CHRONIC UPPER AIRWAY INFECTIONS

The role of p15E-like proteins and defects in cell-mediated immunity

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This study was carried out at the departments of Otorhinolaryngology/Head and Neck Surgery (chairman: prof.dr. G.B. Snow) and Pathology (chairman: prof.dr. C.J.L.M. Meijer), Free University Hospital, Amsterdam.

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
dr. C. Datema,
hoogleraar aan de faculteit der letteren,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der geneeskunde
op vrijdag 11 december 1992 te 15.30 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105
door

Robert Antonius Scheeren

geboren te Leiden

Promotoren	: prof.dr. G.B. Snow
	prof.dr. C.J.L.M. Meijer
Copromotor	: dr. S. van der Baan
Referent	: prof.dr. E.C.M. Hoefsmit

Aan Adrienne en mijn ouders

Contents

Voorwoord

Aan het onderzoek, zoals beschreven in dit proefschrift, hebben velen een bijdrage geleverd. De goede en plezierige samenwerking tussen de afdelingen Keel-, Neus- en Oorheelkunde en Pathologie van het Academisch Ziekenhuis van de Vrije Universiteit te Amsterdam stond hierin centraal.
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Chapter 1

General Introduction

The respiratory tract consists of the nasal cavity, the paranasal sinuses, the middle ear cavity, the pharynx, the larynx and continues through the trachea into the bronchi, bronchioli and finally via the ducti alveolares in the alveoli. A division between the lower and upper respiratory tract can be made with the larynx interposed between the two parts (1). Infections of the upper respiratory tract are predominantly present in the nose and paranasal sinuses. In this thesis, upper respiratory tract infections are defined as infections primarily localized in the nose and paranasal sinuses.

Infections of the upper respiratory tract are frequently present in man. In the United States of America, sinusitis is the most common health complaint, afflicting approximately 31,2 million persons each year (2). Chronic upper airway infections interfere with one's quality of life and are predominantly characterized by nasal obstruction, purulent nasal discharge, headache and anosmia. In The Netherlands, during the period from 1989 untill 1990 (2 years-period), 6.4% (mean), approximately 960.000 persons, of the dutch population had complaints due to chronic infections of the upper airways (3). For comparison, the prevalence of other chronic diseases in the dutch population in the same period were (diagnosis code according to the vademecum of health statistics of The Netherlands 1992): asthma, chronic bronchitis or chronic aspecific respiratory disease, 5.7%; hypertension, 7.3%; diabetes mellitus, 2.0%; chronic spinal affections, 7.8%; migraine, 4,9%.

The influence of upper airway infections (acute and chronic) on the dutch society is enormous. In 1989, these infections resulted for the dutch population in 192.933 spells of sickness and 2.1 X 10^6 days of absence (3).

Paranasal disease is involved in the pathogenesis of lower airway disease. The association with bronchial asthma is well known (4,5). Several factors (bacterial seeding, diminished air-conditioning, hypersensitivity for bacterial products, ß-adrenergic blockade, parasympathetic reflex) may have a role in this association. Improvement of lungfunction after endoscopic sinus surgery for chronic sinusitis (6) and nasal polyps (4,7) has been reported.

Many research has been done on the pathogenesis of allergic and non-allergic rhinosinusitis (8-10) and nasal polyps (11). Several factors, such as modifications of nasal secretions, deficiency of mucociliary

clearance, immunodeficiencies and anatomic abnormalities may contribute to the pathogenesis. Recently, attention has also been focussed on cellular immunity in patients with chronic rhinosinusitis (12,13).
Since the reports of Stammberger (14) and Messerklinger (15) about the role of the ethmoid in the pathogenesis of chronic sinus disease, endoscopic sinus surgery received an important place in the treatment of chronic sinus disease. Present therapy includes endoscopic sinus surgery with the removal of the inflamed mucosa and establishing adequate drainage via opening of physiologic ostia (16,17), frequently followed by topical corticosteroids.

I. Anatomy of the nose and paranasal sinuses

The nose

The internal nose consists of the nasal vestibule and the nasal cavities and is divided from front to back by the nasal septum (figure 1). The septum has a cartilagineous and an osseous part. The total length of the internal nose is 10-12 cm (from the tip to the pharyngeal wall) and is linked via the choanal openings with the nasopharynx. On each side there are three turbinates (superior, middle and inferior) which project from the lateral wall into the nose and narrow the nasal cavity.

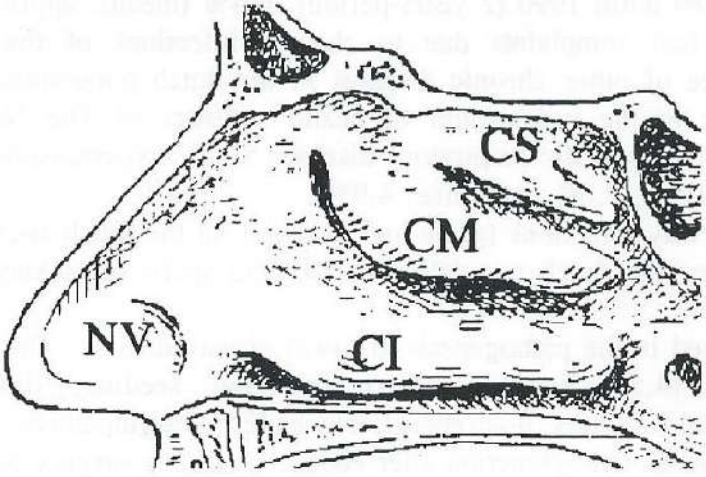

Figure 1: Sagital section of the lateral wall of the nose. CS: concha superior; CM: concha media; CI: concha inferior; NV: nasal vestibulum

Most openings of the paranasal sinuses are under the superior and, especially under the middle turbinate. The internal ostium (or nasal valve), which is the narrowest part of the internal nose, separates the nasal vestibule from the nasal cavity. The olfactory epithelium, a specialized neural tissue which contains the olfactory receptors, occupies approximately 1 cm^2 of the top and both sides of each nasal cavity between the septum and the lateral nasal wall.

The paranasal sinuses

The paranasal sinuses are air-filled spaces in the skull that surround the nasal cavity. Usually four sinuses on each side of the head are present, which also lie immediately adjacent to the orbit and to the dura of the anterior cranial fossa. The sinuses are developed during fetal life and early childhood and their expansion continues during early adulthood. ***The maxillary sinus***, the largest of the paranasal sinuses, is located in the body of the maxilla. The anterior wall is related to the face, the sinus is separated from the pterygopalatine fossae by its posterior wall. The floor is the alveolar process of the maxilla, the roof is the floor of the orbit. The medial wall is the lateral wall of the nasal cavity and is mainly formed by the maxilla and additional by the lacrimal bone, the ethmoid bone, the inferior concha and the perpendicular plate. The maxillary sinus drains by one or more openings into the middle meatus of the nasal cavity via the hiatus semilunaris. ***The ethmoidal sinus***, which consists of numerous small cavities (the socalled ethmoidal cells), is located between the eyes and the nasal chamber. A thin bony plate, the lamina papyracea, separates the sinus from the eyes. An anterior and a posterior part can be distinguished in this labyrinth of cells, separated by the basilar plate of the medial concha. The anterior ethmoidal cells drain into the middle meatus, the posterior cells drain into the superior meatus.
The frontal sinuses are situated in the forehead region and may be absent on one or both sides (18,19). The sinus is mainly developed after birth and growth is slow, so that radiological presence of the sinus can be seen at the age of 6 years (20). The frontal sinuses vary greatly and are frequently of different size. The septum, which separates the two sides, is not always located in the midline. Sometimes, there are more septa. The frontal sinus is connected with the middle meatus by the frontonasal duct, which may or may not be continuous with the ethmoidal infundibulum (21). ***The sphenoid sinus*** is located under the sella turcica. The two sides are separated by a septum which is often situated besides the midline (22). The entrance of the sphenoid sinus to the nasal cavity is frequently formed by the spheno-ethmoidal recess, a splitlike opening between the posterior ethmoid cells and the sphenoid cells.
The ostiomeatal complex (anterior ethmoid-middle meatal complex) is the area which provides the drainage of the frontal, anterior ethmoidal and maxillary sinuses (23). This area is located lateral to the anterior half of the middle turbinate and consists of the uncinate process, the hiatus semilunaris, the

anterior half of the middle turbinate and consists of the uncinate process, the hiatus semilunaris, the ethmoid bulla, the rest of the anterior ethmoid cells, the maxillary infundibulum and the frontal recess. Anatomical variations or pathologic lesions that block or narrow this region, are thought to play an important role in the pathogenesis of paranasal disease (17). The anatomy is illustrated in figure 2.

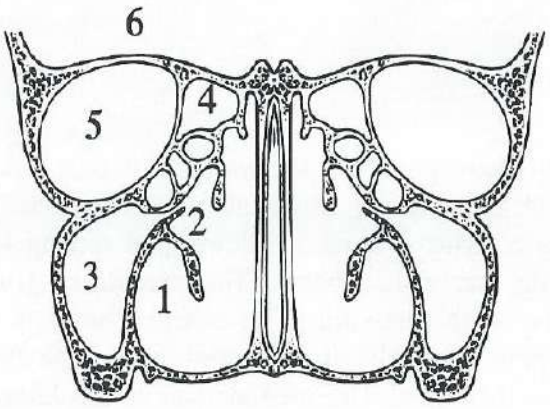

Figure 2: Coronal section of the nose and paranasal sinuses at the level of the anterior ethmoid cells.
1: inferior meatus; 2: middle meatus; 3: maxillary sinus; 4: ethmoid sinus; 5: orbit; 6: frontal sinus.

II. Histology of the (para)nasal mucosa

The mucosa present in the nasal and paranasal cavities consists of epithelium usually covered with a mucuslayer, the basement membrane which separates the epithelium from the lamina propria and the laminia propria itself. Stratified squamous epithelium is present in the vestibulum of the nose and the first millimeters of the nasal cavity. The rest of the nasal cavity and the paranasal sinuses are mainly covered with ciliated columnar epithelium and/or partially stratified cuboidal epithelium (24). Ciliated cells are found in patches on the respiratory epithelium which consists also of non-ciliated cells with microvilli, basal cells and goblet cells (figure 3) (25). Furthermore, migratory cells as antigen presenting cells (Langerhans cells, dendritic cells), lymphocytes, eosinophils, neutrophils and mast cells can be found in the respiratory epithelium.

these exposures, turnover of the cells occurs and respiratory epithelial cells are presumed to be renewed in about 3 to 8 weeks (26). The lamina propria is situated between the basement membrane and the underlying, supportive tissue. Two layers can be distuingished, a superficial and a deeper layer. The superficial layer is cell-rich, collagen poor and contains most of the mucus glands. The deeper layer which lies on the supporting skeleton contains only a few cells (fibroblasts) and is collagen rich. Several inflammatory cell types can be found in the lamina propria: lymphocytes, plasma cells, eosinophils, mast cells, neutrophils, and antigen-presenting cells (Langerhans cells, dendritic cells).

