlying the maturation of mononuclear phagocytes.

The available findings and hypotheses prompted us to study in more detail the effects of human rIL-4 and dexamethasone on the maturation of PBM *in vitro*. We also studied the effects of rIL-2, because functional IL-2 receptors have been described on freshly isolated PBM recently [21,22]. *In vitro* culture of PBM is known to be associated with maturation and may therefore be considered as a model to study maturation associated changes in phenotype and effects of various stimuli [12,23,24]. Here, the immunological phenotype and function of PBM during culture were compared with those of AM. The results of this study demonstrate that IL-4 accelerates the maturation of PBM *in vitro*, whereas glucocorticoids inhibit maturation and counteract the IL-4 effects. The results support the hypothesis that both cytokines and steroid hormones are involved in a balanced regulation of the phenotype and function of monocytic cells *in vivo*.

MATERIALS AND METHODS

Isolation of PBM

Human peripheral blood monocytes were isolated from 500 ml blood from healthy volunteers as described earlier [25]. Briefly, mononuclear cells were separated by density centrifugation with a blood component separator. Next, the mononuclear cells were fractionated into lymphocytes and monocytes by centrifugal elutriation. The monocyte preparation was over 95% pure and contained more than 98% viable cells as judged by trypan blue exclusion. Lymphocytes and granulocytes constituted less than 2% and 3% of the monocyte preparation, respectively.

Isolation of AM

All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. Bronchoalveolar lavage (BAL) was performed in 10 healthy, non-smoking volunteers after premedication with thiazinamium and local anesthesia using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection, the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles, whereafter the BAL cells were isolated by centrifugation at 4°C at a force of 400 g. More than 90% of the BAL cells appeared to be macrophages as judged by May-Grünwald Giemsa staining. Before analysis of the surface membrane determinants the cells were washed twice with a phosphate-buffered saline solution (PBS; 300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated bovine serum albumin (BSA) (Organon Teknika, Oss, The Netherlands).

Detection of surface membrane determinants

For immunofluorescence stainings of PBM and AM the following monoclonal antibodies (mAb) were used: CD13 [Q20 (IgG2a); Dr. C.E. van der Schoot, Amsterdam, The Netherlands], CD14 [UCHM1 (IgG2a); Dr. P.C.L. Beverly, London, U.K.], CD23 [Tü1 (IgG1); Biotest, Dreieich, Germany] and RFD9 (IgG1; Dr. L.W. Poulter, London, U.K.). Isotype matched antibodies which were directed against idiotypic determinants on a B cell lymphoma cell line and did not react with monocytes were used as control antibodies. Fifty µl of the PBM cell suspension (2 x 10^6 cells/ml) was incubated for 30 min at 4°C with 50 µl of one of the, optimally titrated, mAb. Incubation was performed in wells of 96-wells microtiter plates. After three washings PBM were incubated with a FITC-labeled goat anti mouse-immunoglobulin antiserum (Nordic, Immunological Laboratories, Tilburg, The Netherlands) for 30 min at 4°C. After another three washings cell pellets were resuspended for analysis of the fluorescence intensity by means of the FACScan (Becton Dickinson). The fluorescence intensities are expressed as a ratio relative to the background fluorescence intensity of cells stained with an isotype matched control antibody, where a value of 1.0 reflects fluorescence equivalent to background.

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

All standard washings were performed with PBS/0.5% BSA/0.5% sodium azide.

Monocyte cultures

PBM were cultured in a modified Iscove's medium as described previously [26], in which BSA was replaced by human serum albumin. In some experiments this medium was supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin-free (defined as less than 1.0 ng/ml of endotoxin as quantified by the Limulus amebocyte lysate assay). PBM (4 x 10^6/ml) were cultured at 37°C, 5% CO_2 and 100% humidity in Teflon bags (Janssen's MNL, St-Niklaas, Belgium) for 1 to 6 days. For detection of surface membrane determinants, PBM were harvested from the Teflon bags and culture supernatant was removed after centrifugation. Subsequently, the PBM were washed twice with PBS/0.5% BSA/0.5% sodium azide. The viability as determined by trypan blue exclusion exceeded 85%. No significant

differences in the numbers of cells recovered after culture in the presence of different supplements could be observed.

Cytokines

rIL-4 was a generous gift from Dr. K. Arai (DNAX Research Institute, Palo Alto, CA, USA). rIL-2 was kindly provided by Eurocetus (Amsterdam, The Netherlands).

Anti rIL-4

A polyclonal rabbit anti-IL-4 antiserum was used. This antiserum, as has been described earlier [27], blocks the biologic activity of IL-4.

Dexamethasone

Dexamethasone micronisatum used in these experiments was obtained from Duchefa b.v. (Haarlem, The Netherlands). A stock solution of 2×10^{-3} M dexamethasone in ethanol was prepared. The concentration of ethanol during culture was less than 0.25% in each experiment.

RU 38486

The glucocorticoid antagonist RU 38486 [28] was kindly provided by Roussel Uclaf (Romainville, France). A stock solution of 2×10^{-3}M RU 38486 in ethanol was prepared. The concentration of ethanol during culture was less than 0.25% in each experiment.

Staining for endogenous acid phosphatase

Activity of endogenous acid phosphatase was determined using the Burnstone method [29]. Briefly, cytocentrifuge preparations of AM, freshly isolated and cultured PBM were incubated in a mixture of naphthol AS-BI phosphate (Sigma, St. Louis, MO) as substrate and diazotized pararosaniline (Sigma) as coupling agent in acetate-barbiturate buffer (pH 5.0) at 37°C for 30 min. After washing in PBS the slides were mounted in glycerin-gelatin (Merck, Darmstadt, Germany) and evaluated using light microscopy.

RESULTS

Immunophenotype of freshly isolated PBM and AM

The most prominent differences in immunophenotype between PBM and AM concerned the expression of RFD9 and CD14 (UCHM1) antigen (Ag) (Table 1). RFD9 was virtually absent on the PBM, whilst 85% of the AM expressed this membrane antigen. In contrast, most of the PBM were positive for CD14 (UCHM1), whereas only 23% of the AM was positive.

Table 1. Percentages of freshly isolated PBM and AM positive for various cell membrane determinants.

	PBM[a]	AM[b]
CD14 (UCHM1)	87 ± 9[c]	23 ± 15
CD23 (Tü1)	0 ± 1	0 ± 1
RFD9	0 ± 1	85 ± 23

a. Freshly isolated peripheral blood monocytes were stained with specific mAb and FITC-labeled goat anti mouse-immunoglobulin antiserum as detailed under *Materials and Methods*. Cells were evaluated with a FACScan.

b. Freshly isolated alveolar macrophages (AM) were stained as described in footnote a. Cells were evaluated using a Zeiss fluorescence microscope equipped with phase-contrast facilities.

c. Data are expressed as mean ± SD of 10 independent experiments.

Effects of IL-2, IL-4 and dexamethasone on the expression of various surface membrane determinants by PBM

PBM were cultured for 60 hours in the presence of either IL-4 (100 U/ml), IL-2 (1000 U/ml) or dexamethasone (5×10^{-7} M), or without either of these supplements. After culture without any supplements, the expression of RFD9 was greatly enhanced (Figure 1 and Table 2). This increase was consistently seen in all our experiments irrespective of donor (n = 6). It could only partially be explained by an increase in cell-size as the forward light scatter signal was changed to a very small extent (data not shown). In most experiments, analysis of the histograms showed the loss of CD14 Ag expression by 35-40% of the PBM after culture (Figure 1 and Table 2). This finding appeared to be donor dependent as the loss of CD14 expression was not observed in all experiments. No differences between $CD14^+$ and $CD14^-$ monocytes were observed when forward and sideward scatter signals were compared.

When IL-4 was added to the culture medium, the changes in CD14 and RFD9 Ag expression were much more pronounced. All our experiments revealed one homogenous $CD14^-$ population after culture in the presence of IL-4. In addition to the earlier reported upregulation of CD23 Ag by IL-4 [30], which was also seen in our experiments, IL-4 also upregulated the CD13 Ag [31].

IL-2 had no effects on the expression of CD14, CD23 or RFD9 Ag (Table 2). Culture of PBM in the presence of dexamethasone (5×10^{-7} M) did not result in the expression of RFD9 Ag. In addition, the whole monocyte population exhibited a relatively lower expression of CD14 Ag, but a CD14-negative subpopulation was not observed (Figure 1 and Table 2).

The above-mentioned results were reason for us to study the maturation of PBM at different times during culture, either with or without addition of IL-4 or dexamethasone, separately or in combination with each other.

Table 2. Effects of *in vitro* culture and the influence of cytokines and glucocorticoids on the expression of surface membrane markers on human blood monocytes.

	culture time (hours)	CD14 (UCHM1)	CD23 (Tü1)	RFD9 (not clustered)
Control	0	17.0[a]	0.6	1.2
Control	60	15.9/1.7[b]	1.1	6.2
IL-4 (100 U/ml)	60	1.5	2.5	10.5
IL-2 (1000 U/ml)	60	21.5/2.0[c]	0.8	6.7
Dexamethasone (5×10^{-7} M)	60	5.8	0.9	1.5

a. Data are expressed as relative fluorescence of the whole monocyte population, i.e. the ratio between the mean linear fluorescence intensity of cells labeled with the relevant antibody and the mean fluorescence intensity of cells labeled with the isotype-matched control antibody. A representative experiment out of 4 is shown.

b. Calculation of one value of relative fluorescence is not possible because both a bright positive (66% of the whole cell population) and a negative (34% of the whole cell population) monocyte population were observed.

c. Calculation of one value of relative fluorescence is not possible because both a bright positive (70% of the whole cell population) and a negative (30% of the whole cell population) monocyte population were observed.

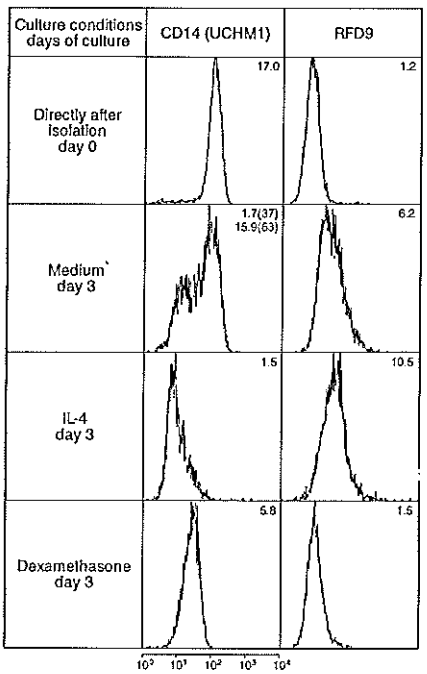

Figure 1. Effect of IL-4 or dexamethasone on the expression of the CD14 and RFD9 Ag on human monocytes after 3 days of culture. The fluorescence intensity of 7500 cells was determined on a log scale with a FACScan. On the abscissa and on the ordinate are given fluorescence intensity and number of cells, respectively. Relative fluorescence was determined as described in the footnotes of table 2, and is stated in the right upper corner of each histogram. When a histogram revealed both a positive and negative population, relative fluorescence of both these populations was determined. In parentheses is shown the percentage of negative and positive cells, respectively. One representative experiment out of four is shown.

Influence of IL-4 and dexamethasone on the expression of the CD14 Ag on PBM during a culture period of 6 days

The down-regulation of the determinant recognized by CD14 was already observed after 1 day of culture in the presence of IL-4 (100 U/ml) (Figure 2). This down-regulation became more pronounced upon further culture. Even after 6 days of culture re-expression of the CD14 Ag could not be detected, indicating that this IL-4 effect was not transient. This IL-4-induced down-regulation could be blocked by an anti-IL-4 antiserum, indicating the specificity of the down-regulation by IL-4. Culture with mock-medium typically revealed that only part of the cell population lost the CD14 Ag. Notably, this negative subpopulation could be demonstrated until 2-3 days after start of the culture, but could not be observed anymore after 6 days of culture. Only one positive population was seen at that point of time.

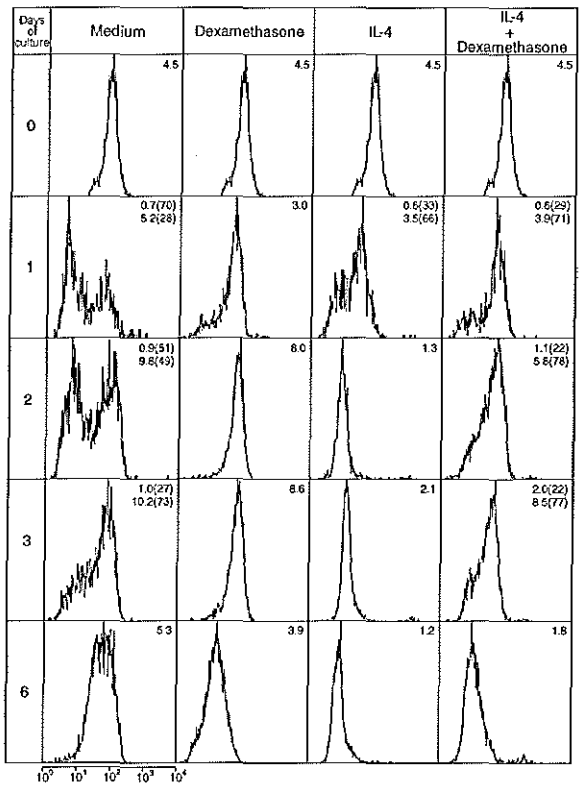

Figure 2. Time course of the inhibition of IL-4-induced down-regulation of the CD14 Ag expression on human monocytes by dexamethasone. Monocytes were cultured for 1, 2, 3 and 6 days without or with 100 U IL-4/ml and/or dexamethasone (5×10^{-7} M). Fluorescence intensity was determined as described in the legend of figure 1. On the abscissa and on the ordinate are given fluorescence intensity and number of cells, respectively. Relative fluorescence was determined as described in the footnotes of table 2, and is stated in the right upper corner of each histogram. When a histogram revealed both a positive and negative population, relative fluorescence of both these populations was determined. In parentheses is shown the percentage of negative and positive cells, respectively. One representative experiment out of three is shown.

When dexamethasone (5×10^{-7} M) was added to the culture medium the appearance of a negative subpopulation was less pronounced and usually seen on day 1 of culture only (Figure 2). Culture of PBM in the presence of both dexamethasone and IL-4 revealed that

the IL-4-induced down-regulation of the CD14 Ag was inhibited. The vast majority of the cells retained the CD14 Ag. This inhibition by dexamethasone was most evident during the first 3 days of culture. Furthermore, this inhibition was dependent on the dexamethasone concentration and was virtually absent when dexamethasone was used in a concentration of 5×10^{-9} M (data not shown). RU 38486 (5×10^{-6} M), an antagonist of glucocorticoids, restored the IL-4-induced down-regulation of the CD14 Ag when cells were cultured with IL-4 and dexamethasone (Table 3).

Influence of IL-4 and dexamethasone on the expression of the CD23 Ag on PBM during a culture period of 6 days

Freshly isolated PBM did not express the CD23 Ag and upon culture this antigen was not acquired spontaneously (Figure 3). However, it has been shown that IL-4 is able to induce its expression [26]. Here we confirm that IL-4 (100 U/ml) is able to induce a transient expression of this antigen. Culture of PBM in the presence of dexamethasone (5×10^{-7} M) was without effect on the expression of the CD23 Ag. On the other hand, induction of CD23 Ag by IL-4 was blocked by dexamethasone. When PBM were cultured with IL-4, dexamethasone and RU 38486, the IL-4-induced expression was restored (Table 3).

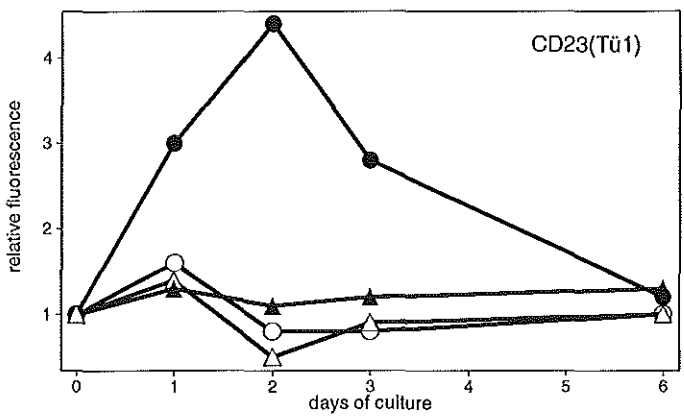

Figure 3. Dexamethasone inhibits the IL-4-induced expression of CD23 Ag on human monocytes. Cells were cultured in medium alone (○), or in medium supplemented with either 100 U IL-4/ml (●), 5×10^{-7} M dexamethasone (△) or both IL-4 and dexamethasone (▲). Relative fluorescence was determined as described in the footnotes of Table 2. A representative experiment out of three is shown.

Influence of IL-4 and dexamethasone on the expression of the antigen recognized by RFD9 on PBM during a culture period of 6 days

The antigen recognized by RFD9 was not present on freshly isolated PBM. Upon

culture PBM became progressively positive for this marker (Figure 4). The appearance of this marker was greatly accelerated by addition of IL-4 to the culture medium. From these IL-4 experiments there seemed to exist a plateau-value for the expression of RFD9 Ag and this value was already reached after 3 days of culture. This level of expression was not (yet) reached after 6 days of culture in medium without IL-4. Dexamethasone alone appeared to have only a minor effect during the first 3 days, but the expression of RFD9 Ag at day 6 was eventually half the expression of the control cells. On the other hand, dexamethasone totally blocked the accelerated appearance of RFD9 Ag induced by IL-4. This inhibitory effect of dexamethasone on the IL-4-induced expression of RFD9 was dependent on the concentration of dexamethasone (data not shown) and could be antagonized by RU 38486 (Table 3).

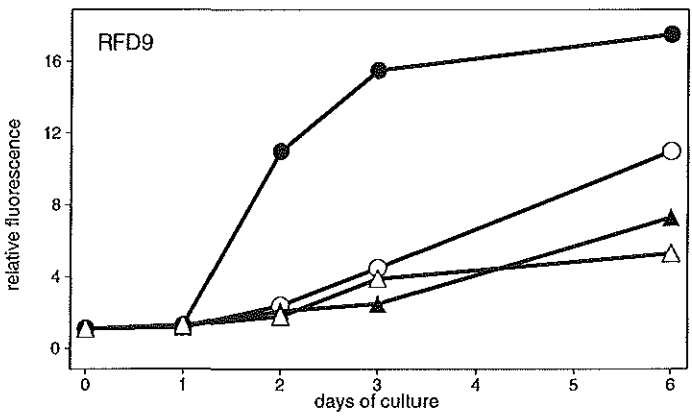

Figure 4. Kinetics of the effects of IL-4 and dexamethasone on the expression of RFD9 Ag on human monocytes. Cells were cultured in medium alone (○), or medium supplemented with either 100 U IL-4/ml (●), 5 x 10^{-7} M dexamethasone (△) or both IL-4 and dexamethasone (▲). Relative fluorescence was determined as described in the footnotes of Table 2. One representative experiment out of three is shown.

Influence of IL-4 and dexamethasone on the expression of endogenous acid phosphatase in PBM

It has been shown that endogenous acid phosphatase is abundant in more mature monocytes/macrophages [32]. Human alveolar macrophages were highly positive for endogenous acid phosphatase (Figure 5A), whilst freshly isolated PBM exhibited only very low levels of this enzyme (Figure 5B). *In vitro* culture of PBM revealed that these cells became progressively positive for endogenous acid phosphatase (Figure 5C), suggesting maturation of the cells. Activity of this enzyme could be enhanced by culture in the presence of IL-4 (100 U/ml) (Figure 5D). Dexamethasone (5 x 10^{-7} M) prevented the additional enhancement of enzyme activity caused by IL-4 (Figure 5E). On the other hand, RU 38486 (5 x 10^{-6} M) restored the influence of IL-4 on acid phosphatase activity when cells were cultured with IL-4 and dexamethasone (Figure 5F).

Table 3. Effect of dexamethasone on the IL-4-induced changes in the expression of surface membrane determinants on human monocytes and the antagonizing effect of RU 38486.

		control			IL-4 (100 U/ml)			IL-4 (100 U/ml) + dexamethasone (5×10^{-7} M)			IL-4 (100 U/ml) + dexamethasone (5×10^{-7} M) + RU 38486 (5×10^{-6} M)		
	day 0	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3	day 1	day 2	day 3
CD13 (Q20)	36.4[a]	92.7	67.0	70.3	216.0	177.1	127.2	117.4	74.6	36.0	160.6	173.9	122.2
CD14 (UCHM1)	28.8	22.8	40.5	16.4	19.7	4.8	1.6	19.6	16.0	6.5	15.6	5.3	1.6
CD23 (Tü1)	1.2	1.4	1.6	1.2	3.5	6.5	3.2	1.4	1.6	1.4	3.4	6.1	6.1
RFD9	1.3	1.2	3.3	6.0	1.2	8.3	14.1	1.4	2.5	2.4	1.3	7.7	19.5

a. Data are expressed as the relative fluorescence described in the footnotes of Table 2. One out of two separate experiments is shown.

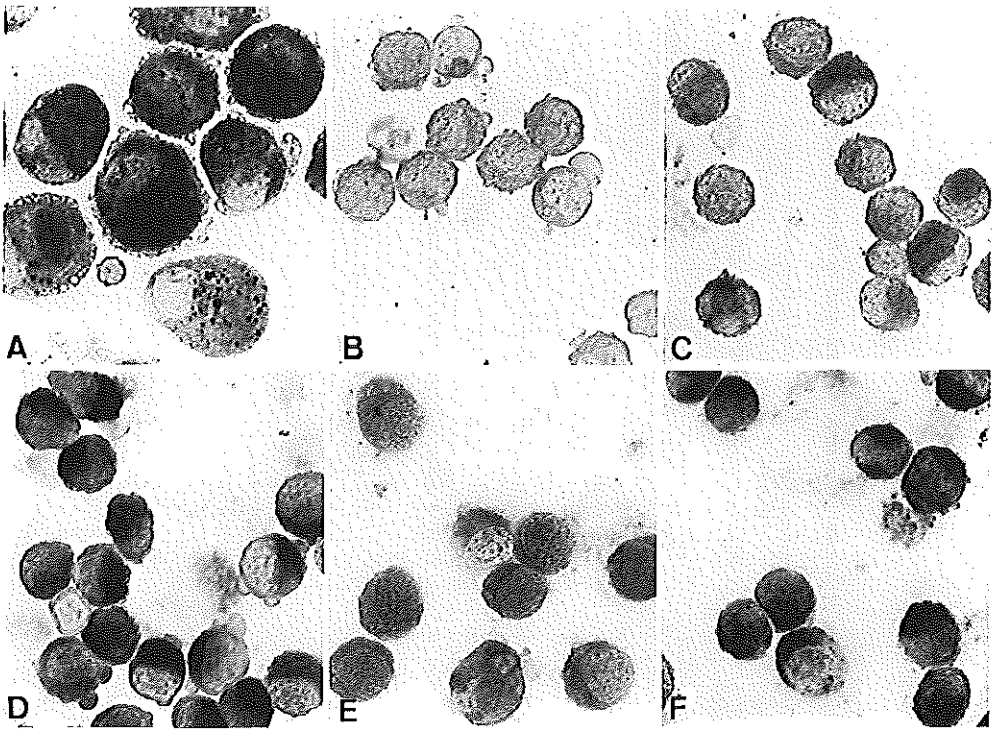

Figure 5. Effects of IL-4, dexamethasone and RU 38486 on the activity of intracellular acid phosphatase in human blood monocytes. Freshly isolated human alveolar macrophages were highly positive for endogenous acid phosphatase (A), whilst freshly isolated blood monocytes (B) were virtually negative for this enzyme. After 2 days of culture, enzyme activity in blood monocytes was enhanced (C). Further enhancement was achieved after 2 days of culture with IL-4 (100 U/ml) (D). This IL-4-induced increase could be antagonized by dexamethasone (5×10^{-7} M) (E), whereas RU 38486 (5×10^{-6} M) restored full IL-4-induced enzyme activity (F). Culture in the presence of RU 38486 (5×10^{-6} M) alone was without effect (data not shown).

DISCUSSION

The present study indicates that interactions between cytokines and glucocorticoids may occur at the level of the maturation of mononuclear phagocytes. We describe that, *in vitro*, IL-4 modulates the immunophenotype and function of PBM, resulting in more mature cells which resemble AM. Dexamethasone is shown to antagonize these IL-4 effects. The significance of these observations is twofold. First, a better understanding of the maturation process of mononuclear phagocytes may lead to possibilities to influence this process. This may be important in the treatment of diseases, in which cellular maturation is derailed or affected. Second, they stress the importance of "cross-talk" between 2 different signalling systems: that of steroid hormones and that of cytokines [33,34].

The maturation of mononuclear phagocytes was studied here by means of the expression of cell membrane markers and endogenous acid phosphatase. Freshly isolated PBM were positive for CD14 and negative for both RFD9 and endogenous acid phosphatase, whereas AM were positive for RFD9 and the phosphatase. A small subpopulation of the AM expressed the CD14 Ag. These differences seem to indicate that the phosphatase and RFD9 Ag are up-regulated when PBM leave the circulation, whilst CD14 Ag is down-regulated. During *in vitro* culture, PBM gradually expressed the RFD9 Ag and part of PBM lost the CD14 Ag, suggesting *in vitro* maturation. The *in vitro* down-regulation of CD14 Ag in the absence of cytokines, has also been described previously [35]. Bažil et al showed in their study, just as we did in ours, that the spontaneous decrease in CD14 Ag expression appeared to be donor-dependent [35]. Other studies observed upregulation [36,37] or unchanged expression [38] of the CD14 Ag. These discrepancies may result from the choice of monoclonal antibody, as the glycoprotein recognized by CD14 mAb has 3 or 4 distinct epitopes [39]. Here we used UCHM1, as this mAb yielded the best way to discriminate between PBM and AM. Furthermore, the discrepancies may result from differences in the culture medium, the way of isolating PBM or the purity of the analyzed cells. The PBM used here were highly purified according to a procedure which did not activate the cells [40], and they were cultured under serum-free conditions, ruling out influences of unidentified serum factors on maturation.

In sarcoidosis, local production of IFN-γ has been suggested to cause down-regulation of the CD14 Ag on AM [41], as IFN-γ has been shown to down-regulate this antigen *in vitro* [35,42,43]. Here we showed that also IL-4 is able to down-regulate the CD14 Ag, adding proof that some IFN-γ effects are similar [16] to IL-4 effects, although in other aspects these cytokines appear to have antagonizing effects [16]. This IL-4-induced down-regulation of CD14 Ag has been reported previously, although the investigators did not interpret this finding in terms of PBM maturation [44]. Te Velde et al mentioned the influence of IL-4 on the maturation of PBM [13]. Here, we confirm her findings, and we further show that 2 other features of maturation, the expression of RFD9 Ag and endogenous acid phosphatase, occur faster and are more pronounced in the presence of IL-4.

Thinking of maturation as an irreversible process, it was surprising to see in our *in vitro* study that after prolonged culture without IL-4, PBM re-expressed CD14 Ag comparable to expression directly after isolation, despite high expression of RFD9 Ag and acid phosphatase. In view of the recently proposed functions of CD14 Ag (see below), also this finding may indicate that CD14 is not an ideal maturation marker. Probably, expression of CD14 Ag represents a certain functional stage, whether or not associated with maturation.

CD14 mAb recognize a 55 kD glycoprotein. Because it maps to chromosome 5 within a region containing other genes encoding cytokines (IL-3, IL-4, IL-5, GM-CSF, M-CSF) and receptors (c-fms), it may function as a receptor important for myeloid differentiation [39,45,46]. It is absent from stem cells, myeloid cells of very early differentiation states and some mature tissue macrophages [46]. On the other hand, it has been reported that the protein may be involved in phagocytosis [47]. Recently, it was suggested that the CD14 Ag could function as a receptor for complexes of LPS and LPS binding protein

[39,48,49]. The IL-4-induced down-regulation of the CD14 Ag may explain why IL-4 inhibits LPS-induced secretion of IL-1β, TNF-α and IL-6 [50,51]: down-regulation of the receptors for complexes of LPS and LBP may thus lead to LPS-unresponsiveness.

Another role for CD14 Ag in the regulation of T cell proliferation has been proposed by Lue et al [52]. They suggested that monocytes may deliver a negative signal to T cells through interaction with a putative ligand for CD14 Ag on T cells. If their model is correct, one should expect that IL-4 is able to intensify T cell proliferation as a result of diminished negative regulatory monocyte-T cell interactions.

Originally, RFD9 has been described to recognize an antigen present on germinal center and granuloma macrophages [53,54]. The function of this antigen has not been established yet, but from our studies the antigen may be considered as a maturation marker. Although this antigen has been reported by Noble et al [54] to be present on only a minority (18 ± 8%) of macrophages in BAL fluid, our study demonstrates that virtually all AM are positive for this marker and that it makes a clear distinction between PBM, which are all negative, and AM. This apparent discrepancy in results may be due to differences in experimental conditions, as in our study unfixed cells were analyzed in suspension using immunofluorescence staining, whilst in other studies immunoperoxidase staining was performed on fixed samples.

IL-4 has been shown to be a cytokine with pleiotropic biologic effects, also affecting monocytes. Te Velde et al indicated that IL-4 seems to be able to induce monocyte maturation [13]. On the other hand, IL-4 is also able to modulate the phenotype and function of monocytes, paradoxically resulting in features of both activation (increased expression of MHC class II Ag [13], increased expression of cell-membrane aminopeptidase-N [31], increased antigen-presenting capacity [55] and induction of FcεRIIb [30]) and de-activation (inhibition of superoxide production [56], inhibition of the release of PGE_2 [57], IL-1β, IL-6 [50] and TNF-α [50,51]). Here we provide further evidence for a role of IL-4 in the maturation of monocytes. These IL-4 effects seemed to be long-lasting, as they could still be observed after 6 days of culture. This is in contrast with the transient IL-4 effects on the expression of FcεRIIb [30] and aminopeptidase-N [31], which are maximum after 2-3 days of culture. These findings suggest two working mechanisms of IL-4 *in vitro*. Effects on cell maturation seem to be sustained, whereas influence on the activation-state of PBM seems to be transient.

Glucocorticoids are hormones with potent anti-inflammatory actions, which mainly result from either a decreased or increased transcription of inflammatory and anti-inflammatory proteins, respectively [58]. Recently, it was suggested that glucocorticoids may modulate monocyte differentiation [18-20]. Using mAb against cell membrane determinants we have provided further evidence that monocyte maturation is inhibited by dexamethasone. In our study, also the IL-4-accelerated maturation and the IL-4-induced expression of CD23 Ag could be antagonized by dexamethasone in a dose-dependent way. These results suggest that also *in vivo* the concerted actions of glucocorticoids and cytokines may regulate the maturation and activation of mononuclear phagocytes. It seems likely that dexamethasone exerts its effects via glucocorticoid receptors, as RU 38486 could effectively reverse the influence of dexamethasone on the IL-4 effects. Recently, it

was suggested that the regulation of transcription mediated by glucocorticoids may interact with various cytokine effects [33,34,59,60]. The signal transduction pathway of cytokines is thought to include the phosphorylation and activation of transcription factors [61]. Part of the "cross-talk" between the glucocorticoid and cytokine actions has been shown to result from the interaction of transcription factors with glucocorticoid receptors [60,62]. Recent studies also reported the interaction between IL-4 and glucocorticoid effects [63,64]. Zubiaga et al showed that IL-4 antagonized the glucocorticoid-induced apoptosis of murine Th2 cells, and that protein kinase C may be involved [63]. Also synergism between IL-4 and glucocorticoids has been reported [64], underlining the complexity of "cross-talk". Therefore, further studies are needed to unravel the precise mechanism of the IL-4/dexamethasone antagonism observed here, and should focus on the phosphorylation of transcription factors and their interaction with glucocorticoid receptors.

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REFERENCES

1. Van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull WHO 1972;46:845-852.
2. Van Furth R. Current view on the mononuclear phagocyte system. Immunobiol 1982;161:178-185.
3. Blussé van Oud Alblas A, van der Linden-Schrever B, van Furth R. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intra-alveolar administration of aerosolized heat-killed BCG. Am Rev Respir Dis 1983;128:276-281.
4. Gordon S, Keshav S, Chung LP. Mononuclear phagocytes: tissue distribution and functional heterogeneity. Curr Opin Immunol 1988;1:26-35.
5. Franklin WA, Mason DY, Pulford K, Falini B, Bliss E, Gatter KC, Stein H, Clarke LC, McGee JO'D. Immunohistological analysis of human mononuclear phagocytes and dendritic cells by using monoclonal antibodies. Lab Invest 1986;54:322-335.
6. Hance AJ, Douches S, Winchester RJ, Ferrans VJ, Crystal RG. Characterization of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. J Immunol 1985;134:284-292.
7. Bitterman PB, Salzman LE, Adelberg S, Ferrans VJ, Crystal RG. Alveolar macrophage replication: one mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. J Clin Invest 1984;74:460-469.
8. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
9. Biondi A, Rossing TH, Bennett J, Todd RF. Surface membrane heterogeneity among human mononuclear phagocytes. J Immunol 1984;132:1237-1243.
10. Hancock WW, Zola H, Atkins RC. Antigenic heterogeneity of human mononuclear phagocytes: immunohistologic analysis using monoclonal antibodies. Blood 1983;62:1271-1279.

11. Ferro TJ, Kern JA, Elias JA, Kamoun M, Daniele RP, Rossman MD. Alveolar macrophages, blood monocytes, and density-fractionated alveolar macrophages differ in their ability to promote lymphocyte proliferation to mitogen and antigen. Am Rev Respir Dis 1987;135:682-687.
12. Akagawa KS, Kamoshita K, Tokunaga T. Effects of granulocyte-macrophage colony-stimulating factor and colony-stimulating factor-1 on the proliferation and differentiation of murine alveolar macrophages. J Immunol 1988;140:3383-3390.
13. Te Velde AA, Klomp JPG, Yard BA, de Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
14. Perussia B, Dayton ET, Fanning V, Thiagarajan P, Hoxie J, Trinchieri G. Immune interferon and leukocyte-conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytic pathway. J Exp Med 1983;158:2058-2080.
15. Becker S. Interferons as modulators of human monocyte-macrophage differentiation. I. Interferon-γ increases HLA-DR expression and inhibits phagocytosis of zymosan. J Immunol 1984;132:1249-1254.
16. Becker S, Daniel EG. Antagonistic and additive effects of IL-4 and interferon-γ on human monocytes and macrophages: effects on Fc receptors, HLA-D antigens, and superoxide production. Cell Immunol 1990;129:351-362.
17. Guyre PM, Munck A. Glucocorticoid actions on monocytes and macrophages, in Schleimer RP, Claman HN, Oronsky A (eds): Anti-inflammatory Steroid Action: Basic and Clinical Aspects. Academic Press, Inc., San Diego, 1989, p 199.
18. Rinehart JJ, Wuest D, Ackerman GA. Corticosteroid alteration of human monocyte to macrophage differentiation. J Immunol 1982;129:1436-1440.
19. Hoff T, Spencker T, Emmendoerffer A, Goppelt-Struebe M. Effects of glucocorticoids on the TPA-induced monocytic differentiation. J Leukocyte Biol 1992;52:173-182.
20. Baybutt HN, Holsboer F. Inhibition of macrophage differentiation and function by cortisol. Endocrinology 1990;127:476-480.
21. Malkovský M, Loveland B, North M, Asherson GL, Gao L, Ward P, Fiers W. Recombinant interleukin-2 directly augments the cytotoxicity of human monocytes. Nature 1987;325:262-265.
22. Ennen J, Ernst M, Flad H-D. The effect of interleukin 2 on Fc-γ receptor function of human monocytes requires specific intercellular interaction. Immunobiol 1989;179:17-32.
23. Andreesen R, Picht J, Löhr GW. Primary cultures of human blood-borne macrophages grown on hydrophobic teflon membranes. J Immunol Methods 1983;56:295-304.
24. Dougherty GJ, McBride WH. Monocyte differentiation in vitro, in Zembala M, Asherson GL (eds): Human Monocytes. Academic Press Ltd, London, 1989, p 49-58.
25. Figdor CG, Bont WS, Touw I, Roos D, Roosnek EE, de Vries JE. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. Blood 1982;60:46-53.
26. Yssel H, de Vries JE, Koken M, van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Methods 1984;72:219-227.
27. Chrétien I, van Kimmenade A, Pearce MK, Bangchereau J, Abrams JS. Development of polyclonal and monoclonal antibodies for immunoassay and neutralization of human interleukin-4. J Immunol Methods 1989;117:67-81.
28. Spitz IM, Bardin CW. Mifepristone (RU 486) - a modulator of progestin and glucocorticoid action. New Engl J Med 1993;329:404-412.
29. Burnstone MS. Histochemical demonstration of acid phosphatases with naphthol AS-phosphatases. J Nat Cancer Inst 1958;21:523-540.
30. Vercelli D, Jabara HH, Lee B-W, Woodand N, Geha RS, Leung DYM. Human recombinant interleukin 4 induces FcεR2/CD23 on normal human monocytes. J Exp Med 1988;167:1406-1416.
31. Van Hal PThW, Hopstaken-Broos JPM, Prins AA, Favalora EJ, Huijbens RJF, Hilvering C, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by interleukin-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1994;153:2718-2728.

32. Musson RA. Human serum induces maturation of human monocytes in vitro. Changes in cytolytic activity, intracellular lysosomal enzymes, and nonspecific esterase activity. Am J Pathol 1983;111:331-340.
33. Schüle R, Evans RM. Cross-coupling of signal transduction pathways: zinc finger meets leucine zipper. Trends in Genetics 1991;7:377-381.
34. Egan SE, Weinberg RA. The pathway to signal achievement. Nature 1993;365:781-783.
35. Bažil V, Strominger JL. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. J Immunol 1991;147:1567-1574.
36. Landmann R, Wesp M, Dukor P. Modulation of interferon-γ-induced major histocompatibility (MHC) and CD14 antigen changes by lipophilic muramyltripeptide MTP-PE in human monocytes. Cell Immunol 1988;117:45-55.
37. Landmann R, Ludwig C, Obrist R, Obrecht JP. Effect of cytokines and lipopolysaccaride on CD14 antigen expression in human monocytes and macrophages. J Cell Biochem 1991;47:317-329.
38. Andreesen R, Brugger W, Scheibenbogen C, Kreutz M, Leser H-G, Rehm A, Löhr GW. Surface phenotype analysis of human monocyte to macrophage maturation. J Leukocyte Biol 1990;47:490-497.
39. Ziegler-Heitbrock HWL, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today 1993;14:121-125.
40. Figdor CG, te Velde AA, Leemans J, Bont WS. Differences in functional, phenotypical and physical properties of human peripheral blood monocytes (mo) reflect their various maturation stages. In Oppenheim JJ, Jacobs DM (eds.), Leucocytes and Host Defense: Progress in Leukocyte Biology, Alan R. Liss, Inc., New York, 1986, p.301-306.
41. Hoogsteden HC, van Dongen JJM, van Hal PThW, Delahaye M, Hop W, Hilvering C. Phenotype of blood monocytes and alveolar macrophages in interstitial lung disease. Chest 1989;95:574-577.
42. Firestein GS, Zvaifler NJ. Down regulation of human monocyte differentiation antigens by interferon-γ. Cell Immunol 1987;104:343-354.
43. Jayaram Y, Buckle AM, Hogg N. The Fc receptor, FcRI, and other activation molecules on human mononuclear phagocytes after treatment with interferon-gamma. Clin exp Immunol 1989;75:414-420.
44. Lauener RP, Goyert SM, Geha RS, Vercelli D. Interleukin 4 down-regulates the expression of CD14 in normal human monocytes. Eur J Immunol 1990;20:2375-2381.
45. Goyert SM, Ferrero E, Rettig WJ, Yenamandra AK, Obata F, Le Beau MM. The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science 1988;239:497-500.
46. Goyert SM, Tesio L, Ashman LK, Ball E, Bažil V, Garrido F, Hogg N, Hořesjší V, Howell A, Jayaram Y, Look AT, Ruiz-Cabello F, Schmitt D, Schneider M, van der Schoot CE. Report on the CD14 cluster workshop. In Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr (eds). Leukocyte Typing IV. White Cell Differentiation Antigens. Oxford University Press, 1989, p 789-794.
47. Schneider EM, Lorenz J, Kögler G, Wernet P. Modulation of monocyte function by CD14-specific antibodies in vitro. In Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, von dem Borne AEGKr (eds). Leukocyte Typing IV. White Cell Differentiation Antigens. Oxford University Press, 1989, p 794-795.
48. Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. Science 1990;249:1429-1431.
49. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431-1433.
50. Te Velde AA, Huijbens RJF, Heije K, de Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990;76:1392-1397.
51. Essner R, Rhoades K, McBride WH, Morton DL, Economou JS. IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J Immunol 1989;142:3857-3861.

52. Lue K-H, Lauener RP, Winchester RJ, Geha RS, Vercelli D. Engagement of CD14 on human monocytes terminates T cell proliferation by delivering a negative signal to T cells. J Immunol 1991;147:1134-1138.
53. Munro CS, Campbell DA, Collings LA, Poulter LW. Monoclonal antibodies distinguish macrophages and epithelioid cells in sarcoidosis and leprosy. Clin Exp Immunol 1987;68:282-287.
54. Noble B, Du Bois RM, Poulter LW. The distribution of phenotypically distinct macrophage subsets in the lungs of patients with cryptogenic fibrosing alveolitis. Clin Exp Immunol 1989;76:41-46.
55. Zlotnik A, Fischer M, Roehm N, Zipori D. Evidence for effects of interleukin 4 (B cell stimulatory factor 1) on macrophages: enhancement of antigen presenting ability of bone marrow-derived macrophages. J Immunol 1987;138:4275-4279.
56. Abramson SL, Gallin JI. IL-4 inhibits superoxide production by human mononuclear phagocytes. J Immunol 1990;144:625-630.
57. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. Proc Natl Acad Sci USA 1989;86:3803-3807.
58. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
59. Cato ACB, König H, Ponta H, Herrlich P. Steroids and growth promoting factors in the regulation of expression of genes and gene networks. J Steroid Biochem Molec Biol 1992;43:63-68.
60. Ponta H, Cato ACB, Herrlich P. Interference of pathway specific transcription factors. Biochim Biophys Acta 1992;1129:255-261.
61. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253-262.
62. Pfahl M. Nuclear receptor/AP-1 interaction. Endocr Rev 1993;14:651-658.
63. Zubiaga AM, Munoz E, Huber BT. IL-4 and IL-2 selectively rescue Th cell subsets from glucocorticoid-induced apoptosis. J Immunol 1992;149:107-112.
64. Wu CY, Sarfati M, Heusser C, Fournier S, Rubio-Trujillo M, Peleman R, Delespesse G. Glucocorticoids increase the synthesis of immunoglobulin E by interleukin 4-stimulated human lymphocytes. J Clin Invest 1991;87:870-877.

IL-1β GENE EXPRESSION IN CULTURED HUMAN BRONCHOALVEOLAR MACROPHAGES FROM SMOKERS AND NONSMOKERS, AND MODULATION BY IL-4 AND DEXAMETHASONE*

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SUMMARY

Mononuclear phagocytes are key regulators of immune and inflammatory responses. This function results, to a great extent, from their ability to produce a large variety of mediators, e.g. the cytokine IL-1. It is not clear yet whether the capacity of mature mononuclear phagocytes like alveolar macrophages (AM) to release IL-1 is limited as compared with the relatively immature peripheral blood monocytes (PBM). This lack of clarity queries whether the inhibition of AM to produce IL-1 is an actual anti-inflammatory mechanism of glucocorticoids in the lung. Further studies are needed to clarify this issue.

In this study, IL-1β gene expression was studied in freshly isolated and cultured human AM, and compared with PBM and PMA/LPS-stimulated U937 cells. Furthermore, the influences of glucocorticoids and IL-4 on IL-1β gene expression were studied. AM and PBM were cultured under serum-free conditions. Northern blot analysis was used to study mRNA expression, whereas dot blot analysis was applied to semiquantitate mRNA levels.

Freshly isolated AM showed low levels of IL-1β mRNA as compared with stimulated U937 cells. However, upon culture AM exhibited a great capacity to increase IL-1β mRNA levels, which increased 64-128 fold after 1 day of culture. Thereafter, IL-1β mRNA decreased, but even after 3 days of culture levels were still higher than in freshly isolated AM. Both AM from smokers and nonsmokers exhibited comparable kinetics of IL-1β mRNA expression. Freshly isolated PBM were found to have higher IL-1β mRNA levels than freshly isolated AM, but showed a smaller increase upon culture. Furthermore, this increase in PBM was not as long lasting as in AM. Both glucocorticoids and IL-4 were able to inhibit the culture-associated increase in IL-1β mRNA levels. The glucocorticoid-induced inhibition was counteracted by the glucocorticoid antagonist RU 38486, indicating that this inhibition was mediated via specific glucocorticoid receptors.

These results show conclusively the relatively great capacity of AM to increase their expression of the IL-1β gene, and stress the potential role of AM in inflammatory lung diseases. Part of the anti-inflammatory mechanisms of glucocorticoids can be explained by their influence on IL-1β mRNA expression in AM. The effects of IL-4 on IL-1β mRNA expression stress the potential role of IL-4 as an anti-inflammatory cytokine *in vivo*.

INTRODUCTION

Initially, IL-1 was described as a factor in culture supernatants of human peripheral blood adherent leukocytes that promoted thymocyte proliferation (1). Then it was found that monocytic cells rather than lymphocytes produced this factor, and that besides thymocytes also other target cells are susceptible to IL-1 (2). Nowadays, the IL-1 family, which includes IL-1α, IL-1β and IL-1RA, is known to have a broad range of actions in inflammatory and immune responses, and to be produced by a diverse array of cell types and tissues (3,4).

AM have been suggested to play an important role in the pathogenesis of several inflammatory lung diseases (5,6). On the one hand, AM may downregulate local

inflammation in the lung (7), e.g. through the production of IL-1RA (8). On the other hand, AM may exhibit critical pro-inflammatory activities. Direct tissue injury may result from secretion of proteolytic enzymes and oxygen radical species (9). Furthermore, AM are able to secrete powerful pro-inflammatory cytokines as TNF and IL-1 (3). These two cytokines act in a synergistic fashion in inducing inflammation, shock, or even death. Initially, the proinflammatory activities of AM appeared relatively limited, as the production of IL-1β by both freshly isolated and LPS-stimulated AM was found to be much smaller than by PBM (10,11). However, recent studies showed that AM contain a relatively large reservoir of intracellular pro-IL-1β after stimulation with LPS. These findings turned AM again into potential, powerful inducers of inflammation, and stressed that differences in IL-1 production by freshly isolated AM and PBM may be based on the method applied to measure the IL-1 protein (12,13). Moreover, culture conditions (in particular the presence of serum factors in culture medium) may interfere with the kinetics of IL-1 protein or mRNA production. The extent of IL-1 production by AM remains therefore controversial. Consequently, also the supposed crucial role of AM in initiating pulmonary inflammation and the supposed macrophage-mediated working mechanism of anti-inflammatory drugs may have to be reconsidered.

These conflicting results prompted us to study the kinetics of IL-1β gene expression in freshly isolated human AM and AM cultured under serum-free conditions. Furthermore, we studied the effects of dexamethasone, a known anti-inflammatory drug, and IL-4, a potential anti-inflammatory cytokine, on IL-1β gene expression.

MATERIALS AND METHODS

Isolation of monocytes/macrophages

Human AM were obtained as described elsewhere (14). BAL was performed in healthy volunteers, both smokers and non-smokers. All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. At least 90% of the BAL cells appeared to be monocytes/macrophages as judged by May-Grünwald Giemsa staining. Human PBM were isolated from 500 ml blood from healthy volunteers as described elsewhere (15). The monocyte preparation was over 95% pure as judged by May-Grünwald Giemsa staining. After isolation, some of the AM or PBM were used for preparation of total RNA, whereas the rest of the cells were cultured in the presence of either IL-4, dexamethasone (a synthetic glucocorticoid), RU 38486 (a glucocorticoid receptor antagonist), a combination of dexamethasone and RU 38486, or without one of these additions.

Cell lines

U937, a human monocytic cell line, originally described by Sundström and Nilsson (16) was maintained as described previously (14). To induce IL-1β production, cells were stimulated with PMA/LPS as described earlier (17). Unstimulated and PMA/LPS-stimulated U937 cells served as negative and positive controls, respectively, with regard to the presence of IL-1β mRNA.

CHAPTER 5.3

Recombinant IL-4, dexamethasone and RU 38486

Human rIL-4 was a generous gift from Dr. H.F.J. Savelkoul from our department and Dr. K. Arai (DNAX Research Institute, Palo Alto, CA). Dexamethasone was purchased from Duchefa b.v. (Haarlem, The Netherlands). The glucocorticoid antagonist RU 38486 was kindly provided by Roussel Uclaf (Romainville, France).

Culture of cells

PBM and AM were cultured under serum-free conditions as described elsewhere (14) for up to 7 days. In most experiments cells were maintained in T75 Falcon tissue culture flasks (Becton Dickinson, Plymouth, UK), whereas in some experiments cells were cultured under non-adherent conditions in Teflon bags (Janssen's MNL, St-Niklaas, Belgium). For isolation of total RNA from cells cultured in Teflon bags, cells were separated from culture supernatants by centrifugation under RNase-free conditions. Subsequently, cells were washed twice with PBS. Cell pellets were homogenized with 0.5 ml of solution D (18) and cooled to 4°C on iced water. If cells were cultured under adherent conditions, they were washed twice with PBS, then homogenized in the culture flasks with 0.5 ml of solution D and cooled as described above.

Preparation of total cellular RNA, Northern blot and dot blot analysis

Total cellular RNA was isolated from U937 cells, freshly isolated or cultured AM and PBM largely according to Chomczynski and Sacchi (18). For Northern blot analysis, total RNA (5-20 μg) was separated by electrophoresis in a 1%-agarose gel, subsequently vacuum transferred onto a nylon membrane (NY-13N; Schleicher and Schuell, Dassel, Germany), and hybridized with an IL-1β probe, which was labeled according to the Klenow-oligonucleotide method (19). This IL-1β cDNA probe, a 1.3 kb *Pst* I fragment, was kindly provided by Genetics Institute (Cambridge, MA). After hybridization, the membranes were washed and exposed to Fuji NIF-RX films (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens. The quality and amounts of RNA applied were controlled by rehybridization of the blots with a GAPDH probe (20). For dot blot analysis, a series of total RNA dilutions was vacuum transferred onto a nylon membrane (see below). Thereafter, the membrane was handled as described for Northern blot analysis.

Semiquantification of IL-1β mRNA levels by RNA dot blotting

Dot blotting was largely performed as described previously (21). Briefly, 1 volume of total RNA was dissolved in 1 volume 15 x SSC (1 x SSC = 0.15 M NaCl/15 mM Nacitrate, pH = 7) and 0.5 volume 37% formaldehyde, and incubated for 15 min at 60°C. Next, serial 1:2 or 1:1.58 dilutions of total RNA in 15 x SSC were applied with a Biodot microfiltration apparatus onto a nylon membrane, which had been soaked in 20 x SSC. A serial dilution of total RNA from PMA/LPS-stimulated U937 cells was used as a reference and was also applied to each membrane containing serial dilutions of RNA from experiments with AM or PBM. After aspiration, RNA was fixed to the membrane with a 254 nm UV crosslinker. Prehybridization, hybridization, washing, and autoradiography were performed as described above for Northern blotting. Semiquantification of IL-1β mRNA levels in the different AM and PBM experiments was achieved by comparison of the serial dilutions of RNA from AM or PBM with the serial dilutions of reference RNA from stimulated U937 cells.

RESULTS

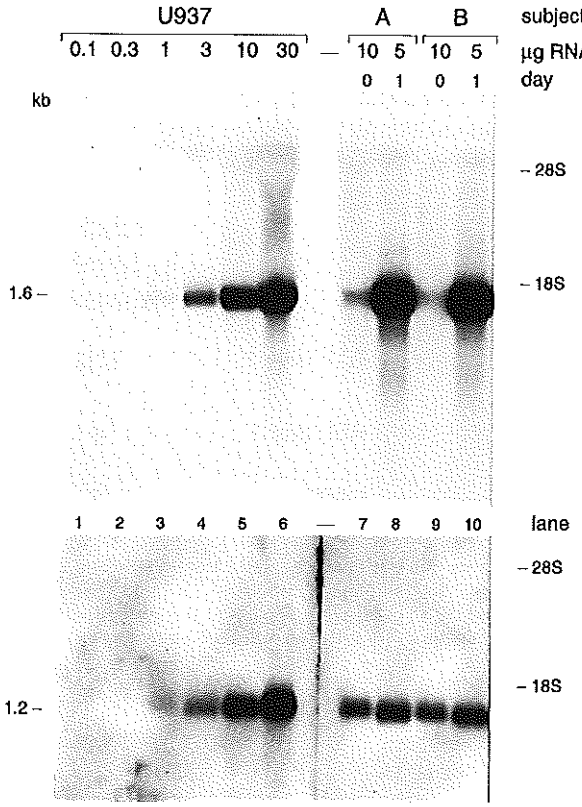

Figure 1. Expression of IL-1β mRNA in AM from two nonsmoking subjects A and B as compared with PMA/LPS-stimulated U937 cells. Lanes 1 to 6 contain 0.1, 0.3, 1, 3, 10, and 30 µg, respectively, of total RNA from U937 cells, and were used as a reference to estimate expression of IL-1β mRNA in freshly isolated (lanes 7 and 9) or cultured (lanes 8 and 10) AM. IL-1β mRNA expression in freshly isolated AM turned out to be many times lower than in AM cultured for 1 day. Therefore, lanes 7 and 9 were loaded with 10 µg, whereas lanes 8 and 10 were loaded with 5 µg of total RNA. The upper panel shows hybridization with the IL-1β cDNA probe. The lower panel shows the same membrane rehybridized with the GAPDH cDNA probe.

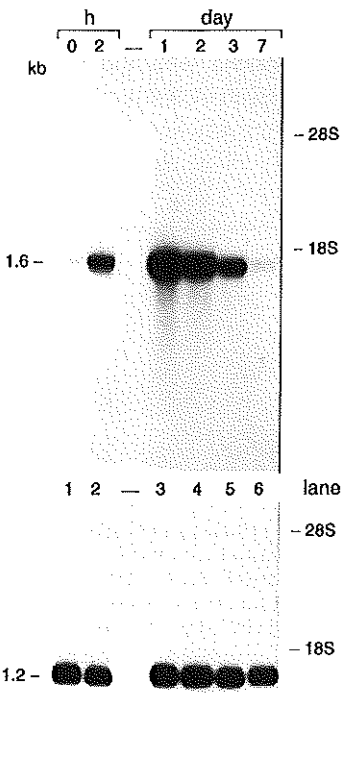

Figure 4. Expression of IL-1β mRNA in freshly isolated and cultured AM from a smoking subject. Lanes were loaded with 15 µg of total RNA obtained from freshly isolated AM or AM cultured for 2 hours, or 1, 2, 3 or 7 days. The upper panel shows hybridization with the IL-1β cDNA probe, whereas the lower panel shows the same membrane rehybridized with the GAPDH cDNA probe.

Levels of IL-1β mRNA in freshly isolated AM and PBM as compared with PMA/LPS-stimulated U937 cells

Freshly isolated AM expressed IL-1β mRNA, but in all our experiments (n = 7) this basal

expression appeared low (16-64 times as low) as compared with PMA/LPS-stimulated U937 cells (Figures 1 and 2). IL-1β mRNA expression in these U937 cells was used as a reference expression in all experiments. Freshly isolated PBM also expressed IL-1β mRNA. Their basal expression was consistently higher than the basal expression in AM, and was comparable with the expression in PMA/LPS-stimulated U937 cells (Figure 3). Basal expression of IL-1β mRNA in AM from nonsmokers did not differ from basal expression in AM from smokers (data not shown).

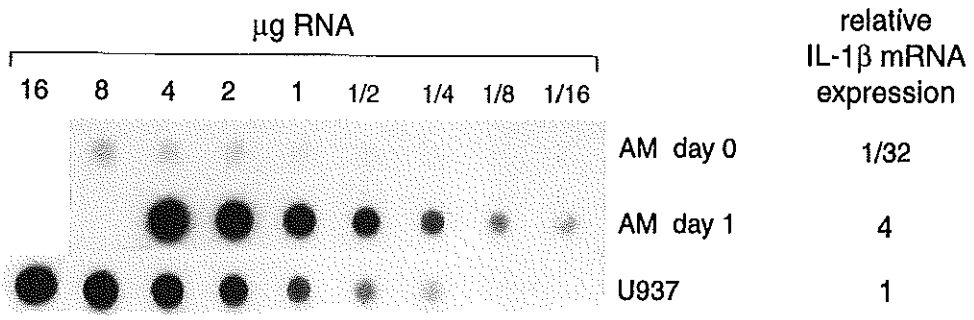

Figure 2. Semiquantification of IL-1β mRNA expression by dot blot analysis in AM from a smoking subject as compared with PMA/LPS-stimulated U937 cells. Serial 1:2 dilutions of total RNA [isolated from freshly isolated AM (day 0), AM cultured for 1 day (day 1), and U937 cells] were applied to the wells. Blots were probed with the IL-1β cDNA probe. The right column indicates IL-1β mRNA expression relative to expression in PMA/LPS-stimulated U937 cells.

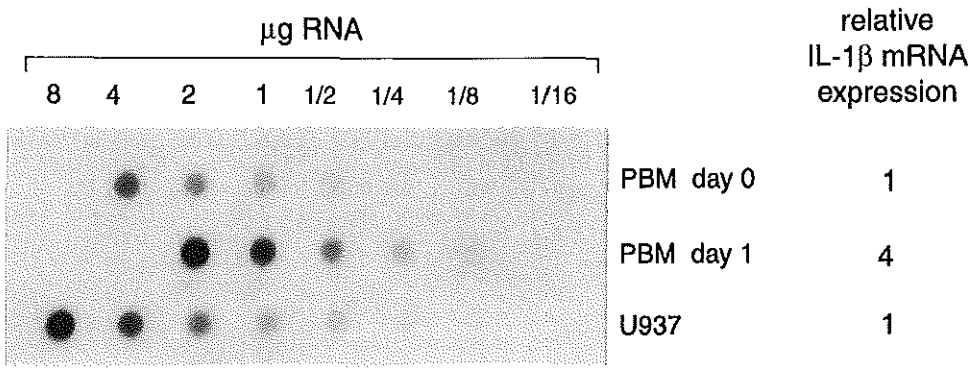

Figure 3. Semiquantification of IL-1β mRNA expression by dot blot analysis in PBM from a healthy volunteer as compared with PMA/LPS-stimulated U937 cells. Dot blot procedures are the same as mentioned in the legend to Figure 2. The right column indicates IL-1β mRNA expression relative to expression in PMA/LPS-stimulated U937 cells.

Kinetics of IL-1β mRNA expression in cultured AM and PBM

Upon culture of AM, a consistent marked increase in IL-1β mRNA expression was observed in all our experiments (n = 7). Already after 2 hours of culture an increase in IL-1β mRNA level was observed (Figure 4, lane 2). Semiquantitative analysis of IL-1β mRNA expression revealed that already after 2 hours of culture expression in AM almost equalled the expression in PMA/LPS-stimulated U937 cells (Figure 5). Maximal levels of IL-1β mRNA were observed after 1 day of culture. On this day, levels were higher than in PMA/LPS-stimulated U937 cells and were 64-128 times as high as compared with levels in freshly isolated AM (Figures 4 and 5). After 3 days of culture, levels of IL-1β mRNA were still higher than the levels in freshly isolated AM (Figure 4). Both in AM from smokers and nonsmokers a significant increase in IL-1β mRNA upon culture was seen, which was maximal on day 1 of culture. Culture of PBM also revealed an increase in IL-1β mRNA expression, although this increase was not seen in every experiment and was not as large as in AM (data not shown). Furthermore, in PBM the culture-associated increase in IL-1β mRNA expression was not as lasting as in AM. Already after 2 days of culture, IL-1β mRNA levels were comparable with freshly isolated PBM.

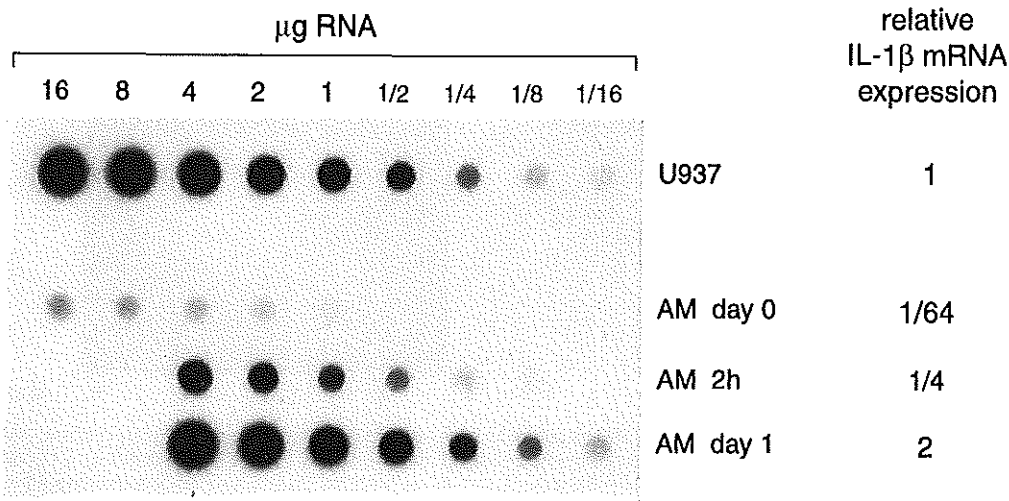

Figure 5. Semiquantification of IL-1β mRNA expression by dot blot analysis in AM from the same subject as in Figure 4. Serial 1:2 dilutions of total RNA [isolated from freshly isolated AM (day 0), AM cultured for 2 hours (2h), AM cultured for 1 day (day 1), and U937 cells] were applied to the wells. The right column indicates IL-1β mRNA expression relative to the expression in PMA/LPS-stimulated U937 cells.

Influence of dexamethasone on the kinetics of IL-1β mRNA expression in cultured AM

Culture of AM in the presence of dexamethasone inhibited the culture-associated increase in IL-1β mRNA levels. As compared with the IL-1β mRNA expression in freshly isolated AM, there was still an increase in expression after 1 day of culture, but this increase was consistently and markedly smaller than the increase in AM cultured without dexamethasone (Figures 6 and 7). Semiquantification of IL-1β mRNA expression in a representative experiment out of seven is shown in Figure 7. After 1 day of culture, IL-1β mRNA expression increased 160 fold (line 1). Culture in the presence of dexamethasone reduced this increase to 40 times (line 4) (75% reduction). After 2 days of culture these increases were 40 and 16 fold, respectively (lines 7 and 10) (60% reduction). This dexamethasone-associated inhibition of increase in IL-1β mRNA expression was mediated through glucocorticoid receptors, as RU 38486 antagonized this effect of dexamethasone (Figure 6, lane 3; Figure 7, line 3): culture of AM in the presence of both dexamethasone and RU 38486 revealed the same increase in IL-1β mRNA expression as compared with control cultures (Figure 6, lane 3 and 1, respectively; Figure 7, line 3 and 1, respectively).

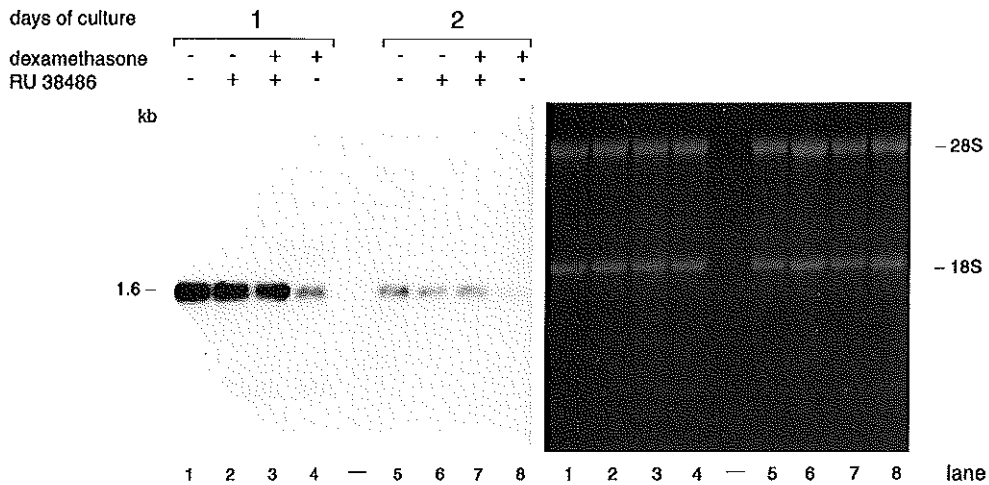

Figure 6. Northern blot analysis of the effects of dexamethasone on IL-1β mRNA expression in cultured AM. Total RNA was prepared from AM cultured in either control medium (lanes 1 and 5), RU 38486 (5×10^{-6} M; lanes 2 and 6), dexamethasone (5×10^{-7} M; lanes 4 and 8), or both RU 38486 and dexamethasone (lanes 3 and 7). Each lane was loaded with 5 μg of total RNA. The left panel shows hybridization with the IL-1β cDNA probe. The right panel shows ethidiumbromide staining of the gel after electrophoresis to visualize equal loading per lane.

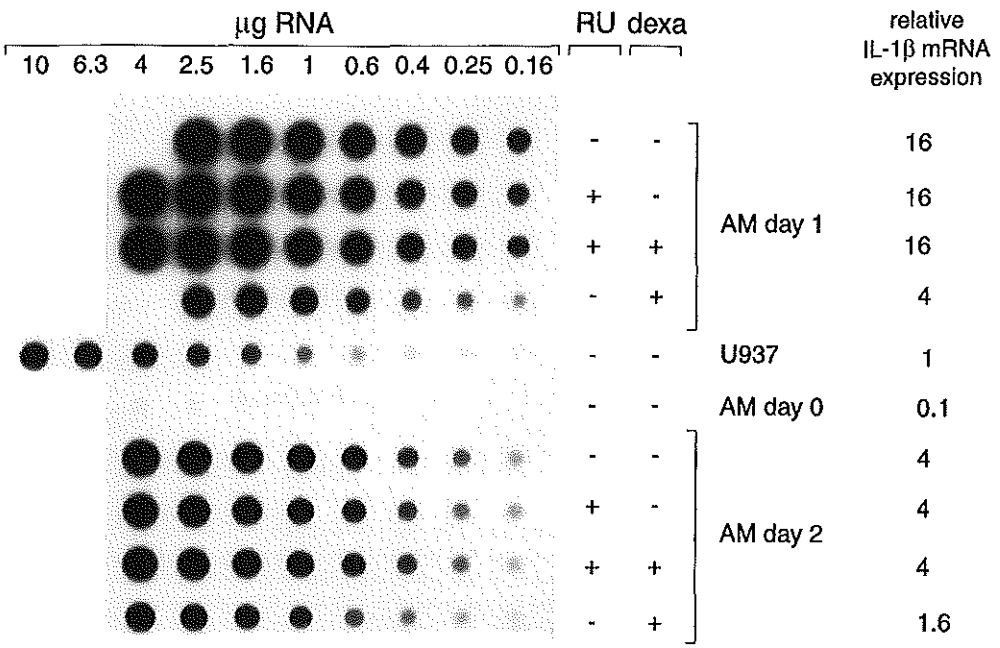

Figure 7. Semiquantification of IL-1β mRNA expression by dot blot analysis in dexamethasone-treated AM from the same subject as in Figure 6. Serial 1:1.58 dilutions of total RNA (obtained from freshly isolated AM (day 0), AM cultured for 1 day (day 1), AM cultured for 2 days (day 2), and U937 cells) were applied to the wells. AM were cultured in either control medium (– –), medium with RU 38486 (+ –), medium with dexamethasone (– +), or medium with both RU 38486 and dexamethasone (+ +). The right column indicates IL-1β mRNA expression relative to the expression in PMA/LPS-stimulated U937 cells.

Influence of IL-4 on the kinetics of IL-1β mRNA expression in cultured AM and PBM

Culture of PBM in the presence of IL-4 revealed a reduction in IL-1β mRNA expression as compared with the control cultures, as was described previously (22). This reduction was most evident on day 1 of culture (data not shown). This IL-4 effect was also observed in AM. In those cells, the IL-4-mediated suppression was even more distinct, probably as a result of the higher IL-1β mRNA levels during culture as compared with PBM. Even on day 3 of culture, the inhibitory effect of IL-4 on IL-1β mRNA expression was still observed in AM (Figure 8, lines 6 and 7). Semiquantification of the effects of IL-4 on IL-1β mRNA expression in a representative experiment out of 3 is shown in Figure 8. IL-1β mRNA expression increased 128 fold after 1 day of culture (line 2). IL-4 inhibited this increase, which resulted in only an 8 fold increase (more than 90% reduction) after 1 day of culture (line 3).

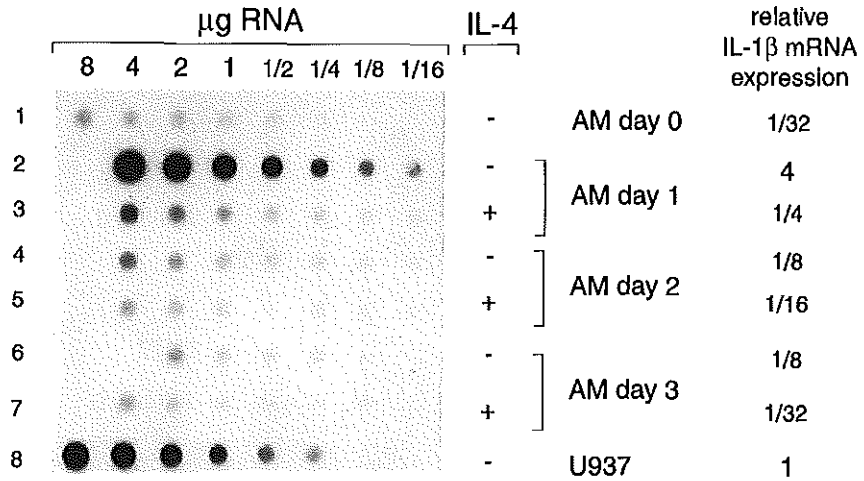

Figure 8. Semiquantification of IL-1β mRNA expression by dot blot analysis in IL-4-treated AM. Serial 1:2 dilutions of total RNA [prepared from freshly isolated AM (day 0), AM cultured for 1, 2 or 3 days (day 1, 2 and 3, respectively), and U937 cells] were applied to the wells. AM were cultured in either control medium (-) or in the presence of 100 U IL-4/ml (+). The far right column indicates IL-1β mRNA expression relative to the expression in PMA/LPS-stimulated U937 cells.

DISCUSSION

Monocytes/macrophages are considered as a pivotal cell type in the initiation and perpetuation of inflammation in several diseases. This vision is concluded from their ability to function as antigen presenting cells, to secrete a great variety of inflammatory mediators, and to influence other inflammatory cells (5,6,23). Against this background, AM have been studied extensively to elucidate their role in (inflammatory) lung diseases. This cellular knowledge is being used in medicine to understand how glucocorticoids may exert their clinical anti-inflammatory effects (24). Furthermore, products that exhibit potential anti-inflammatory effects *in vitro* may be applied in the treatment of diseases, eventually. A good example of the latter is represented by IL-4 which has been shown to have potential anti-inflammatory effects *in vitro* (22,25,26), and is being used in clinical studies nowadays (27,28).

In this study, the regulation of IL-1β gene expression was analyzed in human AM. The literature shows conflicting data as to whether or not AM are an important source of IL-1β. Recent findings suggest that IL-1β production by AM has been underestimated because conventional ELISA's are unable to detect the 35 kDa pro-IL-1β molecule (12,13). These findings strengthened the potential role of AM in inflammation, and prompted us to re-examine the expression of the IL-1β gene in AM. In this study, three aspects of IL-1β gene expression in AM were evaluated. First, we studied the kinetics of IL-1β mRNA expression

in cultured AM from smokers and nonsmokers. Second, we examined the influence of glucocorticoids on this expression. Third, we evaluated the effects of IL-4.

The basal expression of the IL-1β gene in freshly isolated AM appeared relatively low as compared with freshly isolated PBM or stimulated U937 cells. These findings correspond to the conclusions of Wevers et al (10). However, AM showed an increased expression upon culture, eventually resulting in higher expressions of IL-1β mRNA than in PBM and U937 cells. This may indicate that also *in vivo*, under certain conditions, AM may be able to increase the production of IL-1β to a greater extent than any other cell type. The role of AM in the initiation of inflammation in the lung may therefore be more important than it was suggested on the basis of former studies (10). PBM also showed a transient increase in IL-1β mRNA expression upon culture, but this increase was less pronounced and of shorter duration. These findings stress the functional differences between AM and PBM. Differences in the stage of maturation/differentiation within the mononuclear phagocyte system, to which both AM and PBM belong, may underlie such functional differences (14).

The spontaneous increase in IL-1β mRNA upon culture of AM and PBM may result from mRNA stabilization or an increased transcription. The ultimate trigger of this spontaneous increase remains unknown. Serum components were excluded, as cells were cultured in serum-free medium. Cellular adherence as a cause is also unlikely, as the spontaneous increase was observed both during culture in flasks (adherent conditions) and during culture in Teflon bags (non-adherent conditions). It has been reported that culture of PBM under both non-adherent and serum-free conditions induced a spontaneous increase in MHC class II (29) and CD13 Ag expression (25). It appears that in those culture systems, and also ours, PBM and AM become activated. This activation may result from e.g. cellular products or low levels of LPS.

AM from smokers and nonsmokers differ in morphology, immunophenotype and function (30,31). It was shown previously that smoking decreased IL-1 secretion by LPS-stimulated AM, and it was suggested that smoking impaired the release of intracellular IL-1 rather than the production of IL-1 (31). We found no differences in the expression of IL-1β mRNA between AM from smoking and nonsmoking subjects. These results are consistent with the idea that smoking decreases IL-1 production via a post-transcriptional mechanism. Upon culture, AM from both smokers and nonsmokers showed a transient increase in IL-1β mRNA levels. Even with the technique of mRNA semiquantification used in our experiments, we were unable to detect differences between AM from smokers and nonsmokers.

Part of the anti-inflammatory effects of glucocorticoids has been shown to result from modulation of the production of various inflammatory cytokines (32,33). Expression of IL-1 by U937 cells and PBM has been reported to decrease upon treatment with glucocorticoids (34,35). In the current study, these findings were confirmed in more mature mononuclear phagocytes, i.e. AM. This effect of glucocorticoids was mediated via glucocorticoid receptors, as RU 38486 antagonized the decrease in IL-1β mRNA levels. Either reduced transcription or increased mRNA instability may underlie the glucocorticoid-mediated decrease in IL-1β mRNA levels. In PBM, only stability of IL-1β mRNA was markedly decreased by dexamethasone (35), whereas in U937 cells expression of IL-1 was inhibited

both transcriptionally and post-transcriptionally (34). The mechanism of glucocorticoid-mediated IL-1β mRNA decrease in AM is currently being studied in our laboratory. AM are nowadays considered as a potential reservoir of intracellular IL-1β as a result of large amounts of pro-IL-1β. This reservoir may be activated and released in the presence of the appropriate milieu (13). Therefore, modulation of IL-1β gene expression by glucocorticoids in AM may be of more importance than previously considered. Our findings indicate that in the lung part of the anti-inflammatory actions of glucocorticoids may be mediated via AM.

IL-4 was recently found to modulate the gene expression (22) and secretion (22,36) of cytokines. In those studies, it was demonstrated that IL-4 suppressed LPS-induced mRNA and protein accumulation for IL-1β in human PBM. Little is known about the effects of IL-4 on AM. Results in PBM can not be extrapolated on AM thoughtlessly, because it has been reported that effects of cytokines on target cells may depend on their stage of maturation (14). The results on PBM presented here confirm the results of Vannier et al (22). In addition, we show that also in AM IL-4 suppresses IL-1β gene expression. Time-course experiments demonstrated that IL-4 reduced IL-1β levels for a prolonged period of time (> 3 days). This IL-4-mediated inhibition of the spontaneous, culture-associated increase in IL-1β mRNA levels may be due to decreased transcription or enhanced degradation of IL-1β mRNA. Future nuclear run-off transcription experiments and mRNA half-life experiments may clarify this point. Indirect evidence that IL-4 modulates transcription comes from other studies which described IL-4 effects on mRNA transcription of IL-1 RA (37) and IL-2 (38). However, IL-4 may also accelerate mRNA decay, as was demonstrated in recent studies on MIP-α (39). Our findings may be considered as further evidence of the potential anti-inflammatory properties of IL-4.

Recently, Galve-de Rochemonteix et al described the effects of IL-4 on the regulation of IL-1α, IL-1β and IL-1RA production by AM (8). In that study, AM were obtained from patients with pulmonary cell carcinoma immediately after surgery. AM were cultured in the presence of 5% FCS. A transient increase in IL-1β mRNA levels was shown upon culture of AM with a peak at 3 h. Furthermore, IL-4 was shown to increase IL-1RA production and IL-1RA mRNA levels, but IL-4 did not affect the IL-1β production or IL-1β mRNA levels. In our study, AM were obtained from healthy volunteers and were cultured in medium without serum. A spontaneous, transient increase in IL-1β mRNA was demonstrated with a peak at day 1 of culture. After 1 day, IL-1β mRNA levels were 64-128 fold higher than in freshly isolated AM. Even after 3 days of culture, levels were still higher than in freshly isolated AM. These different results may arise from differences in either the patient population (healthy volunteers versus cancer patients), the method of performing BAL (whether or not after surgery), or the culture conditions (whether or not in the presence of serum). Furthermore and in contrast to the previous study, in our study a consistent decrease in IL-1β mRNA was induced by IL-4. This discrepancy in findings may also result from the above mentioned differences between the two studies. An important factor may be the presence of serum in the culture medium, as serum factors (e.g. glucocorticoids or cytokines) may antagonize IL-4 effects.

In conclusion, the results of this study stress that AM are potential modulators of

inflammation as illustrated by their ability to increase steady state IL-1β mRNA levels substantially under certain environmental condition. Furthermore, both glucocorticoids and IL-4 were shown to decrease IL-1β mRNA levels. These findings help us to understand the anti-inflammatory mechanisms of glucocorticoids in the lung, and stress the potential role of IL-4 as an anti-inflammatory cytokine.

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REFERENCES

1. Gery I, Gershon RK, Waksman BH. Potentiation of the T-lymphocyte response to mitogens. J Exp Med 1972;136:128-142.
2. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. There is more than one interleukin 1. Immunol Today 1986;7:45-56.
3. Dinarello CA. The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome. J Infect Dis 1991;163:1177-1184.
4. Dinarello CA, Wolff SM. The role of interleukin-1 in disease. New Engl J Med 1993;328:106-113.
5. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
6. Fels AOS, Cohn ZA. The alveolar macrophage. J Appl Physiol 1986;60:353-369.
7. Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. J Exp Med 1993;177:397-407.
8. Galve-de Rochemonteix B, Nicod LP, Chicheportiche R, Lacraz S, Baumberger C, Dayer J-M. Regulation of interleukin-1ra, interleukin-1α, and interleukin-1β production by human alveolar macrophages with phorbol myristate acetate, lipopolysaccharide, and interleukin-4. Am J Respir Cell Mol Biol 1993;8:160-168.
9. Nathan CF. Secretory products of macrophages. J Clin Invest 1987;79:319-326.
10. Wewers MD, Rennard SI, Hance AJ, Bitterman PB, Crystal RG. Normal human alveolar macrophages obtained by bronchoalveolar lavage have a limited capacity to release interleukin-1. J Clin Invest 1984;74:2208-2218.
11. Kern JA, Lamb RJ, Reed JC, Elias JA, Daniele RP. Interleukin-1-beta gene expression in human monocytes and alveolar macrophages from normal subjects and patients with sarcoidosis. Am Rev Respir Dis 1988;137:1180-1184.
12. Wewers MD, Pope HA. IL-1β measurements using proIL-1β specific ELISA confirm alveolar macrophage as potential reservoir of intracellular IL-1β. Am Rev Respir Dis 1993;147:A230.
13. Herzyk DJ, Berger AE, Allen JN, Wewers MD. Sandwich ELISA formats designed to detect 17 kDa IL-1β significantly underestimate 35 kDa IL-1β. J Immunol Meth 1992;148:243-254.
14. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.
15. Figdor CG, Bont WS, Touw I, De Roos J, Roosnek EE, De Vries JE. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. Blood 1982;60:46-53.

16. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565-577.
17. Baldari CT, Telford JL. The intracellular precursor of IL-1β is associated with microtubules in activated U937 cells. J Immunol 1989;142:785-791.
18. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
19. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991;198:1-92.
20. Benham FJ, Hodgkinson S, Davies KE. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosomes defines a multigene family. EMBO J 1984;3:2635-2640.
21. Nooter K, Sonneveld P, Oostrum R, Herweijer H, Hagenbeek T, Valerio D. Overexpression of the *mdr 1* gene in blast cells from patients with acute myelocytic leukemia is associated with decreased anthracycline accumulation that can be restored by cyclosporin-A. Int J Cancer 1990;45:263-268.
22. Vannier E, Miller LC, Dinarello CA. Coordinated antiinflammatory effects of interleukin 4: Interleukin 4 suppresses interleukin 1 production but up-regulates gene expression and synthesis of interleukin 1 receptor antagonist. Proc Natl Acad Sci USA 1992;89:4076-4080.
23. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
24. Russo-Marie F. Macrophages and the glucocorticoids. J Neuroimmunol 1992;40:281-286.
25. Van Hal PThW, Hopstaken-Broos JPM, Prins A, Favaloro EJ, Huijbens RJF, Hilvering C, Figdor CG, Hoogsteden HC. Potential indirect anti-inflammatory effects of IL-4. Stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N activity (CD13; EC 3.4.11.2). J Immunol 1994;153:2718-2728.
26. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiiflammatory effects of interleukin 4: Suppression of human monocyte tumor necrosis factor α, interleukin 1, and prostaglandin E_2. Proc Natl Acad Sci USA 1989;86:3803-3807.
27. Maher DW, Davis I, Boyd AW, Morstyn G. Human interleukin-4: An immunomodulator with potential therapeutic applications. Prog Growth Factor Res 1991;3:43-56.
28. Wong HL, Lotze MT, Wahl LM, Wahl SM. Administration of recombinant IL-4 to humans regulates gene expression, phenotype, and function in circulating monocytes. J Immunol 1992;148:2118-2125.
29. Te Velde AA, Klomp JPG, Yard BA, De Vries JE, Figdor CG. Modulation of phenotypic and functional properties of human peripheral blood monocytes by IL-4. J Immunol 1988;140:1548-1554.
30. Hoogsteden HC, Van Hal PThW, Wijkhuijs JM, Hop W, Verkaik APK, Hilvering C. Expression of the CD11/CD18 cell surface adhesion glycoprotein family on alveolar macrophages in smokers and nonsmokers. Chest 1991;100:1567-1571.
31. Soliman DM, Twigg HL. Cigarette smoking decreases bioactive interleukin-6 secretion by alveolar macrophages. Am J Physiol 1992;263:L471-L478.
32. Munck A, Mendel DB, Smith LI, Orti E. Glucocorticoid receptors and actions. Am Rev Respir Dis 1990;141:S2-S10.
33. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray MA, Anderson WH, eds. Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:593-617.
34. Knudsen PJ, Dinarello CA, Strom TB. Glucocorticoids inhibit transcriptional and posttranscriptional expression of interleukin-1 in U937 cells. J Immunol 1987;139:4129-4134.
35. Amano Y, Lee SW, Allison AC. Inhibition by glucocorticoids of the formation of interleukin-1α, interleukin-1β, and interleukin-6: Mediation by decreased mRNA stability. Mol Pharmacol 1993;43:176-182.
36. Te Velde AA, Huijbens RJF, Heije K, De Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1β, tumor necrosis factor α, and IL-6 by human monocytes. Blood 1990;76:1392-1397.
37. Orino E, Sone S, Nii A, Ogura T. IL-4 up-regulates IL-1 receptor antagonist gene expression and its production in human blood monocytes. J Immunol 1992;149:925-931.

38. Schwarz EM, Salgame P, Bloom BR. Molecular regulation of human interleukin 2 and T-cell function by interleukin 4. Proc Natl Acad Sci USA 1993;90:7734-7738.
39. Standiford TJ, Kunkel SL, Liebler JM, Burdick MD, Gilbert AR, Strieter RM. Gene expression of macrophage inflammatory protein-1 alpha from human blood monocytes and alveolar macrophages is inhibited by interleukin-4. Am J Respir Cell Mol Biol 1993;9:192-198.

INVERSE MODULATION OF LIPOCORTIN-1 (ANNEXIN-1) AND IL-1β GENE EXPRESSION BY DEXAMETHASONE IN CULTURED HUMAN BRONCHOALVEOLAR MACROPHAGES*

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*This chapter is submitted for publication.

SUMMARY

Glucocorticoids are powerful anti-inflammatory drugs, which are used in the treatment of many diseases including certain inflammatory lung diseases. Modulation of transcription of both pro- and anti-inflammatory proteins in a variety of cell types is nowadays considered as the most important working mechanism of glucocorticoids. Alveolar macrophages (AM) are thought to have a central function in the regulation of inflammatory processes in the lung. On the one hand, they may initiate or perpetuate inflammation by production of certain cytokines, e.g. IL-1. On the other hand, they may inhibit inflammation via the production of proteins with potentially anti-inflammatory properties, e.g. lipocortins. As AM express glucocorticoid receptors, the inflammatory activities of AM may be regulated by glucocorticoids. The glucocorticoid-mediated induction of lipocortin gene expression could be demonstrated in only a few reports, and therefore requires further study.

Here we have focussed on the gene regulation of an anti-inflammatory protein, lipocortin-1, by glucocorticoids in human AM, and we compared this, under the same experimental conditions, with the gene regulation of a pro-inflammatory cytokine IL-1β. AM were obtained from healthy volunteers by bronchoalveolar lavage and cultured for up to 3 days under serum-free conditions. Northern blot and dot blot analysis were employed to study mRNA expression and to semiquantitate mRNA levels. Lipocortin-1 mRNA in AM was compared with BEAS S6 cells, whereas IL-1β gene expression in AM was compared with peripheral blood monocytes (PBM) or PMA/LPS-stimulated U937 cells.

Freshly isolated AM contained low levels of IL-1β mRNA as compared with PBM and U937. Upon culture, AM exhibited a large, but transient increase in IL-1β mRNA levels. After one day of culture, levels had increased 64 to 128 fold. After 3 days of culture, IL-1β mRNA levels were still higher than in freshly isolated AM. Dexamethasone inhibited this culture-associated increase. Upon culture, PBM also showed a transient increase in IL-1β mRNA, which was much smaller than in AM. Under the same experimental conditions, dexamethasone induced an increase in lipocortin-1 mRNA in AM, but only after one day of culture. The glucocorticoid antagonist RU 38486 counteracted the effects of dexamethasone on both IL-1β and lipocortin-1 mRNA levels, indicating that these effects were receptor-mediated.

These results show that part of the anti-inflammatory activity of glucocorticoids in the lung can be explained by their influence on IL-1β and lipocortin-1 mRNA expression in AM. Furthermore, effects of glucocorticoids on lipocortin-1 mRNA were only seen up to one day of culture, whereas effects on IL-1β mRNA were still observed after 3 days of culture. These differences between kinetics of IL-1β and lipocortin-1 mRNA regulation indicate that glucocorticoids can regulate different target genes differently. These data may clarify the results in the literature with regard to the induction of lipocortins by glucocorticoids.

INTRODUCTION

Pulmonary diseases with a major inflammatory component are usually treated, although sometimes with varying degrees of success, with anti-inflammatory drugs. Glucocorticoids are extremely powerful anti-inflammatory drugs which have been used clinically since 1949 (1). It is known that glucocorticoids can influence many aspects of the different cell types regulating the inflammatory processes in certain lung diseases (2), but their subcellular working mechanisms are, even today, only partially understood (3,4). The cell types involved in inflammatory processes interact with each other extensively, either through direct cell-cell contact or soluble mediators, both of which mechanisms can be modulated by glucocorticoids (5-8). However, further studies on the working mechanisms of glucocorticoids are needed in order to understand these mechanisms more fully and thus develop treatment strategies with fewer unwanted side effects.

Central to inflammatory and immunologic processes are mononuclear phagocytes. In these cells, glucocorticoids are thought both to inhibit the production of inflammatory mediators such as IL-1 (9,10) and to induce anti-inflammatory proteins such as lipocortins (11-13). IL-1 has been proposed to play a key role in inflammatory diseases (14,15), and the anti-inflammatory effects of glucocorticoids are likely to include interaction with IL-1 production (9,10). One member of the lipocortin (annexin) family, lipocortin-1 (annexin-1), has been described as inducible by glucocorticoids in rat peritoneal macrophages (11). If so, induction of this protein may represent one working mechanism of glucocorticoids, as lipocortin-1 has been shown to possess anti-inflammatory activity (for a recent review: 16). Many studies confirmed this hypothesized mechanism (12,13,17), and also recent studies showed the involvement of lipocortin-1 in the anti-inflammatory action of glucocorticoids (18,19). However, some authors failed to reproduce the glucocorticoid-mediated induction of lipocortin-1 (20-22), and therefore further studies are needed to understand the nature of the conflicting data on the inducibility of lipocortins by glucocorticoids.

Alveolar macrophages (AM), a special type of mononuclear phagocytes, are known to exhibit both pro- and anti-inflammatory properties which make them potent regulators of inflammatory processes in lung diseases (23-25). Furthermore, there is evidence that AM possess functional glucocorticoid receptors (26). However, many of the hypothesized working mechanisms of glucocorticoids in human AM are extrapolated results from studies in other mononuclear cells. Therefore, further studies in human AM are needed to elucidate the precise working mechanisms of glucocorticoids in the treatment of lung diseases in which AM are a major cellular component.

In this study we have focussed on the influence of glucocorticoids on the kinetics of gene expression of lipocortin-1 and IL-1β in both freshly isolated and cultured human alveolar macrophages. Glucocorticoids were shown to increase transiently levels of lipocortin-1 mRNA as well as to reduce levels of IL-1β mRNA.

CHAPTER 5.4

MATERIALS AND METHODS

Isolation of monocytes/macrophages

Human AM were obtained as described elsewhere (27). BAL was performed in healthy volunteers, both smokers and nonsmokers. None of these volunteers were being treated with glucocorticoids at the time of investigation. BAL was performed between 8.30 and 11.30 a.m. to minimize the influence of the physiological circadian rhythm of cortisol. All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. At least 90% of the BAL cells appeared to be monocytes/macrophages as judged by May-Grünwald Giemsa staining. Human PBM were isolated from 500 ml blood from healthy volunteers as described elsewhere (28). The monocyte preparation was over 95% pure as judged by May-Grünwald Giemsa staining. After isolation, some of the AM or PBM were used for preparation of total RNA, whereas the rest of the cells were cultured in the presence of either a synthetic glucocorticoid (in casu dexamethasone), a glucocorticoid antagonist (in casu RU 38486), a combination of dexamethasone and RU 38486, or without one of these additions.

Cell lines

U937, a human monocytic cell line, originally described by Sundström and Nilsson (29), was maintained as described previously (27). To induce IL-1β production, cells were stimulated with PMA/LPS as described previously (30). Unstimulated and PMA/LPS-stimulated U937 cells served as negative and positive controls, respectively, with regard to the presence of IL-1β mRNA. BEAS S6 cells, a human bronchial epithelial cell line, were kindly provided by Dr. M.M. Verheggen from our department. They were maintained as described earlier (31), and were used as a positive control with regard to the presence of lipocortin-1 mRNA.

Dexamethasone and RU 38486

Dexamethasone was purchased from Duchefa b.v. (Haarlem, The Netherlands). The glucocorticoid antagonist RU 38486 was kindly provided by Roussel Uclaf (Romainville, France). Stock solutions in ethanol of 2×10^{-3} M RU 38486 and 2×10^{-3} M dexamethasone were used for preparation of final solutions in culture medium.

Culture of cells

PBM and AM were cultured as described elsewhere (27) for up to 3 days. Culture media were serum- and glucocorticoid-free (unless indicated). In most experiments cells were maintained in T75 Falcon tissue culture flasks (Becton Dickinson, Plymouth, UK), whereas in some experiments cells were cultured under non-adherent conditions in Teflon bags (Janssen's MNL, St-Niklaas, Belgium). For isolation of total RNA from cells cultured in Teflon bags, cells were separated from culture supernatants by centrifugation under RNase-free conditions. Subsequently, cells were washed twice with PBS. Cell pellets were homogenized with 0.5 ml of solution D (32) and cooled to 4°C on iced water. If cells were cultured under adherent conditions, they were washed twice with PBS, then homogenized in the culture flasks with 0.5 ml of solution D and cooled as described above.

Probes

The IL-1β cDNA probe was kindly provided by Genetics Institute (Cambridge, MA). This probe was a

1.3 kb *Pst* I fragment, and hybridized to a RNA species of 1.6 kb. The lipocortin-1 cDNA probe was kindly provided by Dr. B.P. Wallner (Biogen Research Corp, Cambridge, MA). This probe was a 1.3 kb *Eco* RI fragment and hybridized to a RNA species of approximately 1.4 kb. The GAPDH probe was a 0.7 kb *Eco* RI - *Pst* I fragment, hybridizing to a 1.2 kb band (33).

Preparation of total cellular RNA, Northern blot and dot blot analysis

Total cellular RNA was isolated from U937 cells, and freshly isolated or cultured AM and PBM largely according to Chomczynski and Sacchi (32). For Northern blot analysis, total RNA (5-20 μg) was separated by electrophoresis in a 1%-agarose gel, subsequently vacuum transferred onto a nylon membrane (NY-13N; Schleicher and Schuell, Dassel, Germany), fixed to the membrane with a 254 nm UV crosslinker and hybridized with the IL-1β or lipocortin-1 probe, which were labelled using to the Klenow-oligonucleotide method (34). After hybridization, the blots were washed and exposed to Fuji NIF-RX films (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens. In some experiments, hybridization of the membrane with the IL-1β probe was followed by rehybridization with the lipocortin-1 probe. This procedure excluded differences in mRNA levels caused by differences in the amount of applied RNA, and enabled us to relate IL-1β and lipocortin-1 mRNA levels in the same samples. The quality and amounts of RNA applied were controlled by rehybridization of the blots with the GAPDH probe. For dot blot analysis, a series of total RNA dilutions was vacuum transferred onto a nylon membrane (see below). Thereafter, the membrane was handled as described for Northern blot analysis.

Semiquantification of IL-1β and lipocortin-1 mRNA levels by RNA dot blotting

Dot blotting was largely performed as described previously (35). Briefly, 1 volume of total RNA was dissolved in 1 volume 15 x SSC (1 x SSC = 0.15 M NaCl/15 mM Nacitrate, pH = 7) and 0.5 volume 37% formaldehyde, and incubated for 15 min at 60°C. Next, serial 1:2 or 1:1.58 dilutions of total RNA in 15 x SSC were applied with a Biodot microfiltration apparatus onto a nylon membrane, which had been soaked in 20 x SSC. A serial dilution of total RNA from PMA/LPS-stimulated U937 cells was used as a reference and was applied to each membrane containing serial dilutions of RNA from experiments with AM or PBM. After aspiration, RNA was fixed to the membrane with a 254 nm UV crosslinker. Prehybridization, hybridization, washing, and autoradiography were performed as described above for Northern blotting. Semiquantification of IL-1β or lipocortin-1 mRNA levels in the different AM and PBM experiments was achieved by comparison of the serial dilutions of RNA from AM or PBM with the serial dilutions of reference RNA from stimulated U937 and BEAS S6 cells, respectively. Arbitrarily, the expression of IL-1β mRNA in AM after one day of culture without dexamethasone and the expression of lipocortin-1 mRNA in freshly isolated AM were set at 100%.

RESULTS

Kinetics of IL-1β mRNA expression in cultured AM

Freshly isolated AM expressed IL-1β mRNA, but at low levels as compared with PMA/LPS-stimulated U937 cells or freshly isolated PBM. However, upon culture this expression increased tremendously. Northern blot analyses revealed a transient increase in the expression of IL-1β mRNA (Figure 1), which was seen in all our experiments (n = 11).

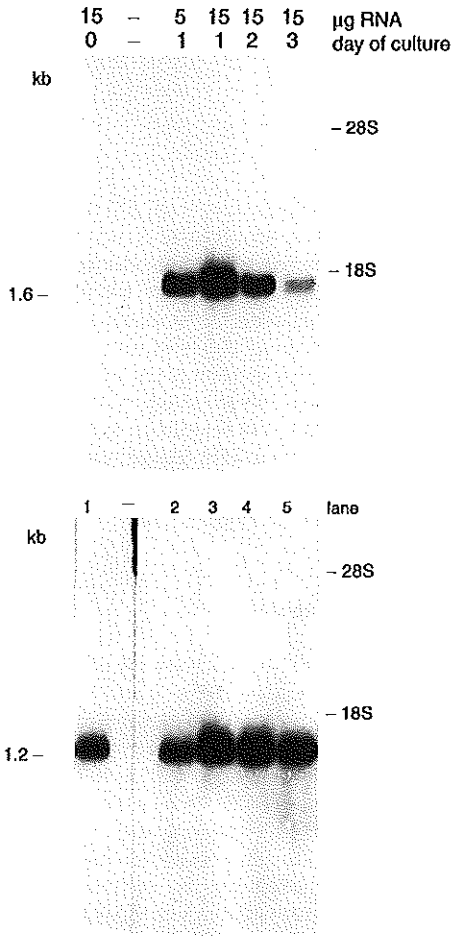

Figure 1. Expression of IL-1β mRNA in AM from a smoking subject. The upper panel shows hybridization with the IL-1β cDNA probe. IL-1β mRNA expression in cultured AM was many fold higher than in freshly isolated AM. Therefore, both 5 and 15 µg of total RNA from AM cultured for one day (lanes 2 and 3, respectively) were used in this Northern blot analysis. For the same reason, exposure time to the film after hybridization was restricted to two hours and 40 min in this experiment, even though it was, consequently, not possible to demonstrate the presence of IL-1β mRNA in freshly isolated AM (lane 1). The lower panel shows the same membrane rehybridized with the GAPDH cDNA probe.

Maximal expression was observed after one day of culture (Figure 1, lane 3). Thereafter, expression of IL-1β mRNA diminished, but even after 3 days of culture expression was still higher than in freshly isolated AM. Expression of GAPDH mRNA remained unchanged during culture, although in some experiments we observed a slight increase in GAPDH mRNA expression (lower panel of Figure 1). To semiquantitate the kinetics of IL-1β mRNA expression during culture, dot blot analysis was performed. With this technique, it was seen in AM from both smokers and nonsmokers that after one day of culture IL-1β mRNA expression was 20 to 500 fold as high as compared with the expression in freshly isolated AM (Figures 2, 3 and Table 1). Usually, the expression after one day of culture was higher than the expression in stimulated U937 cells.

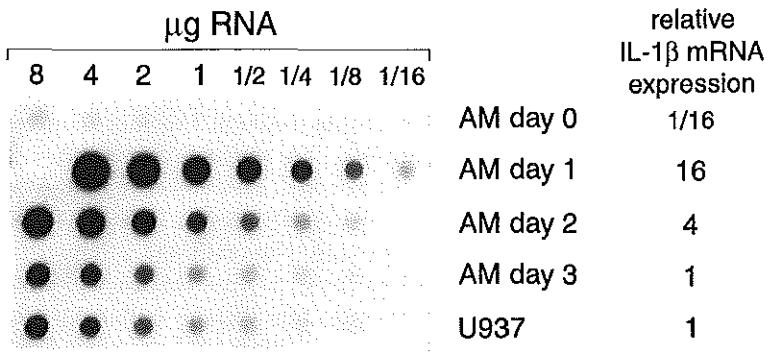

Figure 2. Semiquantification of IL-1β mRNA expression by dot blot analysis in AM from the same smoking subject as in Figure 1, and comparison with PMA/LPS-stimulated U937 cells. Serial 1:2 dilutions of total RNA [isolated from freshly isolated AM (day 0), cultured AM (for 1, 2, or 3 days, respectively) and U937 cells] were applied to the wells. Blots were probed with the IL-1β cDNA probe. The right column indicates IL-1β mRNA expression relative to the expression in PMA/LPS-stimulated cells.

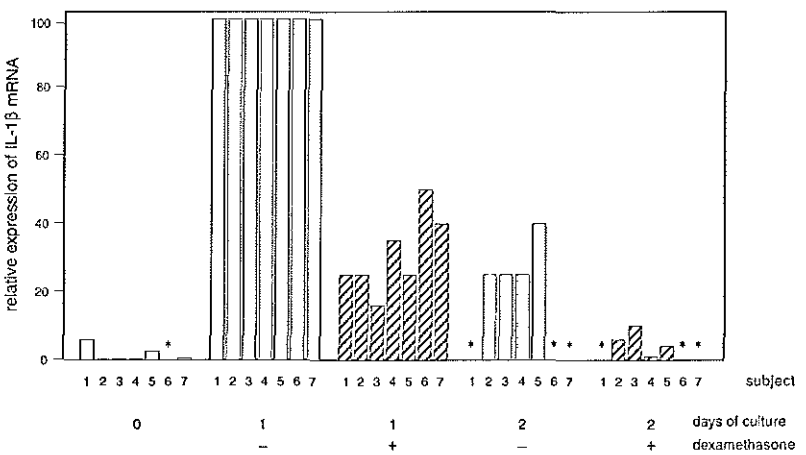

Figure 3. Relative expression of IL-1β mRNA in freshly isolated and cultured human AM (n = 7), and the influence of glucocorticoids on this expression. The expression at day 1 of culture without dexamethasone is arbitrarily set at 100%. Subject numbers correspond with subject numbers in Figure 6.
*: not determined.

Influence of dexamethasone on the kinetics of IL-1β mRNA in AM during culture

Culture of AM in the presence of dexamethasone reduced the increase in IL-1β mRNA expression as compared with AM cultured in control medium. This inhibition was already seen after one day of culture (n = 7; Figure 4A). To semiquantitate this influence of dexamethasone on IL-1β mRNA expression, dot blot analysis was performed. After one day of culture in the presence of dexamethasone, IL-1β mRNA levels were 16 to 50% of the levels in AM cultured for one day in control medium [Figures 3 and 5 (upper panel), and Table 1]. The effect of dexamethasone was also observed after two days of culture [n = 4; Figures 3, 4A and 5 (upper panel)], and even after four days of culture (n = 2; data not shown). This inhibitory effect of dexamethasone was concentration dependent (data not shown) and could be antagonized with RU 38486 [Figures 4A and 5 (upper panel), and Table 1]. In most experiments, dexamethasone was used in a concentration of 5×10^{-7} M, which was non-toxic and resulted in maximal effects. RU 38486 was always used in a tenfold excess.

Table 1. Influence of dexamethasone on the kinetics of IL-1β gene expression in cultured human AM.[a]

subject[b]	days of culture	culture conditions			
		–[c]	dexamethasone	RU 38486 + dexamethasone	RU 38486
3	0	0.4			
3	1	100[d]	16	100	63
3	2	25	10	16	16
4	0	0.4			
4	1	100[d]	35	100	63
4	2	25	1	16	10

a. Total RNA was prepared from freshly isolated or cultured AM. Each sample was dot-blotted in 1:1.58 dilutions. Relative IL-1β mRNA expression in each sample was determined by comparison with the expression in stimulated U937 cells.
b. Subject numbers correspond with subject numbers in Figures 3 and 6, and Table 2.
c. Either freshly isolated AM or AM cultured in control medium.
d. Expression after 1 day of culture in control medium was arbitrarily set at 100%.

Kinetics of lipocortin-1 mRNA expression in cultured AM

Freshly isolated AM expressed lipocortin-1 mRNA. This expression was of equal magnitude to expression in the epithelial cell line BEAS S6. Whereas expression of IL-1β mRNA increased upon culture, lipocortin-1 mRNA expression showed no such a spontaneous increase [Figures 4C, 5 (lower panel) and 6].

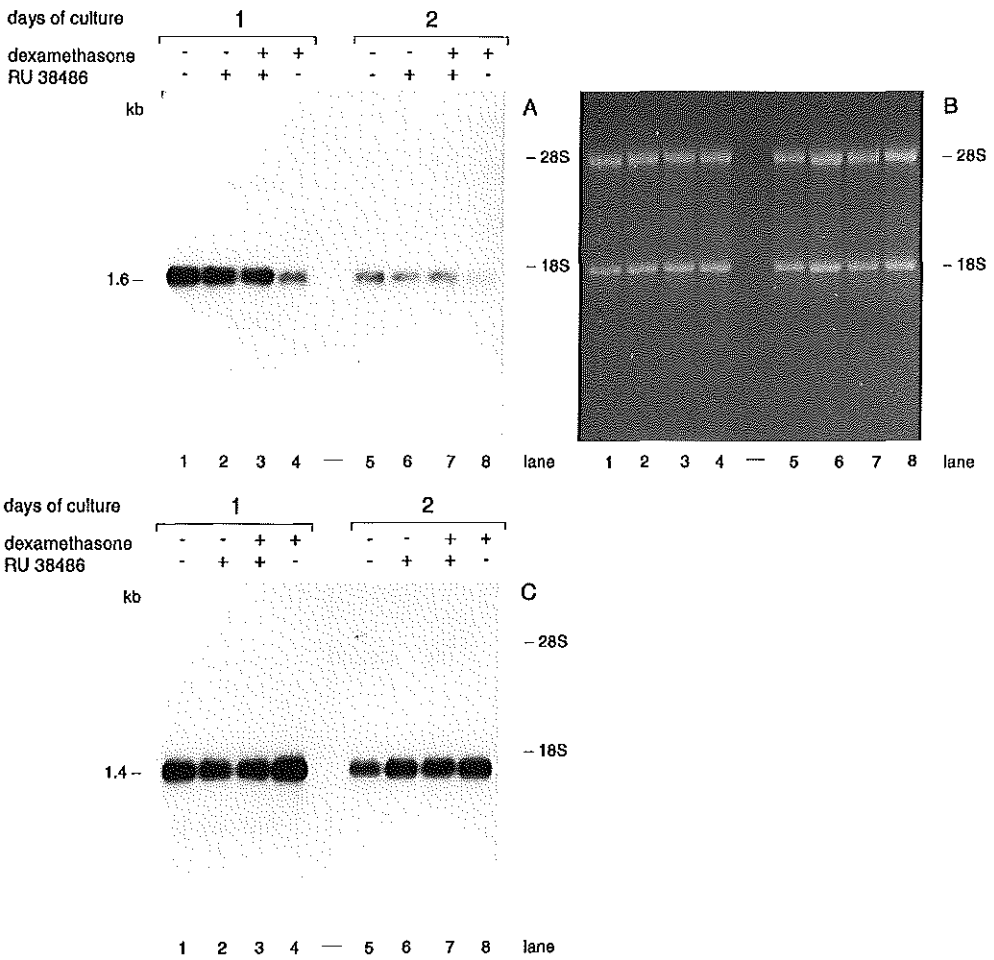

Figure 4. Northern blot analysis of the effects of dexamethasone on IL-1β mRNA (panel A) and lipocortin-1 mRNA (panel C) expression in cultured AM. Total RNA was prepared from AM cultured in either control medium (lanes 1 and 5), RU 38486 (5 x 10^{-6}M; lanes 2 and 6), dexamethasone (5 x 10^{-7}M; lanes 4 and 8), or both RU 38486 and dexamethasone (lanes 3 and 7). Each lane was loaded with 5 µg of total RNA. Panels A and C show hybridization of the same membrane with the IL-1β and lipocortin-1 cDNA probes, respectively. Panel B shows ethidiumbromide staining of the gel after electrophoresis to visualize equal loading per lane.

Influence of dexamethasone on the expression of lipocortin-1 mRNA in AM

After one day of culture in the presence of dexamethasone (5 x 10^{-7} M), expression of lipocortin-1 mRNA was found to be consistently higher than expression in AM cultured

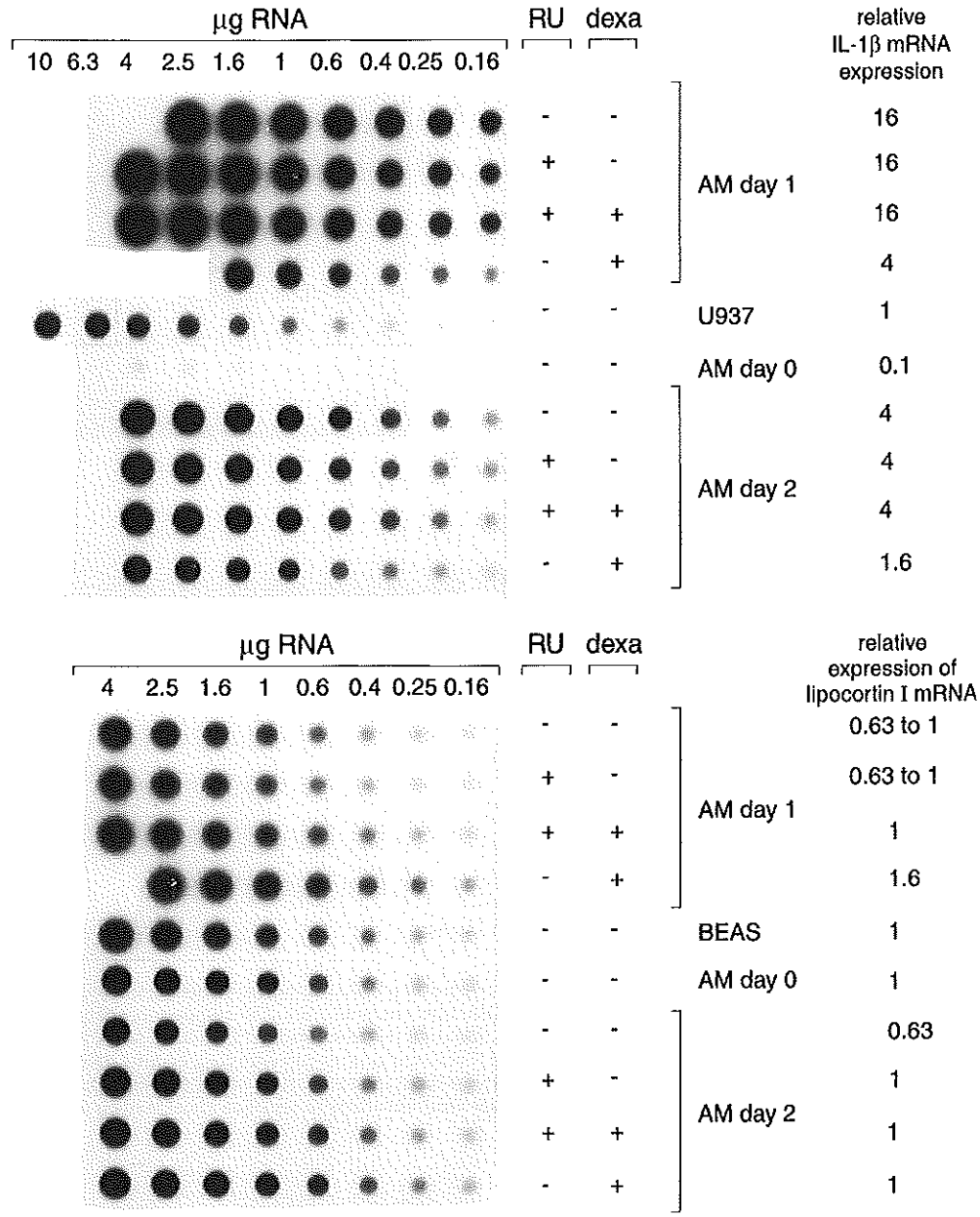

Figure 5. Semiquantification of IL-1β mRNA (upper panel) and lipocortin-1 mRNA (lower panel) expression by dot blot analysis in dexamethasone-treated AM. AM are from the same smoking subject as in Figure 4. Serial 1:1.58 dilutions of total RNA [obtained from freshly isolated AM (day 0), cultured AM (for 1 and 2 days,

respectively), and control cell lines (U937 in the upper, and BEAS S6 in the lower panel)] were applied to the wells. AM were cultured in either control medium (- -), medium with RU 38486 (+ -), dexamethasone (+ -), or both RU 38486 and dexamethasone (+ +). The right columns in A and B indicate IL-1β and lipocortin-1 mRNA expression relative to the expression in PMA/LPS-stimulated U937 cells and BEAS S6 cells, respectively. The upper panel shows hybridization with the IL-1β cDNA probe, whereas the lower panel shows the same membrane rehybridized with the lipocortin-1 cDNA probe.

in control medium [n=5; Figures 4C, 5 (lower panel) and 6]. Dot blot analysis to semiquantitate the expression of lipocortin-1 mRNA revealed a 60 to 150% increase after one day of incubation with dexamethasone as compared with the control experiment (Figure 6 and Table 2). This dexamethasone-induced increase in mRNA expression could be antagonized by RU 38486 [Figures 4B and 5 (lower panel), and Table 2]. In contrast to the effect of dexamethasone on IL-1β mRNA expression, which was still present on day 2 of culture, lipocortin-1 mRNA expression was elevated in only 2 of the 3 subjects studied after two days of incubation with dexamethasone (Figure 6). A consistent dexamethasone-induced increase in mRNA expression, as was observed on day 1, was not observed on day 2.

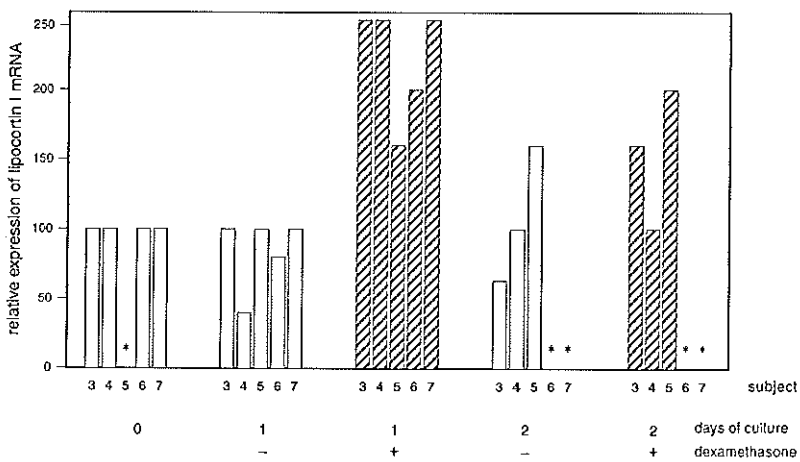

Figure 6. Relative expression of lipocortin-1 mRNA in freshly isolated and cultured human AM (n=5), and the influence of glucocorticoids on this expression. The expression in freshly isolated AM is arbitrarily set at 100%. Subject numbers correspond with subject numbers in Figure 3.
*: not determined.

Table 2. Influence of dexamethasone on the kinetics of lipocortin-1 gene expression in cultured human AM.[a]

subject[b]	days of culture	culture conditions			
		–[c]	dexamethasone	RU 38486 + dexamethasone	RU 38486
3	0	100[d]			
3	1	100	250	100	80
3	2	63	158	158	100
4	0	100[d]			
4	1	40	250	159	100
4	2	100	100	159	100

a. Total RNA was prepared from freshly isolated or cultured AM. Each sample was dot-blotted in 1:1.58 dilutions. Relative expression of lipocortin-1 mRNA in each sample was determined by comparison with the expression in the epithelial cell line BEAS S6.
b. Subject numbers correspond with subject numbers in Figures 3 and 6, and Table 1.
c. Either freshly isolated AM or AM cultured in control medium.
d. Expression in freshly isolated AM was arbitrarily set at 100%.

DISCUSSION

This study shows that glucocorticoids are able to induce lipocortin-1 mRNA in human alveolar macrophages *in vitro*, but that this induction is time-dependent, which may explain the conflicting results from the literature. Using the glucocorticoid antagonist RU 38486 it was shown that these effects are mediated via glucocorticoid receptors. Furthermore, under the same *in vitro* conditions IL-1β gene expression was downregulated by glucocorticoids. These findings are important because glucocorticoids are widely used anti-inflammatory agents. Their precise working mechanisms are still largely unknown, although recent studies have unravelled some of their cellular effects (2,3,7,8). It is known that glucocorticoids can modulate mRNA transcription, resulting in either increased or decreased protein synthesis. Many inflammatory proteins, in particular inflammatory cytokines, are downregulated by glucocorticoids (7,8). Downregulation of IL-1β gene expression by glucocorticoids, which has been demonstrated previously in human monocytes and the human monocytic cell line U937 (9,10), was confirmed in human AM in this study. The glucocorticoid-mediated induction of lipocortins was once proposed as an important aspect of the working mechanisms of glucocorticoids, but since then only some studies showed a clear induction of lipocortin-1 mRNA, whereas others did not (11-13,17-22,36,37). The reason for these apparent contradictory results may be obvious differences in experimental conditions [*in vitro* (13,20) versus *in vivo* studies (12,13,38)], differences in cell type studied (monocytes/macrophages, epithelial cells), differences in species studied [rats (11,17), mice (13,21), or rabbits (38) versus humans (12,20,39)], or still others [e.g. differences in differentiation state of the cells studied (40) or differences in culture conditions]. Here we show that the glucocorticoid-induced upregulation of lipocortin-1 mRNA occurs over a defined time course, which may explain why it has not been observed

in all previous studies.

In early studies in rats, it was shown that glucocorticoids induced lipocortin-1 mRNA in peritoneal macrophages *in vivo* (11). However, it is unclear whether glucocorticoids had the same effects in humans. Studies on steroid-inducibility *in vivo* are difficult in man because of the presence of endogenous glucocorticoids; effects of administered glucocorticoids will be superimposed upon the physiological effects of endogenous glucocorticoids, and may therefore be difficult to detect. These limitations can be bypassed in animal studies by adrenalectomy or treatment with glucocorticoid antagonists (41). Only few studies describe effects of chronic treatment with glucocorticoid antagonists in man (16,42). However, recently it was shown that glucocorticoids could induce lipocortin-1 *in vivo* in peripheral blood leukocytes (12), BAL cells (39), and BAL-fluid (43). Ambrose et al showed that human AM contain lipocortin-1, and their levels of lipocortin-1 were higher than in peripheral blood monocytes (39). They suggested that expression of lipocortin-1 may be dependent of the maturation state of mononuclear phagocytes, which was also suggested in a study on U937 cells (40). Furthermore, they showed that glucocorticoids increased the amounts of lipocortin-1 in a dose-dependent manner, although the highest dexamethasone concentrations used in that study are generally accepted as in vast excess of therapeutic doses and toxic (39). Lipocortin-1 mRNA levels were not studied. These findings at the protein level of lipocortin-1 contrast with studies which failed to demonstrate effects of dexamethasone on the expression of lipocortin-1 mRNA in human AM (20). To explain the observed induction of lipocortin-1 in other studies, Brönnegård et al suggested that this may result from posttranslational effects of glucocorticoids (20). On the other hand, glucocorticoids were shown to induce both expression of lipocortin-1 mRNA and secretion of lipocortin-1 in U937 cells (10,40).