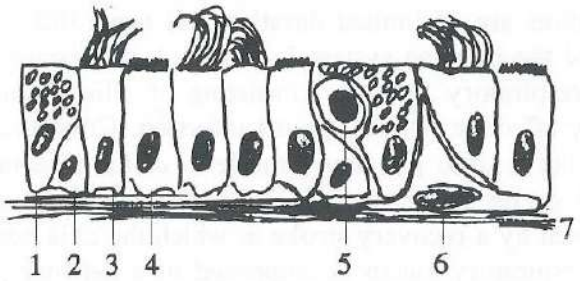

Figure 3: Respiratory epithelium. 1: goblet cell; 2: basal cell; 3: ciliated cell; 4: non-ciliated cell; 5: intra-epithelial lymphocyte; 6: antigen-presenting cell with dendritic processes; 7:basement membrane.

III. Functional aspects of the nose and paranasal sinuses

The nose is responsible for filtering, humidifying and heating the inhaled air, in order to protect the more vulnerable lower respiratory tract. In addition, the nose also provides the sense of smell. Recently, it has been suggested that sensory information from receptors in the nasal passage is involved in controlling the shift of breathing route (oral vs nasal breathing) (27).

The functional role of the paranasal sinuses has long been in dispute and several theories have been proposed (28). Functions ascribed to the sinuses are vocal resonance, humidification, heat exchange, lightening the skull, protection of the brain and craniofacial development. However, these theories are based on traditional evolutionary ideas of ape/man transition and none of them is completely convincing (28).

IV. Defense mechanisms of the upper respiratory tract

In man, the mechanisms underlying resistance to infection are complicated and effective. Therefore, most infections are of limited duration and leave little damage because of the individual's immune response. Before the immune system is activated, mechanical barriers form the first line of defense in the airways. The respiratory mucosa, consisting of ciliated and non-ciliated cells, with the overlying mucus is an highly effective barrier against infection. Ciliated cells are found in patches in the respiratory epithelium from the nose to just above the level of the alveolar sacs (25). The ciliated cells in each patch beat at 15 to 20 cycles per second in a coordinated fashion (29). The motion occurs in a rapid forward phase followed by a recovery stroke in which the cilia bent slowly to the starting position.

The respiratory mucus is composed of a network of high molecular weight glycoproteins and one of its main functions is to constitute a barrier protecting the epithelial cells against invasion and injury by microorganisms and toxic agents (30). The superficial part of the mucus layer (the 'gel') is viscous and is transported by the ciliary action. The deeper, or periciliary, part (the 'sol') is not transported. When the 'sol' layer is too thick (e.g. during viral infection), the cilia do not reach the 'gel' layer and efficient transport will not be achieved. Furthermore, the respiratory mucus contains several proteins, such as immunoglobulins, especially sIgA (31), lactoferrin and lysozyme (32) which possess bacteriostatic and bactericidal properties. Microorganisms which enter the upper airways are predominantly eliminated by the mucociliary transport, which is the fundamental function of the mucociliary system (33). For adequate transport, well functioning cilia and a proper mucus layer are neccessary. Clearance is achieved by the sequential coordinated action of cilia resulting in movement of mucus, containing adherent materials, towards the pharynx. Movement is directed posteriorly from the nasal airway and upward from the lung (34).

Failure of this mucociliary clearance often results in chronic infection which is seen for example in primary ciliary dyskinesia (35) a disorder with abnormal ciliary ultrastructure and abnormal ciliary activity (36) and cystic fibrosis, a multiorgan disorder resulting in abnormal thickening of exocrine secretions including the mucus of the respiratory tract (37-40).

If there are any defects in these mechanical barriers, the respiratory mucosa can be injured by microorganisms and subsequently an inflammatory reaction will follow. During this inflammatory reaction, inflammatory cells enter the tissues via the postcapillary venules and a non-specific or innate immune response and a specific or adaptive immune response can occur (table 1). Both responses work together in close harmony. The non-specific immune response will be effective first but is not improved by repeated contact with any particular pathogen. In contrast, the specific immune system can specifically recognize a pathogen after a single infection and the specific immune response will be enhanced after reinfection with the same pathogen.

	humoral	cellular
mechanical barriers	mucus	ciliated respiratory epithelium
non-specific immunity	lactoferrin lysozyme complement	phagocytic cells - neutrophils - monocytes - macrophages eosinophils
specific immunity	immunoglobulins	antigen presenting cells T and B lymphocytes

Table 1: Defense mechanisms of the respiratory tract.

Inflammatory reaction and the non-specific immune response

Defects in the epithelial lining of the respiratory tract can be caused by physical or chemical agents and by microorganisms as bacteria, viruses, fungi or protozoa. The majority of acute infections of the upper airways is viral in origin (41). Viral respiratory pathogens may significantly alter local and/or systemic physical and immune defence mechanisms, paving the way for secondary bacterial invasion (42). Commensal bacteria, such as streptococci, staphylococci, and Haemophilus influenzae in particular are considered to act as pathogens (43,44).

The inflammatory reaction is a local response to tissue injury caused by a variety of noxious stimuli. The principal function of the inflammatory reaction is elimination of the noxious stimuli, thus limiting the spread of the local lesion and restoration of the tissue integrity. During the inflammatory reaction vasoactive and chemotactic factors are produced leading to hyperaemia, increased vascular permeability and infiltration of inflammatory cells. After 24 hours granulocytes enter the site of inflammation followed later by macrophages and lymphocytes. When the infecting microorganisms are quickly neutralized, the inflammatory response is called acute. The characteristic infiltrating celltype is the neutrophilic granulocyte. When the cause of the inflammation is not neutralized, then the inflammatory response is called chronic and the main infiltrating cell types are lymphocytes, macrophages, plasma cells, eosinophils and mast cells.

Phagocytic cells, including neutrophils and monocytes/macrophages, contain hydrolytic and proteolytic enzymes and generate reactive oxygen species, all designed to eliminate and digest invading organisms and cell debris, thus limiting tissue damage. However, the release of lysosomal enzymes and reactive oxidants may also contribute to cell injury and tissue damage (45). Neutrophils also have the potential to release products as prostaglandins, thromboxanes, leukotriene B_4 and platelet activating factor which may contribute to the result of the inflammatory response. Airway macrophages, like other tissue macrophages, arise from circulating monocytes which mature into macrophages during their presence within the airway system (46-48). Macrophages become activated by locally produced cytokines (a.o. IFN-τ). In contrast to non-activated macrophages, activated macrophages have much stronger cytotoxic activity against microorganisms by the secretion of oxygen radicals, nitrogen oxides and proteinases. Furthermore, macrophages have the capability to synthesize and release inflammatory products as thromboxane A_2, platelet activating factor (PAF), leukotriene B_4, interleukins (IL-1, IL-6), tumor necrosis factor (TNF-α), and granulocyte monocyte colony stimulating factor (GM-CSF) for regulating the inflammatory response (49-53).
Eosinophilic granulocytes have cytoplasmic granules and are attracted to the site of inflammation by mediators released by mast cells, eosinophils, macrophages and T lymphocytes (54-56). Activation of eosinophils will result in the production and release of inflammatory products like platelet activating factor, leukotriene C_4, and oxygen radicals (57). Eosinophils are thought to play a role in allergic disease (58,59) and in the development of nasal polyps (60-62).

Specific immune response

In general, four types of hypersensitivity reactions can occur by exposure to antigen (63). These reactions can be divided into *humoral* and *cellular* reactions. Humoral immunity includes the production of antibodies by B cells, reactive against a given antigen. The type of reactions (type I-III) which depends on interactions of antigen with antibody are usually called immediate or intermediate reactions. Type I, immediate hypersensitivity, is mediated by IgE and plays a role in the pathogenesis of allergic rhinopathy and atopic asthma. Type II hypersensitivity refers to cytotoxic antibodies (autoantibodies) produced by the host to his own tissues (e.g. Goodpasture's syndrome, haemolytic anaemias). In type III hypersensitivity reactions, immune complexes are involved which cause tissue damage (Arthus reaction). This kind of reaction is involved in some types of glomerulonefritis and vasculitis.
Cellular immunity, or type IV hypersensitivity, is a T cell dependent immune reaction. Two types of reactions mediated by different T cell subsets are involved in type IV hypersensitivity: 1. delayed type hypersensitivity (DTH) initiated by $CD4^+$ T cells and 2. cellular cytotoxicity mediated by $CD8^+$ T cells.

Type IV reactions are initiated by the exposure of sensitized T cells to specific antigens complexed to MHC molecules. In DTH, MHC class II molecules are involved, in cellular cytotoxicity MHC class I molecules. In DTH $CD4^+$ T cells secrete cytokines, leading to the recruitment of other cells especially macrophages which are the major effector cells. In cell mediated cytotoxicity, cytotoxic $CD8^+$ T cells exert the effector function.

In specific mucosal immunity, secretory IgA (sIgA) plays an important role in protecting mucosal surfaces against viruses and bacteria by blocking specific antibody sites on microbial cell walls, thus preventing adherence to the epithelium. sIgA is a dimer linked by a J-chain produced by mucosal B cells and provided with a secretory piece which is secreted by epithelial cells. In chronic inflammatory reactions of the upper upper airway mucosa, cellular immune reactivity is of importance. This reactivity comprises cellular cytotoxicity and DTH reactions. The cells involved in cellular immunity will be further discussed with special attention to the dendritic cell as antigen presenting cell.

Cellular aspects of delayed type hypersensitivity

1. Dendritic cells

In the initiation of T cell dependent immune responses, dendritic cells (DC) play an important role. They are antigen presenting cells par excellence and stimulate T lymphocytes (64-67). To the dendritic cell series belong: Langerhans cell (LC) of the skin, the veiled cell in afferent lymph, the interdigitating cell of the T cell areas of lymphoid organs and the thymic medulla, the lymphoid DC from lymphoid organs and the DC isolated from the peripheral blood. DC exhibit distinct characteristics like a typical dendritic morphology and the presence of acid phosphatase in a juxtanuclear spot in the cytoplasm (68). Furthermore, DC express several molecules on their surface which are important for efficient antigen presentation, i.e. adhesion receptors and MHC class I and class II molecules. Analysis of the expression of adhesion receptors on the cell surface of blood DC revealed that these cells express lymphocyte function antigen (LFA)-1 ($CD11_a$/CD18), ICAM-1 (CD54), LFA-3 (CD58) and CD44 (69). These cell adhesion receptors play a critical role in the initial, antigen-independent, phase of T cell adhesion to DC (67,70). The interactions are predominantly mediated through binding of the integrin LFA-1 with the Ig superfamily member ICAM-1 (69,71). Additional binding via the LFA3/CD2 pathway has been described for the tonsillar system (71).

If antigen-independent cell binding is strong enough, antigen-dependent adhesion will occur via the interaction of MHC molecules and antigenic peptides and the T cell recptor (TcR). DC abundantly express MHC class I and class II molecules. These molecules form with processed antigenic peptides a

complex on the surface of the DC which is recognized by the TcR on T cells, either CD8 on the cytotoxic T cell (MHC class I mediated response) leading to cellular cytotoxicity or CD4 on the T-helper-cell (MHC class II mediated response) leading to DTH-reaction. Subsequently, T cell activation will follow.
In contrast to macrophages, which are also efficient antigen presenting cells (72,73), DC do not produce interleukin-1 (IL-1) (74,75), although membrane associated IL-1-like activity involved in the activation of T cells has been mentioned (76). Furthermore, DC have slight phagocytic capacity.