These apparent contradictory findings suggest that experimental conditions may determine whether glucocorticoid effects on lipocortin-1 gene expression will be observed or not. Serum factors in the culture medium or pretreatment with cytokines or LPS may modulate the cellular response to glucocorticoids with regard to lipocortin-1 gene expression (36,37). In the current study, monocytes and AM were cultured under serum-free conditions, and thus we can exclude the presence of unidentified serum factors which were previously suggested to synergise with glucocorticoids to promote lipocortin-1 induction (36). Compelling evidence that the induction of lipocortin-1 mRNA in our study is glucocorticoid-specific, comes from the observed antagonizing effects of the glucocorticoid antagonist RU 38486. Furthermore, our results show that incubation time determined whether a glucocorticoid-induced modulation of expression will be observed or not. This is illustrated by our findings that the glucocorticoid effects on IL-1β mRNA expression were observed for 4 days, but those on lipocortin-1 for only 24 hours. The kinetics of lipocortin-1 mRNA or protein induction *in vitro* is sparsely described in other studies (13,20,39). Brönnegård et al studied lipocortin-1 mRNA expression in human alveolar macrophages treated for 6 hours with dexamethasone (20). Induction of lipocortin-1 mRNA was not observed, but they did not mention whether mRNA expression was studied after longer incubations. On the other hand, metallothionein II mRNA was induced after 6 hours culture in the presence of dexamethasone. The latter is mentioned as their

positive control, but it is clear from the differences between IL-1β and lipocortin-1 mRNA kinetics shown here that glucocorticoids can regulate different target genes differently. Ambrose et al studied the induction of lipocortin-1 at the protein level in human alveolar macrophages (39). Kinetics were not mentioned, but they observed an increase after 24 hours of incubation. Wong et al described time-course experiments in mouse fibroblasts (13). As in this study, they found the largest increase in lipocortin-1 mRNA level after 24 hours of incubation with dexamethasone. In *in vivo* experiments, effects of glucocorticoids on lipocortin-1 expression appear to be faster. Wallner et al showed induction of lipocortin-1 mRNA within 2 hours (11). Peers et al described an increase in the amount of lipocortin-1 protein even within 30 min, which may suggest a mechanism different from the classic nuclear working mechanism of glucocorticoids (44). Summarizing, all these results stress the importance of time-course experiments in lipocortin studies.

The results presented in this study clearly demonstrate that glucocorticoids influence the gene expression in AM with regard to proteins involved in inflammatory processes. Gene expression of the pro-inflammatory cytokine IL-1β is down-regulated by glucocorticoids, whilst under the same experimental conditions glucocorticoids increase lipocortin-1 gene expression. To our knowledge, this is the first time that these two aspects of the anti-inflammatory actions of glucocorticoids have been demonstrated together *in vitro* in human AM. Furthermore, these findings strongly suggest that one of the ways in which glucocorticoids may modulate lipocortin-1 levels is by regulation of gene transcription.

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REFERENCES

1. Hench PS, Kendall EC, Slocumb CH, Polley HF. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc Staff Meet, Mayo Clin 1949;24:181-197.
2. Schleimer RP. Effects of glucocorticoids on inflammatory cells relevant to their therapeutic applications in asthma. Am Rev Respir Dis 1990;141:S59-S69.
3. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
4. De Waal RMW. The anti-inflammatory activity of glucocorticoids. Mol Biol Rep 1994;19:81-88.
5. Schleimer RP, Claman HN, Oransky A, eds. Anti-inflammatory steroid action: basic and clinical aspects. San Diego: Academic Press, Inc. 1989:132-258.

6. Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc Natl Acad Sci USA 1992;89:9991-9995.
7. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray MA, Anderson WH, eds. Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:593-617.
8. Boumpas DT, Paliogianni F, Anastassiou ED, Balow JE. Glucocorticosteroid action on the immune system: molecular and cellular aspects. Clin Exp Rheumatol 1991;9:413-423.
9. Amano Y, Lee SW, Allison AC. Inhibition by glucocorticoids of the formation of interleukin-1α, interleukin-1β, and interleukin-6: mediation by decreased mRNA stability. Mol Pharmacol 1993;43:176-182.
10. Knudsen PJ, Dinarello CA, Strom TB. Glucocorticoids inhibit transcriptional and post-transcriptional expression of interleukin-1 in U937 cells. J Immunol 1987;139:4129-4134.
11. Wallner BP, Mattaliano RJ, Hession C, Cate RL, Tizard R, Sinclair LK, Foeller C, Chow EP, Browning JL, Ramachandran KL, Pepinsky RB. Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature 1986;320:77-81.
12. Goulding NJ, Godolphin JL, Sharland PR, Peers SH, Sampson M, Maddison PJ, Flower RJ. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. Lancet 1990; 335: 1416-1418.
13. Wong WT, Frost SC, Nick HS. Protein-synthesis-dependent induction of annexin I by glucocorticoid. Biochem J 1991;275:313-319.
14. Oppenheim JJ, Kovacs EJ, Matsushima K, Durum SK. There is more than one interleukin 1. Immunol Today 1986;7:45-56.
15. Dinarello CA, Wolff SM. The role of interleukin-1 in diseases. New Engl J Med 1993;328:106-113.
16. Goulding NJ, Guyre PM. Regulation of inflammation by lipocortin 1. Immunol Today 1992;13:295-297.
17. Ambrose MP, Hunninghake GW. Corticosteroids increase lipocortin I in alveolar epithelial cells. Am J Respir Cell Mol Biol 1990;3:349-353.
18. Perretti M, Ahluwalia A, Harris JG, Goulding NJ, Flower RJ. Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse. J Immunol 1993;151:4306-4314.
19. Perretti M, Flower RJ. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. J Immunol 1993;150:992-999.
20. Brönnegård M, Andersson O, Edwall D, Lund J, Norstedt G, Carlstedt-Duke J. Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol Endocrinol 1988;2:732-739.
21. Beyaert R, Suffys P, Van Roy F, Fiers W. Inhibition by glucocorticoids of tumor necrosis factor-mediated cytotoxicity. Evidence against lipocortin involvement. FEBS Lett 1990;262:93-96.
22. Northup JK, Valentine-Braun KA, Johnson LK, Severson DL, Hollenberg MD. Evaluation of the antiinflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J Clin Invest 1988;82:1347-1352.
23. Herscowitz HB. In defense of the lung: paradoxical role of the pulmonary alveolar macrophage. Ann Allergy 1985;55:634-648.
24. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
25. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
26. Van Hal PThW, Wijkhuijs JM, Hoogsteden HC, Mulder E. Functional glucocorticoid receptors in human alveolar macrophages. Submitted for publication.

27. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcεRIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.
28. Figdor CG, Bont WS, Touw I, De Roos J, Roosnek EE, De Vries JE. Isolation of functionally different human monocytes by counterflow centrifugation elutriation. Blood 1982;60:46-53.
29. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565-577.
30. Baldari CT, Telford JL. The intracellular precursor of IL-1β is associated with microtubules in activated U937 cells. J Immunol 1989;142:785-791.
31. Lechner JF, La Veck MA. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J Tissue Culture Meth 1985;9:43-48.
32. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
33. Benham FJ, Hodgkinson S, Davies KE. A glyceraldehyde-3-phosphate dehydrogenase pseudogene on the short arm of the human X-chromosomes defines a multigene family. EMBO J 1984;3:2635-2640.
34. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes. Part I: Basic and technical aspects. Clin Chim Acta 1991;198:1-92.
35. Nooter K, Sonneveld P, Oostrum R, Herweijer H, Hagenbeek T, Valerio D. Overexpression of the *mdr 1* gene in blast cells from patients with acute myelocytic leukemia is associated with decreased anthracycline accumulation that can be restored by cyclosporin-A. Int J Cancer 1990;45:263-268.
36. Flower RJ. Lipocortin. Prog Clin Biol Res 1990;349:11-25.
37. Browning JL, Ward MP, Wallner BP, Pepinsky RB. Studies on the structural properties of lipocortin-1 and the regulation of its synthesis by steroids. Prog Clin Biol Res 1990;349:27-45.
38. Náray-Fejes-Tóth A, Fejes-Tóth G, Fischer C, Frölich JC. Effect of dexamethasone on *in vivo* prostanoid production in the rabbit. J Clin Invest 1984;74:120-123.
39. Ambrose MP, Bahns C-LC, Hunninghake GW. Lipocortin I production by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;6:17-21.
40. Solito E, Raugei G, Melli M, Parente L. Dexamethasone induces the expression of the mRNA of lipocortin 1 and 2 and the release of lipocortin 1 and 5 in differentiated, but not undifferentiated U-937 cells. FEBS Lett 1991;291:138-244.
41. Vishwanath BS, Frey FJ, Bradbury M, Dallman M, Frey BM. Adrenalectomy decreases lipocortin-I messenger ribonucleic acid and tissue protein content in rats. Endocrinol 1992;130:585-591.
42. Laue L, Lotze MT, Chrousos GP, Barnes K, Loriaux DL, Fleisher TA. Effect of chronic treatment with the glucocorticoid antagonist RU 486 in man: toxicity, immunological, and hormonal aspects. J Clin Endocrinol Metab 1990;71:1474-1480.
43. Ambrose MP, Hunninghake GW. Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. J Appl Physiol 1990;68:1668-1671.
44. Peers SH, Smillie F, Elderfield AJ, Flower RJ. Glucocorticoid- and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes *in vivo*. Br J Pharmacol 1993;108:66-72.

FUNCTIONAL GLUCOCORTICOID RECEPTORS IN HUMAN ALVEOLAR MACROPHAGES*

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SUMMARY

Alveolar macrophages (AM) play a major role in the regulation of inflammatory responses in the lung, and are thought to be a primary target for glucocorticoid therapy. The variable clinical response to glucocorticoids in the treatment of various inflammatory lung diseases may be explained by a variable cellular response of AM to glucocorticoids, although literature is conflicting about the presence of specific glucocorticoid receptors in human AM. The present study was designed to determine whether human AM possessed a single class of functional glucocorticoid receptors. Established methods, which use a ^{3}H-labeled ligand to estimate glucocorticoid receptor numbers per cell and the dissociation constant of these receptors, were hampered by extremely high nonspecific binding when AM were analyzed. Typically, the nonspecific binding was 80-95% of the total binding. As receptor numbers could be easily determined in other cell types, this high nonspecific binding appeared to be typical of AM and may be caused by phagocytosed dust, carbon particles or tobacco smoke products. To reduce this nonspecificity, we eventually introduced an immunoprecipitation procedure after incubation of AM with various concentrations of ^{3}H-labeled dexamethasone, with the intention to precipitate the ligand-receptor complex excluding ligand bound to nonspecific binding sites. This resulted in an adequate reduction of the nonspecific binding to only 10-20% of the total binding. Using this procedure, AM were found to have $3.9 \pm 2.0 \times 10^3$ (mean ± SD; n = 3) specific glucocorticoid receptors per cell and the dexamethasone K_d was 7.0 ± 1.5 nM (mean ± SD; n = 3). For comparison, the monocytic cell line U937 and the uterine cervix carcinoma cell line NHIK 3025 were studied. These cell lines were found to have $6.8 \pm 0.9 \times 10^3$ (mean ± SD; n = 4) and $19.2 \pm 2.5 \times 10^3$ (mean ± SD; n = 3) glucocorticoid receptors per cell, respectively. Their dexamethasone K_d's were 3.5 ± 0.5 nM and 3.1 ± 0.6 nM, respectively. Furthermore, it was shown in this study that glucocorticoid-induced inhibition of cell spreading upon culture of AM was dependent on the dexamethasone concentration and could be antagonized by the glucocorticoid antagonist RU 38486. These observations demonstrate that also human AM express a single class of specific glucocorticoid receptors, through which cellular effects of glucocorticoids are mediated. We therefore conclude that AM may be an actual, direct target for glucocorticoid therapy in inflammatory lung diseases. Further studies are needed to confirm the putative role of the glucocorticoid receptor system in AM in the clinical responsiveness to glucocorticoids.

INTRODUCTION

Since the identification of the macrophage, its classical and most important function was considered to phagocytose waste and foreign particles (1). Accordingly, alveolar macrophages (AM) in the lungs of heavy smokers contain large amounts of phagocytosed carbon particles and tobacco smoke products (2,3). In addition to this scavenger function, macrophages are nowadays known to have important immunologi-

cal functions, which turn macrophages into a central cell type in initiating and perpetuating inflammation (3,4). These functions require interactions with other cells and molecules, and may take place through the release of numerous secretory products (e.g. cytokines, enzymes, and biologically active lipids) and the expression of several surface receptors (e.g. cytokine receptors, MHC class II, and adhesion molecules). Therefore, AM are thought to play a major role in airway inflammation in lung diseases as asthma and chronic obstructive pulmonary diseases (COPD) (3-6), although there is evidence that other cell types (T lymphocytes, bronchial epithelium, mast cells and granulocytes) may also contribute to the inflammatory reaction (7-9).

Glucocorticoids are frequently used in the treatment of inflammatory lung diseases. The precise nature of their working mechanism is still unknown (10,11). Because of the above-mentioned role of AM in airway inflammation and because the function of monocytes/macrophages can be influenced by glucocorticoids (12,13), glucocorticoids may suppress airway inflammation by influencing AM. Other evidence for a monocyte/macrophage-mediated effect of glucocorticoids in asthma stems from studies in which a cellular defect of the monocyte/macrophage lineage was suggested in glucocorticoid-resistant asthmatic patients (14,15). However, it remains to be elucidated whether the reported effects of glucocorticoids on monocytes/macrophages result from a direct, glucocorticoid receptor-mediated (16,17) or a non receptor-mediated (18) mechanism. Another actual possibility concerns an indirect effect via mediators released by other, glucocorticoid sensitive cell types. To substantiate a direct monocyte/macrophage-mediated effect of glucocorticoids, evidence for specific, saturable and functional glucocorticoid receptors in these cells is still a prerequisite. Studies which describe estimations of the number of glucocorticoid receptors in human AM are rare, ambiguous regarding the presence of a single class of high affinity glucocorticoid receptors, and are severely hampered by nonspecific binding (19-21). In this report a procedure for the estimation of glucocorticoid receptors in human AM is described and it is shown that these receptors are functional. The method employs precipitation of the ligand-receptor complex with a receptor-specific monoclonal antibody. The major advantage of this immuno receptor assay comes from the by-passing of nonspecific binding. As the number and quality of steroid receptors in target cells may determine the extent of glucocorticoid responsiveness (22,23), this assay create new openings for studies on the role of glucocorticoid receptors, or transcription factors interacting with them, in the variable clinical response to glucocorticoids (24).

MATERIALS AND METHODS

Cell lines

U937 cells, an immature monocytic cell line and originally described by Sundström et al. (25) were maintained at concentrations of 0.25-1.0 x 10^6/ml in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with FCS (10%; GIBCO, Paisley, UK), penicillin G sodium (10^2 U/ml; Gist-Brocades, Delft, The Netherlands) and streptomycin-sulfate (0.1 mg/ml; Biochrom KG, Berlin, Germany). The cell line NHIK

3025, derived from a carcinoma of the human uterine cervix and originally described by Nordbye and Oftebro (26), was cultured in DMEM supplemented with FCS (10%), penicillin G sodium (10^2 U/ml), streptomycin-sulfate (0.1 mg/ml) and non-essential amino acids.

Isolation of AM

All lavage studies were approved by the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. Bronchoalveolar lavage (BAL) was performed in healthy, smoking volunteers after premedication with thiazinamium and local anesthesia using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe, and four aliquots of 50 ml sterile saline solution were infused and aspirated immediately in a siliconized specimen trap placed on melting ice. Immediately after collection the BAL fluid was strained through a sterile nylon gauze to trap large mucous particles, whereafter the BAL cells were isolated by centrifugation at 4°C at a force of 400 g. More than 90% of the BAL cells appeared to be macrophages as judged by May-Grünwald Giemsa staining.

Culture of AM

AM were cultured in a modified Iscove's medium (GIBCO, Paisley, UK) as described previously (27), in which BSA is replaced by human serum albumin supplemented with 2% autologous heat-inactivated serum. The culture medium was found to be endotoxin-free (defined as less than 1.0 ng/ml of endotoxin as quantified by the Limulus amoebocyte lysate assay). AM ($1\text{-}2 \times 10^6$/ml) were cultured at 37°C, 5% CO_2 and 100% humidity under adherent conditions in flat bottom wells of 24-wells culture plates (Becton Dickinson, NJ) for 1 to 4 days.

Steroids

Nonradioactive dexamethasone was obtained from Duchefa b.v. (Haarlem, The Netherlands). A stock solution of 2×10^{-3} M dexamethasone in ethanol was prepared. ^{3}H-labeled dexamethasone (1,2,4,6,7 [^{3}H] dexamethasone; specific activity 81 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). The glucocorticoid antagonist RU 38486 was kindly provided by Roussel Uclaf (Romainville, France). A stock solution of 2×10^{-3} M RU 38486 in ethanol was prepared. The concentration of ethanol during culture of AM was less than 0.25% in each experiment. The concentration of ethanol during incubations for the receptor assay was less than 1% in each sample.

Glucocorticoid receptor assay without immunoprecipitation procedure

Glucocorticoid receptor density and dissociation constant were determined according to established methods (28,29). A whole cell assay, rather than a binding assay on cellular extracts, was chosen for, as the former assay requires smaller numbers of cells (cellular yields from BAL are limited) and minimizes proteolysis which may lead to loss of receptors. Freshly isolated AM, U937 or NHIK cells were washed with a phosphate-buffered saline solution (PBS) (300 mosmol; pH 7.4) and resuspended at a density of $8\text{-}10 \times 10^6$ cells/ml. A series of seven 1:2 dilutions (200 µl) of ^{3}H-labeled dexamethasone was prepared. For measurements of nonspecific binding, a parallel series of 1:2 dilutions of ^{3}H-labeled dexamethasone plus a 100-fold excess nonradioactive dexamethasone was prepared. Incubation was initiated by adding 200 µl of the cell suspension to each of the 14 tubes. Final concentrations of ^{3}H-labeled dexamethasone

were 0.5-32 nM. Incubations were carried out in polypropylene tubes for 90 min at 21°C. These conditions were shown earlier to allow adequate equilibration between free and receptor-bound steroid (28). After this incubation of the cells, three 100 µl aliquots of each incubation mixture (determination in triplicate) were transferred to separate tubes. Fifty µl of the incubation mixture was used to verify the concentration of ^{3}H-labeled dexamethasone. One ml of ice-cold PBS was added to each 100 µl aliquot and the samples were centrifuged (400 g, 5 min, 4°C). Supernatants were removed and cell pellets were resuspended in 1 ml of ice-cold PBS and left on ice for 30 min to reduce nonspecific binding. Next, the samples were centrifugated and the supernatants were discarded. The cell pellets were transferred to scintillation vials and radioactivity was determined by liquid scintillation counting. Specific binding was calculated as the difference between the totally bound radioactivity and the nonspecifically bound radioactivity at each glucocorticoid concentration. Nonspecific binding was calculated from the aliquots containing the 100-fold excess nonradioactive dexamethasone, assuming that nonspecific binding was nonsaturable and linearly related to the concentration of free glucocorticoid.

Immuno glucocorticoid receptor assay

Freshly isolated AM, U937 cells or NHIK cells were washed once and resuspended in TEG buffer (40 mM Tris-HCl, 1 mM EDTA, 10% glycerol; pH 7.4). Cells (200 µl; 8-10 x 10^6/ml) were incubated with 200 µl of ^{3}H-labeled dexamethasone (final concentration 1-32 nM) in polypropylene tubes for 90 min at 21°C. Nonspecific binding was determined in a parallel series of incubation of cells with radiolabeled dexamethasone plus a 100-fold excess nonradioactive dexamethasone. After this incubation, two 150 µl aliquots of each incubation mixture (determination in duplicate) were transferred to separate 1.5-ml Eppendorf tubes. Fifty µl of the incubation mixture was used to verify the concentration of ^{3}H-labeled dexamethasone. All subsequent procedures were performed at 0-4°C, unless otherwise indicated. Next, 250 µl of a solution of protease inhibitors (final concentrations bacitracin (Aldrich Chemie, Brussels, Belgium) 500 µM, phenylmethylsulfonylfluoride 600 µM (Sigma, St. Louis, MO), dithiothreitol 10 mM (Sigma), leupeptine 500 µM (Sigma)) was added to each 150 µl aliquot. The samples were freezed/thawed (-196°C/10°C) three times to lyse cells. Thereafter, the samples were centrifuged (15.000 g, 15 min, 4°C) and supernatants were frozen (-20°C) and used for immunoprecipitation later. Pellets were resuspended in 1 ml NaOH (1 M), incubated at 60°C for 30 min and frozen (-20°C) for determination of DNA content later.

Agarose coated with goat anti mouse-immunoglobulin antibodies (Sigma) was incubated with the monoclonal antibody F52.24.4 overnight (4°C) with rotation [The monoclonal antibody F52.24.4 was raised against the homologous part of the DNA-binding domain of different steroid hormone receptors. Details about the generation and characterization of this monoclonal antibody are described elsewhere (30)]. After this incubation the agarose was centrifuged (400 g, 5 min) and washed three times with PBS. The agarose was resuspended in PBS and 100 µl of this solution was added to the thawed supernatants and incubated with rotation for 2 hours at 4°C. Thereafter, 0.5 ml PBS was added to the incubation mixture. Subsequently, the samples were centrifuged (2200 g, 1 min, 4°C) and washed with 1 ml of PBS three times. Finally, the tips of the Eppendorf tubes containing the pellets were cut off, and radioactivity was determined by liquid scintillation counting.

RESULTS

Glucocorticoid receptors in cell lines

Previous studies have reported the presence of glucocorticoid receptors in U937

cells (31). In our assay conditions using the unadapted receptor assay, we could also demonstrate specific binding of ^{3}H-labeled dexamethasone to these cells. A typical binding curve is shown in figure 1A. The binding data were replotted according to the Scatchard technique and these replotted data generated a straight line (Figure 1B). This suggested that dexamethasone is binding to a single class of receptor sites of uniform affinity and allowed to estimate the total number of specific binding sites per cell (R; x intercept) and the equilibrium dissociation constant (K_d; negative inverse of the slope). U937 cells appeared to have 17.1 ± 5.6 x 10^3 (mean ± SD; n = 4) sites per cell, while the K_d was 5.3 ± 1.0 nM (n = 4) (Table 1). It was shown earlier (32) that NHIK cells also contain specific binding sites for glucocorticoids. In the present study NHIK cells showed to have far greater numbers of specific binding sites per cell than U937 cells, 86.0 ± 6.3 x 10^3 (n = 3). The K_d was not different from the K_d in U937 cells, 4.4 ± 1.9 nM (n = 3) (Table 1).

Table 1. Numbers of glucocorticoid receptors and their K_d values in U937 cells, NHIK cells and alveolar macrophages (AM).[a]

	U937	NHIK	AM
R [b]	18.2 ± 5.1 [c]	86.0 ± 6.3	n.d. [d]
K_d [e]	5.3 ± 1.0	4.4 ± 1.9	n.d.

a. Glucocorticoid receptor density and K_d value were determined using the established receptor assay without immunoprecipitation procedure as described in the section *Materials and Methods*.
b. R: number of binding sites per cell (x 10^3).
c. Results are shown as mean ± standard deviation of four (U937 and AM) or three (NHIK) independent experiments.
d. Using the unadapted assay, number of binding sites and dissociation constant could not be determined in alveolar macrophages.
e. K_d: dissociation constant (nM).

Glucocorticoid receptors in alveolar macrophages

There are some reports concerning glucocorticoid receptors in human alveolar macrophages (19-21). However, those reports are all hampered by a high nonspecific binding, which, fundamentally, makes hard conclusions impossible. In this study, it was not possible to estimate reliably the number of glucocorticoid receptors or their dissociation constant in AM using the unadapted receptor assay. In many experiments the nonspecific binding was as high as the total binding (Table 2). Additional wash procedures after the 90 min incubation, the use of androgens as antagonist, or cell membrane lysis were all unsuccessful in reducing nonspecific binding adequately (data not shown). To circumvent these difficulties, we introduced an immuno receptor assay in our studies. Using this assay, the effects of nonspecific binding were by-passed and reliable estimation of specific binding in AM proved to be possible (Table 3 and figure 2). AM from healthy volunteers showed to have 3.9 ± 2.0 x 10^3 specific binding sites per cell (n = 3). The corresponding K_d was 7.0 ± 1.5 nM (n = 3).

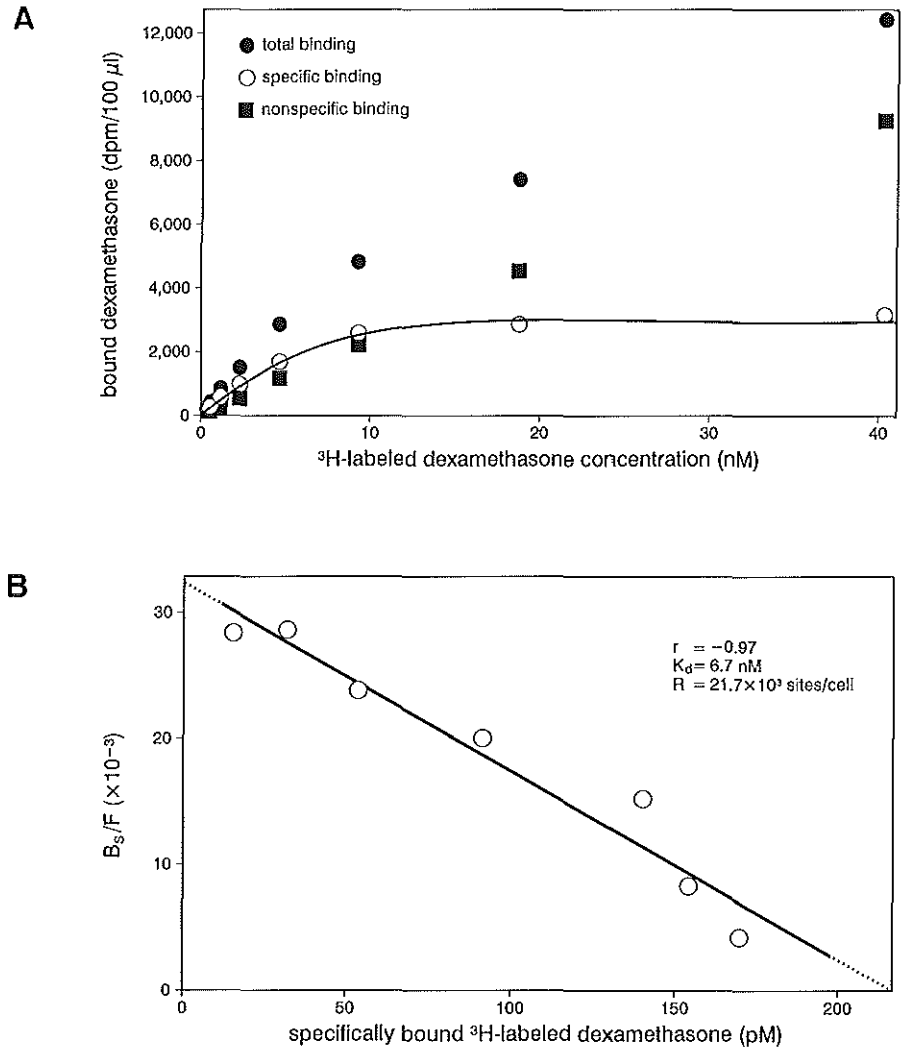

Figure 1. (A) Binding curves of ^{3}H-labeled dexamethasone for U937 cells as determined with the receptor assay without immunoprecipitation procedures. The specific binding (○) is represented by the difference between the total binding (●) and nonspecific binding (■). On the ordinate is given the binding of ^{3}H-labeled dexamethasone (dpm/100 µl of incubation mixture). (B) Scatchard plot of the specific binding of ^{3}H-labeled dexamethasone to U937 cells. On the abscissa is given the amount of specifically bound ^{3}H-labeled dexamethasone (dpm/100 µl of incubation mixture), while on the ordinate is given the ratio of the amount of specifically bound ^{3}H-labeled dexamethasone (B_s) to the amount of free ^{3}H-labeled dexamethasone (F). One representative experiment out of four is shown. The negative inverse of the slope and the x intercept were used to calculate the dissociation constant (K_d) and the number of glucocorticoid receptors per cell (R), respectively.

Table 2. Binding of ^{3}H-labeled dexamethasone in alveolar macrophages.

Concentration of ^{3}H-labeled dexamethasone (nM)	Total binding	Nonspecific binding	Specific binding	Specific binding / Total binding x 100%
0.5	286 [a]	237	49 [b]	17
1.0	607	523	84	14
2.0	1,333	1,127	206	15
4.0	2,060	2,186	— [c]	—
8.0	4,645	4,318	327	7
16.0	9,696	8,882	814	8
32.0	21,599	18,923	2,676	12

a. Data are given as counts per minute and determined using the established assay without immunoprecipitation procedure as described in the section *Materials and Methods*. One representative experiment out of ten is shown.
b. Specific binding should be calculated by subtracting the nonspecific binding from the total binding.
c. Calculation of specific binding is not possible, because total and nonspecific binding are virtually identical.

Table 3. Binding of ^{3}H-labeled dexamethasone in alveolar macrophages using the immuno receptor assay.

Concentration of ^{3}H-labeled dexamethasone (nM)	Total binding	Nonspecific binding	Specific binding	Specific binding / Total binding x 100%
1.0	167 [a]	36	131 [b]	79
2.0	288	46	242	84
4.0	466	66	400	86
8.0	743	99	644	87
16.0	950	186	764	80
32.0	1,236	308	928	75

a. Data are given as counts per minute. One representative experiment out of three is shown.
b. Specific binding should be calculated by subtracting the nonspecific binding from the total binding.

Characterization of the immuno receptor assay

To compare the characteristics of AM as obtained with the immuno receptor assay with those of U937 cells and NHIK cells, both cell lines were also put to the immuno receptor assay. Under the conditions of this assay, U937 and NHIK cells showed to have $6.8 \pm 0.9 \times 10^3$ (n=4) and $19.2 \pm 2.5 \times 10^3$ (n=3) specific binding sites per cell, respectively. The K_d's were 3.5 ± 0.5 nM (n=4) and 3.1 ± 0.6 nM (n=3), respectively (Table 4). To test whether the immuno receptor assay could discriminate between small differences in number of specific binding sites per cell, we estimated the number of glucocorticoid receptors per cell in a mixture (1:1) of NHIK and U937 cells. This mixture produced $12.1 \pm 2.2 \times 10^3$ (n=3) specific binding sites per cell (expected number $13.0 \pm 1.3 \times 10^3$) and 3.0 ± 0.3 nM as K_d (Table 4). Therefore, our immuno receptor assay proved to produce reliable results and enabled to determine at least a difference of 5300 receptors per cell.

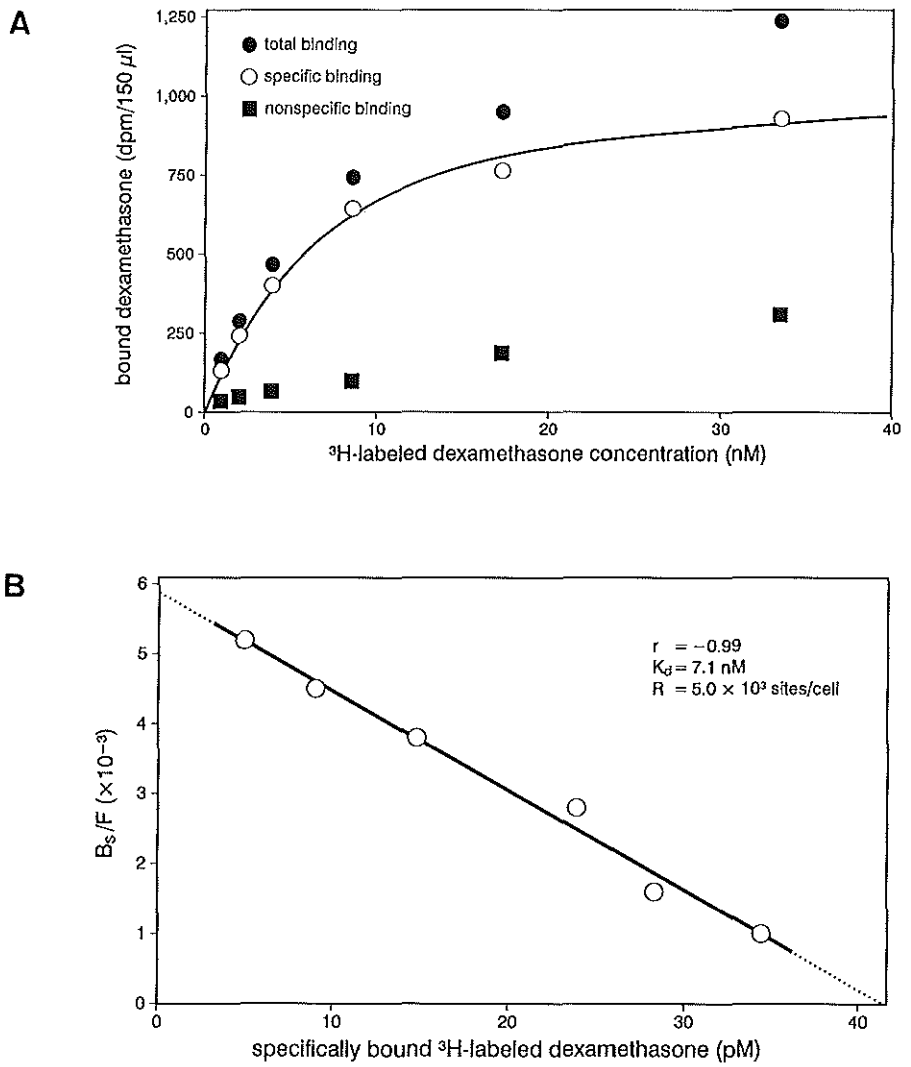

Figure 2. (A) Binding curves of ^{3}H-labeled dexamethasone for human alveolar macrophages (AM) as determined with the immuno receptor assay. The specific binding (○) is represented by the difference between the total binding (●) and nonspecific binding (■). (B) Scatchard plot of ^{3}H-labeled dexamethasone specifically bound to human AM. On the ordinate is given the ratio of the amount of specifically bound ^{3}H-labeled dexamethasone (B_s) to the amount of free ^{3}H-labeled dexamethasone (F). In both (A) and (B) the data from table 3 are used. One representative experiment out of three is shown. The negative inverse of the slope and the x intercept were used to calculate the dissociation constant (K_d) and the number of glucocorticoid receptors per cell (R), respectively.

Table 4. Numbers of glucocorticoid receptors and their K_d values in U937 cells, NHIK cells and alveolar macrophages (AM) as estimated in the immuno receptor assay.

	U937	NHIK	U937 + NHIK (1:1)	AM
R [a]	6.8 ± 0.9 [b]	19.2 ± 2.5	12.1 ± 2.2	3.9 ± 2.0
K_d [c]	3.5 ± 0.5	3.1 ± 0.6	3.0 ± 0.3	7.0 ± 1.5

a. R: number of binding sites per cell (x 10^3).
b. Results are shown as mean ± standard deviation of four (U937) or three (NHIK, U937 + NHIK, AM) independent experiments.
c. K_d: dissociation constant (nM).

Glucocorticoid receptors in alveolar macrophages are functional

As specific glucocorticoid receptors were clearly demonstrated in AM when the immuno receptor assay was used, we were interested to find out whether these receptors were functional or not. Several studies report influences of glucocorticoids on AM functions, but it is not known whether these influences are either mediated directly via glucocorticoid receptors in the AM themselves, or via mediators released by other cell types, or have to be considered as non-receptor mediated influences.

AM cultured under adherent conditions exhibited a specific morphology. Freshly isolated AM appeared round upon examination with phase contrast microscopy, but after three days of culture most of the AM were spread or spindle-shaped (Figure 3A). However, only few AM were spindle-shaped when they were cultured in the presence of dexamethasone (5 x 10^{-7} M) (Figure 3B). Spreading and appearance of cellular protrusions seemed to be inhibited by glucocorticoids. This inhibitory effect of dexamethasone on the culture-induced change in morphology was dependent on the concentration of dexamethasone and could be antagonized by RU 38486 (5 x 10^{-6} M), a known antagonist of glucocorticoids (Figure 3C). This suggests that dexamethasone exerted its effect via glucocorticoid receptors.

DISCUSSION

Studies on glucocorticoid receptors in human alveolar macrophages are rare and conflicting with respect to the presence of a single class of high affinity receptors (19-21). Established methods to determine the dissociation constant and receptor density are hampered by high nonspecific binding (20), which makes it impossible, or at least unreliable, to calculate specific binding as the difference between total and nonspecific binding. Studies on glucocorticoid receptors are easily affected by nonspecific binding, because the affinity of the receptor for its ligand is relatively low as compared with other steroid receptors.

Also in our study it was impossible to determine numbers of glucocorticoid

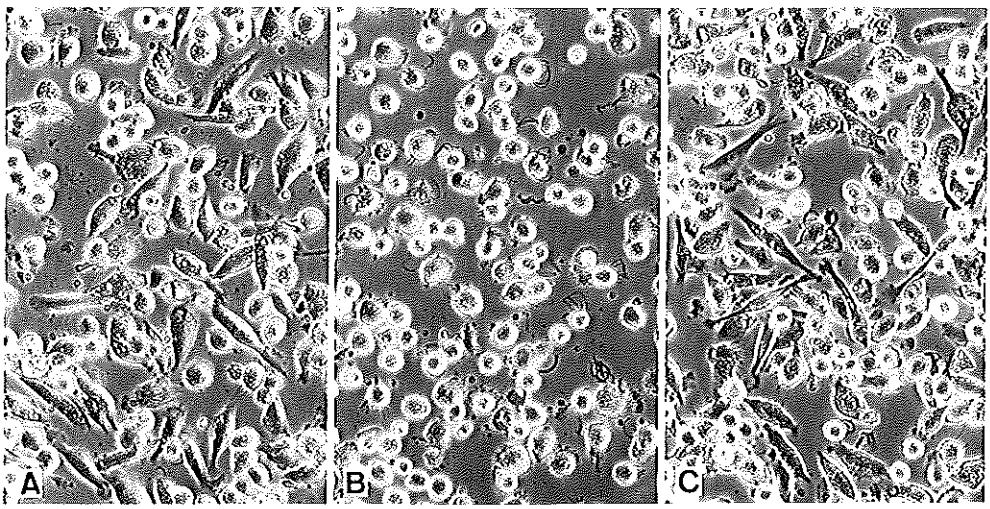

Figure 3. Phase contrast morphology of human alveolar macrophages (AM) cultured for three days *in vitro* under adherent conditions (A). More than 70% of the AM exhibit a spindle-shaped morphology. The acquisition of this morphology is inhibited by dexamethasone (5×10^{-7} M) (B). Most cells retain the rounded morphology of freshly isolated AM. Culture in the presence of both dexamethasone (5×10^{-7} M) and RU 38486 (5×10^{-6} M) resulted in the same percentage of spindle-shaped AM as culturing in medium without supplements (C). Culture in the presence of RU 38486 (5×10^{-6} M) alone had no effect on the morphology of AM (data not shown).

receptors and their K_d values in AM from healthy volunteers using an established glucocorticoid receptor assay, which did yield excellent results when a monocytic or an uterine cervix carcinoma cell line was used. Nonspecific binding was extremely high in AM and often in the same range as total binding. Also a recent study by Lacronique et al. (20) reported high nonspecific binding (85-90% of total binding). Their results seemed to indicate nonsaturable receptors in AM, although they mentioned that their results may be caused by methodologic problems. If human AM would indeed possess nonsaturable glucocorticoid receptors, they would represent an exceptional cell type, as both AM of other species (33,34) and human peripheral blood monocytes (the precursors of AM) (33,35,36) do contain a single class of high affinity receptors.

From our first experiments we learned that AM, especially from smokers, showed a high nonspecific binding. AM from smokers are known to contain large amounts of carbon particles. As carbon/charcoal is able to bind steroids in general (37), we reasoned that this could act as a second pool of high affinity binding sites besides the hypothesized glucocorticoid receptor in AM. Also other dust particles inhaled by smokers and nonsmokers and phagocyoszed by AM may disturb the outcome of the receptor assay, because they may bind the applied steroid nonspecifically. Theoretically, nonspecific binding can be reduced by stringent wash procedures or cell membrane lysis after the 90 min incubation, or by the use of nonspecific steroid antagonists after the

90 min incubation. None of these methods proved to be successful in reducing nonspecific binding in human AM. Eventually, high nonspecific binding, which interfered with reliable estimation of specific binding sites, could be circumvented effectively by introducing immunoprecipitation of glucocorticoid receptors after incubation with the radioactive ligand. With this, the nonspecific binding in AM was reduced to only 10-20% of the total binding. However, estimation of the numbers of glucocorticoid receptors per cell in U937 and NHIK cells revealed that specific binding sites seemed to be underestimated when the immuno receptor assay was used. In U937 and NHIK cells only 40% and 22% of the specific binding sites could be demonstrated respectively, when the immuno receptor assay was used in stead of the established method. This apparent underestimation may be caused by two factors. First, the immuno receptor assay implies more procedures which may lead to partial loss of complexes of receptors and their ligand. Second, part of the radioactivity may be bound to the pellet after cell lysis and this is not put to immunoprecipitation. As the established receptor assay was inappropriate for studies on human AM, we accepted a possible, partial loss of signal and considered the advantage of low nonspecific binding and reproducible specific binding as first interest in our studies. Eventually, this enabled us to determine indisputably specific binding sites in human AM. Recently, a method in studies on estrogen receptors in rats also involving immunoprecipitation was described elsewhere (38). The authors also reported the small background of nonspecific binding as a major advantage of their method.

Ozaki et al. (19) could demonstrate saturable glucocorticoid receptors in AM, but they did not mention to what extent binding appeared to be nonspecific. In their assay, AM were incubated with ^{3}H-labeled prednisolone for 2 hours at 4°C. In the present study AM were incubated with ^{3}H-labeled dexamethasone for 90 min at 21°C. Both incubation temperature and choice of ligand determine the K_d value and may influence nonspecific binding. We did not use prednisolone in this study, but triamcinolone used in some of our experiments gave similar results as dexamethasone (data not shown). The study of Ozaki et al. (19) showed that human AM contained $9.8 \pm 4.1 \times 10^3$ specific binding sites for ^{3}H-labeled prednisolone per cell. The K_d value of these cells was 9.0 ± 4.9 nM. In patients with IPF they found a reduced number of specific binding sites per cell ($3.2 \pm 2.0 \times 10^3$), although Lacronique et al. (20) could not demonstrate a difference in the pattern of dexamethasone binding by AM between normal subjects and IPF patients. These apparent discrepancies may be explained by methodologic problems resulting from high nonspecific binding. In the present study this problem was eliminated, and AM from healthy volunteers showed to have $3.9 \pm 2.0 \times 10^3$ specific binding sites per cell.

High nonspecific binding of glucocorticoids in AM *in vivo* may have therapeutical consequences. If part of the effects of therapeutic glucocorticoids in inflammatory lung diseases is mediated via the glucocorticoid receptors of AM, nonspecific binding to phagocytosed carbon may interfere with the binding to receptors. The presence of a large number of nonspecific binding sites may contribute to the need of higher therapeutical doses to achieve clinical improvement or may function as a depot.

We also provided evidence that the glucocorticoid receptors in AM are functional. In mice it was shown that glucocorticoids inhibit phagocytosis and cell spreading in cultures of resident peritoneal macrophages (39). It was suggested that the membrane function required for phagocytosis and spreading was disrupted by a steroid-induced factor. In the present study it was shown that also human AM in culture demonstrate marked changes in morphology. Furthermore, inhibition of these morphological changes upon culture of AM could be achieved by glucocorticoids. This inhibition was dependent on the concentration of dexamethasone and could be antagonized by RU 38486. These findings strongly suggest that dexamethasone exerts this effect by binding to glucocorticoid receptors.

In summary, we demonstrated that human AM possess a single class of high affinity glucocorticoid receptors, which can be saturated. To our knowledge, this is the first time glucocorticoid receptors are described in human AM, without the comments that high nonspecific binding may have influenced interpretation of the specific binding. Strong reduction of the nonspecific binding proved to be a prerequisite feature of the method to determine receptor numbers. The method may prove to be helpful in studies on glucocorticoid responsiveness in inflammatory lung diseases. Furthermore, it was shown that these glucocorticoid receptors were functional, which is consistent with the hypothesis that AM are a direct target for glucocorticoid therapy in many inflammatory lung diseases.

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REFERENCES

1. Ham AW, Cormack DH. The origins, morphologies, and functions (including immunological functions) of the cells of loose connective tissue. In: Ham AW, Cormack DH, Histology. Philadelphia, JB Lippincott Company, 1979, 225-259.
2. Marcy TW, Merrill WW. Cigarette smoking and respiratory tract infection. Clin Chest Med 1987;8:381-391.
3. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
4. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
5. Herscowitz HB. In defence of the lung: paradoxical role of the pulmonary alveolar macrophage. Ann Allergy 1985;55:634-648
6. Fels AOS, Cohn ZA. The alveolar macrophage. J Appl Physiol 1986;60:353-369.
7. Rochester CL, Rankin JA. Is asthma T-cell mediated? Am Rev Respir Dis 1991;144:1005-1007.
8. Marini M, Soloperto M, Mezzetti M, Fasoli A, Mattoli S. Interleukin-1 binds to specific receptors on human bronchial epithelial cells and upregulates granulocyte/macrophage colony-stimulating factor synthesis and release. Am J Respir Cell Mol Biol 1991;4:519-524.

9. Schleimer RP. Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic applications in asthma. Am Rev Respir Dis 1990;141:S59-S69.
10. Munck A, Mendel DB, Smith LI, Orti E. Glucocorticoid receptors and actions. Am Rev Respir Dis 1990;141:S2-S10.
11. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray MA, Anderson WH, eds., Mediators of pulmonary inflammation. New York, M Dekker Inc, 1991, 593-617.
12. Rinehart JJ, Sagone AL, Balcerzak SP, Ackerman GA, LoBuglio AF. Effects of corticosteroid therapy on human monocyte function. New Eng J Med 1975;292:236-241.
13. Guyre PM, Munck A. Glucocorticoid action on monocytes and macrophages. In: Schleimer RP, Claman HN, Oronsky A, eds., Anti-inflammatory steroid action: basic and clinical aspects. San Diego, Academic Press Inc, 1989, 199-225.
14. Poznansky MC, Gordon ACH, Douglas JG, Krajewki AS, Wyllie AH, Grant IWB. Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients. Clin Sci 1984;67:639-645.
15. Poznansky MC, Gordon ACH, Grant IWB, Wyllie AH. A cellular abnormality in glucocorticoid resistant asthma. Clin Exp Immunol 1985;61:135-142.
16. Beato M. Gene regulation by steroid hormones. Cell 1989;56:335-344.
17. Miesfeld RL. Molecular genetics of corticosteroid action. Am Rev Respir Dis 1990;141:S11-S17.
18. Duval D, Durant S, Homo-Delarche F. Non-genomic effects of steroids. Interactions of steroid molecules with membrane structures and functions. Biochim Biophys Acta 1983;737:409-442.
19. Ozaki T, Yasuoka S, Nakayama T, Tsubura E. Glucocorticoid receptors in human alveolar macrophages and peripheral blood cells. Clin Exp Immunol 1982;47:505-511.
20. Lacronique JG, Rennard SI, Bitterman PB, Ozaki T, Crystal RG. Alveolar macrophages in idiopathic pulmonary fibrosis have glucocorticoid receptors, but glucocorticoid therapy does not suppress alveolar macrophage release of fibronectin and alveolar macrophage derived growth factor. Am Rev Respir Dis 1984;130:450-456.
21. Van Hal PThW, Mulder E, Hoogsteden HC, Hilvering C, Benner R. Glucocorticoid receptors in alveolar macrophages: methodological aspects of the determination of the number of glucocorticoid receptors per cell. Agents Actions 1989;26:128-131.
22. Lippman M. Clinical implications of glucocorticoid receptors in human leukemia. Am J Physiol 1982;243:E103-E108.
23. Homo-Delarche F. Glucocorticoid receptors and steroid sensitivity in normal and neoplastic human lymphoid tissues: a review. Cancer Res 1984;44:431-437.
24. Corrigan CJ, Brown PH, Barnes NC, Szefler SJ, Tsai JJ, Frew AJ, Kay AB. Glucocorticoid resistance in chronic asthma: glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids *in vitro*. Am Rev Respir Dis 1991;144:1016-1025.
25. Sundström C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565-577.
26. Nordbye K, Oftebro R. Establishment of four new cell strains from human uterine cervix.I. Exp Cell Res 1969;58:458-460.
27. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Meth 1984;72:219-227.
28. Steiner AE, Wittliff JL. A whole-cell assay for glucocorticoid binding sites in normal human lymphocytes. Clin Chem 1985;31:1855-1860.
29. Crabtree GR, Smith KA, Munck A. Glucocorticoid receptors. In: Catovsky D, ed. Methods in hematology: the leukemic cell. New York, Churchill Livingstone, 1981, 252-269.

30. Veldscholte J, Berrevoets CA, Zegers ND, Van der Kwast TH, Grootegoed JA, Mulder E. Hormone-induced dissociation of the androgen receptor-heat-shock protein complex: Use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. Biochemistry 1992;31:7422-7430.
31. Kontula K, Andersson LC, Paavonen T, Myllylä G, Teerenhovi L, Vuopio P. Glucocorticoid receptors and glucocorticoid sensitivity of human leukemic cells. Int J Cancer 1980;26:177-183.
32. Mulder E, Peters MJ, De Vries J, Van der Molen HJ, Østgaard K, Eik-Nes KB, Oftebro R. Androgen receptor specificity and growth response of a human cell line (NHIK 3025). Mol Cell Endocrinol 1978;11:309-323.
33. Werb Z, Foley R, Munck A. Interaction of glucocorticoids with macrophages: identification of glucocorticoid receptors in monocytes and macrophages. J Exp Med 1978;147:1684-1694.
34. George SC, Gudewicz PW, Jaffe RC. Glucocorticoid binding to normal and activated alveolar lung cells. Lung 1980;157:57-64.
35. Lippman M, Barr R. Glucocorticoid receptors in purified subpopulations of human peripheral blood lymphocytes. J Immunol 1977;118:1977-1981.
36. Lane SJ, Lee TH. Glucocorticoid receptor characteristics in monocytes of patients with corticosteroid-resistant bronchial asthma. Am Rev Respir Dis 1991;143:1020-1024.
37. Heimer GM, England DE. Enterohepatic recirculation of oestriol: inhibition by activated charcoal. Acta Endocrinol 1986;113:93-95.
38. Bettini E, Maggi A. A rapid method for the quantitation of estrogen receptors in small amounts of tissue. J Imm Methods 1991;144:87-91.
39. Becker J, Grasso RJ. Suppression of phagocytosis by dexamethasone in macrophage cultures: inability of arachidonic acid, indomethacin, and nordihydroguaiaretic acid to reverse the inhibitory response mediated by a steroid-inducible factor. Int J Immunopharmac 1985;7:839-847.

CHAPTER 6

MONOCYTES/MACROPHAGES AND GLUCOCORTICOID THERAPY

6.1 Introduction

6.2 Cell surface antigen expression by peripheral blood monocytes in allergic asthma: results of 2.5 years therapy with inhaled beclomethasone dipropionate

6.3 Eicosanoids and lipocortin-1 in BAL-fluid in asthma: effects of smoking and inhaled glucocorticoids

CHAPTER 6.1

INTRODUCTION

In chapters 1.3, 2.3, 2.4 and 2.5, we discussed new insights in the cellular and subcellular working mechanisms of glucocorticoids. In chapter 5, we presented data on the modulation of phenotype and function of human mononuclear phagocytes by glucocorticoids *in vitro*, suggesting that glucocorticoids may exert their clinical effects, at least partly, by influencing monocytes and macrophages *in vivo*. Therefore, we were interested to find out whether treatment of asthmatic patients with inhaled glucocorticoids resulted in effects on peripheral blood monocytes and alveolar macrophages similar to the effects observed *in vitro*. These ideas to study the cellular *in vivo* effects of inhaled glucocorticoids were discussed with the Dutch Chronic Non-Specific Lung Disease Study Group, who compared in a 2.5 year study the effects of inhaled glucocorticoids with inhaled anticholinergic agents (1,2). At the end of this long-term trial, we were allowed to analyze both characteristics of the bronchoalveolar lavage fluid and the expression of cell membrane antigens on peripheral blood monocytes. Moreover, we were able to correlate our findings with lung function parameters, e.g. FEV_1 and PC_{20}. The major clinical conclusion from this study was: "the addition of an inhaled glucocorticoid - but not an inhaled anticholinergic agent - to maintenance treatment with a β_2-agonist (terbutaline) substantially reduced morbidity, hyperresponsiveness, and airways obstruction in patients with a spectrum of obstructive airways disease" (1). The precise cellular base of these clinical glucocorticoid effects has still to be elucidated.

REFERENCES

1. Kerstjens HAM, Brand PLP, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, Bleecker ER, Dekhuijzen PNR, De Jong PM, Mengelers HJJ, Overbeek SE, Schoonbrood DFME, and the Dutch Non-Specific Lung Disease Study Group. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. New Engl J Med 1992;237:1413-1419.
2. Brand PLP, Kerstjens HAM, Postma DS, Sterk PJ, Quanjer PhH, Sluiter HJ, Dijkman JH, Van Herwaarden CLA, Hilvering C, Jansen HM, Koëter GH, Kreukniet J, and the Dutch Non-Specific Lung Disease Study Group. Long-term multicentre trial in chronic nonspecific lung disease: methodology and baseline assessment in adult patients. Eur Respir J 1992:5:21-31.

CELL SURFACE ANTIGEN EXPRESSION BY PERIPHERAL BLOOD MONOCYTES IN ALLERGIC ASTHMA: results of 2.5 years therapy with inhaled beclomethasone dipropionate*

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SUMMARY

At present, inhaled glucocorticoids are widely accepted as the therapy of choice in chronic asthma. Treatment with inhaled glucocorticoids significantly suppresses local airway inflammation in asthmatics, but may also have systemic effects, e.g. a reduction of the number of circulating hypodense eosinophils or a down-modulation of HLA-DR antigen (Ag) expression by T lymphocytes in peripheral blood. However, the effect of long-term therapy with inhaled glucocorticoids on peripheral blood monocytes (PBM), which are the precursors of the most numerous cell type in the lung, the alveolar macrophage, have not yet been evaluated. We therefore investigated the expression of various cell surface Ag on PBM from non-smoking patients with allergic asthma who were treated for 2.5 years with a β_2-agonist plus either inhaled glucocorticoids (beclomethasone dipropionate, BDP) ($n=4$) or an anticholinergic (AC) or placebo ($n=8$). We compared the results with healthy volunteers ($n=7$). Long-term treatment of allergic asthmatics with inhaled BDP, but not AC or placebo therapy, was associated with a significantly lower CD11b Ag expression ($p<0.04$) and higher expression of CD13, CD14 and CD18 Ag ($p<0.05$, $p<0.02$ and $p<0.04$, respectively) when compared with the healthy control subjects ($n=7$). In addition, PBM of asthmatic patients treated with inhaled BDP expressed an almost two-fold higher level of CD14 Ag on their cell surface than PBM of patients treated with AC or placebo. No significant differences in the expression of CD16, CD23, CD25, CD32 and CD64 Ag or HLA-DR were observed between PBM from the different patient groups or healthy controls. Taken together, this study shows that the expression of at least several cell surface Ag on PBM from allergic asthmatics differs, after long-term therapy with inhaled BDP, from patients treated with AC or placebo.

INTRODUCTION

One of the major histopathological findings in asthma is chronic inflammation of the airways. In bronchial mucosal biopsies, this inflammation is characterized by an accumulation of mononuclear phagocytes, eosinophils, mast cells and T lymphocytes (1-4). These inflammatory cells have also been described in the bronchoalveolar lavage fluid (BALF) (5-7), which, in addition, has been shown to contain elevated levels of various inflammatory mediators (7,8). It is generally believed that the airway inflammation in asthma results from the concerted actions of the different types of inflammatory cells and their products, and underlies some of the clinical symptoms (1,3,5).

The most numerous cell type in both the normal and asthmatic lung is the alveolar macrophage (AM). This cell type is nowadays known to play a central role in initiating, perpetuating, and reducing inflammatory processes (9-12). Monocytes and macrophages recovered from BALF of asthmatics are highly activated (13-15), manifested in the release of a large variety of mediators which in turn modulate the inflammatory responses of eosinophils, mast cells and T cells (7,16). Furthermore, it has been

reported that AM of asthmatic patients express elevated levels of low affinity receptors for IgE (CD23) (17), which are capable of inducing cellular activation in reponse to specific allergen.

At present, glucocorticoids are the most effective therapy for controlling airway inflammation and clinical symptoms in chronic asthma (18,19). The precise mechanisms by which glucocorticoids reduce airway inflammation are not yet fully understood, but it is known that they modulate gene expression by binding to specific glucocorticoid-responsive elements in DNA (20,21). One of the major mechanisms by which glucocorticoids inhibit inflammation is direct suppression of cytokine production by mononuclear phagocytes and T cells (22) or the induction of anti-inflammatory proteins such as lipocortin-1 (23). Additionally, glucocorticoids inhibit the production of pro-inflammatory mediators indirectly by suppressing the activity of several enzymes involved in the production of these mediators.

Therapy with inhaled glucocorticoids for one to 4 months results in a significant decrease in the numbers of macrophages, eosinophils, mast cells, and T cells in the bronchial epithelium and submucosa of asthmatic patients (24-27). Only mild systemic effects have been reported with a daily dose of 1000-2000 μg of inhaled glucocorticoids. Numbers of peripheral blood eosinophils have been shown to be reduced after treatment with inhaled glucocorticoids (19,28). The effect of long-term treatment with inhaled glucocorticoids on peripheral blood monocytes (PBM), the precursors of AM, has not yet been evaluated. Identification of the effects of therapy with inhaled glucocorticoids on peripheral blood cells may be important, as increased numbers of eosinophils and activated T cells are detected in blood of asthmatic patients, in addition to the infiltration of inflammatory cells in the airways (29,30).

In this report, we analyzed the expression of various cell surface Ag by PBM of non-smoking patients with allergic asthma who were treated for 2.5 years with an inhaled β_2-agonist plus either inhaled beclomethasone dipropionate (BDP), or an anticholinergic or placebo. The patients were participants in the Dutch double-blind placebo-controlled CNSLD study (31,32). Our data indicate that PBM of patients treated with inhaled BDP, in contrast to PBM of patients treated with anticholinergic or placebo, show significant changes in the expression of CD11b, CD13, CD14 and CD18 Ag. In addition, PBM of patients treated with inhaled BDP expressed significantly more CD14 Ag on their cell surface than PBM of patients who were treated with bronchodilator or placebo.

MATERIALS AND METHODS

Subjects

Twelve non-smoking allergic asthmatic patients (participating in the Dutch double-blind placebo-controlled CNSLD study) (31,32) and 7 healthy volunteers were studied. The characteristics of each patient are described in Table 1. The diagnosis of asthma was made according to the criteria of the American Thoracic Society (33) and was based on a history of attacks of breathlessness and wheezing without chronic cough or sputum production. Chronic was defined as more than three months per year.

At entry to the study, all patients showed a 20% decrease in FEV_1 resulting from inhalation of a provocative concentration of histamine of ≤ 8 mg/ml (32,34). Allergy was defined as at least 2 positive wheal and flare reactions to skin prick tests with 12 common aeroallergens, or a positive test to house dust mite (32). All patients but one had a baseline reversibility ≥10% of predicted (one patient had a reversibility of 4%). Control subjects (3 male, median age 27; 4 female, median age 23) were healthy volunteers, taking no medication, who had no history of allergy or asthma and who had negative skin prick tests. Ethical approval for the study protocol was obtained from the Medical Ethics Committees of the participating centres. All subjects gave written informed consent.

Table 1. Patient characteristics.

Patient number	Gender	Age (years)	Baseline FEV_1 (% of predicted)	After 2.5 years FEV_1 (% of predicted)	Baseline PC_{20} (mg/ml)	After 2.5 years PC_{20} (mg/ml)
glucocorticoid group						
1	M	36	46.3	64.5	0.13	1.46
2	F	44	75.0	88.5	0.06	0.45
3	F	45	49.2	69.6	0.05	0.21
4	F	60	59.7	90.3	0.06	0.96
median		44.5	54.5	79.1	0.06	0.71
anticholinergic/placebo group						
1	F	41	54.3	53.1	0.03	0.04
2	M	42	48.7	56.5	0.24	0.87
3	M	24	61.5	54.7	0.19	0.58
4	M	44	63.5	66.3	0.02	0.05
5	M	50	65.1	62.7	0.28	0.18
6	M	57	81.6	42.3	0.14	0.04
7	F	38	57.0	43.6	0.13	0.06
8	M	26	90.0	61.5	0.13	0.02
median		41.5	62.5	55.6	0.14	0.06

Study design

Details of the study design have been described previously (32). Briefly, after a double-blind randomization patients were treated with the β_2-agonist terbutaline (2 puffs of 250 µg q.i.d.) plus either: (A) inhaled glucocorticoids BDP, 2 puffs of 100 µg q.i.d., (B) the anticholinergic bronchodilator (ipratropium bromide), 2 puffs of 20 µg q.i.d., or (C) placebo q.i.d. At the end of the study, no significant differences with regard to FEV_1 and PC_{20} were found between the groups receiving either ipratropium bromide or placebo. Therefore, the data of these groups were pooled for analysis as one single group (designated as anticholinergic/placebo group). Heparinized venous blood of patients was collected after completing 2.5 years of treatment. Heparinized blood of healthy volunteers served as control.

Isolation of peripheral blood mononuclear cells

Mononuclear cells from heparinized venous blood were isolated by Ficoll density centrifugation

(Ficoll Paque; density 1.077 g/ml; Pharmacia, Uppsala, Sweden) for 15 min at room temperature (centrifugal force 1000 g). Peripheral blood mononuclear cells (PBMNC) were washed twice at 4 °C using PBS (300 mosmol; pH 7.8) supplemented with 0.5% heat-inactivated BSA (Organon Teknika; Turnhout, Belgium) and 0.05% w/v NaN_3 (PBS/BSA/NaN_3). The cell concentration was adjusted to $5x10^6$ cells/ml.

Immunofluorescence staining

Monoclonal antibodies (mAb) used in this study are listed in Table 2. CD11 and CD18 mAb were used because of the central role of leukocyte adhesion molecules in the recruitment of blood cells to inflammatory sites (35). Cell surface aminopeptidase-N can be recognized by CD13 mAb, and may play a role in the inactivation of some inflammatory mediators (36,37). CD14 mAb were used because they recognize the receptor for LPS binding protein, which may determine the cellular responsiveness to bacterial products (38). mAb recognizing Fcγ receptors (CD16, CD32, CD64) were used because these receptors play a role in the response of cells to specific antigens (39). The presence of low affinity receptors for IgE, recognized by CD23 mAb, may lead to activation of cells in response to specific allergens (40). Both the expression of the α-chain of the IL-2 receptor (CD25) and HLA-DR are markers of cellular activation (27). RFD9 recognizes a cell membrane determinant present on alveolar macrophages, but virtually absent on blood monocytes, and may be used as a marker of maturation (41). Irrelevant isotype-matched antibodies were used as controls. Reagents were diluted to optimal concentration in PBS/BSA/NaN_3. All incubations (30 min each) were carried out on ice. Each incubation was followed by two washes with PBS/BSA/NaN_3 at 4 °C. For single-colour stainings, aliquots of $2.5x10^5$ PBMNC (in a volume of 50 µl) were first incubated with 50 µl mAb, washed, and subsequently incubated with FITC-conjugated goat anti-mouse Ig (GAM-FITC; CLB, Amsterdam, The Netherlands). After the last washing procedure, the cells were resuspended in PBS/BSA/NaN_3. For two-colour stainings, 50 µl PBMNC were incubated with both a FITC-conjugated and PE-conjugated mAb, washed and eventually resuspended in FACSFLOW™ (Becton Dickinson, Sunnyvale, CA).

Flow cytometry

Flow cytometric analyses were performed using a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. Residual erythrocytes, dead cells and debris were excluded from analysis by electronic gating on the basis of forward and perpendicular light scatter. At least 7,500 events were acquired. Cell surface fluorescence was analyzed after setting scatter gates on either the monocyte or lymphocyte fraction. Within the monocyte gate, cell surface fluorescence was expressed as molecules equivalent to soluble FITC (MESF). MESF values ($x10^4$) were obtained by interpolating cell surface fluorescence to a standard curve prepared using microspheres of known fluorescence intensities (Flow Cytometry Standards, Research Triangle Park, NC). MESF values were corrected for background fluorescence by subtracting the MESF values obtained with isotype-matched control antibodies. Within the lymphocyte gate, results were expressed as the percentage of positively stained cells relative to an isotype-matched control antibody. Subpopulations of T lymphocytes were identified by two-colour labelling.

Statistical analysis

Differences between groups in either the percentage of positively stained lymphocytes or the level of cell surface fluorescence by monocytes were compared using the two-tailed Mann-Whitney U test. Differences associated with p values < 0.05 were regarded as statistically significant.

Table 2. Monoclonal antibodies used in this study.

mAb	clone (isotype)	source
CD3	Leu-4 (IgG_1)	BD[a]
CD4	Leu-3a (IgG_1)	BD
CD8	Leu-2a (IgG_1)	BD
CD11b	44 (IgG_1)	Dr. N. Hogg[b]
CD13	Q20 (IgG_{2a})	Dr. C.E. van der Schoot[c]
CD14	UCHM1 (IgG_{2a})	Dr. N. Hogg
CD15	VIM-D5 (IgM)	Dr. W. Knapp[d]
CD16	Leu-11b (IgM)	BD
CD18	LFA-1/1 (IgG_1)	CLB, Amsterdam, The Netherlands
CD20	B1 (IgG_{2a})	Coulter Clone, Hialeah, FL
CD23	TÜ1 (IgG_1)	Biotest, Dreieick, Germany
CD25	IL-2R(IgG_1)	BD
CD32	IV.3 (IgG_{2b})	Medarex Inc., West Lebanon, NH
CD64	32.2 (IgG_1)	Medarex Inc.
HLA-DR	L243 (IgG_{2a})	BD
anti-macrophage Ag	RFD9 (IgG_1)	Dr. L.W. Poulter[e]
isotype control	(IgG_1)	BD
isotype control	(IgG_{2a})	BD
CD1[f]	Leu-6 (IgG_{2b})	BD

a. Becton Dickinson, Sunnyvale, CA.
b. London, UK.
c. CLB, Amsterdam, The Netherlands.
d. Vienna, Austria.
e. Royal Free Hospital School of Medicine, London, UK.
f. Used as IgG_{2b} isotype control.

RESULTS

Long-term treatment with inhaled BDP in asthma is associated with differences in cell surface Ag expression by PBM compared with bronchodilator therapy or placebo

The expression of various cell surface Ag by PBM from both non-smoking allergic asthmatics treated for 2.5 years with either inhaled BDP or anticholinergic/placebo, and healthy volunteers is summarized in Table 3. PBM from patients who received anticholinergic/placebo did not show any significant alterations in the levels of cell surface Ag expression when compared with PBM from healthy controls. In contrast, PBM from patients treated with inhaled BDP differed significantly in the level of expression of CD11b ($p<0.04$), CD13 ($p<0.05$), CD14($p<0.02$), and CD18 ($p<0.04$) Ag when compared with PBM from healthy controls (Figure 1). A reduced level of CD11b Ag expression was observed, whereas the expression of CD13, CD14, and CD18 Ag was higher. The only significant difference between both groups of patients concerned the level of CD14 Ag expression; PBM from patients who had received inhaled BDP expressed significantly higher levels of CD14 Ag than PBM from asthmatic patients who had received anticholinergic/placebo (Table 3, Figure 1). Both groups of patients and the control group, however, did not differ in the relative numbers of $CD14^+$

monocytes in peripheral blood (data not shown). Taken together, these results demonstrate that long-term therapy with inhaled BDP alters the expression of some cell surface Ag on PBM. The most pronounced change is the enhanced expression of CD14 Ag.

Table 3. Specific cell surface fluorescence by peripheral blood monocytes.

	control group (n=7)	anticholinergic/placebo group (n=8)	glucocorticoid group (n=4)
PBMNC			
% CD15$^+$	0.7 (0.5-2.0)[a]	1.8 (0.4-5.5)	1.6 (0.5-6.8)
Monocytes			
CD11b	19.2 (9.6-27.1)[a,b]	12.6 (2.1-26.4)	7.6 (4.1-13.0) [c/p<0.04]
CD13	5.3 (1.5-6.4)	5.4 (3.6-12.9)	8.2 (6.1-18.7) [c/p<0.05]
CD14	13.0 (9.4-18.7)	11.9 (7.9-19.7)	20.4 (13.7-21.5) [c/p<0.02] [d/p<0.03]
CD16	1.1 (0.6-2.3)	0.9 (0.6-1.6)	1.3 (0.7-1.6)
CD18	8.5 (5.7-11.0)	13.8 (6.3-33.4)	12.5 (8.8-16.1) [c/p<0.04]
CD23	1.0 (0.9-1.6)	1.1 (0.5-2.2)	1.3 (0.7-1.8)
CD25	0.9 (0.7-1.3)	0.9 (0.5-1.3)	1.1 (0.6-1.6)
CD32	11.7 (6.6-15.0)	13.4 (7.2-19.9)	14.0 (11.5-17.6)
CD64	7.7 (4.3-10.4)	8.4 (4.2-12.4)	7.0 (6.3-10.8)
RFD9	1.3 (0.9-1.4)	1.4 (0.6-2.4)	1.8 (1.7-2.0) [c/p<0.01]
HLA-DR	5.9 (3.8-6.6)	6.8 (4.8-21.4)	7.0 (5.7-9.3)

a. Median values (range).
b. MESF values, corrected for background fluorescence by isotype-matched control Ab, were calculated using a calibration line obtained with microspheres of known fluorescence intensities. Statistical analysis was performed using the two-tailed Mann-Whitney U test.
c. Significantly different from control group.
d. Significantly different from anticholinergic/placebo group.

The expression of RDF9 by PBM of asthmatics treated with inhaled BDP was significantly increased when compared with healthy controls ($p<0.01$). However, this specific cell surface fluorescence fell within two-fold the fluorescence intensity of an isotype-matched control mAb, suggesting that monocytes express negligible levels of RFD9 Ag. No differences in the expression of CD16 (FcγRIII), CD23 (FcεRII), CD25 (IL-2R), CD32 (FcγRII) and CD64 (FcγRI) were observed between both groups of patients and healthy controls. The expression of CD16, CD23, and CD25 Ag fell within the two-fold the background fluorescence of isotype-matched control mAb, comparable with the expression of RFD9 Ag.

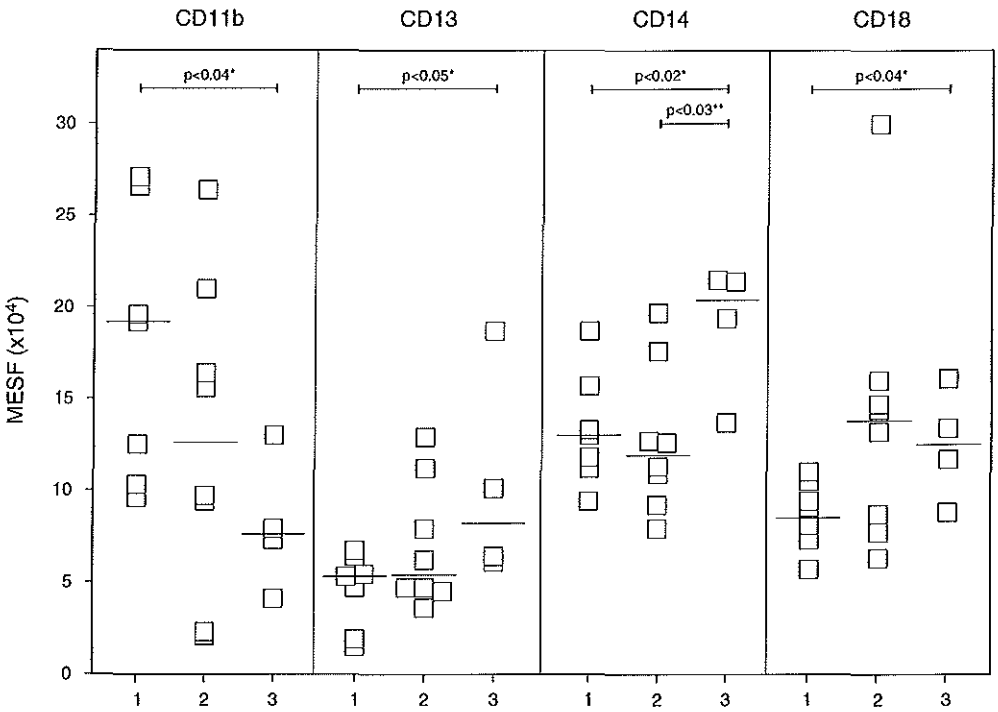

Figure 1. Expression of CD11b, CD13, CD14 and CD18 cell surface Ag (expressed as MESF x10⁴) by PBM. Median values are represented as a horizontal dash (—).
* Value of glucocorticoid group (3) differs significantly from control group (1).
** Value of glucocorticoid group (3) differs significantly from anticholinergic/placebo group (2).

Long-term inhaled therapy and differences in subsets of peripheral blood lymphocytes

In addition to analyzing the cell surface Ag expression on PBM, we investigated whether long-term therapy with inhaled BDP is associated with differences in the percentage of T and B lymphocytes in peripheral blood (Table 4). No differences were observed in the percentages of either $CD4^+$ or $CD8^+$ T cells. However, both groups of patients had higher percentages of $HLA\text{-}DR^+$ T cells in their peripheral blood than healthy controls. Treatment with inhaled BDP did not alter the percentage of these activated T cells. Interestingly, patients who received bronchodilator/placebo, but not the patients treated with inhaled BDP, had a significantly higher percentage of B lymphocytes ($CD20^+$ cells) in their peripheral blood compared with healthy controls (Table 4).

Table 4. Lymphocyte subpopulations in peripheral blood.

	control group (n=7)	anticholinergic/placebo group (n=8)	glucocorticoid group (n=4)
Lymphocytes			
% $CD20^+$	4.2 (3.1-9.1)[a]	7.2 (3.5-10.1) [b ($p<0.04$)]	7.6 (3.9-16.4)
% $CD3^+$	67.4 (57.1-82.2)	68.3 (52.6-81.9)	73.5 (53.4-79.0)
% $CD3^+/CD4^+$	34.5 (22.0-44.1)	40.6 (29.1-53.9)	38.0 (32.1-40.9)
% $CD3^+/CD8^+$	23.8 (16.3-30.2)	21.2 (11.3-35.7)	33.0 (13.9-42.7)
% $CD3^+/HLA\text{-}DR^+$	1.0 (0.4-2.1)	2.0 (1.2-9.3) [b ($p<0.03$)]	5.6 (2.0-8.8) [b ($p<0.02$)]
ratio CD4/CD8	1.6 (0.7-2.1)	1.7 (0.8-3.6)	1.2 (0.8-2.6)

a. Median values (range).
b. Significantly different from control group.

DISCUSSION

In this study, we show that the expression of cell surface Ag on PBM from allergic asthmatics treated for 2.5 years with inhaled glucocorticoids (BDP, 800 μg daily) differs from those treated with bronchodilator or placebo. Long-term treatment of asthmatic patients with inhaled glucocorticoids was associated with a significantly reduced expression of CD11b Ag on PBM, and a significantly increased expression of CD13, CD14, and CD18 Ag compared with healthy control subjects. In addition, PBM of patients treated with inhaled glucocorticoids expressed an almost two-fold higher level of CD14 Ag on their cell surface compared with PBM of patients who received anticholinergic/placebo.

Glucocorticoids were introduced in 1949 as a new and promising drug in the treatment of inflammatory diseases (42). Shortly thereafter, local administration of glucocorticoids was commenced to by-pass the unwanted systemic side effects (43). Nowadays, inhaled glucocorticoids are widely accepted as the treatment of choice in chronic asthma (28). Several reports showed that inhaled glucocorticoids are safe and almost free of systemic effects. However, high doses of inhaled glucocorticoids may still influence the hypothalamus-pituitary-adrenal axis (28,44). More evidence for systemic effects of inhaled glucocorticoids comes from studies on circulating eosinophils and T cells. Circulating hypodense eosinophils, which have been shown to exhibit a great inflammatory potential, were reduced in asthmatic subjects after short-term treatment with inhaled glucocorticoids (45,46). Furthermore, a small but significant reduction in HLA-DR expression by peripheral blood T cells has been reported after 6 weeks of therapy with inhaled glucocorticoids (27). To our knowledge, modulation of PBM by inhaled glucocorticoids has not been described up till now.

Leukocyte adhesion molecules, among which the CD11/CD18 family of β_2 integrins, play an important role in the recruitment of cells from peripheral blood to the site of inflammation as well as in other immunological and inflammatory processes that require direct cellular interactions (35,47,48). Both the expression and function of adhesion

molecules are augmented in response to inflammatory mediators and cytokines (49). The reduction in CD11b Ag expression by PBM observed here in asthmatic subjects treated with inhaled glucocorticoids, may have indirectly resulted from the local glucocorticoid-mediated reduction in inflammatory mediators in the lung. Alternatively, systemically absorbed BDP may be directly responsible for the modulation of CD11b Ag expression. In contrast to a reduced expression of CD11b, a slightly but significantly elevated CD18 Ag expression was seen on PBM of asthmatics treated with inhaled glucocorticoids when compared with PBM of healthy control subjects. The CD18 Ag is found on the cell surface in association with one of three different α chains, i.e. the CD11a, CD11b, or CD11c Ag (47). Since the expression of CD11a and CD11c Ag were not analyzed in this study, we do not know whether the observed increase in CD18 Ag expression coincides with an increased expression of CD11a and/or CD11c Ag.

Long-term treatment of asthmatics with inhaled BDP was associated with an increased expression of CD13 Ag by PBM compared with healthy controls. CD13 Ag, a cell membrane-bound aminopeptidase-N, has been shown to play an important role in modulating the activity of regulatory oligopeptides (36). An increase in CD13 Ag expression may therefore enhance the cell's capacity to inactivate harmful inflammatory peptides. In this context, it is noteworthy that CD13 Ag expression by purified PBM increases upon *in vitro* culture in the presence of IL-4, suggesting that this cytokine, like glucocorticoids, has potential anti-inflammatory effects (37,50). Future studies are needed to determine whether the increased expression of CD13 Ag observed here is accompanied by an increased aminopeptidase-N activity.

The CD14 Ag expression by PBM of patients treated with inhaled glucocorticoids was significantly increased compared with both patients who received anticholinergic/placebo and healthy control subjects. It has been shown that CD14 Ag can function as a receptor for lipopolysaccharide (LPS) (38). In addition, a role for CD14 Ag has been implicated in the adhesion of monocytes to cytokine-activated endothelial cells (51). CD14 Ag expression decreases upon differentiation/maturation of monocytes into AM (38). This process can be inhibited by glucocorticoids (41,52). Therefore, it may be that in this study the interference of inhaled BDP with the maturation of PBM manifests itself in the increase of CD14 Ag expression.

In this study, we also show that all asthmatic patients, treated with either inhaled glucocorticoids or anticholinergic/placebo, had significantly increased percentages of HLA-DR$^+$ T cells in their peripheral blood when compared with healthy control subjects. Long-term treatment with inhaled BDP did not reduce the percentage of circulating activated (HLA-DR$^+$) T lymphocytes in both groups of patients. Recently, Wilson et al. (27) showed in an uncontrolled study that patients treated for 6 weeks with inhaled BDP showed a reduction in the percentage of activated T cells. Compared with our study, they used a higher dose of BDP and they did not compare their result with patients who did not receive inhaled glucocorticoids. In the present study, we also observed that patients treated with bronchodilator/placebo, but not the patients treated with inhaled BDP, had a higher percentage of B lymphocytes in peripheral blood compared with healthy controls. Therefore, treatment with inhaled glucocorticoids may

have influenced the proportion of circulating B lymphocytes in our group of patients.

In summary, our data demonstrate that long-term therapy with inhaled glucocorticoids coincides with an altered expression of several cell surface Ag on PBM of allergic asthmatics. Future studies are needed to determine whether these changes in cell surface Ag expression are part of the anti-inflammatory action of inhaled glucocorticoids, and whether they correlate with the glucocorticoid-induced improvement in FEV_1, PC_{20} and clinical symptoms.