2. T lymphocytes

T lymphocytes are found in the normal (para)nasal mucosa (77). In chronic sinusitis, infiltration of lymphocytes in the mucosa is one of the most characteristics findings (78). Functionally, T lymphocytes play an important role in the regulation of the immune response. They can be divided into two major functional subgroups on the basis of expression of the phenotype markers CD4 (helper/inducer) or CD8 (suppressor/cytotoxic). $CD8^+$ cells are primarily concerned with the recognition and killing of virus-infected cells (79-81). They recognize antigen in combination with MHC class I molecules on the infected cells.
$CD4^+$ cells have different functions in controlling the immune response and they are able to recognize antigen in combination with MHC class II molecules on the surface of antigen presenting cells. After recognition of the antigen/MHC complex they can release protein mediators called cytokines. These cytokines can regulate the differentiation, recruitment, accumulation and activation of specific effector cells (neutrophils, macrophages, eosinophils) at mucosal surfaces. If the T cell recognizes antigen/MHC on a B cell, they can release cytokines which activate the B cells to divide and differentiate. In mice, a functional dichotomy of activated $CD4^+$ cells can be made with regard to their respective patterns of cytokine synthesis (82). Th1 cells secrete interleukin-2 (IL-2), interferon-τ (IFN-τ) and tumour necrosis factor-ß (TNF-ß), but not IL-4, 5 and 6. Th2 cells secrete IL-4, 5, 6 and 10 and TGF-ß, but not IL-2, IFN-τ and TNF-ß. Other cytokines including IL-3 and granulocyte macrophage-colony stimulating factor (GM-CSF) are secreted by both cell types. Th1 cells mediate delayed type hypersensitivity (DTH) reactions and suppress IgE synthesis (83) whereas Th2 cells mediate antibody response and allergic inflammation by the secretion of IL-3,4 and 5 which favor IgE synthesis and activation of mast cells and eosinophils (84-87). In man, this dichotomy is less distinct than in mice. In common with macrophages, lymphocytes are sensitive to the inhibitory effects of glucocorticosteroids, which predominantly inhibit the cytokine release (88).

V. Immunosuppression

Patients who suffer from immunodeficiencies are characterized by: (1) recurrent infections of multiple organ systems including the respiratory tract (89); (2) an abnormal high infection rate and abnormal long duration of the infections; (3) infections caused by microorganisms which are considered to be low pathogenic.

Suppression of the host immune function can be caused by a variety of mechanisms. These mechanisms can be divided into (I) congenital (primary) immunodeficiencies and (II) acquired (secondary) immunodeficiencies.

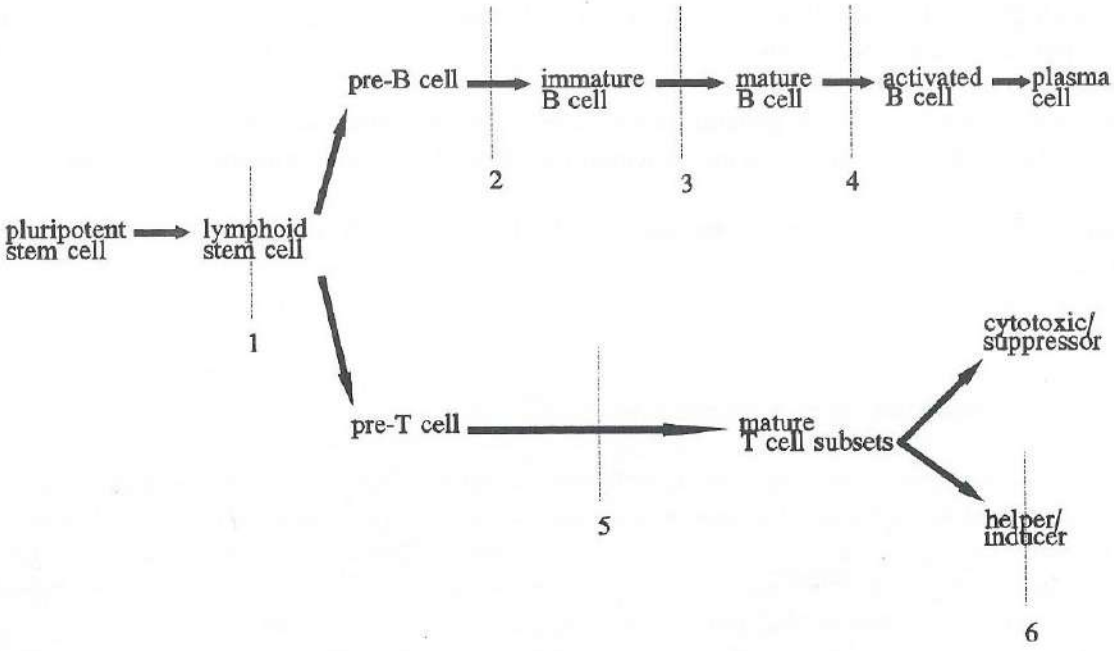

Figure 4: Relationship between primary immunodeficiencies and lymphocyte development. Numbers indicate cells or steps affected in various immunodeficiency states. 1: severe combined immunodeficiency; 2: Bruton's agammaglobulinaemia; 3: isolated IgA deficiency, affects only immature IgA positive B cells; 4: one form of common variable immunodeficiency; 5: Digeorge's syndrome; 6: AIDS.

1. Primary immunodeficiencies

Primary immunodeficiency syndromes can be divided into those affecting specific immunity (B cell immunodeficiencies, T cell immunodeficiencies, combined immunodeficiencies) (see figure 4) and those affecting the non-specific immune system (phagocyte defects, complement defects) (see also table 1).

1.1 Primary immunodeficiencies affecting lymphocyte development

B cell immunodeficiencies can vary from agammaglobulinaemie to selective Ig-deficiencies and therefore show a wide variety of clinical manifestations. Some patients have recurrent bacterial airway infections, sepsis or meningitis, whereas others have no increased frequency of infection rate. The most common primary immunodeficiency syndrome is a selective IgA-deficiency with failure to secrete IgA (90). Infections are mainly those of the upper respiratory tract.
T cell immunodeficiencies are in general more severe and patients are subject to viral and unusual infections such as pneumocystic carinii pneumonia. Partial T cell immunodeficiencies have been described (12).
Immunodeficiencies which interfere completely with T and B cell development result in a severe combined immunodeficiency syndrome (SCID) without any useful antibody response. These patients have the worst prognosis and treatment consists of HLA-matched bone marrow transplantation (91).

1.2. Primary immunodeficiencies affecting non-specific immunity

Phagocyte function defects can range from defective cellular adhesion properties (leucocyte adhesion deficiency syndrome) to impaired chemotaxis (lazy leucocyte syndrome) and deficient intracellular killing (Chediak-Higashi syndrome, chronic granulomatous disease). These syndromes are characterized by recurrent or persistent infections with microorganisms such as Staphylococcus aureus, Haemophilus influenzae, Streptococcus pneumoniae and opportunists such as aspergillus (89,92).
The complement pathway is a group of circulating proteins which are necessary for antigen recognition and antibody formation. Congenital deficiencies of one of the complement proteins are rare and increased susceptibility to infection results mainly from C3 deficiency. Furthermore, deficiencies can lead to collagen-vascular disease such as Systemic Lupus Erythematosus (SLE).

2. Secondary immunodeficiencies

Immunodeficiencies may be acquired, secondary to various disease processes or drug effects. Acquired immunosuppression can be mediated by the generation of (1) antigen-specific suppressor T lymphocytes or by (2) antigen non-specific mechanisms.

2.1. Antigen-specific immunosuppression

Suppression of the immune system can be mediated by the generation of antigen-specific suppressor T cells. This has been well documented for experimental immunological models (93) as for clinical melanoma (94,95). These cells were shown to be CD 8^+ T cells.
An additional antigen-specific suppressor pathway is the antigen specific stimulation of Th2 cells. Stimulated Th2 cells produce lymphokines which can inhibit Th1 cell function. Among these lymphokines, IL-10 and transforming growth factor (TGF-ß) may be important. TGF-ß inhibits T cell proliferation and differentiation, and cytotoxic T cell function (96). IL-10 has suppressive activity on the synthesis of effector cytokines released from Th1 cells (IFN-τ, IL-2) and on various effector T cell functions, including DTH (97). In this mechanism, the induction of suppression is antigen-specific but the suppression itself is antigen-independent.

2.2. Non-specific immunosuppression

Protein deficiency, due to malnutrition, causes defects in cell-mediated immunity and is the commonest cause of secondary immunodeficiencies worldwide. Similar defects can be seen in cachexic state of disseminated cancer.
(retro)Viral infection can result in depression of immunological function. Examples are infections with Epstein-Barr virus resulting in infectious mononucleosis, HIV-infections resulting in AIDS or AIDS-related complex with the depletion of $CD4^+$ lymphocytes and infections with Herpes simplex type I.
Iatrogenic causes of immunodeficiencies include therapy with corticosteroids or other immunosuppressive and cytotoxic drugs and radiotherapy. Corticosteroids, which are frequently used in the treatment of chronic airway disease (98), have inhibitory activity on the production of IL-2 and IFN-τ by T-cells (99), diminish the chemotaxis and adherence of eosinophils (100) and inhibit the proliferation of mast cells (99)
Non-specific soluble factors. Several soluble factors are known to mediate suppression of the immune

functions in an antigen non-specific way. Most of these factors are associated with tumor growth (101-103). These factors include prostaglandin E2 (104), transforming growth factor-ß (105), gangliosides and lipoprotein antigens (106) and retroviral products like p15E (107). Several immune functions can be inhibited by these factors: TGF-ß and gangliosides suppress IL-2 dependent proliferation; prostaglandin E2 suppresses NK cell activity and leucocyte chemotaxis; p15E suppresses monocyte chemotaxis. Since retroviral p15E-like proteins are related to the presence of defects in cell mediated immunity, also seen in patients with chronic upper airway infections, this will be discussed in detail.

Immunosuppression by retroviral p15E-like proteins

Retroviral infections are often accompanied by suppression of the immune system (108). This has been well documented in human HIV-infection (109,110) as well as in leukemias caused by murine and feline leukemia viruses (108,111-113). Immunosuppressive proteins produced by retroviruses play a role in this immunosuppression (114,115). Mathes and coworkers found that the purified feline leukemia virus (FeLV) hydrophobic transmembrane envelope protein p15E has antiproliferative activity on lymphocytes (116,117). p15E is encoded by the *env*-gene of murine (MuLV) and feline (FeLV) leukemia viruses (118).
It has now been demonstrated that p15E inhibits lymphocyte proliferation (112,116,119) macrophage accumulation (120,121), IL-2 secretion (113,119,122) and monocyte chemotaxis (123).
Immunosuppressive proteins isolated from human (123) and murine tumors (107), human malignant pleural effusions (123), and serum from patients with head and neck cancer (124), chronic purulent rhinosinusitis (13) and autoimmune disorders (125) have been shown to have antigenic similarities with p15E, and have inhibitory activity on IL-2 production, lymphocyte proliferation and chemotaxis of mononuclear phagocytes. Furthermore, expression of p15E has been found on non-virus-induced tumor cells (126-128) as well as on activated normal peripheral blood lymphocytes (126). These observations suggest that p15E-like proteins are not exclusively expressed in tumors but can also be found in other tissue and might even, under certain circumstances, be expressed in normal tissues.
In mice, endogenous MuLV-p15E genes have been demonstrated (129). Down-regulation of these genes leads to up-regulation of immune-effector cell activity (129). Furthermore, the endogenous p15E sequences are similar to those in infectious MuLV. Both in mouse and man tissue-specific and age-dependent expression of retroviral transcripts have been noted (130,131). Since env-genes that share homology with retroviral p15E have been found in human DNA (132,133) and the presence of mRNA derived from these genes has been detected in most normal tissues and tumor cell lines (134) an endogenous origin of the p15E-like proteins is likely. Moreover, endogenous retroviral sequences reside

in the genome of all vertebrate species thus far examined, including humans (135). The majority of these sequences are structurally defective and are not correlated with a manifest retroviral infection. However, activation of these sequences, subsequently followed by production of proteins as p15E, might initiate cell dysfunction.