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REFERENCES

1. Jeffery PK, Wardlaw AJ, Nelson FC, Collins JV, Kay AB. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. Am Rev Respir Dis 1989;140:1745-1753.
2. Djukanovic R, Roche WR, Wilson JW, Beasley CRW, Twentyman OP, Howarth PH, Holgate S. Mucosal inflammation in asthma. Am Rev Respir Dis 1990;142:434-457.
3. Bradley BL, Azzawi M, Jacobson M, Assoufi B, Collins JV, Irani A-MA, Schwartz LB, Durham SR, Jeffery PK, Kay AB. Eosinophils, T-lymphocytes, mast cells, neutrophils and macrophages in bronchial biopsies from atopic asthma: comparison with non-asthma and normal controls and relationship to bronchial hyperresponsiveness. J Allergy Clin Immunol 1991;88:661-674.
4. Poston RN, Chanez P, Lacoste JY, Litchfield, Lee TH, Bousquet J. Immunohistochemical characterization of the cellular infiltration in asthmatic bronchi. Am Rev Respir Dis 1992;145;918-921.
5. Wardlaw AJ, Dunette S, Gleich GJ, Collins JV, Kay AB. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. Am Rev Respir Dis 1988;137:62-69.
6. Robinson DS, Bentley AM, Hartnell A, Kay AB, Durham SR. Activated memory T helper cells in bronchoalveolar lavage fluid from patients with atopic asthma: relation to asthma symptoms, lung function, and bronchial responsiveness. Thorax 1993;48:26-32.
7. Smith DL, Deshazo RD. Bronchoalveolar lavage in asthma. An update and perspective. Am Rev Respir Dis 1993;148:523-532.
8. Arm JP, Lee TH. The pathobiology of bronchial asthma. Adv Immunobiol 1992;51:323-382.
9. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science 1987;236:551-557.
10. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
11. Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cells function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. J Exp Med 1993;177:397-407.
12. Poulter LW, Janossy G, Power C, Screenan S, Burke C. Immunological/physiological relationship in asthma: potential regulation by lung macrophages. Immunol Today 1994;15:258-261.
13. Kelly C, Ward C, Stenton CS, Bird G, Hendrick DJ, Walters EH. Number and activity of inflammatory cells in bronchoalveolar lavage fluid in asthma and their relation to airway hyperresponsiveness. Thorax 1988;43:684-692.

14. Rankin JA. The contribution of alveolar macrophages to hyperreactive airway disease. J Allergy Clin Immunol 1989;83:722-729.
15. Chanez P, Vignola AM, Lacoste P, Michel FB, Godard P, Bousquet J. Increased expression of adhesion molecules (ICAM-1 and LFA-1) on alveolar macrophages from asthmatic patients. Allergy 1993;48:576-580.
16. Robinson DS, S Ying, AM Bentley, Q Meng, J North, SR Durham, AB Kay and Q Hamid. Relationship among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. J Allergy Clin Immunol 1993;92:397-403.
17. Williams J, Johnson S, Mascali JJ, Smith H, Rosenwasser LJ, Borish L. Regulation of low-affinity IgE receptor (CD23) expression on mononuclear phagocytes in normal and asthmatic subjects. J Immunol 1992;149:2823-2829.
18. Barnes PJ. A new approach to the treatment of asthma. New Engl J Med 1989;321:1517-1527.
19. Barnes PJ, Pederson S. Efficacy and safety of inhaled corticosteroids in asthma. Am Rev Respir Dis 1993;148:S1-S26.
20. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray MA, Anderson WH, eds. Lung biology in health and disease (Volume 54). Mediators of pulmonary inflammation. New York: M Dekker, Inc. 1991:593-617.
21. De Waal RMW. The anti-inflammatory activity of glucocorticoids. Mol Biol Rep 1994;19:81-88.
22. Schleimer RP. Effects of glucocorticoids on inflammatory cells relevant to their therapeutic applications in asthma. Am Rev Respir Dis 1990;141:S59-S69.
23. Goulding NJ, Guyre PM. Glucocorticoids, lipocortins and the immune response. Curr Opin Immunol 1993;5:108-113.
24. Laitinen LA, Laitinen A, Haahtela T. A comparative study of the effects of an inhaled corticosteroid, budenoside, and of a β_2-agonist, terbutaline, on airway inflammation in newly diagnosed asthma: a randomized, double-blind, parallel-group controlled trial. J Allergy Clin Immunol 1992;90:32-42.
25. Jeffery PK, Godfrey RW, Ädelroth E, Nelson F, Rogers A, Johansson S-Ä. Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma: a quantitative light and electron microscopy study. Am Rev Respir Dis 1992;45:890-899.
26. Trigg CJ, Manolitsas ND, Wang J, Calderón MA, McAulay A, Jordan SE, Herdman MJ, Jhalli N, Duddle JM, Hamilton SA, Devalia JL, Davis RJ. Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. Am J Respir Crit Care Med 1994;150:17-22.
27. Wilson JW, Djukanovic R, Howarth PH, Holgate ST. Inhaled beclomethasone dipropionate downregulates airway lymphocyte activation in atopic asthma. Am J Respir Crit Care Med 1994;149:86-90.
28. Barnes PJ. Inhaled glucocorticosteroids for asthma. New Engl J Med 1995;332:868-875.
29. Corrigan CJ, Kay AB. CD4 T-lymphocyte activation in acute severe asthma: relationship to disease severity and atopic status. Am Rev Respir Dis 1990;141:970-977.
30. Walker C, Virchow Jr J-C, Bruijnzeel PLB, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. J Immunol 1991;146:1829-1835.
31. Kerstjens HAM, Brand PLP, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, Bleecker ER, Dekhuijzen PNR, de Jong PM, Mengelers HJJ, Overbeek SE, Schoonbrood DFME, and the Dutch CNSLD Study Group. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airway disease. New Engl J Med 1992;327:1413-1419.
32. Brand PLP, Kerstjens HAM, Postma DS, Sterk PJ, Quanjer PH, Sluiter HJ, Dijkman JH, van Herwaarden CLA, Hilvering C, Jansen HM, Koëter GH, Kreukniet J, and the Dutch CNSLD Study Group. Long-term multicentre trial in chronic nonspecific lung disease: methodology and baseline assessment in adult patients. Eur Respir J 1992;5:21-31.
33. ATS Task Group. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease and asthma. Am Rev Respir Dis 1987;136:225-231.

34. Cockroft DW, Killan DN, Mellon JJA, Hargreave FE. Bronchial hyperreactivity to inhaled histamine: a method and clinical survey. Clin Allergy 1977;7:235-243.
35. Montefort S, Holgate ST. Adhesion molecules and their role in inflammation. Resp Med 1991;85:91-99.
36. Kenny AH, O'Hare MJ, Gusterson BA. Cell-surface peptidases as modulation of growth and differentiation. Lancet 1989;2:785-787.
37. Van Hal PThW, Hopstaken-Broos JPM, Prins A, Favaloro EJ, Huijbens RJF, Hilvering C, Figdor CG, Hoogsteden HC. Potential anti-inflammatory effects of IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N activity (CD13; EC 3.4.11.2). J Immunol 1994;153:2718-2728.
38. Ziegler-Heitbrock HWL, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today 1993;14:121-125.
39. Van de Winkel JGJ, Anderson CL. Biology of human immunoglobulin G Fc receptors. J Leukocyte Biol 1991;49:511-524.
40. Joseph M, Tonnel A-B, Torpier G, Capron A, Arnoux B, Benveniste J. Involvement of immunoglobulin E in the secretory processes of alveolar macrophages from asthmatic patients. J Clin Invest 1983;71:221-230.
41. Van Hal PThW, Hoogsteden HC, Te Velde AA, Wijkhuijs JM, Figdor CG, Hilvering C. IL-4-induced maturation of monocytes/macrophages is inhibited by glucocorticoids. Submitted.
42. Hench PS, Kendall EC, Slocumb CH, Polley HF. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc Staff Meet, Mayo Clin 1949;24:181-197.
43. Clark TJH. Effect of beclomethasone dipropionate delivered by aerosol in patients with asthma. Lancet 1972;i:1361-1364.
44. Fabbri L, Burge PS, Croonenborgh L, Warlies F, Weeke B, Ciaccia A, Parker C. Comparison of fluticasone propionate with beclomethasone dipropionate in moderate to severe asthma treated for one year. International study group. Thorax 1993;48:817-823.
45. Evans PM, O'Connor BJ, Fuller RW, Barnes PJ, Chung KF. Effect of inhaled corticosteroids on peripheral eosinophil counts and density profiles in asthma. J Allergy Clin Immunol 1993;91:643-649.
46. Kuo HP, Yu TR, Yu CT. Hypodense eosinophil number relates to clinical severity, airway hyperresponsiveness and response to inhaled corticosteroids in asthmatic subjects. Eur Respir J 1994;7:1452-1459.
47. Springer TA. Adhesion receptors of the immune system. Nature 1990;346:425-434.
48. Haskard DO, Lee TH. The role of leukocyte-endothelial interactions in the accumulation of leukocytes in allergic inflammation. Am Rev Respir Dis 1992;145:S10-S13.
49. Bochner BS, Undem BJ, Lichtenstein LM. Immunological aspects of allergic asthma. Annu Rev Immunol 1994;12:295-335.
50. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N (CD13) and FcRIIb (CD23) expression by IL-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1402.
51. Beekhuizen H, Blokland I, Corsel-van Tilburg AJ, Koning F, van Furth R. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. J Immunol 1991;147:3761-3767.
52. Rinehart JJ, Wuest D, Ackermann GA. Corticosteroid alteration of human monocyte to macrophage differentiation. J Immunol 1982;129:1436-1440.

EICOSANOIDS AND LIPOCORTIN-1 IN BAL-FLUID IN ASTHMA: effects of smoking and inhaled glucocorticoids*

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SUMMARY

Both smoking and asthma are associated with inflammatory changes in the lung, which may be suppressed with the help of exogenous anti-inflammatory drugs or by the defence system of the body itself. Lipocortin-1 (Lc-1) is an anti-inflammatory protein in respiratory tract secretions. We report an inverse correlation between extracellular Lc-1 concentration and the bronchoconstrictor prostaglandin D_2 (PGD_2) ($r_s = -0.597$, $p < 0.05$, $n = 15$) in bronchoalveolar lavage fluid (BALF) from allergic asthmatics, together with a positive relationship between BALF Lc-1 concentration and PC_{20} ($r_s = 0.720$, $n = 15$, $p < 0.01$) in these subjects. We quantified Lc-1 concentration in BALF from asthmatic patients and found no significant difference between those receiving inhaled glucocorticoids (2x100 μg beclomethasone q.i.d. for 2.5 y; median 186 ng Lc-1/mg albumin, n = 6) and those who were not (median 126 ng Lc-1/mg albumin, n = 12), perhaps because inhaled drugs deposit predominantly in central airways which are poorly represented in BAL. Both asthmatic and healthy volunteers who smoked had higher levels of Lc-1 in their BALF than their non-smoking counterparts (e.g. asthmatic smokers, median 317 ng Lc-1/mg albumin, $n = 10$; asthmatic non-smokers, median 162 ng Lc-1/mg albumin, $n = 18$; $p < 0.05$), perhaps because smokers' lungs contain more alveolar macrophages, cells which release Lc-1. We observed a positive correlation between BALF Lc-1 and BAL cell number ($r_s = 0.821$, $n = 16$, $p < 0.001$). Increased extracellular Lc-1 may be part of a protective response of the lung to inflammatory insult. Control of PGD_2 levels may be one mechanism by which Lc-1 suppresses inflammation.

INTRODUCTION

Inflammation is a key feature of many diseases of the respiratory tract and hence, there have been numerous studies to examine the effects of factors such as tobacco smoke (1-3) and inhaled allergens (4,5) which can initiate inflammatory processes in the lung. In addition, there have been many studies on the mechanisms of maintenance and resolution of these inflammatory processes, either spontaneously or drug-induced.

In 1950, glucocorticoids were introduced in the treatment of asthma. Nowadays, we know that the suppression of chronic airway inflammation underlies the beneficial effects of exogenous glucocorticoids on bronchial hyperresponsiveness (BHR) in asthma (6). This chronic airway inflammation can be characterized by increased numbers and altered profiles of inflammatory cells and by elevated levels of inflammatory mediators in bronchoalveolar lavage fluid (BALF) and bronchial biopsies (4,5). More recently, it has been suggested that also physiological levels of endogenous glucocorticoids contribute to the control of immune function, because it was shown that treatment with the glucocorticoid antagonist RU 38486 caused a reversible generalized exanthem in man (7). Glucocorticoids modulate the transcription of genes for both pro- and anti-inflammatory proteins (8), but their precise working mechanisms in the inhibition of inflammation are still incompletely understood. Since lipocortin-1 (Lc-1) was identified and cloned, it has been reported by several research

groups to exhibit clear anti-inflammatory properties both *in vitro* and *in vivo* (9-11; see 12 for a recent review). The induction of this anti-inflammatory protein has been proposed to mediate, at least partially, some of the anti-inflammatory effects of glucocorticoids (12,13). However, both the glucocorticoid-inducibility and the exact anti-inflammatory mechanisms of Lc-1 are still under discussion (14,15). The anti-inflammatory actions of Lc-1 are thought to include direct or indirect inhibition of the action of phospholipase A_2, and thus the production of pro-inflammatory arachidonic acid metabolites (9) including those thought to play a role in the pathogenesis of asthma (16) and smoking-related lung diseases (3). Furthermore, Lc-1 may modulate cell functions by binding to specific cell surface receptors (17) and thus require secretion into the extracellular compartment for some or all of its biological activity. Recently, it was hypothesized that Lc-1, as a mediator of the anti-inflammatory effects of glucocorticoids, may function as a "barrier" to inappropriate inflammatory and autoimmune responses (18). It has been shown in rats that physiological levels of endogenous glucocorticoids both have a pharmacological effect on Lc-1 and regulate Lc-1 in the absence of inflammation (19).

Lc-1 is present in abundance in lung homogenates. It can be detected in human BALF (20,21) suggesting that it is released onto the epithelial lung surface. Treatment with oral glucocorticoids can increase Lc-1 both extracellularly (20) and in BAL cells (22). Additionally, nonspecific inflammatory stimuli such as paraffin oil and LPS may also upregulate Lc-1 levels (13,23). To date, there have been no studies on the effects of inhaled glucocorticoids on cellular or extracellular Lc-1 levels in the lung of any species, even though this route of administration provides effective control of asthma symptoms for many subjects. Therefore, the present study was undertaken to investigate the effects of a) inhaled glucocorticoid therapy and b) cigarette smoke, a nonspecific inflammatory stimulus known to increase eicosanoid levels in BALF, on extracellular Lc-1 and a number of inflammatory parameters in the epithelial lining fluid of patients with allergic asthma. BALF was chosen as starting-material, as BAL has been proven to be an excellent and safe tool with which to study inflammatory aspects of lung diseases.

MATERIALS AND METHODS

STUDY A: SUBJECTS WITH ALLERGIC ASTHMA

Patient characteristics

Twenty-eight allergic asthmatic patients (8 women and 20 men; median age 41, range 21-62 yr), both smokers (1 woman and 9 men; median age 42, range 21-62 yr) and nonsmokers (11 men and 7 women; median age 40, range 25-61 yr) were randomly sampled in two participating centers, Groningen and Rotterdam, of the Dutch CNSLD study group (24,25). Patients who had stopped smoking at least 6 months before the start of the study were included in the nonsmokers group. The diagnosis of asthma was based upon a history of attacks of breathlessness and wheezing without chronic (i.e. for more than 3 months per year) cough or sputum production, according to the criteria of the American Thoracic Society (26). Allergy was defined as a positive skin prick test to house-dust mite or to at least two out of twelve other common aeroallergens [mean wheal size $\geq$ 70% of the histamine wheal size (24)]. At entry to the study, all patients

showed airway hyperresponsiveness defined as a 20% decrease in FEV_1 caused by inhalation of a histamine concentration (PC_{20}) of ≤ 8 mg/ml (24). Baseline reversibility of all patients was ≥ 10% of predicted. Patients were treated double-blind with the inhaled β_2-agonist terbutaline (2 puffs of 250 µg q.i.d.) plus either the inhaled glucocorticoid beclomethasone dipropionate (2 puffs of 100 µg q.i.d.; n = 12, 6 smokers), the anticholinergic bronchodilator ipratropium bromide (2 puffs of 20 µg q.i.d.; n = 8, 2 smokers) or placebo q.i.d. (n = 8, 2 smokers). At the end of the study, no significant differences with regard to FEV_1, PC_{20} or any of the biochemical parameters studied were found between the groups using either the β_2-agonist plus anticholinergic bronchodilator or the β_2-agonist plus placebo. Therefore, their data were eventually pooled for analysis as one single group (n = 16, 4 smokers). Patient characteristics are shown in Table 1. The study protocol was approved by the Medical Ethics Committee of all participating centers, and all patients gave written informed consent. Further details of the study methods have been described previously (24).

Table 1. Patient characteristics.

Patient number	Sex[a]	Age	Smoking status	Inhaled glucocorticoids	After 2.5 year	
					FEV_1 (% predicted)	PC_{20} (mg/ml)
1	F	44	–	–	45.4	0.04
2	M	45	–	–	63.2	0.05
3	M	25	–	–	59.5	0.58
4	M	26	–	–	59.0	0.02
5	F	37	–	–	47.2	0.06
6	M	57	–	–	68.0	0.04
7	M	31	–	–	71.9	0.02
8	M	32	–	–	67.5	0.13
9	M	30	–	–	96.4	0.42
10	M	38	–	–	44.6	0.02
11	M	42	–	–	65.9	0.87
12	M	50	–	–	54.0	0.18
13	F	32	–	+	53.4	0.10
14	M	61	–	+	91.7	5.38
15	M	37	–	+	60.3	1.46
16	F	45	–	+	86.9	0.45
17	F	61	–	+	93.2	0.96
18	F	45	–	+	70.4	0.21
19	M	21	+	–	72.1	0.96
20	M	36	+	–	65.2	0.70
21	M	58	+	–	55.6	2.00
22	M	48	+	–	n.d.[b]	n.d.
23	M	54	+	+	61.4	2.55
24	M	40	+	+	77.4	0.96
25	M	29	+	+	64.4	1.16
26	F	62	+	+	56.1	0.05
27	M	43	+	+	82.1	0.40
28	M	31	+	+	92.4	1.79

a. M : male F : female b. n.d. : not determined

Bronchoalveolar lavage

At the end of the 2.5 year study, BAL was performed 2 to 7 days after the last follow-up visit at which FEV_1 and PC_{20} were determined. BAL was performed at the end of the 2.5 year study in the same period of year (between August and December) in both centers, before breaking the code. BAL was done 30 min after premedication with atropine (0.5 mg i.m.) and 2 puffs of 250 µg terbutaline. Local anesthesia was achieved using a lidocaine (2%, w/v) spray. The bronchoscope was placed in wedge position in the right middle lobe. Four aliquots of 50 ml sterile and prewarmed (37°C) phosphate buffered saline were instilled, and aspirated immediately into a siliconized specimen trap placed on iced water. Immediately after collection, BALF was strained through a sterile gauze to trap large mucus particles, after which the cells were separated from the fluid by centrifugation at 4°C and 400g. Differential cell counts of the BAL cells were done after May-Grünwald Giemsa staining. Supernatants were stored at -70°C or lower until biochemical analysis.

Quantitation of eicosanoids

The following eicosanoids were assayed in BALF using radioimmunoassays as described previously (3): prostaglandin (PG) D_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ (a metabolite of prostacyclin, PGI_2), thromboxane (Tx) B_2 (a metabolite of TxA_2), leukotriene (LT) B_4, and LTC_4.

STUDY B: HEALTHY SUBJECTS

Subject characteristics

Twelve healthy women (5 smokers, median age 37, range 31-44 yr; 7 nonsmokers, median age 31, range 24-45 yr), who denied symptoms of pulmonary diseases, were hospitalized for laparoscopy. They did not use any steroidal or nonsteroidal anti-inflammatory drugs. BAL was performed during general anesthesia for laparoscopy. The bronchoscope was introduced via the endotracheal tube, and placed in wedge position in the right middle lobe. From this point, BAL was performed as described above for the asthma patients. All women gave informed consent, and the protocol was approved by the local Medical Ethics Committee.

BOTH STUDIES

Protein analyses

Lc-1 levels were assayed using a sensitive, specific ELISA for human Lc-1 as described previously (27). Albumin and total protein were determined by rocket immuno-electrophoresis using specific polyclonal antisera (28) and the Lowry method (29), respectively. All analyses were performed blind to the clinical and treatment status of the subject.

Statistical analysis

For data from asthmatic subjects, an Analysis of Variance (ANOVA) was used where multiple conditions were compared. Variables for which significant differences were found or significant trends observed were also analysed by simple comparisons between groups using unpaired Student's t-tests. Data from healthy subjects were analysed by unpaired Student's t-test only. Relationships between parameters were examined using the Spearman Rank Correlation Coefficient. Statistical significance was taken as $p < 0.05$. Differences

which were statistically significant with unpaired t-tests were also significant at the $p < 0.05$ level in a Mann-Whitney U test.

RESULTS

STUDY A: ASTHMATIC PATIENTS

Cell and fluid recovery

More lavage fluid was recovered from the lungs of glucocorticoid-treated non-smokers than from those not inhaling glucocorticoids (Table 2). There were no other significant differences in fluid recovery between groups.

More cells were present in the BALF from smokers than non-smokers and a higher proportion of the cells were AM in smokers; these differences were statistically significant when the data from the subjects receiving and those not receiving inhaled glucocorticoids were pooled, whilst the difference in cell number was also significant when the subjects not treated with glucocorticoids were considered alone. No significant differences were observed in either BAL cell number or cell profile between the groups treated with glucocorticoids and those receiving other treatments (Table 2).

Table 2. Fluid and cell recoveries of BALF from asthmatic subjects.

Smoking status	Steroid status	n	Fluid (ml)	BAL cells (x 10^6)	AM (%)
–	–	12	73 (30–135)	3.5 (0.4–10.0)	90 (62-98)
–	+	6	123 (65–144)[a]	11.0 (0.9–31.0)	82 (70-100)
+	–	4	85 (60–160)	21.1 (2.4–30.7)[a,b]	95 (94-97)
+	+	6	98 (60–127)	27.4 (4.0–41.8)[b]	97 (90-98)

Data are shown as median values with the limits of the range in parentheses.

a. > untreated non-smokers, $p<0.05$, Student's t-test.

b. > pooled data from non-smokers, $p<0.05$, ANOVA.

Protein and albumin

Concentrations of total protein and albumin were lower in BALF collected from glucocorticoid-treated asthmatic subjects than from asthmatic subjects given no glucocorticoids; these differences were statistically significant in smokers even though there was no difference in fluid recovery between these groups (Table 3). There were no

differences in albumin/total protein ratios between any of the groups.

Table 3. Total protein, albumin and lipocortin-1 concentrations in BALF from asthmatic subjects.

Smoking status	Steroid status	n	Total protein (μg/ml)	Albumin (μg/ml)	Albumin/protein (μg/μg)	Lc-1 (ng/ml)
–	–	12	130 (25–180)	28 (12–32)	0.20 (0.11–0.50)	3 (1–16)
–	+	6	91 (53–155)	28 (15–42)	0.28 (0.24–0.53)	5 (2–9)
+	–	4	223 (53–810)	44 (22–65)	0.23 (0.08–0.41)	13 (4–33)[b]
+	+	6	76 (48–163)[a]	22 (14–39)[a]	0.24 (0.20–0.44)	7 (3–26)

Data are shown as median values with the limits of the range in parentheses.
a. < untreated smokers, $p < 0.05$, Student's t-test.
b. > non-smokers, $p < 0.05$, Student's t-test.

Eicosanoids

There was considerable inter-subject variation in the concentrations of the eicosanoids measured, particularly in PGD_2, TxB_2 and LTB_4 (Table 4). 6-Keto $PGF_{1\alpha}$ varied significantly between groups, smokers having more of this prostacyclin metabolite in their BALF than non-smokers (Table 4). Furthermore, glucocorticoid-treated subjects who did not smoke had lower levels of LTC_4 in their BALF compared to non-smoking patients receiving other treatments (Table 4).

Table 4. Eicosanoid concentrations in BALF from asthmatic subjects.

Smoking status	Steroid status	PGD_2 pg/ml	$PGF_{2\alpha}$ pg/ml	TxB_2 pg/ml	LTC_4 pg/ml	LTB_4 pg/ml	$6kPGF_{1\alpha}$ pg/ml
–	–	77 (20–200)	19 (11–25)	76 (1–141)	16[a] (6–53)	75 (21–138)	16 (10–28)
–	+	35 (17–138)	14 (7–36)	41 (3–194)	7 (1–12)	32 (23–279)	12 (7–21)
+	–	48 (27–54)	21 (8–53)	10 (1–112)	11 (3–12)	48 (10–171)	31[b] (15–66)
+	+	31 (17–172)	14 (10–29)	38 (7–67)	13 (5–16)	97 (23–171)	28[b] (10–62)

Data are shown as medians with the limits of the range in parentheses.
a. > non-smokers using inhaled glucocorticoids.
b. > non-smokers from equivalent treatment groups, > non-smokers when ± steroid data pooled, $p < 0.05$, Student's t-test, ANOVA.

Lipocortin-1

In asthmatic subjects who did not use inhaled glucocorticoids, extracellular Lc-1 was significantly greater in BALF from smokers than from non-smokers whether the data were expressed per unit of fluid recovered (Table 3) or as ratios over albumin (Figure 1A) or total protein (data not shown). When the data were expressed per million BAL cells, there was no significant difference between smokers and non-smokers (Figure 1B). Interestingly, no significant difference between smokers and non-smokers in extracellular Lc-1 (expressed ng/ml) was observed in the glucocorticoid-treated asthmatics (Table 3). In contrast to smoking, glucocorticoid treatment had no significant effect on extracellular Lc-1 (Table 3, Figure 1A,B).

Relationships between Lc-1 and other parameters

When the data from all asthmatic subjects were pooled irrespective of smoking and treatment status, there was a significant correlation between BAL cell number and both total Lc-1 ($n=27$, $r_s=0.825$, $p<0.001$) and 6-keto $PGF_{1\alpha}$ ($n=26$, $r_s=0.565$, $p<0.01$).

When the data from only those asthmatic patients not taking glucocorticoids were pooled, there was a positive relationship between total Lc-1 and both fluid recovery ($n=15$, $r_s=0.796$, $p<0.01$) and BAL cell number ($n=16$, $r_s=0.821$, $p<0.001$). Similarly, in the absence of exogenous glucocorticoid, there was a positive correlation between Lc-1 concentration and both total protein ($n=15$, $r_s=0.574$, $p<0.05$) and albumine ($n=15$, $r_s=0.942$, $p<0.001$) concentrations. Interestingly, in the subjects not receiving glucocorticoids, Lc-1 (however expressed) was inversely related to the PGD_2 concentration (e.g. Lc-1/ml vs PGD_2/ml, $n=15$, $r_s=-0.597$, $p<0.05$; Lc-1/albumin vs PGD_2/ml, $r_s=-0.783$, $p<0.01$), but to no other eicosanoid (Figure 2). Furthermore, in the subjects not inhaling glucocorticoids, positive correlations were found between FEV_1 and Lc-1 expressed as a ratio either over albumin ($n=15$, $r_s=0.471$, $p<0.05$) or over total protein ($n=15$, $r_s=0.586$, $p<0.05$). PC_{20} was also significantly correlated to Lc-1/albumin ($n=15$, $r_s=0.645$, $p<0.01$) and Lc-1 concentration ($n=15$, $r_s=0.720$, $p<0.01$; Figure 3). In asthmatic patients receiving glucocorticoid treatment, none of these relationships was significant, except that between Lc-1 and cell number ($n=11$, $r_s=0.838$, $p<0.01$).

STUDY B: SUBJECTS WITH HEALTHY LUNGS

Cell and fluid recovery

Although there were no significant differences in fluid recovery between smokers and non-smokers with healthy lungs, significantly more BAL cells were recovered from smokers than from non-smokers (Table 5). Irrespective of smoking status, more than 90 % of the cells were AM.

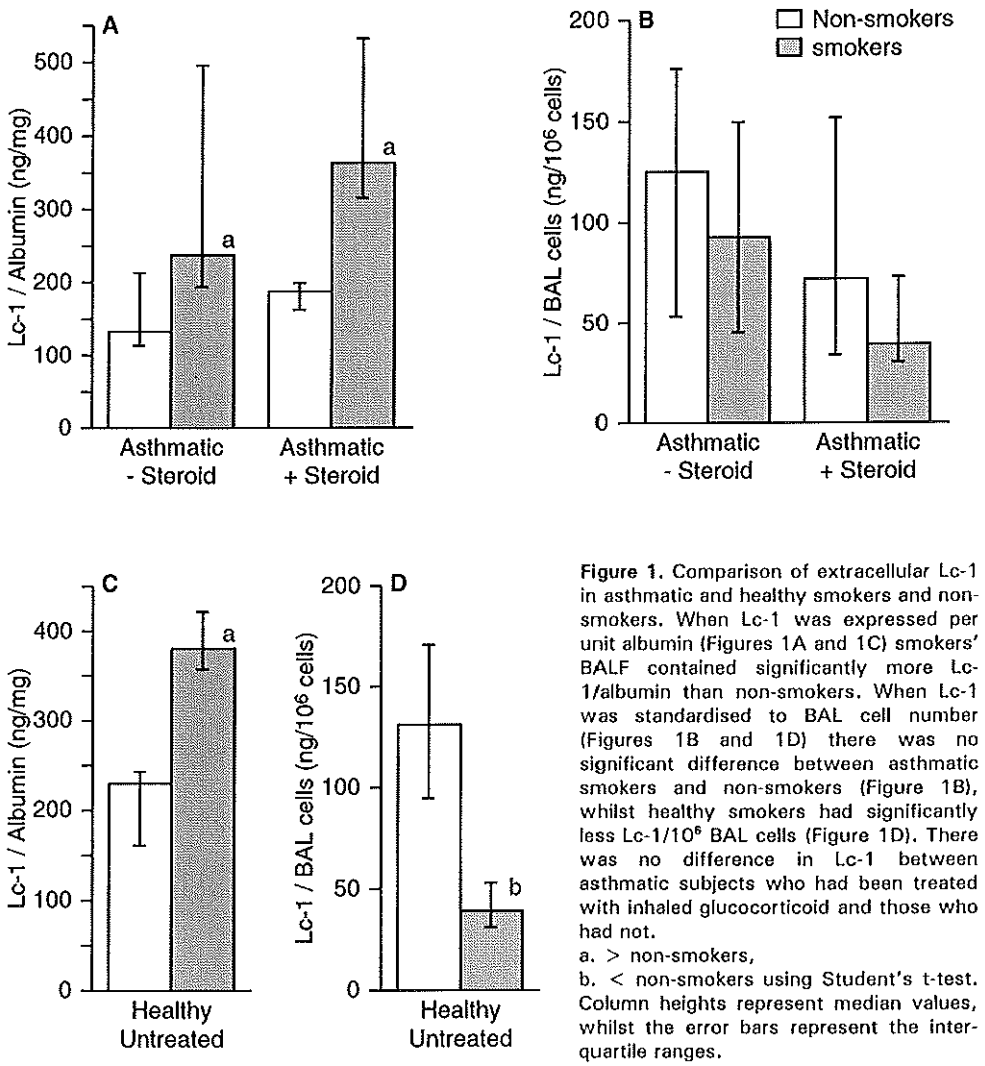

Figure 1. Comparison of extracellular Lc-1 in asthmatic and healthy smokers and non-smokers. When Lc-1 was expressed per unit albumin (Figures 1A and 1C) smokers' BALF contained significantly more Lc-1/albumin than non-smokers. When Lc-1 was standardised to BAL cell number (Figures 1B and 1D) there was no significant difference between asthmatic smokers and non-smokers (Figure 1B), whilst healthy smokers had significantly less Lc-1/10^6 BAL cells (Figure 1D). There was no difference in Lc-1 between asthmatic subjects who had been treated with inhaled glucocorticoid and those who had not.
a. > non-smokers,
b. < non-smokers using Student's t-test. Column heights represent median values, whilst the error bars represent the inter-quartile ranges.

Protein and albumin

Neither total protein nor albumin concentrations, nor albumin/total protein ratios differed significantly between smokers and non-smokers with healthy lungs (Table 5).

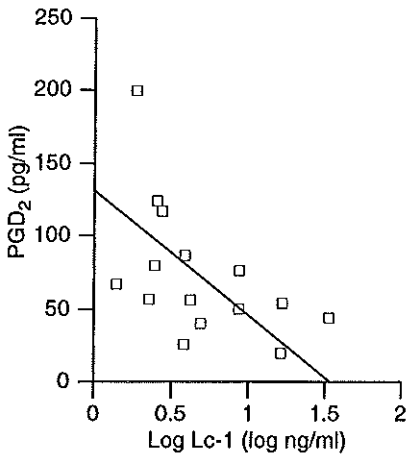

Figure 2. Relationship between extracellular Lc-1 and PGD_2 in BALF from asthmatic subjects not treated with inhaled glucocorticoid. Lc-1/ml BALF data are shown as logarithms for ease of illustration. Data from both smokers and non-smokers are included (n = 15).

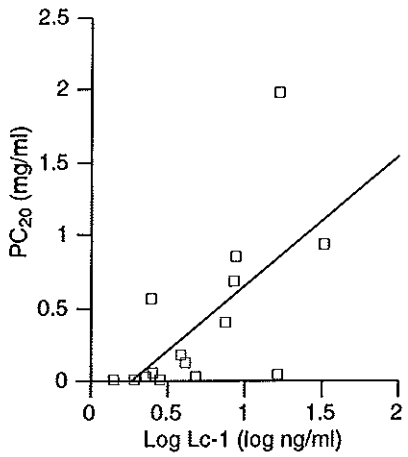

Figure 3. Relationship between extracellular Lc-1 in BALF and PC_{20} of asthmatic subjects not treated with inhaled glucocorticoid. PC_{20} values were determined at the end of the 2.5 y study period. Lc-1/ml BALF data are shown as logarithms for ease of illustration. Data from both smokers and non-smokers are included (n = 15).

Table 5. Fluid, cell and protein recoveries in BALF from subjects with healthy lungs.

Smoking status	n	Fluid (ml)	BAL cells (x 10^6)	Total protein (μg/ml)	Albumin (μg/ml)	Albumin/ total protein (μg/μg)	Lc-1 (ng/ml)
–	7	125 (110–160)	6.5 (4.7–16.8)	115 (95–148)	34.5 (20.7–70.1)	0.313 (0.175–0.553)	7 (5–10)
+	5	130 (90–150)	46.6[a] (30.0–71.0)	130 (78–212)	43.3 (20.9–47.5)	0.365 (0.158–0.555)	16[a] (8–21)

a.> non-smokers, $p < 0.05$, Student's t-test.

Lipocortin-1

As in asthmatic patients, there was more extracellular Lc-1 in BALF from healthy smokers than non-smokers whether the data were expressed per millilitre (Table 5) or as a ratio to albumin (Figure 1C) or total protein (data not shown). However, in contrast to the asthmatic patients, there was significantly less extracellular Lc-1/10^6 BAL cells in the BALF from healthy smokers than from non-smokers (Figure 1D).

Relationships between Lc-1 and other parameters

When data from smokers and non-smokers was pooled, there was a strong positive correlation between total extracellular Lc-1 recovery and BAL cell number ($n=12$, $r_s=0.9091$, $p<0.001$), but no significant relationship with any other parameter.

DISCUSSION

In this study, we have demonstrated that, in both control subjects without lung disease and patients with allergic asthma, there is a positive relationship between extracellular Lc-1 in BALF and BAL cell numbers. This relationship may explain why tobacco smokers have more extracellular Lc-1 in their airspaces than non-smokers and may suggest that BAL cells are the principle source of the Lc-1 found in respiratory tract secretions, even though other lung cell types contain and may secrete this protein as well (30,31). In addition, we observed that in the lung lavage of asthmatic subjects who were not treated with inhaled glucocorticoids, there was an inverse correlation between extracellular Lc-1 and the bronchoconstrictor PGD_2, and a positive correlation between Lc-1 and both FEV_1 and PC_{20}, suggesting that there may be a link between these variables.

Lc-1 has been shown to have anti-inflammatory effects in a variety of *in vivo* and *in vitro* models, including some involving the lung (9). It has been suggested that one of the anti-inflammatory mechanisms of Lc-1 may be to reduce the activity of phospholipase A_2, either directly or indirectly (9,12) and hence, the synthesis of inflammatory mediators derived from arachidonic acid. The inverse correlation between Lc-1 and the eicosanoid PGD_2 observed here, would support this hypothesis, but the fact that none of the other 5 eicosanoids measured in this study was similarly related to Lc-1, might suggest a rather more selective mechanism of action for the protein. PGD_2 is a mediator which may contribute to the early bronchoconstrictive reaction following allergen challenge in allergic asthma (16) and thus, could be a determinant of BHR. This would be supported by the observation that the higher the PGD_2, the more responsive the airways to histamine as measured by the PC_{20} (manuscript in preparation). Thus, this study suggests that, in allergic asthmatic subjects not given exogenous glucocorticoids, Lc-1 may contribute to protection of the airways from histamine-induced bronchoconstriction; one possible mechanism for this could be via PGD_2 regulation.

Lc-1 is made both by secretory cells in the lung (30,31) and by AM (22,32). Thus, the increase in extracellular Lc-1 in smokers' BALF could result from increased production of respiratory tract secretions, from the raised macrophage population, from activation of Lc-1-producing cells, or a combination of these possibilities. However, Lc-1/protein tended to be higher in asthmatic smokers than non-smokers ($p=0.065$) and was significantly higher in control smokers than non-smokers ($p<0.05$), suggesting that the increased extracellular Lc-1 was not due simply to a non-specific increase in protein secretion. The strong positive correlation between Lc-1 and BAL cell number in both healthy subjects and those with asthma, would suggest the macrophage as the major source of Lc-1 in the respiratory tract

secretions, supporting the hypothesis of Ambrose and colleagues (32) to this effect. Furthermore, there was no significant difference in extracellular Lc-1/10^6 BAL cells from asthmatic smokers and non-smokers, again indicating that the extracellular Lc-1 is a reflection of BAL cell number in these patients. Interestingly, this was not the case in the non-asthmatic volunteers, since in this group smokers had significantly less extracellular Lc-1/10^6 cells than non-smokers. Thus, it may be that in the healthy lung of smokers each cell secretes less Lc-1, or that Lc-1 is released only by a subpopulation of AM, but that the greater cell number means that extracellular Lc-1 rises.

The regulation of Lc-1 secretion into the epithelial lining fluid is not fully understood. Oral glucocorticoids have been shown to increase both extracellular Lc-1 in BALF (20,21) and intracellular levels in BAL cells (22). In addition, two studies in glucocorticoid-depleted (adrenalectomized) rats have shown that Lc-1 in peritoneal leukocytes and lung homogenates can be increased by non-specific stimuli, namely paraffin oil (13) and lipopolysaccharide (23). Thus, it is possible that tobacco smoke also non-specifically induces an increase in Lc-1 production and/or secretion. Alternatively, the increase in extracellular Lc-1 observed in the current study could result from circulating cortisol. However, it is unknown whether circulating cortisol is increased in smokers. Furthermore, it is known that the promoter sequence of the gene for Lc-1 has regions which are similar to those found in acute phase proteins (33), so other factors could contribute to the control of Lc-1 expression either directly, or indirectly.

In the present investigation, levels of extracellular Lc-1 in asthmatic patients who had been taking inhaled glucocorticoids were not different from those in asthmatics who had not taken inhaled glucocorticoids, even though both total protein and albumin were reduced in these subjects, and significantly so in smokers. This suggests that whilst inhaled glucocorticoids reduced the levels of other proteins, Lc-1 levels were maintained, but, in contrast to previous studies using oral or intravenous glucocorticoids (20-22), were not elevated above control values. The doses administered by inhalation may be insufficient to stimulate Lc-1 synthesis or secretion, or the glucocorticoid may be deposited and act in the upper and central airways which are poorly represented by conventional BAL (34). Alternatively, it is possible that Lc-1 synthesis and/or release is not glucocorticoid-inducible in patients with allergic asthma. This seems less likely, since De Caterina et al (22) observed an increase in cellular Lc-1 in BAL cells from asthmatic subjects following treatment with oral glucocorticoids. Interestingly, there were no significant correlations between Lc-1, PGD_2 and lung function parameters in glucocorticoid-treated subjects, such as those seen in no-steroid treated patients, supporting the hypothesis that inhaled glucocorticoids suppress respiratory tract inflammation by multiple mechanisms. The data presented in this paper suggest that one relevant mechanism may be the suppression of LTC_4 release (Table 4). A future longitudinal study in which BAL is performed in the same subject both before and after treatment with inhaled glucocorticoid should enable us to address this problem more fully.