VI. Clinical tests for the assessment of the immune response

The immune response can be divided into three stages:
1. *The recognition phase* in which antigen is processed by antigen presenting cells and subsequently presented to T cells. Most B cells will not respond directly upon recognition of their antigen and an additional T cell signal is required.
2. *The proliferation and production phase.* After antigen- presentation T cells will undergo clonal expansion, while B cells will expand and mature to antibody-secreting plasma cells.
3. *The effector phase.* Antigen-specific effector T cells and antibodies are involved in the process to eliminate antigen. T cells regulate the B cell response, are cytotoxic, or secrete lymphokines (e.g. IL-2, IFN-τ, GM-CSF) which trigger inflammatory reactions by attracting phagocytes (monocytes/macropgahes, neutrophils) and lymphocytes to the site of reaction.
In clinical practice, tests are available which evaluate cell function in each of the three stages of the immune response.
The *quantification* of cell populations (lymphocytes, neutrophils, monocytes) can easily be calculated from the total and differential white cell counts. Lymphocyte subpopulations express different cell markers and monoclonal antibodies are used to identify these subsets. After incubation with specific immunoglobulin (antibody), followed by fluorescein-conjugated second antibody to immunoglobulin, cells can be counted with a fluorescein activated cell sorter (FACS) or fluorescent microscope.

ad 1. The recognition phase:
Dendritic cells initiate primary T cell responses. The induction of these responses occur in cell aggregates or clusters. The capability of dendritic cells to form clusters can be measured in the cluster assay. Furthermore, the capacity of antigen-presenting cells to induce T cell proliferation can be measured in the mixed leukocyte reaction (MLR). In this test, allogeneic lymphoid cells can be used and stimulation is MHC class II dependent. The numbers of formed clusters correlate with the capability of dendritic cells to induce lymphocyte proliferation in the MLR.

ad 2. The proliferation and production phase:
The proliferative response of specific lymphocytes after exposure to their corresponding antigen or mitogen can be measured by radioactive thymidine incorporation into DNA.
The lymphokine production of T cells after exposure to antigen can be assessed by using monoclonal antibodies directed against these lymphokines. Furthermore, the production of the macrophage migration inhibition factor (MIF) can be evaluated by the microdroplet agarose assay in which the inhibition of movement of human U937 cells is measured.

ad 3. The effector phase:
The production of lymphokines by stimulated T cell attract phagocytes (chemotaxis). Chemotaxis is the directional movement of cell towards a chemoattractant (e.g. fMLP). It can be measured by the polarization assay (123). The polarization of monocytes towards chemoattractants is an early event that precedes the chemotactic response. The polarization assay is a rapid method to measure chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (136).
Phagocytosis is the ingestion of antigenic particles. This ingestion can be measured by incubation of the phagocytes with bacteria or latex beads. The intracellular enzyme activity can be measured by intracellular bacterial killing.

An assessment of the three stages of the immune response together can be measured by the in vivo *delayed type hypersensitivity skin tests:*
When an antigen is recognized after intradermal injection, swelling and redness of the skin will appear after 48-72 hour, and then fades away after several days. This delayed type hypersensitivity reaction is a T cell dependent phenomenon in which specific T cells produce lymphokines upon exposure to antigen for which the individual is already sensitized. These tests are frequently used for screening of tuberculosis (mantoux tests).
Delayed type hypersensitivity can also be measured by performing patch tests in which antigen is absorbed through the skin. These tests are essential in determining which antigen is responsible for contact dermatitis. Skin testing assumes that the patients have been previously sensitized to the antigen (e.g. candidal antigen, streptococcal antigen).

In patients with chronic purulent rhinosinusitis, normal lymphocytes counts and subsets ratio's have been demostrated. Furthermore, the mitogen-induced, as well as the antigen-induced lymphocyte proliferation was not defective in these patients when compared to the values found in healthy controls (13). For that

reason, an analysis of lymphocytes-subsets and lymphocyte proliferation was not performed in this study. Cell mediated immunity was evaluated by the dendritic cell cluster assay, the monocyte polarization and DTH skin tests upon commensal antigens.

VII. Aim of the study

Several factors, such as anatomic abnormalities, defects in mucociliary clearance, modifications of nasal secretions, defects in humoral and cellular immunity, may contribute to the pathogenesis of chronic upper airway infections. In patients with chronic purulent rhinosinusitis, defects in cellular immunity, i.e. diminished delayed type hypersensitivity skin reactions upon commensal antigens, defective production of macrophage migration inhibition factor (MIF) and decreased monocyte chemotaxis (12,13) have been shown.
This thesis focusses on the role of p15E-like proteins and their related defects in cell mediated immunity in the pathogenesis of chronic upper airway infections.

The presence of retroviral p15E-like serum proteins (13,137) is related to these defects in cellular immunity. Furthermore, it has been demonstrated that p15E-like proteins can also be found in patients with head and neck cancer (123,124) and that these proteins are expressed in several tumor cell lines and activated lymphocytes (126). An endogenous origin of p15E-like proteins is suggested since sequences have been found in human DNA that share homology with p15E (132,133). However, the role of p15E-like proteins in the regulation of the immune response is still unclear. We, therefore, analyzed the expression of p15E-like proteins in normal, malignant, and inflamed human tissues. The expression has been correlated with the presence of p15E-like serum proteins in patients with head and neck cancer and chronic upper airway infections ***(Chapter 2)***.

Defects of cellular immunity, present in chronic purulent rhinosinusitis, are related to retroviral p15E-like proteins. However, it is unknown if these defects and p15E-like proteins are specific for chronic purulent rhinosinusitis, or that they can also be found in other patients characterized by chronic upper airway infections? Moreover, it is unknown whether the respiratory tract infections are primarely caused by the, already present, immunosuppressive proteins or that these proteins are produced during these infections? Therefore, we analyzed several parameters of the cellular immune response viz., delayed type hypersensitivity skin test upon commensal bacterial antigen and the chemotactic responsiveness of monocytes, in three different patient groups, all characterized by chronic recurrent upper airway

infections of different aetiology and pathogenesis. The data concerning the parameters of cellular immunity were related to the presence of immunosuppressive p15E-like proteins in patient's serum ***(Chapter 3)***.

In chronic upper airway infections, defects in the induction stage of the immune response are not reported yet. Hence, we studied whether defects could also be found at the induction stage of the immune response. Dendritic cells play an important role in the induction of the immune response and they initiate T cell responses which occur in cell clusters of DC and T cells. The molecular basis of blood DC-T cell clustering is described in ***Chapter 4***, showing the LFA-1/ICAM-1 as the major pathway. The analysis of the cluster capability of blood DC in patients with chronic upper airway infections is described in ***Chapter 5***. The results of the DC cluster capability were related to the presence of retroviral p15E-like serum proteins in these patients.

Since it has been shown that the expression of p15E-like proteins is predominantly limited to the mucosa of the upper airways and is significantly higher in patients with chronic upper airway infections (chapter 2) it has been suggested that the inflamed (para)nasal mucosa is the production site of p15E-like proteins in chronic upper airway infections. Thus, treatment of chronic upper airway infections (endoscopic removal of the inflamed mucosa and postoperatively application of topical corticosteroids) should decrease the presence and bioactivity of retroviral p15E-like proteins in the serum of these patients and subsequentially restores the cellular immune defects. In ***Chapter 6*** we describe the improvement of the dendritic cell cluster capability and monocyte chemotaxis after treatment (endoscopic sinus surgery followed by postoperatively application of topical steroids) in patients with chronic upper airway infections. This improvement is related to the decrease of inhibitory activity of p15E-like serum proteins. The results support the concept that the inflamed (para)nasal mucosa is the production site of retroviral p15E-like proteins in chronic upper airway infections.

Chapter 2

Distribution of retroviral p15E-related proteins in neoplastic and non-neoplastic human tissues, and their role in the regulation of the immune response

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Summary

In patients with head and neck carcinomas and in patients with chronic purulent upper airway infections low molecular weight, retroviral p15E-like, factors are found. These factors are responsible for partial defects in the cellular immune response. We studied the distribution of these p15E-related proteins in neoplastic, inflamed and normal human tissues and related these findings with the presence of p15E-like factors in patients' sera. Demonstration of p15E-like proteins in sera of patients with upper airway infections and of patients with head and neck carcinomas correlated exclusively with the presence of p15E in normal and pathologic epithelium of the upper respiratory tract. p15E was not demonstrated in epithelia of other localisations. Our results suggest that chronic stimulation or neoplastic transformation of the epithelia of the upper respiratory tract stimulates the production of p15E-like proteins leading to their reported immunosuppressive actions.

Introduction

Retroviral infection can result in suppression of the immune response. This has been well documented in leukemias caused by murine and feline leukemia viruses (1-4). The retroviral transmembrane envelope protein p15E plays an important role in this phenomenon (5). Immunosuppressive proteins isolated from human (6) and murine tumors (5), human malignant pleural effusions (6) and serum from patients with head and neck carcinomas (7) and patients with chronic purulent rhinosinusitis (8) have been shown to have antigenic similarities with p15E, and exert an inhibitory effect on IL-2 production, lymphocyte proliferation and chemotaxis of mononuclear phagocytes. The in vitro chemotactic responsiveness of monocytes has been shown to be very sensitive to these p15E-like proteins (6-8).
Since both in mouse and man tissue-specific and age-dependent expression of retroviral transcripts have been noted (9,10) and sequences have been found in human DNA (11,12) that share homology with p15E, an endogenous origin of p15E-like proteins is suggested. However, a detailed analysis of the distribution of p15E-like proteins in human tissues has never been reported. Furthermore, the role of these proteins in the regulation of the normal immune response still remains unclear. We, therefore, studied the expression of p15E-like proteins in normal, malignant and inflamed human tissues. In addition, we related this expression to p15E-mediated immunosuppressive activity in serum.

Material and methods

Tissues

Neoplastic and non-neoplastic tissues were obtained from surgical procedures. Tissues also were derived from autopsies performed within six hours after death. Tissues were snap frozen and stored in liquid nitrogen.

Sera

Sera were obtained from the following patients groups:
a. Healthy controls (n=25, 13 female, ages ranging from 22-40 years, mean 29 years) without known diseases.
b. Patients with carcinoma of the cervix uteri (n=10, ages ranging from 32-75 years, mean 57 years; clinico-pathological stage ranging from I^A-IV^A).

c. Patients with Morbus Crohn, based on clinico-pathological, radiological and endoscopical criteria as previously described (13) (n=10, 7 females, 3 males, ages ranging from 22-46 years, mean 32 years). d. Patients with Primary Ciliary Dyskinesia (PCD), based on abnormal ciliary motility (14) and abnormal ciliary ultrastructure (15) (n=22, 8 females, 14 males, ages ranging from 2-58 years, mean 23 years).
e. Patients with Polyposis Nasi (PPN) i.e. recurrent upper airway infections and the presence of nasal polyps at the moment of surgery or endoscopic examination (n=28, 16 females, 12 males, ages ranging from 18-66 years, mean 42 years).
f. Patients with Chronic Purulent Rhinosinusitis (CPR) i.e. recurrent upper airway infections not responding to adequate antibiotic treatment or surgical procedures, and without the presence of nasal polyps at the time of surgery or endoscopic examination (n=41, 27 females, 14 males, ages ranging from 7-71 years, mean 34 years).
No patient with CPR or PPN had abnormal ciliary motility or abnormal ciliary ultrastructure. Furthermore, the leucocyte counts were within normal range and no defects in humoral immunity were present.
h. Patients with head and neck squamous cell carcinoma (H/N CA),(n=10, 3 females, 7 males, age ranging from 55-81 years, mean 65 years; TNM classification ranging from T2N0-T3N3).