The concentrations of Lc-1 observed in BALF from subjects with healthy lungs in this study are significantly higher than those from another study on volunteers by one of us (34). Differences in the number of BAL cells recovered do not account for the differences

in Lc-1 between the two studies. Sex may be one relevant factor, as all the volunteers in the current study were female, whereas those in the earlier study were male. In addition, the use of general anaesthesia may have stimulated Lc-1 release from cells, whilst the posture of the subject during lavage may also have affected the relative proportions of cells and extracellular material recovered in the lavage fluid.

Interestingly, the PGI_2 metabolite 6-keto $PGF_{1\alpha}$ was significantly increased in smokers. This may be due to an increase in the parent compound, leading to more PGI_2 available for metabolism. Alternatively, an increase in metabolic activity or in prostanoid-carrier systems compared to non-smokers may explain this finding. Ninety-five percent of circulating PGI_2 normally passes the pulmonary circulation without being metabolized; this may not be the case in smokers. If prostanoid-carrier systems or metabolic pathways are upregulated by smoking, more PGI_2 may be captured and metabolized by the lung.

In summary, we have observed an increase in extracellular Lc-1 in smokers which we hypothesize to be part of a self-protecting mechanism to limit respiratory tract inflammation. We have confirmed that inhaled glucocorticoids down-regulate total protein and albumin in BALF. Finally, we present preliminary evidence suggesting that Lc-1 levels may be related to improved FEV_1's and PC_{20}'s in allergic asthma, perhaps via PGD_2 regulation. The effect of inhaled glucocorticoid on Lc-1, and the role of Lc-1 and PGD_2 in the aetiology of allergic asthma, may be worthy of a more detailed investigation using subjects as their own control in a prospective, double blind longitudinal study.

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REFERENCES

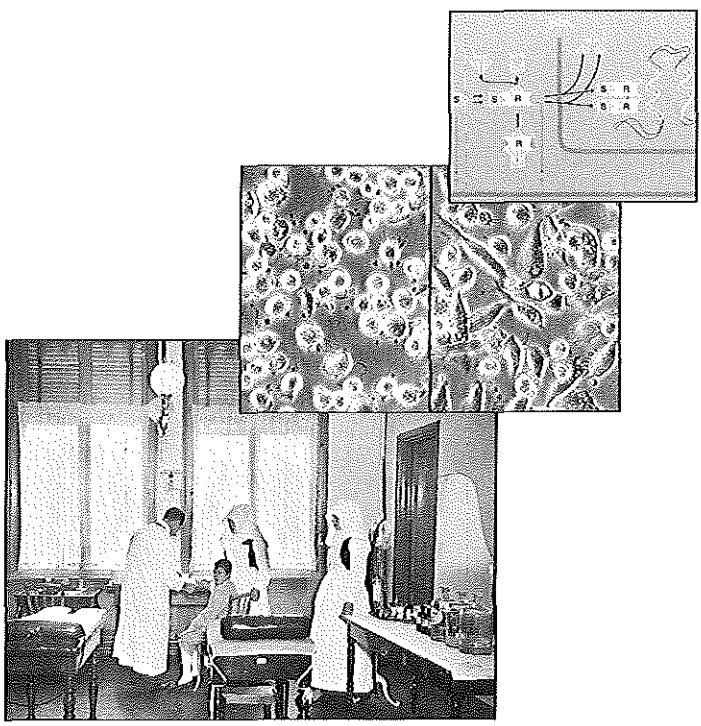
1. Hoogsteden HC, van Hal PThW, Wijkhuijs JM, Hop W, Verkaik APK, Hilvering C. Expression of the CD11/CD18 cell surface adhesion glycoprotein family on alveolar macrophages in smokers and nonsmokers. Chest 1991;100:1567-1571.
2. McCrea K, Ensor J, Bleecker E, Hasday J. Bronchoalveolar lavage fluid (BALF) from tabacco smokers contains more interleukin-8 (IL-8) and less interleukin-6 (IL-6) than nonsmokers. Am Rev Respir Dis 1993;147:A753.
3. Zijlstra FJ, Vincent JE, Mol WM, Hoogsteden HC, van Hal PThW, Jongejan RC. Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and non-smokers. Eur J Clin Invest 1992;22:301-306.
4. Leff AR, Hamann KJ, Wegner CD. Inflammation and cell-cell interactions in airway hyperresponsiveness. Am J Physiol 1990;260:L189-L206.

5. Robinson DS, Ying S, Bentley AM, Meng Q, North J, Durham SR, Kay AB, Hamid Q. Relationship among numbers of bronchoalveolar lavage cells expressing messenger ribonucleic acid for cytokines, asthma symptoms, and airway methacholine responsiveness in atopic asthma. J Allergy Clin Immunol 1993;92:397-403.
6. Juniper EF, Kline PA, Vanzieleghem MA, Ramsdale EH, O'Byrne PM, Hargreave FE. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. Am Rev Respir Dis 1990;142:832-836.
7. Laue L, Lotze MT, Chrousos GP, Barnes K, Lynn Loriaux D, Fleisher TA. Effect of chronic treatment with the glucocorticoid antagonist RU 486 in man: toxicity, immunological and hormonal aspects. J Clin Endocrinol Metab 1990;71:1474-1480.
8. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
9. Cirino G, Flower RJ, Browning JL, Sinclair LK, Pepinsky RB. Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. Nature 1987;328:270-272.
10. Perretti M, Flower RJ. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. J Immunol 1993;150:992-999.
11. Relton JK, Strijbos PJLM, O'Shaughnessy CT, Carey F, Forder RA, Tilders FJH, Rothwell NJ. Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. J Exp Med 1991;174:305-310.
12. Flower RJ, Rothwell NJ. Lipocortin 1: cellular mechanisms and clinical relevance. Trends Pharmacol Sci 1994;15:71-76.
13. Peers SH, Smillie F, Elderfield AJ, Flower RJ. Glucocorticoid and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leukocytes *in vivo*. Brit J Pharmacol 1993;108:66-72.
14. Brönnegård M, Andersson O, Edwall D, Lund J, Norstedt G, Carlstedt-Duke J. Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol Endocrinol 1988;2:732-739.
15. Northup JK, Valentine-Braun KA, Johnson LK, Severson DL, Hollenberg MD. Evaluation of the antiinflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J Clin Invest 1988;82:1347-1352.
16. Barnes PJ, Chung KF, Page CP. Inflammatory mediators. In: Page CP, Barnes PJ, eds. Pharmacology of asthma. Berlin Heidelberg: Springer-Verlag, Handb Exp Pharmacol 1991;98:53-106.
17. Goulding NJ, Luying P, Guyre PM. Characteristics of lipocortin 1 binding to the surface of human peripheral blood leukocytes. Biochem Soc Trans 1990;18:1237-1238.
18. Goulding NJ, Guyre PM. Regulation of inflammation by lipocortin 1. Immunol Today 1992;13:295-297.
19. Vishwanath BS, Frey FJ, Bradbury M, Dallman MF, Frey BM. Adrenalectomy decreases lipocortin-I messenger ribonucleic acid and tissue protein content in rats. Endocrinology 1992;130:585-591.
20. Ambrose MP, Hunninghake GW. Corticosteroids increase lipocortin I in BAL fluid from normal individuals and patients with lung disease. J Appl Physiol 1990;68:1668-1671.
21. Smith SF, Tetley TD, Datta AK, Smith T, Guz A, Flower RJ. Lipocortin-1 distribution in bronchoalveolar lavage from healthy human lung: effect of prednisolone. J Appl Physiol (in press).
22. De Caterina R, Sicari R, Giannessi D, Paggiaro PL, Paoletti P, Lazzerini G, Bernini W, Solito E, Parente L. Macrophage-specific eicosanoid synthesis inhibition and lipocortin-1 induction by glucocorticoids. J Appl Physiol 1993;75:2368-2375.
23. Smith T, Smith SF, Flower RJ, Buckingham JC. Endotoxin and the expression of lipocortins in central and peripheral tissues of the rat. Brit J Pharmacol 104:218P, 1991 (abstract).
24. Brand PLP, Kerstjens HAM, Postma DS, Sterk PJ, Quanjer PhH, Sluiter HJ, Dijkman JH, Van Herwaarden CLA, Hilvering C, Jansen HM, Koëter GH, Kreukniet J and the Dutch CNSLD Study Group. Long-term multicentre trial in chronic nonspecific lung disease: methodology and baseline assessment in adult patients. Eur Respir J 1992;5:21-31.

25. Kerstjens HAM, Brand PLP, Hughes MD, Robinson NJ, Postma DS, Sluiter HJ, Bleecker ER, Dekhuijzen PNR, De Jong PM, Mengelers HJJ, Overbeek SE, Schoonbrood DFME and the Dutch Chronic Non-specific Lung Disease Study Group. A comparison of bronchodilator therapy with or without inhaled corticosteroid therapy for obstructive airways disease. New Engl J Med 1992;327:1413-1419.
26. Task Group appointed by the ATS Scientific Assembly on Clinical Problems. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease (COPD) and asthma. Am Rev Respir Dis 1987;136:225-244.
27. Smith SF, Goulding NJ, Godolphin JL, Tetley TD, Roberts CM, Guz A, Flower RJ. An assay for the assessment of lipocortin 1 levels in human lung lavage fluid. J Immunol Meth 1990;131:119-125.
28. Smith SF, Guz A, Cooke NT, Burton GH, Tetley TD. Extracellular elastolytic activity in human lung lavage: a comparative study between smokers and non-smokers. Clin Sci 1985;69:17-27.
29. Lowry OH, Roseborough JJ, Farr AL, Randall RJ. Protein measurement with the Folin-phenol reagent. J Biol Chem 1951;193:265-275.
30. Ambrose MP, Hunninghake GW. Corticosteroids increase lipocortin I in alveolar epithelial cells. Am J Respir Cell Mol Biol 1990;3:349-353.
31. Jacquot J, Dupuir F, Elbtaourri H, Hinnrasky J, Antonicelli F, Haye B, Puchelle E. Production of lipocortin-like proteins by cultured human tracheal submucosal gland cells. FEBS Lett 1990;274:131-135.
32. Ambrose MP, Bahns C-LC, Hunninghake GW. Lipocortin 1 production by human alveolar macrophages. Am J Respir Cell Mol Biol 1992;6:17-21.
33. Browning JL, Ward MP, Wallner BP, Pepinsky RB. Studies on the structural properties of lipocortin and the regulation of its synthesis by steroids. In: Melli M, Parente L, eds. Cytokines and lipocortins in inflammation and differentiation. New York: Wiley-Liss, 1990:27-45.
34. Smith SF, Guz A, Winning AJ, Cooke NT, Burton GH, Tetley TD. Comparison of human lung surface protein profiles from the central and peripheral airways sampled using two regional lavage techniques. Eur Respir J 1988;1:792-800.

CHAPTER 7

CONCLUDING REMARKS



CONCLUDING REMARKS

Probably no other cell type is having a more important part in so many different physiological and pathological processes than mononuclear phagocytes (1-3). In concert with many other cell types, mononuclear phagocytes are able to initiate, perpetuate, and suppress inflammation (4,5). Inflammation is a major component of many diseases, and sometimes a patient needs treatment to relieve symptoms of inflammation. Anti-inflammatory treatment aims to influence the function of cells participating in the inflammatory response. Glucocorticoids are clinically being used in the treatment of inflammatory diseases since 1949 (6), but their precise cellular and subcellular working mechanisms are still not known (7). The very opposite concerns the field of cytokines: starting from a more or less clear understanding of their cellular working mechanisms, cytokines are now beginning to be used in the treatment of patients (8,9). The studies presented in this thesis were aimed at, on the one hand, delineating cellular and subcellular working mechanisms of glucocorticoids at the level of mononuclear phagocytes, in order to understand their well-known anti-inflammatory properties. On the other hand, we studied *in vitro* effects of IL-4 on mononuclear phagocytes with the intention of evaluating possible *in vivo* roles and therapeutical applications of IL-4.

IL-4 was initially described to influence the differentiation of resting B cells. Nowadays we know that the function of many other cell types can be modulated by IL-4. Its influence on mononuclear phagocytes concerns various functional aspects. Some of these influences are transient, whereas others have a more prolonged or permanent effect. In the investigations presented in this thesis, IL-4 effects were studied in, as best as possible, homogenous cell populations which were cultured under serum-free conditions. Therefore, the IL-4 effects observed here under well-defined conditions could not be attributed to unidentified serum factors or products of other cell types. The effects of IL-4 on mononuclear phagocytes can be subdivided in 3 main groups.

First, IL-4 affects the process of maturation *in vitro*. In the presence of IL-4, blood monocytes acquire morphological characteristics of the more mature macrophages: they increase greatly in cell size with a higher cytoplasm to nucleus ratio, and develop cytoplasmic protrusions. Furthermore, IL-4 induces a more mature immunophenotype: the expression of CD14 Ag is decreased, whilst the expression of RFD9 Ag is upregulated. Acid phosphatase, another marker of mononuclear phagocyte maturation, is also induced by IL-4. These maturation-associated changes appeared to be permanent.

Second and concomitant with the induction of maturation, cell surface markers associated with activation of mononuclear phagocytes are affected by IL-4. The upregulation of MHC class II, CD13 and CD23 Ag may have important functional implications. The increased expression of class II Ag may explain the IL-4-induced increase in antigen-presenting capacity observed by others. An increased IgE-mediated cytotoxic activity may result from the IL-4-induced increase in FcεRIIb (CD23) expression. The IL-4-mediated increase in CD13 Ag expression is accompanied with an enhanced aminopeptidase activity, through which cells may be turned into better processors of protein mediators. In line with this postulated function of aminopeptidase-N, this enzyme was

shown recently to degrade IL-8 and inactivate its chemotactic activity *in vitro* (10). All these effects of IL-4 on mononuclear phagocytes appeared transient and reversible, in contrast to the prolonged maturation-associated actions of IL-4.

Third, IL-4 decreases the production of various mediators by mononuclear phagocytes, suggesting reduction of activation. In our studies, we showed a transient downregulation of the expression of the IL-1β gene in cultured human AM, whilst others observed an IL-4-mediated inhibition of the secretion of IL-1β, IL-6, IL-8 and TNF-α, and an upregulation of IL-1RA. These findings can be interpreted as potential anti-inflammatory actions of IL-4.

These three different aspects of IL-4 actions on mononuclear phagocytes stress the pleiotropy of cytokines, but they interfere with a clear idea of the *in vivo* effects of IL-4: how is an organism to cope with a cytokine that exhibits both activating and de-activating actions. In this matter, it is important to realize that the above-mentioned effects of IL-4 were observed in a definitely inadequate model of the *in vivo* situation: effects of a single cytokine were studied on an isolated cell population, whereas *in vivo*, IL-4 will act in concert with a wide range of cytokines and other mediators. Additionally, influences mediated via either direct cell-cell or cell-matrix contact may be important *in vivo*. Future studies have to concentrate also on the latter aspects of cytokine effects, in order to really get to the bottom of the function of IL-4 *in vivo*. A glimpse of the *in vivo* complexity is exemplified by our finding that the IL-4 effects depend on the stage of maturation of mononuclear phagocytes (Table 1).

Table 1. Differential effects of IL-4 on the expression of CD13 and CD23 Ag in human mononuclear phagocytes in different stages of maturation.

Cell membrane Ag	immature monocytic cell lines	peripheral blood monocytes	alveolar macrophages
CD13	–[a]	↑[b]	↑
CD23	↑	↑	–

a. no effect on expression.
b. increased expression.

Some effects of IL-4 may be therapeutically useful, whereas other actions may be considered as unwanted "side" effects. The anti-inflammatory effects of IL-4 are receiving a lot of attention from clinical immunologists (11-13). Recent studies showed that IL-4 inhibited arthritis in bacterial cell wall-induced erosive polyarthritis in rats (12). Another study in rats showed protective effects of IL-4, and also IL-10, against experimental immune complex-induced lung injury (13). IL-13 has been suggested to be an anti-inflammatory cytokine similar to IL-4 (14). Clinical application of IL-4, and anti-inflammatory cytokines in general, may depend on the selection of beneficial effects, which may be achieved by creating a particular context for IL-4.

All the observed effects of IL-4 result from the modulation of gene expression. To achieve this, the binding of IL-4 to specific IL-4R on the cell membrane has to be linked to nuclear processes. As IL-4 itself is unable to pass the cell membrane, a cellular signalling

pathway is needed to transmit the extracellular presence of IL-4 to the nucleus. The first step in this signalling process is the binding of IL-4 to its cell membrane receptor. This IL-4R falls into a large family of structurally related receptors for cytokines (15). The IL-4R is a heterodimer, and its γC chain is also utilized by other members of the receptor family (IL-2R, IL-7R, and probably IL-9R and IL-13R). Upon binding of IL-4 to its receptor, the activation of (receptor-associated), intracellularly located, tyrosine kinases is induced. This may lead to the phosphorylation and activation of a cascade of other kinases, eventually resulting in the activation of transcription factors. Some transcription factors may be activated by kinases in the nucleus. Others may be activated in the cytoplasm, after which they are transported to the nucleus. In the nucleus, activated transcription factors may, either alone or in concert, modulate the transcription of target genes.

Modulation of transcription is also the base of the cellular effects induced by glucocorticoids. Unlike cytokines, glucocorticoids are able to pass the cell membrane without the help of specific receptors on the cell surface. The binding of glucocorticoids to their intracellular receptors activates these receptors to interact as homodimers with specific DNA sequences. Eventually, this interaction results in modulated transcription of particular target genes.

In the studies presented here, it was shown that glucocorticoids affect mononuclear phagocytes *in vitro*. Therefore, the clinical effects of glucocorticoids may be mediated, at least partially, via similar actions on this cell type. Characteristics of maturation (expression of acid phosphatase, RFD9 Ag and CD14 Ag) and activation (IL-1β gene expression) were found to be inhibited by glucocorticoids. Sometimes, a clear distinction between the influences of glucocorticoids on these two cellular processes is difficult. One explanation may be that, upon maturation, responsiveness to activation signals is acquired. Consequently, glucocorticoid-mediated inhibition of maturation can also inhibit activation. This interrelation can be discussed in the light of two recent studies (16,17). Maturation of the monocytic cell line U937 can be induced by phorbol esters (PMA or TPA). In one study, TPA was shown to inhibit cell proliferation and to induce cell clustering and the release of lysozyme, reactive oxygen radicals, and prostanoids (16). Furthermore, TPA down-regulated the expression of the proto-oncogens c-*myc* and c-*myb*, which is related to maturation of hematopoietic cell lines (16). Dexamethasone inhibited the effects of TPA on cell clustering, and the production of lysozyme, oxygen radicals, and prostanoids. However, the TPA-mediated effects on cell proliferation and the expression of c-*myc* and c-*myb* were unaffected by dexamethasone. It was concluded that glucocorticoids interfered with TPA-induced functions of U937, which are typical for activated mononuclear phagocytes, whilst maturation and concomitant growth inhibition were not impaired. Another study showed that PMA inhibited proliferation of U937 cells, and induced morphological changes (cytoplasmic extensions) and adherence to plastic (17). Cortisol mainly inhibited the PMA-induced morphological changes and reduced the proportion of cells that became adherent. Cell proliferation was only partially reduced. PMA-treated cells could be activated with LPS to express IL-1β mRNA, whereas untreated cells could not. Cortisol inhibited this LPS-induced expression. It was concluded that glucocorticoids inhibited some characteristics of maturation and activation of U937.

In spite of the different models used in these two studies and ours to investigate the influence of glucocorticoids (the effects of dexamethasone or cortisol on either TPA-, or PMA-treated U937 cells, or IL-4-treated PBM), it may be concluded that inhibition of maturation and activation of mononuclear phagocytes is one of the cellular working mechanisms of glucocorticoids. However, some glucocorticoid actions may be difficult to classify as either a maturational or an activational effect, which underlines our inadequate knowledge of these cellular processes.

It is recognized nowadays that a whole range of transcription factors, including activated glucocorticoid receptors, may be involved in the regulation of gene transcription. Recent studies on the working mechanisms of glucocorticoids revealed that the glucocorticoid-mediated transrepression of the collagenase gene can be explained as a result of the interaction with AP-1 (18-20; for a recent review 21). This repression occurs without signs of glucocorticoid receptor binding to DNA, contrary to glucocorticoid-mediated gene modulation through positive or negative glucocorticoid response elements (GRE). Moreover, this repression is a function of receptor monomers, whilst dimers of glucocorticoid receptors are required for binding to GRE. This was demonstrated in a recent study that described the use of point mutations in the D-loop region in the second zinc finger of the glucocorticoid receptor (Figure 1) (22).

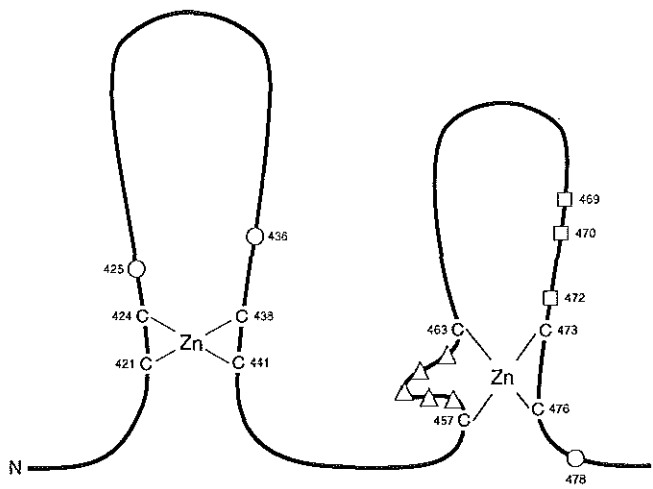


Figure 1. Schematic detail of the DNA-binding domain of the glucocorticoid receptor showing the two zinc fingers, and the amino acids relevant to the functions of this domain. Numbers refer to the sequence of the amino acids in this part (adapted from reference 19).

C : cysteine
N : amino-terminal part of the domain
Zn : zinc
Δ : amino acid required for dimerization (D-loop)
O : amino acid required for interaction with AP-1
□ : amino acid contributing to transactivation

This region plays an important part in the dimerization of receptors and, consequently, in the binding to GRE. Dimerization was prevented as a result of the point mutations, but the ability to repress AP-1 activity was unaffected. Therefore, gene modulation via GRE binding and transrepression via AP-1 interaction appear to be related to distinct regions of the glucocorticoid receptor. Furthermore, the choice of the ligand may also determine the kind of glucocorticoid effect: certain glucocorticoid analogues (e.g. RU 38486) that are able to bind to the glucocorticoid receptor, are unable to induce binding of the receptor to GRE. This explains the reduced or absent transactivation activity. However, these analogues still mediate the AP-1-associated transrepression, which demonstrates again that DNA binding is not required for this type of transrepression. These findings suggest that upon ligand binding, the changes in 3-dimensional structure of the receptor depend on the type of glucocorticoid analogue. The ultimate 3-dimensional structure determines whether the receptor is capable of interaction with either, as a dimer, GRE or, in monomeric form, certain transcription factors. This may enable us to develop synthetic glucocorticoids with more specific effects or without the most serious side effects, and thus may have an important impact on the clinical use of glucocorticoids.

FUTURE STUDIES

The concerted actions of a great variety of different cell types are essential in the initiation and perpetuation of the chronic airway inflammation of asthma. Today, the importance of cytokines in these cellular interactions is an accepted fact (23). Furthermore, there is increasing recognition that certain genes are important in the pathogenesis of asthma and the regulation of allergic responses. Recent studies suggest that several genes on chromosome 5q are central in the control of IgE production and inflammation in asthma (24). This part of chromosome 5 contains the genes encoding IL-3, IL-4, IL-5, IL-9, IL-13, GM-CSF and M-CSFR. Inhibition of the action of these cytokines may down-regulate the different aspects of the inflammatory processes in asthma. At the protein level, this inhibition may be realized with cytokine-antagonists or other (anti-inflammatory) cytokines, and may be important for a better treatment of asthma in the future (25). At the mRNA level, specific modulation of the expression of one (or a combination) of these genes on chromosome 5q is another promising therapeutic target. This may be realized by synthetic glucocorticoids more selective than the currently used glucocorticoids, or other transcription factors.

We showed that IL-4 is able to modulate phenotype and function of mononuclear phagocytes. Some effects of IL-4 result in an enhanced cellular capacity to process proteins (mediated by cell surface aminopeptidase-N) or a decreased IL-1β gene expression, and are therefore potentially anti-inflammatory. The physiological role of the IL-4-mediated increase in aminopeptidase-N expression is unknown, but it may be involved in the down-regulation of other cell membrane proteins, the degradation or activation of protein mediators (10), and the degradation of extracellular matrix (26). Aminopeptidase-N (26), together with other cell membrane proteases (27,28), has been suggested to play a role in the invasion

and metastasis of tumor cells. From the findings presented in this thesis it may be hypothesized that IL-4 in the micro-environment of tumor cells may lead to modulation of their metastatic properties. Recently it was found that aminopeptidase-N degraded IL-8 (10). It would therefore be interesting to study whether IL-4-treated monocytes or macrophages are better inactivators of IL-8 than untreated cells.

It may be hypothesized that *in vivo* IL-4 modulates IL-8-mediated chemotaxis of neutrophils via the upregulation of aminopeptidase-N expression. Future studies should also focus on the clinical application of these aspects of IL-4, and anti-inflammatory cytokines in general. In the treatment of many diseases, new and more specific anti-inflammatory drugs are needed. Such drugs may be used in stead of, or in combination with, currently available drugs in order to reduce side-effects of the latter. More basic *in vitro* studies will be needed to get to the bottom of the complex *in vivo* phenomena, always fully understanding that interpretation of an isolated *in vitro* result demands an *in vivo* context.

We also studied effects of glucocorticoids on mononuclear phagocytes. Future studies on the working mechanisms of these potent anti-inflammatory drugs should focus on their role in the regulation of gene expression. In the treatment of diseases, doctors daily encounter examples of abnormal cell function resulting from disrupted gene expression. They therefore need tools to modulate the relevant cell function. In the future, these tools will be increasingly based upon modulation of the genetic information and its expression in cells (29,30), although some physicians caution for too much enthusiasm (31). For a few diseases, such as cystic fibrosis, gene therapy has already become a reality in the experimental setting (32). Insight in the interaction mechanisms of the four major classes of transcription factors with DNA is growing and will help to develop new tools (33). Studies on the functions of the heat shock proteins associated with the glucocorticoid receptor, studies on the process of receptor dimerization and on the interactions with other, known and unknown, transcription factors will eventually lead to new and better ways to modulate cell functions with glucocorticoids. The recent finding that certain glucocorticoid analogues prevented interaction of the glucocorticoid receptor with GRE, but did not influence the interaction with AP-1, may be a base for the development of more specific glucocorticoids (22).

In some patients with asthma, glucocorticoids do not bring about the expected improvement in clinical symptoms (34). This clinical unresponsiveness may result from a defect in the cellular response to glucocorticoids (35-37). A recent study showed that a reduced number of glucocorticoid receptors available for GRE binding may underlie the phenomenon of glucocorticoid resistant asthma (38). The presence of an excess of AP-1 or NF-κB interacting with glucocorticoid receptors was suggested to cause the reduced number of receptors available for binding to DNA (38). Therefore, modulation of the expression of additional transcription factors may indirectly lead to altered glucocorticoid responsiveness. These findings stress the importance of studies on the regulation of gene expression in lung diseases.

REFERENCES

1. Solbach W, Moll H, Röllinghoff M. Lymphocytes play the music but the macrophage calls the tune. Immunol Today 1991;12:4-6.
2. Zembala M, Asherson GL, eds. Human monocytes. London: Academic Press, 1989.
3. Lewis CE, McGee JOD, eds. The macrophage. Oxford: Oxford University Press, 1992.
4. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141:471-501.
5. Strickland DH, Kees UR, Holt PG. Suppression of T-cell activation by pulmonary alveolar macrophages: dissociation of effects on TcR, IL-2R expression, and proliferation. Eur Respir J 1994;7:2124-2130.
6. Hench PS, Kendall EC, Slocumb CH, Polley HF. The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocorticotropic hormone on rheumatoid arthritis. Proc Staff Meet, Mayo Clin 1949:24:181-197.
7. De Waal RMW. The anti-inflammatory activity of glucocorticoids. Mol Biol Rep 1994;19:81-88.
8. Smith II JW, Longo DL, Alvord WG, Janik JE, Sharfman WH, Gause BL, Curti BD, Creekmore SP, Holmlund JT, Fenton RG, Sznol M, Miller LL, Shimizu M, Oppenheim JJ, Fiem SJ, Hursey JC, Powers GC, Urba WJ. The effects of treatment with interleukin-1α on platelet recovery after high-dose carboplatin. New Engl J Med 1993;328:756-761.
9. Teppler H, Kaplan G, Smith KA, Montana AL, Meyn P, Cohn ZA. Prolonged immunostimulatory effect of low-dose polyethylene glycol interleukin 2 in patients with human immunodeficiency virus type 1 infection. J Exp Med 1993;177:483-492.
10. Kanayama N, Kajiwara Y, Goto J, Maradny EEL, Maehara K, Andou K, Terao T. Inactivation of interleukin-8 by aminopeptidase-N (CD13). J Leukocyte Biol 1995;57:129-134.
11. Yano S, Sone S, Nishioka Y, Mukaida N, Matsushima K, Ogura T. Differential effects of anti-inflammatory cytokines (IL-4, IL-10 and IL-13) on tumoricidal and chemotactic properties of human monocytes induced by monocyte chemotactic and activating factor. J Leukocyte Biol 1995;57:303-309.
12. Allen JB, Wong HL, Costa GL, Bienkowski MJ, Wahl SM. Suppression of monocyte function and differential regulation of IL-1 and IL-1ra by IL-4 contribute to resolution of experimental arthritis. J Immunol 1993;151:4344-4351.
13. Mulligan MS, Jones ML, Vaporciyan AA, Howard MC, Ward PA. Protective effects of IL-4 and IL-10 against immune complex-induced lung injury. J Immunol 1993;151:5666-5674.
14. Yanagawa H, Sone S, Haku T, Mizuno K, Yano S, Ohmoto Y, Ogura T. Contrasting effect of interleukin-13 on interleukin-1 receptor antagonist and proinflammatory cytokine production by human alveolar macrophages. Am J Respir Cell Mol Biol 1995;12:71-76.
15. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. Cell 1994;76:253-262.
16. Hoff T, Spencker T, Emmendoerffer A, Goppelt-Struebe M. Effects of glucocorticoids on the TPA-induced monocytic differentiation. J Leukocyte Biol 1992;52:173-182.
17. Baybutt HN, Holsboer F. Inhibition of macrophage differentiation and function by cortisol. Endocrinology 1990;127:476-480.
18. Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 1990;62:1189-1204.
19. Yang-Yen H-F, Chambard J-C, Sun Y-L, Smeal T, Schmidt TJ, Drouin J, Karin T. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell 1990;62:1205-1215.
20. Schüle R, Rangarajan P, Kliewer S, Ransone LJ, Bolado J, Yang N, Verma IM, Evans RM. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell 1990;62:1217-1226.
21. Pfahl M. Nuclear receptor/AP-1 interaction. Endocr Rev 1993;14:651-658.

22. Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC. A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. EMBO J 1994;13:4087-4095.
23. Barnes PJ. Cytokines as mediators of chronic asthma. Am J Respir Crit Care Med 1994;150:S42-S49.
24. Meyers DA, Postma DS, Panhuysen CIM, Xu J, Amelung PJ, Levitt RC, Bleecker ER. Evidence for a locus regulating total serum IgE levels mapping to chromosome 5. Genomics 1994;23:464-470.
25. Anderson GP, Coyle AJ. T_H2 and "T_H2-like" cells in allergy and asthma: pharmacological perspectives. Trends Parmacol Sci 1994;15:324-332.
26. Saiki I, Fujii H, Yoneda J, Abe F, Nakajima M, Tsuruo T, Azuma I. Role of aminopeptidase N (CD13) in tumor-cell invasion and extracellular matrix degradation. Int J Cancer 1993;54:137-143.
27. Vassalli J-D, Pepper MS. Membrane proteases in focus. Nature 1994;370:14-15.
28. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature 1994;370:61-65.
29. Rosenthal N. Regulation of gene expression. New Engl J Med 1994;331:931-933.
30. Knowlton AA. Current concepts in transcription, translation and the regulation of gene expression. A primer for the clinician. Chest 1995;107:241-248.
31. Stoeckle M and reply by Rosenthal N, Schwartz RS. The molecular medicine series. New Engl J Med 1995;332:1240.
32. Johnson LG. Gene therapy for cystic fibrosis. Chest 1995;107(Supplement):77S-82S.
33. Papavassiliou AG. Transcription factors. New Engl J Med 1995;332:45-47.
34. Woolcock AJ. Steroid resistant asthma: what is the clinical definition? Eur Respir J 1993;6:743-747.
35. Carmichael J, Paterson IC, Diaz P, Crompton GK, Kay AB, Grant IWB, Corticosteroid resistance in chronic asthma. Brit Med J 1981;282:1419-1422.
36. Poznansky MC, Gordon ACH, Grant IWB, Wyllie AH. A cellular abnormality in glucocorticoid resistant asthma. Clin Exp Immunol 1985;61:135-142.
37. Sher ER, Leung DYM, Surs W, Kam JC, Zieg G, Kamada AK, Szefler SJ. Cellular mechanism contributing to inadequate response to glucocorticoid therapy. J Clin Invest 1994;93:33-39.
38. Adcock IM, Lane SJ, Brown CR, Peters MJ, Lee TH, Barnes PJ. Differences in binding of glucocorticoid receptor to DNA in steroid-resistant asthma. J Immunol 1995;154:3500-3505.

SUMMARY

Since time immemorial, people are trying to influence the course of diseases. History tells us that insight into pathological processes usually resulted in better ways to cure patients. The reaction of our body against its own or foreign structures is nowadays better understood than some decades ago, thanks to many immunological, biochemical and molecular-biological achievements. At this moment, we believe that both the presentation of antigens and cellular interactions are important components of this reaction of our body. Under certain conditions, mononuclear phagocytes are able to initiate, to perpetuate or to inhibit this reaction. Therefore, mononuclear phagocytes are thought to play an essential role in the development of various diseases, including many lung diseases. The latter is easy to understand, as the lung is in close contact with the potentially hostile external world with numerous pathogenic micro-organisms and foreign substances, from which lung diseases may originate. As a consequence of this, processes are initiated continuously in which mononuclear phagocytes play a role. Great numbers of mononuclear phagocytes, represented by interstitial monocytes/macrophages, dendritic cells and alveolar monocytes/macrophages, are present in the lung. Manipulation of the function of these cells may enable us to influence the development of lung diseases and to cure lung patients.

The base of this thesis is the inflammatory process in the airways of asthma patients. At present, we know that the airway inflammation in asthma is chronically present, although the seriousness may vary in time. Furthermore, it is known that this chronic airway inflammation underlies the bronchial hyperresponsiveness which gives rise to the characteristic symptoms of asthmatic patients. We understand that a variety of different cell types (mononuclear phagocytes, T cells, neutrophils, eosinophils, mast cells and epithelial cells) is important in the initiation and perpetuation of this chronic airway inflammation. Mononuclear phagocytes are thought, however, to play a central role, because of their numerous different cell functions, e.g. their ability to modulate the function of many other cell types. At the beginning of this study, we intended to gain more insight in the functions of mononuclear phagocytes, in particular the functions that are important in the chronic airway inflammation in asthma. Furthermore, we wanted to study both established and new ways to influence these cell functions. On the one hand, we wanted to study the working mechanisms of glucocorticoids. They are being used in the treatment of asthma since 1950, but their working mechanism is still not known precisely. On the other hand, we aimed to study the effects of cytokines, which are known to play a role in the intercellular communication and the modulation of cellular functions. The ultimate goal of this thesis was to gain insight in the modulation of cell functions of mononuclear phagocytes, which may be useful in the treatment of asthma patients. Modulation of mononuclear phagocytes' functions which is theoretically beneficial in the treatment of inflammation, may indeed turn out to be important in the treatment of airway inflammation and the related symptoms in lung patients.

Chapter 1 gives a summary of the present knowledge with regard to mononuclear phagocytes, cytokines and glucocorticoids. At the end of the last century, the phagocytosing capacity of macrophages was described. For a long time, it was thought that phagocytosis was the only function of macrophages. Nowadays we know that

mononuclear phagocytes have many important functions, e.g. their ability to modulate immunological reactions. Both the importance and the therapeutical modulation of mononuclear phagocytes' function in a variety of diseases are studied in many centers. The history of cytokines is shorter compared with macrophages, but their powerful influence on many physiological processes was recognized directly after their discovery. Cytokines are essential in the communication between cells and their concerted actions. As a result, cytokines may be used to influence pathological processes. The clinical use of cytokines is still in its infancy, but is promising. In contrast, glucocorticoids are being used clinically since their first application in the treatment of rheumatoid arthritis in 1950. However, their working mechanism is still not known precisely, although it is thought that their function as modulators of the expression of a great variety of genes of known (e.g. cytokines) and probably also unknown proteins, is the most important. In *chapter 2*, the most important and presently known anti-inflammatory working mechanisms of glucocorticoids are described.

Chapter 3 is a summary of the background of the experiments which are described in the chapters 4, 5 and 6.

In *chapter 4* it is shown that certain functions of mononuclear phagocytes can be modulated by cytokines. Alveolar macrophages are abundantly present in the lung, and the majority of these macrophages is derived from peripheral blood monocytes. These blood monocytes undergo many changes during their migration from the vasculature to the alveoli via the interstitial compartment. Part of these changes may be regarded as maturation. During this process of maturation, the expression of cell surface (glyco)proteins may be affected. The presence of these (glyco)proteins can be visualized with the use of specific monoclonal antibodies. The most important differences in the expression of cell membrane (glyco)proteins between blood monocytes and alveolar macrophages concern the CD14 and RFD9 antigens: the majority of blood monocytes exhibits a high and very low expression of the CD14 and RFD9 antigen, respectively, whereas the inverse is related to alveolar macrophages (*chapter 4.1*). We conclude from these findings that the maturation of blood monocytes to alveolar macrophages is associated with the loss and appearance of CD14 and RFD9 antigens, respectively. In *chapter 5.1* it is described that these maturation-associated changes in monocytes can also be observed during *in vitro* culture. IL-4 is shown to accelerate these changes *in vitro*. These findings support the hypothesis that maturation of mononuclear phagocytes *in vivo* is modulated by environmental factors. Possibly, IL-4 also plays a role in the *in vivo* maturation. Furthermore, these findings suggest that the use of IL-4 or anti-IL-4 may be therapeutically useful. Another interesting *in vitro* effect of IL-4 on mononuclear phagocytes is the upregulation of the expression of aminopeptidase-N. Both blood monocytes and alveolar macrophages express this enzyme on the cell membrane. It can be detected with specific CD13 monoclonal antibodies, and its activity can be demonstrated with a specific enzyme assay. IL-4 increases the expression of this enzyme on the cell membrane of both blood monocytes and macrophages. This increased expression is associated with an increased functional activity of aminopeptidase-N (*chapter 4.2*). IL-4 also induces an increase in the mRNA encoding aminopeptidase-N in both blood monocytes and alveolar macrophages. As this peptidase

may be important in the inactivation of inflammatory mediators, IL-4 may indirectly enhance the anti-inflammatory properties of monocytes and macrophages via an increase in the activity of aminopeptidase-N. In summary, we found that IL-4 on the one hand accelerates the maturation of mononuclear phagocytes, and on the other hand activates these cells resulting in the increased activity of aminopeptidase-N. The latter IL-4-mediated activation could also be observed in endothelial cells (*chapter 4.2*). However, this is not a general IL-4 effect, as IL-4 did neither induce nor increase the expression of CD13 antigen in the precursor cells of blood monocytes. It was interesting to observe that these precursor cells were still responsive to IL-4, and therefore must possess functional IL-4 receptors (IL-4R), because FcεRIIb could be induced in these cells by IL-4. FcεRIIb could also be induced by IL-4 in blood monocytes, but not in alveolar macrophages. Therefore, also the maturation stage of a certain cell type, in addition to the presence of functional cytokine receptors, seems to determine whether it will respond to a particular cytokine or not.

In *chapter 4.4* another aspect of the maturation of mononuclear phagocytes is studied. Cell proliferation can be determined thanks to the presence of a nuclear antigen in proliferating cells, which can be detected with the monoclonal antibody Ki-67. A very small subpopulation of blood monocytes exhibited proliferative activity. The percentage of proliferating alveolar macrophages was also small, but still larger than proliferating blood monocytes. These results were unexpected, as it is generally thought that cell maturation is associated with a reduction in proliferative activity. Therefore, lung tissue appears to create a micro-environment for mononuclear phagocytes which stimulates proliferation. This proliferation-inducing micro-environment seems to be determined by colony-stimulating factors, in particular GM-CSF. If the physiological pulmonary micro-environment really determines proliferation of mononuclear phagocytes, lung diseases may change it: in sarcoidosis, we found a higher percentage of proliferating alveolar macrophages, which may be hypothesized to result from an increase in the local levels of GM-CSF.

In *chapter 5* the effects of glucocorticoids on mononuclear phagocytes are described. As mentioned above, in *chapter 5.1* it is shown that IL-4 is able to accelerate the maturation of blood monocytes. Maturation was studied by means of several parameters as will be mentioned later. Glucocorticoids are shown to inhibit maturation: morphologically they inhibited the stretching of monocytes, the increase in the cytoplasmic expression of acid phosphatase, and on the cell membrane the increase and decrease in expression of RFD9 and CD14 antigen, respectively. In addition, glucocorticoids inhibited the accelerating influence of IL-4 on the maturation. Therefore, the regulation of monocyte/macrophage functions appears to be the result of the interaction between immunological factors and hormones. In *chapter 5.2* it is described that at least part of the anti-inflammatory effects of glucocorticoids may be mediated via a decrease of the gene expression of IL-1β. This mechanism has been described previously with regard to blood monocytes. Our results support the idea that it is valid to apply glucocorticoids in the treatment of lung diseases in which inflammation is induced or perpetuated by IL-1β produced by alveolar macrophages. In the same study it is shown that also IL-4 inhibited the IL-1β gene expression, possibly even better than glucocorticoids. Both this effect of IL-4 and the IL-4-induced increase in aminopeptidase-N activity are indications that some IL-4 effects are

anti-inflammatory. The clinical application of these anti-inflammatory effects of IL-4 may be realized in the near future. It may concluded from the results described in the *chapters 5.1* and *5.2* that IL-4 and glucocorticoids may have either antagonizing or similar effects, but it depends on the cellular parameter studied which of the two effects will be observed.

In *chapter 5.3* another aspect of the anti-inflammatory working mechanisms of glucocorticoids is studied. Here it is described that in alveolar macrophages the expression of lipocortin-1 mRNA is increased by glucocorticoids. Under the same experimental conditions, this effect of glucocorticoids was accompanied by a decrease in IL-1β gene expression. In this chapter, it is also shown that the glucocorticoid effects on lipocortin-1 and IL-1β gene expression also differ with regard to their kinetics. Almost all the cellular effects of glucocorticoids described in the literature demand the presence of functional glucocorticoid receptors. This prerequisite has been discussed in chapter 2 in great detail. It has been unclear for a long time, because of technical problems, whether alveolar macrophages express specific and functional glucocorticoid receptors or not. In *chapter 5.4* it is described and shown conclusively that human alveolar macrophages actually express specific glucocorticoid receptors.

It is studied in *chapter 6* whether glucocorticoids used in the treatment of patients induce cellular effects similar to the effects observed in our *in vitro* experiments. In *chapter 6.1* it is shown that the expression of various cell membrane antigens of blood monocytes from allergic asthmatics, who were treated with inhaled glucocorticoids for 2.5 years, differs from healthy volunteers or patients treated with β_2-agonists or placebo; the expression of CD13, CD14 and CD18 antigens was higher in the patients treated with glucocorticoids than in healthy volunteers. In contrast, CD11b antigen expression was lower. Furthermore, CD14 antigen expression was significantly higher in the patients treated with inhaled glucocorticoids compared with the patients treated with β_2-agonists or placebo. These findings seem to indicate that inhaled glucocorticoids have systemic effects, at least on blood monocytes, in addition to their local effects. Future studies are needed to find out whether these observed effects on blood monocytes are an important part of the clinical anti-inflammatory effects of inhaled glucocorticoids. In *chapter 6.2* various substances are studied in the bronchoalveolar lavage (BAL) fluid of the same patients described in chapter 6.1. The positive correlation between the levels of lipocortin-1 and the numbers of cells in the BAL fluid appeared to be an important finding. Furthermore, the concentration of a arachidonic acid metabolite, the bronchoconstrictor PGD_2, was found to be inversely related to lipocortin-1 levels. We have interpreted this as a possible sign for an anti-inflammatory role of lipocortin-1 in the lung. We also found that smoking patients had more lipocortin-1 in their BAL fluid than non-smoking patients, and this positive correlation between smoking and increased levels of lipocortin-1 was also demonstrated in nonasthmatic, healthy volunteers. This may be explained by the fact that smokers have more cells in their BAL fluid. Lipocortin-1 may have anti-inflammatory properties, and its increased levels in smokers may therefore inhibit the pro-inflammatory effects of cigarette smoke on the airways. In this way, our body seems to cope locally with the noxious components of cigarette smoke. In this study, we could also demonstrate that treatment with inhaled glucocorticoids was associated with a lower concentration of total protein and

albumin in the BAL fluid, whereas levels of lipocortin-1 were unaffected. This may suggest that inhaled glucocorticoids actively maintain the levels of lipocortin-1, whilst other protein components in BAL fluid are downregulated.

In conclusion, the studies described in this thesis show some new insights in the working mechanisms of glucocorticoids and IL-4 with regard to their influence on mononuclear phagocytes. Both cellular maturation and activation are modulated by glucocorticoids and IL-4. The effects of glucocorticoids and IL-4 on activation are comparable, but their effects are opposite with regard to maturation. Starting-point of the studies was the question whether the course of lung diseases could be influenced by interference in the cellular functions of mononuclear phagocytes. The clinical use of glucocorticoids in those lung diseases in which mononuclear phagocytes play a pro-inflammatory role, is supported by our *in vitro* findings. More clinical and basic research is needed to investigate whether IL-4, alone or in combination with other agents, can be applied in a way similar to glucocorticoids. In the treatment of patients, including lung patients, clinicians are trying increasingly to modulate cell functions. These functions are largely determined by the genetic constitution of the cells. Future lung research should include studies on the regulation of the expression of the relevant genes, e.g. with drugs like glucocorticoids and cytokines.

SUMMARY IN DUTCH

Sinds mensenheugenis tracht men ziekteprocessen te beïnvloeden. De geschiedenis leert ons dat meer inzicht in een ziekteproces veelal ertoe leidde dat men beter in staat bleek een dergelijk proces ten goede te keren. De reactie van ons lichaam op lichaamsvreemde structuren, en ook die op lichaamseigen componenten, wordt tegenwoordig, dankzij talloze immunologische, biochemische en moleculair-biologische "wapenfeiten", beter begrepen dan enkele decennia geleden. Men veronderstelt tegenwoordig dat antigeen-presentatie en cellulaire interacties belangrijke onderdelen zijn van die reactie. Mononucleaire fagocyten zijn, onder bepaalde omstandigheden, in staat een dergelijke reactie te initiëren, te onderhouden, of te remmen. Er wordt dan ook gemeend dat mononucleaire fagocyten een essentiële rol spelen in het ontstaan en het beloop van sommige ziekten. Onder die ziekten zijn vele longaandoeningen. Dit laatste is onder meer begrijpelijk, omdat onze longen organen zijn die in direct contact staan met de buitenwereld en derhalve continu bloot staan aan potentieel ziekmakende micro-organismen en lichaamsvreemde stoffen. Hierbij worden onophoudelijk reacties opgeroepen waarbij mononucleaire fagocyten een rol spelen. In de longen komen grote aantallen mononucleaire fagocyten voor in de vorm van interstitiële monocyten/macrofagen, dendritische cellen en alveolaire monocyten/macrofagen. Beïnvloeding van deze cellen biedt de mogelijkheid om het beloop van longziekten te beïnvloeden c.q. patiënten met longaandoeningen te genezen.

In dit proefschrift is het onderliggende ontstekingsproces zoals dat aanwezig is in de luchtwegen van astmapatiënten als uitgangspunt genomen. We weten dat dit ontstekingsproces eigenlijk chronisch aanwezig is bij deze patiënten, hoewel in de tijd de ernst ervan kan fluctueren. Deze chronische ontsteking wordt tevens verantwoordelijk geacht voor de bronchiale hyperreactiviteit welke zo kenmerkend is voor het klachtenpatroon van astmapatiënten. We beseffen dat een breed scala van verschillende celtypen (mononucleaire fagocyten, T-lymfocyten, neutrofiele en eosinofiele granulocyten, mestcellen en epitheel) van belang is bij het ontstaan en in stand houden van deze chronische luchtwegontsteking. Een centrale rol in dezen wordt toebedeeld aan mononucleaire fagocyten, gezien hun vele functies, waaronder hun vermogen de functies van andere celtypen te beïnvloeden. Aan het begin van deze studie stond ons voor ogen om eigenschappen van mononuclaire fagocyten te bestuderen. Met name die eigenschappen die van belang zouden kunnen zijn voor hun functioneren in het hierboven genoemde ontstekingsproces. Daarnaast werd grote waarde gehecht aan het verkrijgen van inzicht in de mogelijkheden tot beïnvloeding van die eigenschappen. Enerzijds door glucocorticoïden die al sinds 1950 bij astma worden gebruikt, maar waarvan het werkingsmechanisme nog deels onopgehelderd is. Anderzijds door cytokinen waarvan thans wordt gedacht dat zij een grote rol spelen in de intercellulaire communicatie en beïnvloeding van cellulaire functies. Uiteindelijk doel van deze studie was om een beter inzicht te krijgen in de beïnvloeding van de functies van mononucleaire fagocyten, waarvan bij de behandeling van astmapatiënten gebruik gemaakt zou kunnen worden. De beïnvloeding van functies van mononuclaire fagocyten die theoretisch een gunstige uitwerking op ontsteking heeft, kan immers van belang zijn voor de behandeling van het ontstekingsproces in de luchtwegen en de daarmee samenhangende klachten.

In *hoofdstuk 1* wordt een samenvatting gegeven van de huidige kennis over

mononucleaire fagocyten, cytokinen en glucocorticoïden. Aan het einde van de vorige eeuw werd het fagocyterende vermogen van macrofagen ontdekt. Lange tijd werd deze eigenschap beschouwd als de enige van belang zijnde. Thans kennen we meerdere belangrijke functies van mononucleaire fagocyten, waaronder hun vermogen om immunologische reacties te beïnvloeden. Zowel het belang als de therapeutische beïnvloeding van hun functies in diverse ziektebeelden zijn hedentendage onderwerp van vele onderzoeksprojecten. Cytokinen hebben een veel kortere geschiedenis, maar hun krachtige invloed op tal van lichaamsprocessen werd eigenlijk direct onderkend. Voor intercellulaire communicatie en samenwerking zijn cytokinen onmisbaar. Cytokinen vormen hiermee een waardevolle ingang om ontspoorde lichaamsprocessen te beïnvloeden. Hun klinische toepassingen staan pas in de kinderschoenen, maar zijn veelbelovend. De klinische toepassingen van glucocorticoïden daarentegen zijn al sinds hun eerste gebruik bij de behandeling van reumatoïde artritis in 1949 bekend. Toch zijn hun werkingsmechanismen nog niet volledig bekend, hoewel hun vermogen om de transcriptie te reguleren van bekende (en mogelijk nog te ontdekken) eiwitten, waaronder vele cytokinen, waarschijnlijk de belangrijkste is. In *hoofdstuk 2* wordt uitgebreid ingegaan op de thans bekende werkingsmechanismen van glucocorticoïden.

Hoofdstuk 3 is een korte omschrijving van de achtergrond van de experimenten zoals die worden beschreven in de hoofdstukken 4, 5 en 6.

Hoofdstuk 4 beschrijft hoe enkele eigenschappen van mononucleaire fagocyten beïnvloed kunnen worden door cytokinen. Alveolaire macrofagen, die in groten getale voorkomen in de longen, zijn voor het merendeel afkomstig van bloedmonocyten. Deze monocyten ondergaan een aantal veranderingen als zij vanuit het bloed, via het interstitium naar de alveolaire ruimten migreren. Die veranderingen kunnen worden gezien als onderdeel van een uitrijpingsproces. Tijdens dit uitrijpen verandert ondermeer de expressie van (glyco)proteïnen op de celmembraan. De aanwezigheid van dergelijke (glyco)proteïnen kan zichtbaar worden gemaakt met behulp van specifieke monoclonale antistoffen. De belangrijkste verschillen in expressie van eiwitten op de celmembraan tussen bloedmonocyten en alveolaire macrofagen betroffen het CD14 en RFD9 antigeen: bloedmonocyten brengen het CD14 antigeen hoog en het RFD9 antigeen laag tot expressie, terwijl dit bij alveolaire macrofagen juist omgekeerd is (*hoofdstuk 4.1*). We concluderen hieruit dat het uitrijpen van bloedmonocyten tot alveolaire macrofagen gepaard gaat met het verdwijnen van het CD14 antigeen en het tot expressie komen van het RFD9 antigeen. In *hoofdstuk 5.1* wordt beschreven dat dit uitrijpingsproces *in vitro* is na te bootsen en dat het bovendien kan worden versneld door IL-4. Deze bevindingen zijn een belangrijke aanwijzing voor de hypothese dat uitrijping van mononucleaire fagocyten *in vivo* kan worden gestuurd door omgevingsfactoren: mogelijk speelt IL-4 ook *in vivo* een rol bij dit proces. Bovendien opent deze bevinding therapeutische mogelijkheden waarbij kan worden gedacht aan beïnvloeding van dit proces met bijvoorbeeld IL-4 of anti-IL-4. Een tweede belangwekkende bevinding die werd gedaan bij de bestudering van effecten van IL-4 op mononucleaire fagocyten bleek het effect op de activiteit van het enzym aminopeptidase-N. Dit enzym komt tot expressie op de celmembraan van zowel bloedmonocyten als alveolaire macrofagen en kan op twee manieren worden aangetoond: enerzijds de aanwezigheid van

het enzym met specifieke monoclonale antistoffen (CD13) en anderzijds de enzymactiviteit met een specifieke enzymassay. IL-4 bleek in staat om de expressie van dit enzym op de celmembraan van bloedmonocyten én alveolaire macrofagen te verhogen. Deze verhoging ging bovendien gepaard met een verhoogde functionele activiteit van aminopeptidase-N (*hoofdstuk 4.2*). Ook het mRNA dat codeert voor aminopeptidase-N bleek na incubatie met IL-4 in verhoogde mate voor te komen in bloedmonocyten en alveolaire macrofagen. Omdat dit enzym mogelijk van belang is bij onder andere de inactivatie van ontstekingsmediatoren, kan IL-4 mogelijk de ontstekingsremmende capaciteiten van mononucleaire fagocyten verhogen. Derhalve lijkt IL-4 enerzijds de maturatie van mononucleaire fagocyten te versnellen, terwijl het anderzijds deze cellen zodanig activeert dat een verhoogde activiteit van het enzym aminopeptidase-N kan worden waargenomen. Dit activerend vermogen van IL-4 blijkt ook waarneembaar op endotheelcellen (*hoofdstuk 4.2*). Toch is deze eigenschap van IL-4 niet van algemene aard, omdat IL-4 niet in staat bleek de expressie van het CD13 antigeen te induceren of te verhogen op voorlopers van bloedmonocyten (*hoofdstuk 4.3*). Dat deze voorlopers echter wel degelijk beïnvloedbaar zijn door IL-4 en dus ook IL-4 receptoren (IL-4R) moeten bezitten, bleek uit het feit dat de FcεRIIb op deze cellen wel induceerbaar was door IL-4. Ook bloedmonocyten waren gevoelig voor deze laatste eigenschap van IL-4. Op alveolaire macrofagen was de FcεRIIb echter niet te induceren met IL-4. Waarschijnlijk wordt de wijze waarop een celtype reageert op de aanwezigheid van een cytokine niet alleen bepaald door de aanwezigheid van functionele cytokine-receptoren, maar hangt deze mede af van andere factoren, waaronder het rijpingsstadium.

In *hoofdstuk 4.4* wordt een ander aspect van de rijping van mononucleaire fagocyten belicht. Proliferatie van cellen kan worden aangetoond middels de expressie van een bepaald antigeen in de kern van cellen. Hiervoor kan de monoclonale antistof Ki-67 worden gebruikt. Een zeer klein percentage van bloedmonocyten bleek in deling te zijn. Het percentage delende cellen onder alveolaire macrofagen bleek ook klein te zijn, maar iets hoger dan onder bloedmonocyten. Deze bevindingen waren eigenlijk onverwacht omdat algemeen wordt aangenomen dat uitrijping van cellen gepaard gaat met een vermindering van de proliferatieve activiteit. Longweefsel lijkt derhalve een zodanig micromilieu voor mononucleaire fagocyten te creëren dat proliferatie wordt gestimuleerd. Een belangrijke factor in dezen lijkt de aanwezigheid van "colony stimulating factors", met name GM-CSF. Als het micromilieu van de longen inderdaad deze invloed heeft, lijkt dit tevens te kunnen worden beïnvloed door ziekte: bij patiënten met sarcoïdose werd namelijk een verhoogd percentage proliferende alveolaire macrofagen aangetroffen, en dit kan mogelijk worden veroorzaakt door een verhoogde lokale concentratie van GM-CSF.

Hoofdstuk 5 behandelt de effecten van glucocorticoïden op mononucleaire fagocyten. Zoals we hierboven reeds meldden, wordt in *hoofdstuk 5.1* beschreven dat IL-4 in staat is maturatie van bloedmonocyten te versnellen, waarbij maturatie werd vervolgd aan de hand van diverse, hieronder nader omschreven parameters. Glucocorticoïden bleken *in vitro* in staat die maturatie te remmen: ze remden morfologisch het strekken van monocyten, de toename van de expressie van zure fosfatase in het cytoplasma, en op de celmembraan de toename van de expressie van het RFD9 antigeen en de afname van de expressie van het CD14 antigeen. Bovendien bleken glucocorticoïden de versnellende invloed van IL-4 op de

maturatie tegen te gaan. Op grond van deze bevindingen lijkt er een wisselwerking te bestaan tussen enerzijds immunologische factoren en anderzijds hormonen met betrekking tot de regulatie van cellulaire activiteiten. In *hoofdstuk 5.2* wordt uiteengezet dat verlaging van de expressie van het gen dat codeert voor IL-1β een werkingsmechanisme kan zijn van de ontstekingsremmende eigenschappen van glucocorticoïden. Deze hypothese was reeds eerder geopperd ten aanzien van bloedmonocyten. Onze bevindingen ondersteunen de gedachte dat het klinische gebruik van glucocorticoïden zinnig is bij die longziekten waarbij alveolaire macrofagen via de produktie van IL-1β een ontsteking induceren. IL-4 bleek in deze studie eveneens een verlaging van de expressie van IL-1β mRNA te bewerkstelligen, en zo mogelijk nog krachtiger dan glucocorticoïden. Deze invloed van IL-4, tezamen met de verhoging van de aminopeptidase-N-activiteit door IL-4, lijkt de werking als potentiële ontstekingsremmer te bevestigen. Mogelijk zullen deze anti-inflammatoire aspecten van IL-4 in de toekomst ook klinische toepassing vinden. Uit de resultaten beschreven in *hoofdstuk 5.1* en *hoofdstuk 5.2* lijkt bovendien geconcludeerd te moeten worden dat, afhankelijk van de cellulaire parameter die wordt bestudeerd, IL-4 en glucocorticoïden soms vergelijkbare en soms tegengestelde effecten hebben.

In *hoofdstuk 5.3* wordt ingegaan op een ander, en in de literatuur omstreden, mechanisme van de ontstekingsremmende effecten van glucocorticoïden. In onze studie werd een toename gezien van lipocortine-1 mRNA in alveolaire macrofagen onder invloed van glucocorticoïden. Dit effect werd waargenomen onder experimentele condities waarbij de expressie van IL-1β mRNA juist werd verlaagd door glucocorticoïden. Tevens wordt in dit hoofdstuk ingegaan op de bevinding dat de effecten van glucocorticoïden op de expressie van het IL-1β en het lipocortine-1 gen verschillen in kinetiek. Voor bijna alle beschreven effecten van glucocorticoïden op een cel dient deze in het bezit te zijn van functionele glucocorticoïd receptoren. Aan deze voorwaarde is uitgebreid aandacht besteed in hoofdstuk 2. Door technische problemen die zijn gerelateerd aan eigenschappen van humane alveolaire macrofagen is het lang onduidelijk geweest of dit celtype in het bezit was van deze receptoren. Dat humane alveolaire macrofagen inderdaad specifieke glucocorticoïd-receptoren bezitten wordt beschreven en onomstotelijk aangetoond in *hoofdstuk 5.4.*

In *hoofdstuk 6* wordt onderzoek beschreven waarbij is geëvalueerd of het therapeutisch gebruik van glucocorticoïden bij patiënten cellulaire veranderingen induceert vergelijkbaar met die welke werden waargenomen in onze *in vitro* studies. In *hoofdstuk 6.1* wordt beschreven dat de bloedmonocyten van allergische astmapatiënten, die inhalatie-glucocorticoïden gebruiken, een expressie vertonen van enkele eiwitten op hun celmembraan die verschilt van gezonde vrijwilligers of patiënten die werden behandeld met een β_2-sympathicomimeticum of placebo: de expressie van CD13, CD14 en CD18 antigenen was hoger in de patiëntengroep die werd behandeld met glucocorticoïden dan bij gezonden, terwijl de expressie van CD11b antigeen juist lager was. Verder bleek dat de expressie van CD14 antigeen significant hoger was in de groep die 2,5 jaar inhalatie-glucocorticoïden had gebruikt dan bij de astmatici die anders werden behandeld. Deze bevindingen lijken in te houden dat inhalatie-glucocorticoïden niet alleen lokaal in de longen, maar ook systemisch effecten hebben, in ieder geval op bloedmonocyten. In hoeverre deze

veranderde expressie van membraaneiwitten door glucocorticoïden ook klinische betekenis heeft, vereist verder onderzoek. In *hoofdstuk 6.2* zijn verschillende aspecten bestudeerd van de bronchoalveolaire lavage (BAL)-vloeistof van dezelfde patiëntengroep die ook in hoofdstuk 6.1 wordt beschreven. Een belangrijke bevinding bleek de positieve correlatie tussen de concentratie lipocortine-1 en het aantal cellen in de BAL-vloeistof. Bovendien bleek de concentratie van één van de metabolieten van arachidonzuur, de bronchoconstrictor PGD_2, negatief te zijn gecorreleerd aan de concentratie van lipocortine-1. We hebben dit geïnterpreteerd als een mogelijke aanwijzing voor een anti-inflammatoire werking van lipocortine-1 in de longen. Tevens werd waargenomen dat rokende patiënten meer lipocortine-1 in hun BAL-vloeistof hebben dan patiënten die niet roken. De relatie tussen roken en verhoogde concentraties aan lipocortine-1 in de BAL-vloeistof kon ook worden aangetoond in gezonde, niet-astmatische proefpersonen. Waarschijnlijk kan deze bevinding worden verklaard doordat bij rokers hogere celaantallen in de BAL-vloeistof worden aangetroffen dan bij niet-rokers. Mogelijk hebben deze verhoogde concentraties aan lipocortine, dat potentieel anti-inflammatoire eigenschappen bezit, een remmende invloed op de ontsteking in de luchtwegen die door roken wordt veroorzaakt. Aldus lijkt ons lichaam in staat spontaan lokaal voor een beschermende reactie te zorgen tegen de schadelijke componenten van sigaretterook. Daarnaast konden we aantonen dat behandeling van allergische astmapatiënten met inhalatie-glucocorticoïden geassocieerd is met lagere concentraties totaal eiwit en albumine in de BAL-vloeistof. Bij de concentratie van lipocortine-1 bleek dat niet het geval, hetgeen kan betekenen dat glucocorticoïden actief de produktie van dit eiwit hoog houden in tegenstelling tot verlaging van de vorming van andere eiwitcomponenten.

Concluderend kunnen we stellen dat het onderzoek dat is beschreven in dit proefschrift een aantal nieuwe inzichten in de werkingsmechanismen van glucocorticoïden en IL-4 heeft opgeleverd met betrekking tot hun invloed op mononucleaire fagocyten. Glucocorticoïden en IL-4 beïnvloeden beide zowel de maturatie, met tegengesteld effect, als de activatie, met gelijkgericht resultaat. Uitgangspunt van het onderzoek was de vraag of het ziektebeloop van patiënten met astma uiteindelijk gunstig zou kunnen worden beïnvloed door ingrijpen in het functioneren van mononucleaire fagocyten. Het gebruik van glucocorticoïden bij longaandoeningen, waarbij mononucleaire fagocyten door hun pro-inflammatoire functies een rol spelen, lijkt te worden gesteund door onze *in vitro* bevindingen. Of ook voor IL-4, al dan niet in combinatie met andere middelen, vergelijkbare toepassingen zijn weggelegd, vereist verder klinisch én basaal onderzoek. In toenemende mate wordt in de kliniek, ook bij behandeling van longziekten, geprobeerd cellulaire functies te beïnvloeden. Deze functies worden grotendeels bepaald door de genetische constitutie. Pulmonologisch onderzoek zal in de toekomst mede gericht moeten zijn op medicamenteuze regulatie van gen-expressie, bijvoorbeeld met behulp van medicamenten zoals glucocorticoïden en cytokinen.

ABBREVIATIONS

ACTH : adrenocorticotropic hormone
ADCC : antibody-dependent cell mediated cytotoxicity
Ag : antigen
AM : alveolar macrophages
AP-1 : activator protein-1
AMDGF : alveolar macrophage-derived growth factor
ARDS : adult respiratory distress syndrome
BAL : bronchoalveolar lavage
BALF : bronchoalveolar lavage fluid
BEAS : a human bronchial epithelial cell line derived from normal human bronchial epithelial cells transformed by infection with adenovirus 12-SV40 hybrid virus
BHR : bronchial hyperresponsiveness
bp : basepair
BSA : bovine serum albumin
cAMP : cyclic adenosine 3′,5′ monophosphate
°C : degree Celsius
CALLA : common acute lymphoblastic leukemia antigen
CD : cluster of designation/cluster of differentiation
CFU : colony forming unit
COPD : chronic obstructive pulmonary diseases
COUP-TF : chicken ovalbumin upstream promoter transcription factor
CREB : cAMP response element binding protein
DNA : deoxyribonucleic acid
EAA : extrinsic allergic alveolitis
EAR : v-erb A-related (protein)
e.g. : exempli gratia (for example)
ELAM : endothelial-leukocyte adhesion molecule
ERE : estrogen response element
ERR : estrogen receptor related
F(ab) : antigen binding fragment
FACScan : fluorescence-activated cell scanner
Fc : crystallizable fragment
$Fc\gamma R$: Fc receptor for IgG
$Fc\varepsilon R$: Fc receptor for IgE
FCS : fetal calf serum
FEV_1 : forced expiratory volume in 1 second
FGF : fibroblast growth factor
FITC : fluorescein isothiocyanate
FMLP : formyl-methionyl-leucyl-phenylalanine
FRC : functional residual capacity
g : unit of gravity
GAM : goat anti-mouse Ig antiserum
G-CSF : granulocyte colony stimulating factor
GM-CSF : granulocyte macrophage stimulating factor
GR : glucocorticoid receptor
GRE : glucocorticoid response element
h : hour
HeLa : name of the cells of the first human continuously cultured carcinoma cell line; acronym of the name of the patient (Henriette Lacks) from whose carcinoma the parent cells were isolated in 1951
HIV : human immunodeficiency virus
HRE : hormone response element
HRR : hormone response region
hsp : heat shock protein
i.c. : in casu (in this case)
ICAM : intercellular adhesion molecule
i.e. : id est (that is)
IFN : interferon
Ig : immunoglobulin
IGF : insulin-like growth factor
IL : interleukin
ISGF3 : IFN-stimulated gene factor-3
kb : kilobase
LAF : lymphocyte-activating factor
LAP : L-leucine-aminopeptidase
LBP : LPS-binding protein
LPS : lipopolysaccharide
mAb : monoclonal antibody
MCP : monocyte chemoattractant protein
M-CSF : macrophage colony stimulating factor
MESF : molecules equivalent to soluble FITC
MHC : major histocompatibility complex
MIF : migration inhibitory factor
MIP : macrophage inflammatory protein
MMTV : mouse mammary tumor virus
MNC : mononuclear cells
mRNA : messenger RNA
n : number in study or group
NAP : neutrophil activating protein

NEP : neutral endopeptidase
NF I : nuclear factor I
NF-κB : nuclear factor-κB
NHIK : cell line derived from a carcinoma of the human uterine cervix
NK cell : natural killer cell
NMS : normal mouse serum
NO : nitric oxide
OTF-1 : octamer transcription factor-1
PAF : platelet-activating factor
PBM : peripheral blood monocytes
PBS : phosphate-buffered saline
PC_{20} : provocative concentration (of a nonspecific agent, e.g. histamine) which causes a 20% decrease in FEV_1
PCP : *Pneumocystis carinii* pneumonia
PCR : polymerase chain reaction
PDGF : platelet-derived growth factor
PE : phycoerythrin
PEEP : positive end-expiratory pressure
PGE_2 : prostaglandin E_2
PIE : pulmonary infiltration with blood eosinophilia
PMA : phorbol myristate acetate
PMN : polymorphonuclear cells
PRE : progesterone response element
q.i.d. : quater in die (four times a day)
r : recombinant (e.g. rIL-4)
R : receptor (e.g. IL-4R)
RA : rheumatoid arthritis
RANTES : regulated upon activation, normal T expressed, and presumably secreted
RNA : ribonucleic acid
RNase : ribonuclease
SD : standard deviation
SLE : systemic lupus erythematosus
SSC : sodiumchloride sodiumcitrate
TGF : transforming growth factor
TNF : tumor necrosis factor
TPA : 12-O-tetradecanoylphorbol-13-acetate
TSC : total static compliance
U : unit
U937 : a human immature monocytic cell line
V_T : tidal volume
Yop : yersinial membrane protein

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Voordat het daadwerkelijke experiment aan de laboratoriumtafel kan worden uitgevoerd is een gedegen voorbereiding van essentieel belang. Hierbij voelden we ons altijd gesteund door Tom Vermetten (veel dank voor je begrip en de daaraan gekoppelde acties als we kwamen uitleggen hoe belangrijk een bepaalde bestelling was voor een experiment), Jopie en Joke Bolman en Elly Hofman (er klonk nooit bezwaar als we verzochten om een extra "rondje" autoclaveren).

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interested to get to the bottom of the subcellular working mechanisms of glucocorticoïds, and I hope our collaboration will continue in the future.

Per 1 januari 1992 moesten mijn werkzaamheden als full time onderzoeker plaatsmaken voor patiëntenzorg in de kliniek, in het kader van mijn opleiding tot arts voor Longziekten en Tuberculose. Toch mocht ik ook in die periode geregeld vertoeven op het laboratorium. Dr. H.S.L.M. Tjen van de afdeling Inwendige Geneeskunde van het Sint-Franciscus Gasthuis wil ik bedanken voor zijn steun zodat ik ook daadwerkelijk enkele uren in de week kon werken aan de afronding van mijn proefschrift. Mijn directe collega's arts-assistenten, Erna Barendrecht, Paul Benner, dr. Luuk Berk, Angelique Boers, Ingrid Bonaparte, Sarah Bovenberg, Jan Nouwen, Ingrid Planken, Huibert Ponsen, Ronald van Rossum, dr. Karin van der Rijt, Frank Slaats en Ad Zanen, wil ik bedanken voor de uiterst plezierige werksfeer in het SFG, het bij tijd en wijlen overnemen van de "suikerpieper" (3002), en het mij leren skiën.

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Rotterdam, augustus 1995

CURRICULUM VITAE AND PUBLICATIONS

CURRICULUM VITAE

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12 maart 1960	: Geboren te Rotterdam
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jan. 1985 - juni 1986	: Student-assistentschap : afdeling Inwendige Geneeskunde III, Academisch Ziekenhuis Rotterdam-Dijkzigt (o.l.v. Prof. dr. S.W.J. Lamberts) Project : beïnvloeding van de groei en hormoonproduktie van hypofyse tumoren bij ratten
aug. 1985	: Doctoraalexamen Geneeskunde Erasmus Universiteit Rotterdam
febr. 1987	: Artsexamen Erasmus Universiteit Rotterdam
april 1987 - febr. 1994	: Werkzaam als arts/wetenschappelijk medewerker op de afdeling Immunologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt en de Erasmus Universiteit Rotterdam (hoofd Prof. dr. R. Benner): - voor het onderwijs Klinische Immunologie aan studenten Geneeskunde - als consulent in de diagnostiek van diffuse longziekten d.m.v. immunologische analyse van cellen in bronchoalveolaire lavagevloeistoffen - in opleiding tot Immunoloog (Stichting voor Opleiding tot Medisch Biologisch Wetenschappelijk Onderzoeker) - met het promotie-onderzoek beschreven in dit proefschrift
aug. 1989	: Doctoraalexamen Biologie, cum laude Rijksuniversiteit Leiden
jan. 1992 - febr. 1994	: Vooropleiding "Inwendige Geneeskunde" Sint-Franciscus Gasthuis, Rotterdam (opleider dr. H.S.L.M. Tjen)
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jan. 1995 - heden	: Honoraire aanstelling als "clinical research fellow" Department of Medicine, Charing Cross and Westminster Medical School, Londen

PUBLICATIONS

1. Van Hal PThW, Leynse B. Over de regeling van het zuur-base evenwicht in het organisme, I en II. Tijdschr Geneesk 1985;41:1413.

2. Lamberts SWJ, Reubi J-C, Uiterlinden P, Zuiderwijk J, Van der Werff P, Van Hal P. Studies on the mechanism of action of the inhibitory effect of the somatostatin analog SMS 201-995 on the growth of the prolactin/adrenocorticotropin-secreting pituitary tumor 7315a. Endocrinology 1986;118:2188-2194.

3. Hoogsteden HC, Van Hal PThW, Van Dongen JJM. Immunologische markers van cellen in bronchoalveolaire lavage (BAL) en perifeer bloed (PB) bij interstitiële longziekten. Bull Fijnvlekkige Longafwijkingen 1988;16:57-60.

4. Van Hal PThW, Mulder E, Hoogsteden HC, Hilvering C, Benner R. Glucocorticoid receptors in alveolar macrophages: methodological aspects of the determination of the number of glucocorticoid receptors per cell. Agents Actions 1989;26:128-131.

5. Hoogsteden HC, Van Dongen JJM, Van Hal PThW, Delahaye M, Hop W, Hilvering C. Phenotype of blood monocytes and alveolar macrophages in interstitial lung disease. Chest 1989;95:574-577.

6. Van Hal PThW, Mulder E, Hoogsteden HC, Hilvering C. 1949-1989: glucocorticoïden, hun receptor en variabele klinische respons. Ned Tijdschr Geneesk 1989;133:1305-1310.

7. Hoogsteden HC, Van Hal PThW. Mediators of the induction of non-allergic pulmonary inflammation. In: Bray M, Handerson WA, Eds. Lung Biology in Health and Disease. New York: M Dekker Inc., 1991:185-277.

8. Van Hal PThW, Hoogsteden HC. Inflammatory mediators and glucocorticoids. In: Bray M, Handerson WA, Eds. Lung Biology in Health and Disease. New York: M Dekker Inc., 1991:593-617.

9. Van Hal PThW, Wijkhuijs JM, Broos JPM, Hoogsteden HC. Monocyten/macrofagen in bronchoalveolaire lavagevloeistoffen. Analyse 1991;46:208-211.

10. Hoogsteden HC, Van Hal PThW, Wijkhuijs JM, Hop W, Verkaik APK, Hilvering C. Expression of the CD11/CD18 cell surface adhesion glycoprotein family on alveolar macrophages in smokers and non-smokers. Chest 1991;100:1567-1571.

11. Hoogsteden HC, Van Hal PThW, Wijkhuijs JM, Hop W, Hilvering C. Expression of the CD11/CD18 surface adhesion glycoprotein family and MHC class II antigen on blood monocytes and alveolar macrophages in interstitial lung diseases. Lung 1992;170:221-233.

12. Zijlstra FJ, Vincent JE, Mol WM, Hoogsteden HC, Van Hal PThW, Jongejan RC. Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and non-smokers. Eur J Clin Invest 1992;22:301-306.

13. Van Daal GJ, Van 't Veen A, So KL, Mouton JW, Smit F, Van Hal PThW, Bergman KCh, Lachmann B. Influence of oral immunization and infection with S. pneumoniae on interferon-gamma and PMN-elastase concentrations in murine bronchoalveolar lavage fluid. Int Arch Allergy Appl Immunol 1992;97:173-177.

14. Van Hal PThW, Hopstaken-Broos JPM, Wijkhuijs JM, Te Velde AA, Figdor CG, Hoogsteden HC. Regulation of aminopeptidase-N(CD13) and $F_c\varepsilon$RIIb(CD23) expression by interleukin-4 depends on the stage of maturation of monocytes/macrophages. J Immunol 1992;149:1395-1401.

15. Hoogsteden HC, Van Hal PThW, Wijkhuijs JM, Hop W, Hilvering C. Differences in expression of monocyte/macrophage surface antigens in peripheral blood and bronchoalveolar lavage cells in interstitial lung diseases. Lung 1993;171:149-160.

16. Van Hal PThW, Hopstaken-Broos JPM, Prins A, Hoogsteden HC, Huijbens RJF, Figdor CG, Hilvering C. Potential, indirect anti-inflammatory effects of IL-4: stimulation of human monocytes, macrophages, and endothelial cells by IL-4 increases aminopeptidase-N-activity (CD13; EC 3.4.11.2). J Immunol 1994;153:2718-2728.

17. Van Hal PThW, Prins A, Verheggen MM, Hoogsteden HC. Werkingsmechanismen van glucocorticoïden. Analyse 1995. Accepted for publication.

18. Van Hal PThW, Wijkhuijs JM, Mulder PG, Hoogsteden HC. Proliferation of mature and immature subpopulations of bronchoalveolar monocytes/macrophages and peripheral blood monocytes. Cell Proliferat 1995. Accepted for publication.

19. Van Hal PThW, Hoogsteden HC, Te Velde AA, Wijkhuijs JM, Figdor CG, Hilvering C. IL-4-induced maturation of monocytes/macrophages is inhibited by glucocorticoids. Submitted.

20. Van Hal PThW, Wijkhuijs JM, Van der Kwast ThH, Zegers ND, Hoogsteden HC, Mulder E. Functional glucocorticoid receptors in human alveolar macrophages. Submitted.

21. Van Hal PThW, Prins A, Hoogsteden HC. IL-1β gene expression in cultured human bronchoalveolar macrophages from smokers and nonsmokers, and modulation by IL-4 and dexamethasone. Submitted.

22. Van Hal PThW, Prins A, Smith SF, Hoogsteden HC. Inverse modulation of lipocortin-1 (annexin-1) and IL-1β gene expression by dexamethasone in cultured human bronchoalveolar macrophages. Submitted.

23. Slieker WAT, Van Hal PThW, Wijkhuijs JM, Hopstaken-Broos JPM, Overbeek SE, Mulder PG, Postma DS, Hoogsteden HC. Cell surface antigen expression by peripheral blood monocytes in allergic asthma: results of 2.5 years therapy with inhaled beclomethasone dipropionate. Submitted.

24. Van Hal PThW, Overbeek SE, Hoogsteden HC, Zijlstra FJ, Murphy K, Oosterhof Y, Postma DS, Guz A, Smith SF. Eicosanoids and lipocortin-1 in BAL-fluid in asthma: effects of smoking and inhaled glucocorticoids. Submitted.

25. Verheggen MM, Van Hal PThW, Hoogsteden HC, Mulder E, Versnel MA. Expression of glucocorticoid receptor mRNA and protein in human bronchial epithelial cell lines detected by Northern blot analysis and ^{3}H-dexamethasone binding studies. Submitted.

26. Verheggen MM, De Bont HIM, Adriaansen-Soeting PWC, Goense BJA, Tak CJAM, Hoogsteden HC, Van Hal PThW, Versnel MA. Expression of lipocortins in human bronchial epithelial cells: effects of IL-1β, TNF-α, LPS and dexamethasone. Submitted.

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