Monoclonal antibodies

The mAb used were: 4F5(IgG2a)(16) and 19F8(IgG2b)(17) both specific for p15E but recognizing different epitopes (16); Control mAb were an IgG2a and IgG2b, secreted by the mouse myeloma P1.17 and MPC11.OUA cell lines respectively, both obtained from the American Type Culture Collection (ATCC, Rockville, Maryland).

Immunoperoxidase staining

Four micrometer thick sections of frozen tissue blocks were prepared with a cryostat (Jung-Reichert, Nussloch, FRG), mounted on poly-L-lysine coated glass slides, air dried, and acetone-fixed during ten minutes at room temperature. The sections were incubated for 60 min at room temperature with mAb, washed, and incubated for 30 min at room temperature with horseradish peroxidase conjugated Rabbit-anti-mouse IgG (Dakopatts, Denmark) in the presence of 5% normal human serum. Subsequently, the sections were weakly counterstained with hematoxylin.
Tissue reactivity was scored for the percentage of positive staining cells and the intensity of staining (-=negative, ±=very weak, +=weak, ++=strong). In the negatively scored tissue samples, there was

no reactivity present with the anti-p15E mAbs or there were no differences in reactivity between the anti-p15E mAbs and the respective control mAbs.

Monocyte isolation from healthy donors

From four different healthy donors monocytes were isolated from the peripheral blood. Buffy coats from 500 ml of human blood were obtained after informed consent from healthy donors. The monocytes were purified by successive isopycnic centrifugation and elutriator centrifugation as previously described [18] with minor modifications (19). These healthy monocytes were used to test the effects of patients' serum fractions.

The determination in patient serum of low molecular weight factors inhibiting the polarization of healthy donor monocytes

Sera were collected from patients by venapuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 minutes at 700 g (molecular weight "cut off point" 25 kD). The residues, the low molecular weight factors (LMWF), were dissolved in phosphate buffered saline (PBS) and stored at -70 °C until further use.
The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes ($1X10^6$/ml) for 15 minutes at 37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

$$\text{inhibition} = \left(1 - \frac{P_2 - P_0}{P_1 - P_0} \right) X\ 100\,\%$$

P_0= % spontaneously polarization
P_1= % polarization after incubation with fMLP alone
P_2= % polarization after incubation with fMLP and LMWF

Serum fractions were tested in triplicate. Addition of serum fractions to non-stimulated (fMLP) donor monocytes did not affect the spontaneous polarization.

Determination of the p15E-like character of patient LMWF's

To validate the p15E-like character of the LMWF's in human serum ,adsorption experiments were carried out by neutralizing the serum fractions before testing in the monocyte polarization assay with a p15E specific monoclonal antibody (19F8) in a final dilution of 1:200 (25 μg/ml) at 4°C for 16h, followed by Amicon ultrafiltration to remove formed immunecomplexes: This adsorption /neutralizing procedure was carried out twice (7). Adsorption experiments carried out with the mAb 4F5 or with 4F5 and 19F8 together did not show any difference in neutralizing the serum fractions as with 19F8 alone. Control experiments were carried out with the isotype matched control mAbs.
Statistical analysis was performed by the Student's t-test.

RESULTS

Reactivity of anti-p15E mAb with normal human tissues

In this study, immunohistochemical staining was performed on frozen sections. Among the normal human tissues tested, the respiratory epithelium cells of nasal mucosa reacted with the anti-p15E mAb. Furthermore, staining was seen in the squamous epithelium surrounding the tonsils at places where histologically keratinisation could easily observed. There was no difference of staining pattern between

Organ		Positive/tested
nasal mucosa:	concha inferior	10/12[1]
	concha media	6/12[1]
lung		0/2
bronchus		0/2
muscle		0/2
skin		0/2
brain		0/2
kidney		0/2
liver		0/2
heart		0/2
stomach		0/2
bladder		0/2
gall bladder		0/2
lymph node		0/5
thymus		0/5
spleen		0/6
adenoid		0/2
tonsil		2/2[2]
esophagus		0/2
vagina		0/1
larynx		0/4
colon		0/2
cervix		0/2
placenta		0/2
thyroid		0/2
adrenal gland		0/2
parotid gland		0/3
pancreas		0/2
ovary		0/2
testes		0/2

Table 1: Frozen tissue reactivity of mAbs directed against p15E with normal human tissue specimens.
In negative scored tissue not a single cell was labeled by 4F5/19F8 or there was no significant difference with the control mAb.
[1] Positive staining in basal cell layer of respiratory epithelium. More than 75% of the cells showed a stainig intensity from +/++.
[2] Positive staining (+/++) in squamous epithelium surrounding the tonsils especially at places were keratinisation could easily be observed.

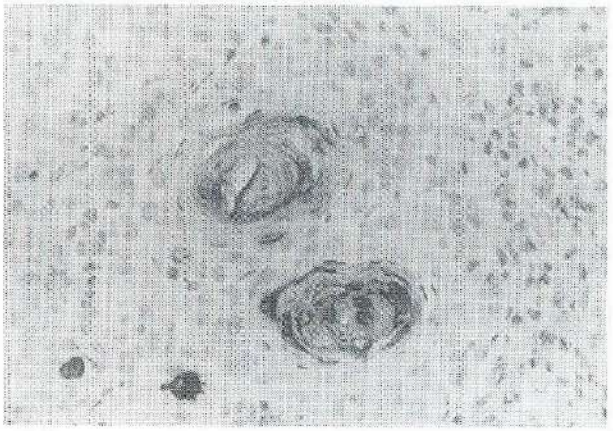

Figure 1[a]: Reactivity with the anti-p15E mAb 4F5 within the well differentiated areas of squamous cell carcinoma of the head and neck (magnification X 400).

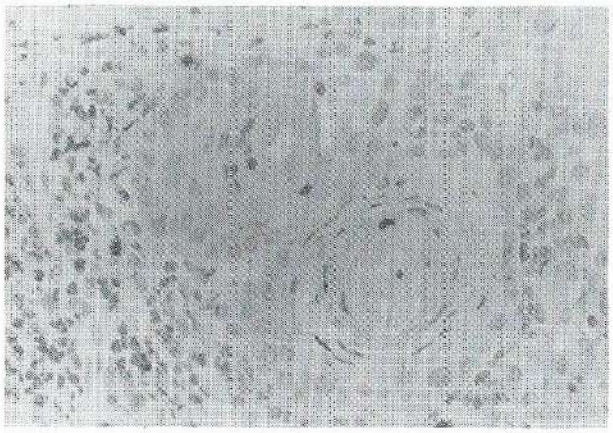

Figure 1[b]: No binding detectable with the control mAb in the same area of squamous cell carcinoma of the head and neck (magnification X 400).

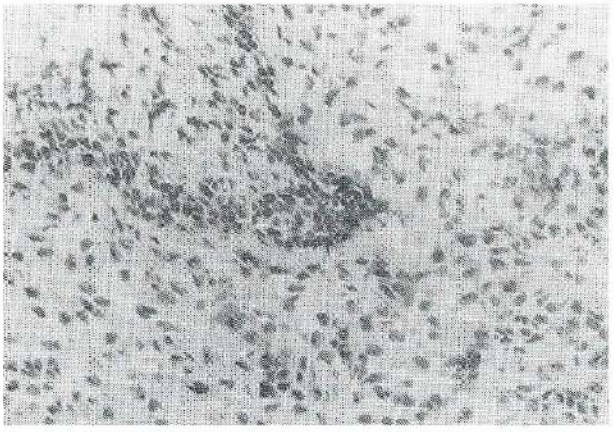

Figure 2[a]: Reactivity with the anti-p15E mAb 4F5 in the basal cell layer of the epithelium of nasal polyps (magnification X 400).

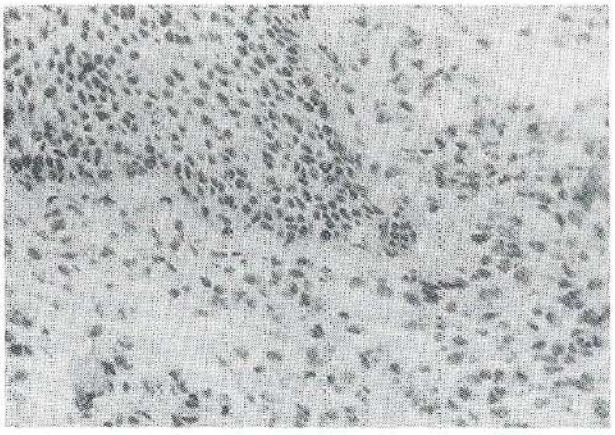

Figure 2[b]: The epithelium of nasal polyps shows no binding with the control mAb (magnification X 400).

the anti-p15E mAb 4F5 and 19F8, although the reactivity with the 4F5 mAb was more intense. No reactivity was found with epithelium from skin, digestive tract or urogenital tract, nor in any other tissue tested, including immunocompetent cells in the lymphoid organs (table 1).

Neoplastic tissue		Positive/tested
squamous cell carcinoma		
	head and neck	13/13[1]
	cervix	0/2
	lung	0/2
	esophagus	0/2
adenocarcinoma		
	colon	0/4
	breast	0/6
	ovary	0/5
	lung	0/1
small cell carcinoma		
	lung	0/1
melanoma		0/4
Hodgkin lymphoma		0/2
non-Hodgkin lymphoma		0/2

Table 2: Frozen tissue reactivity of mAbs directed against p15E with human neoplastic tissues.
[1] Positive staining (+/++) focally in well differentiated areas of the tumor.

Reactivity of anti p15E mAb with human neoplasms and tissue with chronic inflammation

Of all human neoplastic tissues tested, only well differentiated areas of squamous cell carcinoma of the head and neck showed reactivity with the anti-p15E mAb (table 2, figure 1). Again, no difference in binding pattern between 4F5 and 19F8 was detected. In contrast with normal human nasal mucosa, more binding of the anti-p15E mAb was seen in squamous cell carcinoma from the head and neck region. Squamous cell carcinoma from other regions, other type of carcinoma including adenocarcinoma, and small cell carcinoma, melanomas, Hodgkin lymphoma and non-Hodgkin lymphoma did not show any positive staining.
Reactivity of both anti-p15E mAbs was seen with respiratory and squamous epithelium in inflammatory nasal polyps (figure 2) and purulent bronchitis and with epithelium covering inflammation of the middle ear (table 3). Again, more intense staining of the anti-p15E mAb was seen with the chronically inflamed mucosa of the upper airways than with normal human upper airway mucosa. Inflamed tissue from the skin, gut and cervix did not show any binding of the mAbs.

Influence of low molecular weight -retroviral like- serumfactors on fmlp induced monocyte polarization

The results of the inhibition of the fMLP induced polarization of healthy donor monocytes by LMWF of patients with squamous cell carcinoma of the head and neck, patients with squamous cell carcinoma of the cervix uteri, patients with M.Crohn, patients with primary ciliary dyskinesia, patients with polyposis nasi,

Tissue	Positive/tested
inflamed mucosa of nasal polyps	23/23[1]
chronic inflamed mucosa of the middle ear	4/4[1]
chronic inflamed bronchial mucosa in purulent bronchitis	3/3[2]
cholesteatoma of the middle ear	0/1
chronic dermatitis	0/2
ulceratieve colitis	0/2
Morbus Crohn	0/2

Table 3: Frozen tissue reactivity of mAbs directed against p15E with chronic inflamed human tissue specimens.
[1] Positive staining in basal cell layer of respiratory epithelium.
[2] Positive staining in respiratory epithelium of the bronchus
[1,2] More than 75% of the cells showed a staining intensity from +/++.

patients with chronic recurrent purulent rhinosinusitis and of healthy individuals are shown in figure 3. The LMWF of patients with squamous cell carcinoma of the head and neck and of patients with recurrent upper airway infections (polyposis nasi, primary ciliary dyskinesia and chronic recurrent purulent rhinosinusitis) all showed clear significant ($p < 0.001$) inhibition of fMLP induced monocyte chemotaxis. The optimal inhibiting effect of fMLP induced monocyte chemotaxis was seen with the LMWF in a final dilution of 1:60. A limited number of serum samples could additionally be assayed at 1:30 and 1:120 dilutions, and similar differences in inhibition were seen (data not shown). In all tested concentrations, the inhibitory activity observed in serum samples from patients with squamous cell carcinoma was more pronounced in comparison to those observed from non-neoplastic disease. Since, after neutralizing the serum fractions with a p15E specific mAb (19F8) before testing in the monocyte polarization no inhibition on fMLP induced monocyte chemotaxis could be detected, the inhibition was due to the presence of p15E in the LMWFs. In contrast, no significant influence of LMWF of patients with squamous cell carcinoma of the cervix uteri and M.Crohn on the fMLP induced chemotaxis of healthy donor monocytes was detected (figure 3). In addition, there was also no influence of LMWF of patients with adenocarcinoma of the mamma and patients with soft tissue sarcoma in fMLP induced chemotaxis of monocytes from healthy donors (data not shown).

The inhibition-data of the LMWFs of the different patient groups on monocyte chemotaxis correlated well with the outcome of the immunohistochemical staining. P15E-like serum factors could only be detected in patients with pathological conditions in which the tissue specimens showed activity with mAbs directed against p15E. The low expression of p15E-like factors in "normal" human nasal mucosa is apparently not sufficient to produce detectable serum levels, since p15E-like factors were not present in serum of healthy controls.

Discussion

In man, several studies have shown that the chemotactic responsiveness of mononuclear phagocytes is defective in patients with malignancies (6,7) or chronic inflammatory respiratory disease (8). It was demonstrated that LMWFs (Mw< 25 kD) isolated from tumors and serum were responsible for this suppression of chemotactic responses (6). This inhibitory effect could be abolished by blocking of these factors with antibodies directed against p15E, the transmembrane envelope protein of retroviral murine leukemia virus (6,7).

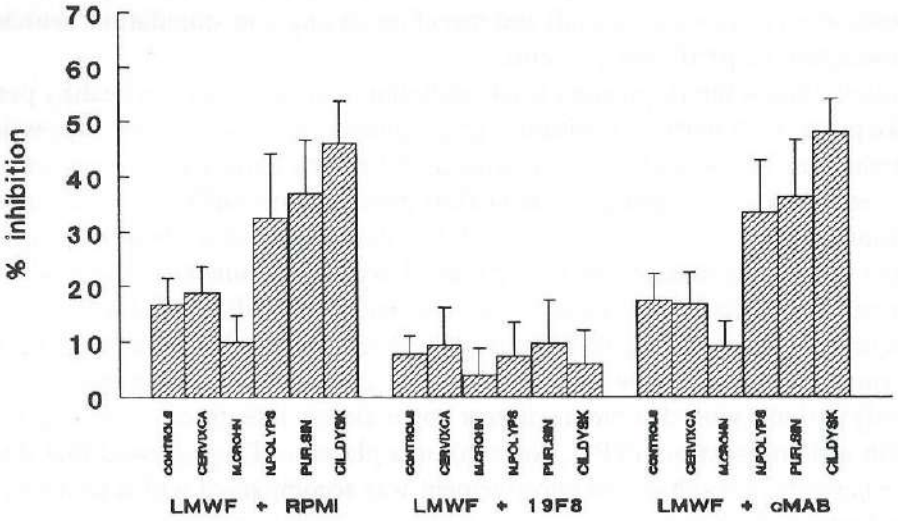

Figure 3: Inhibition of the fMLP-induced polarization of healthy donor monocytes by serumfactors <25kD of healthy controls and patients with respectively carcinoma of the cervix uteri (cervixca), M.Crohn, nasal polyposis (PPN), chronic purulent rhinosinusitis (CPR) or carcinoma of the head and neck region (H/N CA) (mean ± sd). To validate the p15E-like character of the inhibition, the polarization assay was also performed with serumfactors after adsorption with anti-p15E mAb (19F8) or control mAb (cmAb). Inhibition of the polarization was significant ($p < 0.001$) with sera of PPN, CPR, PCD and H/N CA.

Transmembrane envelope proteins related to p15E are assumed to play a role in suppression of the immune system as seen in retroviral infection (6,16,20-22).

In the present study we have shown that the p15E-like proteins are only found in the sera of patients with upper airway diseases (figure 3) and that they can be demonstrated exclusively in normal and pathologic epithelium of the upper respiratory tract. Since env-genes that share homology with retroviral p15E have been found in human DNA and the presence of mRNA derived from these genes has been detected in most normal tissues and tumor cell lines (23) an endogenous origin of the p15E-like proteins is likely. Moreover, endogenous retroviral sequences reside in the genome of all vertebrate species thus far examined, including humans (24). The majority of these sequences are structurally defective and are not correlated with a manifest retroviral infection. However, activation of these sequences, subsequently followed by production of proteins as p15E, might initiate cell dysfunction.

We favor the view that chronic stimulation of the upper respiratory tract epithelium, seen in inflammatory and malignant processes, triggers the production of p15E-like proteins. The quenching effect of these proteins

on the cellular immune response results in a lower inflammatory reaction. This would result in a reduced eradication of noxious stimuli and therefore an ongoing stimulation, which may in turn result in an increased production of p15E-like proteins.
Stimulation of the respiratory tract epithelium will also occur in healthy persons, hence the presence of p15E-like proteins in normal respiratory tract epithelium. However, the expression of p15E-like proteins in normal epithelium is low and these proteins could not be detected in serum of healthy persons. Thus, stimulation of respiratory tract epithelium in healthy persons is not sufficient to produce a detectable serum level of p15E.
Monitoring p15E serum levels can be of diagnostic value since decreased levels of p15E have been found after appropiate therapy, including radical excision of tumor (7) and functional endoscopic nasal sinus surgery (unpublished data). Furthermore, measurement of p15E serum levels can be a tool in early detection of local recurrence of neoplastic or inflammatory disease of the upper airway tract. In addition, measurement of p15E serum levels can also be used in evaluating immunopotentiation therapies as shown by Tas et al (25). In this study patients with chronic, recurrent upper airway infections, refractory to the current therapies, were treated with a thymusextract (TP-1, Serono) or a placebo. They showed that during the TP-1 treatment period of the patients, the subjective improvement was accompanied with a decrease of inhibitory activity of p15E-like proteins in their serum.
In conclusion, we have shown that the presence of p15E-like proteins is limited to the epithelia of the upper respiratory tract. The production of these proteins is triggered by malignant and inflammatory processes and may contribute to immunosuppression. Monitoring p15E serum levels can be of value in the follow-up of patients with neoplastic or chronic inflammatory disease of the upper airway tract.

Addendum

Recently, we have analyzed the distribution of p15E-like proteins in normal tissue from the oral cavity. We could demonstrate the presence of p15E-like proteins in the epithelium of the ducts of the small salivary glands in the buccal mucosa and in the mucosa of the floor of the mouth.

Chapter 3

Defects in cellular immunity in chronic upper airway infections are associated with immunosuppressive retroviral p15E-like proteins

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Summary

Partial defects in cell mediated immunity have been shown in patients with chronic purulent rhinosinusitis. These defects, i.e. impaired delayed type hypersensitivity (type IV) skin reactions upon commensal microorganisms of the upper respiratory tract and impaired chemotactic responsiveness of monocytes, are associated with the presence of immunosuppressive retroviral p15E-like proteins in the serum of these patients.

In the present study, we tested whether partial defects in cellular immunity could also be demonstrated in other groups of patients with chronic upper airway infections. Therefore, three well characterized groups of patients with chronic upper airway infections were investigated: a) patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; b) patients with chronic rhinosinusitis, with normal

functioning cilia and with nasal polyps (PPN); c) patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR).
Our results show that in all three groups the majority (87%) of the patients had defects in cellular immunity associated with the presence of p15E-like proteins in their serum. These results indicate that during chronic infections of the upper respiratory tract immunosuppressive retroviral p15E-like proteins are found, which are probably responsible for the partial immune defects found in these patients.

Introduction

Chronic recurrent upper airway infections are characterized by their high incidence and their tendency to be resistant to therapy. In patients with chronic rhinosinusitis, not responding to adequate treatment with antibiotics and/or surgery, partial defects in cellular immunity were reported: impaired delayed type hypersensitivity (type IV) skin reactions to commensal microorganisms of the upper respiratory tract and impaired chemotactic responsiveness of monocytes (1-4). These defects proved to be associated with the presence of immunosuppressive factors in the serum of these patients homologous to retroviral p15E[4].
Retroviral infection can result in suppression of the immune response. This has been well documented in leukemias caused by murine and feline leukemia viruses (5-7). The retroviral transmembrane envelope protein p15E plays an important role in this phenomenon (8-11). Since sequences have been found in human DNA that share homology with p15E, an endogenous production of p15E-like proteins in man is suggested (12,13). However, the exact relationship between recurrent infections in the upper respiratory tract, the defects in cellular immunity and the presence of the p15E-like proteins in the serum is not known. Are the respiratory infections primarily caused by the, already present immunosuppressive serumfactors or are these factors produced during these infections? In addition, are the presence of cellular immune defects and p15E-like proteins in the sera specific for chronic purulent rhinosinusitsis?
We, therefore, analyzed several parameters of the cellular immune response viz., delayed type hypersensitivity skin test upon commensal bacterial antigen and the chemotactic responsiveness of monocytes, in three different patient groups, all characterized by chronic recurrent upper airway infections: patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; patients with chronic rhinosinusitis, with normal functioning cilia and with nasal polyps (PPN); patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR). In addition, the data concerning the parameters of cellular immunity were related to the presence of immunosuppressive p15E-like proteins in patient's serum.

Material and methods

Patients and healthy controls

a. Healthy controls (n=20, 9 females, 11 males, ages ranging from 22-44 years, mean 33 years) without known diseases.
b. Patients with Primary Ciliary Dyskinesia (PCD), based on abnormal ciliary motility (14) and abnormal ciliary ultrastructure (15) (n=22, 8 females, 14 males, ages ranging from 2-58 years, mean 23 years).
c. Patients with Polyposis Nasi (PPN) i.e. recurrent upper airway infections, with normal functioning cilia, and the presence of nasal polyps at the moment of surgery or endoscopic examination (n=25, 13 females, 12 males, ages ranging from 18-61 years, mean 41 years).
d. Patients with Chronic Purulent Rhinosinusitis (CPR) i.e. recurrent upper airway infections not responding to adequate antibiotic treatment or surgical procedures, with normal functioning cilia, but without the presence of nasal polyps at the time of surgery or endoscopic examination (n=34, 21 females, 13 males, ages ranging from 7-71 years, mean 34 years).
The leucocyte counts of all patients and the healthy controls were within normal range. In addition, the serum levels of immunoglobulins were also within normal range and specific immunoglobulins to bacterial antigen (H. Influenza) were present.

Monoclonal antibodies

The mAb used were: 4F5(IgG2a) (9) and 19F8(IgG2b) (16) both specific for p15E but recognizing different epitopes (9); Control mAb were an IgG2a and IgG2b, secreted by the mouse myeloma P1.17 and MPC11.OUA cell lines respectively, both obtained from the American Type Culture Collection (ATCC, Rockville, Maryland).

Skin test

Delayed responsiveness was tested by intradermal injection of 0,1 ml of 1% Candidal antigen preparation (HAL allergens, Haarlem, the Netherlands) and 0,1 ml of 100 U Streptokinase/Streptodornase antigen preparation (Varidase, Lederle, Wayne, M, USA) in the forearm. The skin reactions (in mm) were read at 30 minutes, 24 and 48 hours and the induration, expressed as the average of two measurements at right angles, was recorded. The maximal induration was seen after 48 hours.

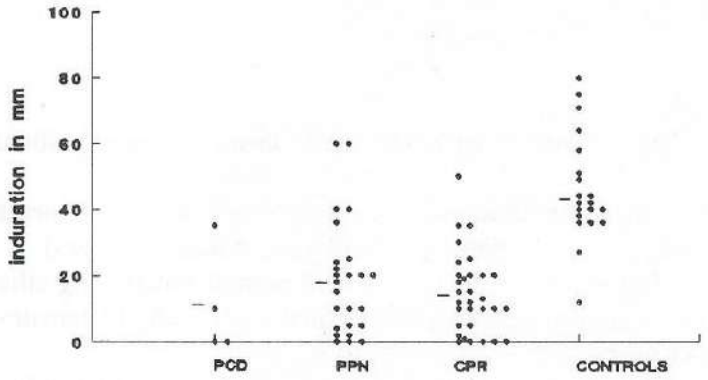

Figure 1a: The delayed skin test reactivity to Candidal antigen (1% solution) of patients with PCD, PPN, CPR and healthy controls (controls). The diameter of the induration was recorded (in mm) 48 hours after intradermal antigen injection and is given for individual patients. The DTH skin test reactivity to Candidal antigen in PCD ($p < 0.01$), PPN ($p < 0.001$) and CPR ($p < 0.001$) is significantly lower than those observed in healthy controls.

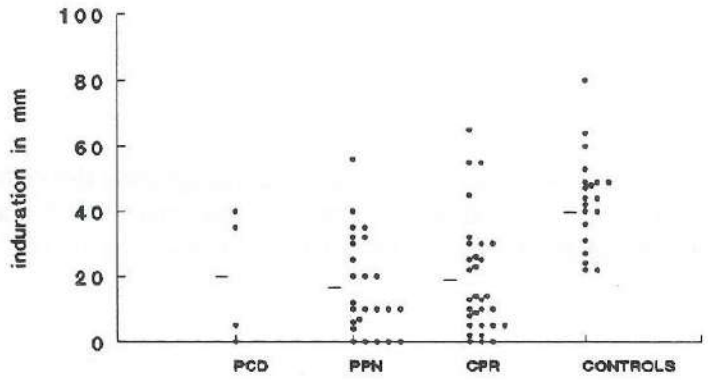

Figure 1b: The delayed skin test reactivity to Streptokinase/Streptodornase antigen (1% solution) of patients with PCD, PPN, CPR and healthy controls (controls). The diameter of the induration was recorded (in mm) 48 hours after intradermal antigen injection and is given for individual patients. The DTH skin test reactivity to Streptokinas/Streptodornase antigen in PCD ($p<0.01$), PPN ($p<0.001$) and CPR ($p<0.001$) is significantly lower than those observed in healthy controls.

Isolation of blood monocytes

Peripheral blood mononuclear cells were isolated by Ficoll-Isopaque (Pharmacia, Diagnostics AC, Uppsala, Sweden) density gradients centrifugation and sequentially washed twice in phosphate buffered saline (PBS), pH= 7,4, containing 0,5% bovine serum albumin (BSA) and counted in suspension employing positive staining with non-specific esterase (NSE) (17). The percentage of NSE-positive cells varied from 5-25%. An enrichment for the monocytes in the Ficoll-Isopaque isolated fraction was obtained by Percoll (Pharmacia, Diagnotics AC, Uppsala, Sweden) gradient centrifugation (18).
After washing, the pellet containing the monocytes was resuspended in medium (RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands)) and carefully underlayed with an equal volume of Percoll 1,063. After centrifugation (40 minutes, 450 g) the cells were collected from the interface, washed twice in medium (10 minutes, 500g) and counted: the suspension now contained 70-95% NSE-positive cells

Polarization Assay

The Cianciolo and Snyderman assay (19) was performed with slight modifications (20). Aliquots (0,2 ml) of the Percoll purified cell suspension

containing 0,2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co, Oxford, CA, USA) containing 0,05 ml of either medium alone or medium with N-formyl-methionyl-leucyl-phenylalanine (fMLP) in a final concentration of 10nM. All experiments were carried out in triplicate.The tubes were incubated in 37°C in a waterbath for 15 minutes. The incubation was stopped by addition of 0,25 ml icecold 10% formaldehyde in 0,05% PBS, pH=7,2. The cell suspensions were kept at 4°C until counting in an hematocytometer using an ordinary light microscope (Zeiss, Germany, magnification X 250). The test was read 'blindly' by two persons: 200 cells were counted from each tube. A cell was considered to be 'polarized' if any of the following characteristics were encountered : elongated or triangular shape, broadened lamellopodia, membrane ruffling. The percentage of polarized cells was calculated as follows:

$$\frac{\%\ \text{total cell polarized}}{\%\ \text{NSE-positive cells}} \times 100\%$$

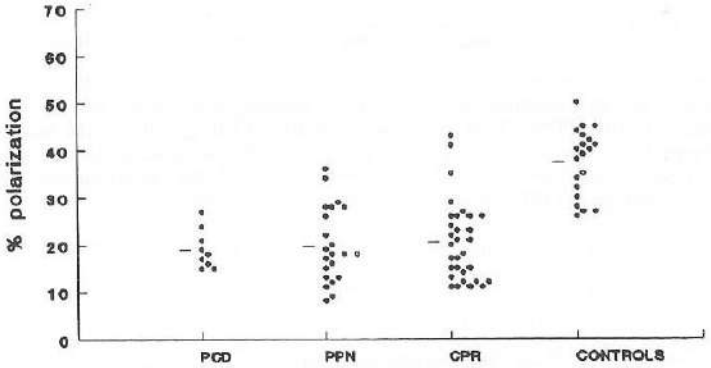

Figure 2: The fMLP-induced polarization of monocytes from patients with PCD, PPN, CPR, and healthy controls (controls). The percentage of polarization is given for individual patients. In PCD, PPN and CPR the monocyte polarization is significantly lower than those observed in healthy controls ($p < 0.001$).

Lymphocytes do not exhibit any polarization activity in this assay (19). The chemotactic responsiveness of a monocyte population is expressed as the percentage polarized cells in the presence of fMLP minus the percentage of polarized cells in the absence of fMLP. The assay has proven to be a rapid method to measure chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (20).

Monocyte isolation from healthy donors

From four different healthy donors monocytes were isolated from the peripheral blood. Buffy coats from 500 ml of human blood were obtained after informed consent from healthy donors. The monocytes were purified by successive isopycnic centrifugation and elutriator centrifugation as previously described (21) with minor modifications (22). These healthy monocytes were used to test the effects of patients' serum fractions.

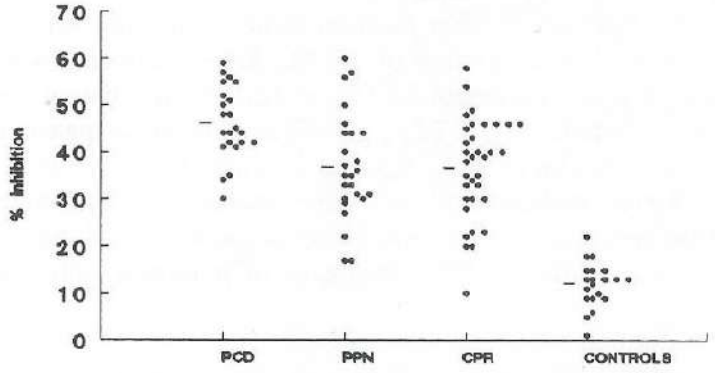

Figure 3: Inhibition of fMLP-induced polarization of healthy donor monocytes by serumfactors < 25kD of patients with PCD (46.1 ± 7.7, mean ± sd), PPN (36.9 ± 11.4) or CPR (37.0 ± 10.7) and healthy donors (controls) (12.0 ± 4.9) respectively. The percentage of inhibition is given for individual patients. Inhibition of the polarization was significant ($p<0.001$) with sera of all patient groups.

The determination in patient's serum of low molecular weight factors inhibiting the polarization of healthy donor monocytes

Sera were collected from patients by venapuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, MA, USA) for 15 minutes at 700 g (molecular weight "cut off point" 25 kD). The residues, the low molecular weight factors (LMWF), were dissolved in phosphate buffered saline (PBS) and stored at -70 °C until further use.

The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes ($1X10^6$/ml) for 15 minutes at 37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

$$\text{inhibition} = \left(1 - \frac{P_2 - P_0}{P_1 - P_0} \right) X\ 100\%$$

P_0= % spontaneously polarization
P_1= % polarization after incubation with fMLP alone
P_2= % polarization after incubation with fMLP and LMWF

Serum fractions were tested in triplicate. Addition of serum fractions to non-stimulated (fMLP) donor monocytes did not affect the spontaneous polarization.

Determination of the p15E-like character of patient LMWF's

To validate the p15E-like character of the LMWF's in human serum, adsorption experiments were carried out by neutralizing the serum fractions before testing in the monocyte polarization assay with a monoclonal antibody directed against p15E (19F8), in a final dilution of 1:200 (25 μg/ml) at 4°C for 16h, followed by Amicon ultrafiltration to remove formed immunecomplexes: this adsorption /neutralizing procedure was carried out twice (20). Adsorption experiments carried out with the mAb 4F5 or with 4F5 and 19F8 together did not show any difference in neutralizing the serum fractions as with 19F8 alone. Control experiments were carried out with the isotype matched control mAbs.

Statistics

Statistical analysis was performed by the Student's t-test.

Results

Delayed type hypersensitivity

If there was a positive response in DTH skin testing, a maximal swelling was present after 48h. A defective response showed no swelling or a diminished swelling (< 20 mm). In patients with CPR or PPN, more than 75% showed a defective skin test response to one (45%) or both (37%) antigens (Figure 1a, 1b). In contrast, only one of the twenty healthy controls (= 5%) had a diminished response to one antigen (Figure 1a). Unfortunately, only four patients with PCD underwent DTH skin testing. One patient had positive reactions to both antigens, the other three patients had negative reactions to one (1 patient) or both antigens (2 patients) (figure 1a, 1b). Delayed skin test reactivity is generally regarded as reflecting the state of protective immunity to chronic bacterial antigen (23). An absent or defective skin test reactivity may therefore be a strong indication of a high susceptibility to these bacteria. DTH skin test reactivity to Candidal antigen (1% solution) in patients with PCD (11.3 $\pm$ 16.5, mean $\pm$ sd, $p<0.01$), PPN (18.7 $\pm$ 17.3, $p<0.001$), CPR (14.4 $\pm$ 12.1, $p<0.001$) is significantly lower than those observed in healthy controls (46.3 $\pm$ 16.5) (figure 1a). DTH skin test reactivity to Streptokinase/Streptodornase antigen (1% solution) of patients with PCD (20.0 $\pm$ 20.4, mean $\pm$ sd, $p<0.01$), PPN (17.0 $\pm$ 15.2, $p<0.001$), CPR (19.0 $\pm$ 17.4, $p<0.001$) is also significantly lower than those observed in healthy controls (43.6 $\pm$ 14.6) (figure 1b).

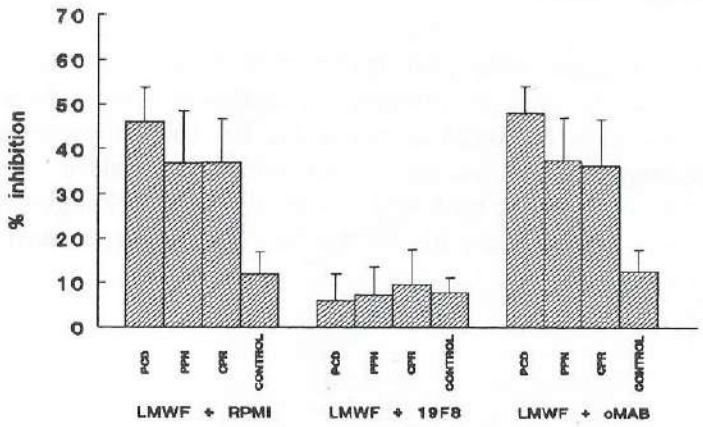

Figure 4: The p15E-like character of inhibition of fMLP-induced polarization of healthy donor monocytes by serumfactors < 25kD of patients with PCD, PPN or CPR is validated by performing the polarization assay with serumfactors after adsorption with anti-p15E mAb (19F8) or control mAb (cMAB) (mean ± sd).

Monocyte Polarization

The monocyte polarization assay was directly performed after the monocytes were isolated from the blood of patients and healthy controls. The results of the assay are shown in figure 2. In all tested patient groups, the majority of the patients showed a significant ($p < 0.001$) decreased monocyte polarization when compared to the values found in healthy controls; PCD: 19.1 ± 4.1 (mean ± sd), PPN: 19.9 ± 7.9, CPR: 20.5 ± 8.5, healthy controls: 37.4 ± 7.0.

Influence of low molecular weight -retroviral like- serumfactors on fMLP induced monocyte polarization

The results of the inhibition of the fMLP induced polarization of healthy, elutriator purified, donor monocytes by LMWF present in serum of patients with chronic recurrent infections of the upper airways are shown in figure 3. The LMWF of patients with CPR, PPN and PCD all showed significant ($p < 0.001$) inhibition of fMLP induced monocyte chemotaxis compared to the inhibitory activity of LMWF found in the serum of healthy donors. Moreover, in patients with PCD, the inhibitory effect was more pronounced ($p < 0.001$) than those observed in CPR or PPN. Since, after neutralizing the serum fractions with a p15E specific mAb (19F8) before testing in the monocyte polarization no inhibition on fMLP induced monocyte chemotaxis could be detected, the inhibition was due to the presence of p15E-like proteins in the LMWFs (figure 4). Figure 5 shows the inverted correlation
(-0.40, $p < 0.01$) between the polarization of patients' monocytes (all groups) and the inhibitory activity of patients' LMWF (all groups) on the polarization of healthy donor monocytes. There was no significant difference of this correlation between the patient groups. The correlation is more pronounced in patients possessing p15E-like serum proteins with large inhibitory activity (>40%). In 90% of these patients, a decreased polarization (<25%) of patients' monocytes was present.

Discussion

Earlier studies reported the presence of defects in DTH skin reactivity upon commensal bacterial antigen and chemotactic responsiveness of monocytes in patients with CPR (1,3,4). These defects could be related to the presence of immunosuppressive, low molecular weight, retroviral p15E-like serum proteins (4). The retroviral transmembrane envelope protein p15E, and transmembrane proteins related to p15E are assumed to play a role in suppression of the immune system as seen in retroviral infection (8,9,11).

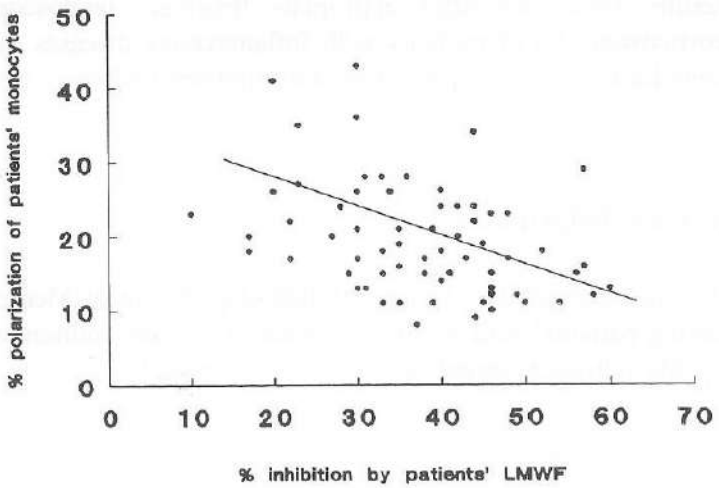

Figure 5: The inverted correlation (coefficient: -0.40, $p < 0.01$)between the polarization of patients' monocytes (all groups) and the inhibitory activity of patients' LMWF (all groups) on the polarization of healthy donor monocytes.

In the present study, we have shown that these defects in cell mediated immunity and the presence of retroviral p15E-like serum proteins are found not only in patients with CPR, but also in other patients with chronic upper airway infections, viz. PCD and PPN. Furthermore, in patients with PCD the inhibitory effect of the p15E-like serum proteins was more pronounced than those observed in patients with CPR or PPN, probably due to the life-long infectious status of the upper respiratory tract epithelium. Since the same defects are detected in 'congenital' (PCD), and in 'acquired' (CPR, PPN) inflammatory diseases of the upper respiratory tract, it is suggested that chronic inflammation of upper airway epithelium in general is associated with defects in cellular immunity. In addition, we have recently shown that the presence of p15E-like proteins is limited to chronically inflamed epithelium of the respiratory tract. In healthy persons, these proteins are only present in the epithelium of the, always stimulated, upper respiratory tract (24). The expression is increased in inflammatory disease of the upper airways. Furthermore, p15E-like proteins could not be detected in patients with chronic inflammatory processes localized elsewhere (e.g. M.Crohn) (24). Therefore we suggest that inflammatory processes of the upper respiratory tract epithelium trigger the production of p15E-like proteins. The immunosuppressive effects of these proteins may then result in a diminished inflammatory reaction. In 90% of the patients possessing p15E-like serum proteins with large inhibitory capacity (>40%), a decreased chemotactic responsiveness of patients' monocytes was present. This could result in a reduced eradication of noxious stimuli and therefore in ongoing inflammation. In this way a vicious circle is present. Preliminary results show that after

results show that after appropiate treatment (endoscopic sinus surgery and postoperatively topical corticosteroids) of patients with inflammatory diseases of the upper airway, reduction of the subjective complaints are accompanied by a diminished inhibitory activity of the p15E-like serum proteins.

Acknowledgements

We like to thank Dr. L. van Alphen (department of Medical Microbiology, University of Amsterdam) for testing patients' sera for the presence of specific immunoglobulins to bacterial antigen, and Drs. J. Nauta for his statistical expertise.

Chapter 4

Adhesion receptors involved in early clustering of blood dendritic cells and T-lymphocytes

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Summary

The relationship of dendritic cells (DC) isolated from the peripheral blood to those of lymphoid tissue is, in terms of maturation and function, incompletely understood. In our present study, we have explored the molecular basis of adhesion of T cells to blood DC. Analysis of the expression of adhesion receptors on the cell surface of blood DC revealed that these cells express LFA-1 (CD11a/18), ICAM-1 (CD54), LFA-3 (CD58), and CD44, but are VLA-4 (CD49d) and VCAM-1 negative. The LFA-1 pathway was found to play a key role in T cells-blood DC adhesion; mAb against both LFA-1 and ICAM-1 strongly inhibited adhesion between those cells. Moreover, a T-cell clone from an LFA-1-deficient patient showed poor binding to blood DC. The important role of LFA-1 in T cell-blood DC adhesion was also supported by the metabolic energy and divalent cation dependence of the interaction. MAb against LFA-3 and CD2 did not inhibit T cell-blood

DC binding. In contrast to the strong inhibition by antibodies to LFA-1 and ICAM-1, antibodies to CD44 enhanced conjugate formation between T cells and blood DC. Together, our results show that the LFA-1/ICAM-1 pathway plays a central role in T cell-blood DC adhesion, a situation like that in T cell adhesion to lymphoid DC. However, unlike lymphoid DC, blood DC do not express VCAM-1 nor use LFA-3 for T-cell binding.

Introduction

Dendritic cells (DC) play an important role in the induction of the immune response. They act as antigen-presenting cells for the initiation of primary T cell responses (1,2). These responses occur in cell aggregates or clusters. In the induction stage of this physical association, T cells bind to DC by an antigen-independent mechanism (3). Subsequently, cell binding is strong enough to facilitate recognition of major histocompatibility complex (MHC) molecules and antigenic peptides by the T cell receptor. During the initial antigen independent phase of T-cell adhesion to DC, cell adhesion receptors play a critical role. Studies of the molecular basis of the interaction between murine T cells and DC (2,4) and between human T cells and tonsil derived DC (5) have shown that adhesion-dependent interactions in these systems are mediated through binding of the integrin LFA-1 with the Ig-superfamily member ICAM-1. In addition, a role for the LFA-3/CD2 adhesion pathway has been described for the tonsillar system (5).
Cells with a dendritic morphology and a high antigen presenting capacity in vitro can be readily isolated from the human peripheral blood. These cells are thought to be a migratory form of the DC of lymphoid tissues (6). However, the relationship of blood DC to those of lymphoid tissues, in terms of maturation and function, is at present incompletely understood. In the present study, we have defined the adhesion receptor profile of blood DC and the molecular basis of blood-DC T-cell adhesion.

Material and methods

T cells

Mononuclear cells were isolated by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were removed by adherence on plastic petri dishes (Falcon Plastics, Oxnard, CA, USA) for 1 hr at 37°C. The non-adherent cells were depleted of B cells by a panning technique (7). In brief,

plastic dishes (Falcon) were coated overnight with 1/100 diluted F(ab) goat anti-human Ig (TAGO, Burlingame, CA, USA) in 0,05 M tris-HCL buffer, pH 9,5. Per coated plate, $1x10^7$ mononuclear cells were applied. The plates were incubated at 4°C for 1 hr and the non-adherent cells were harvested. The percentages of surface Ig+ and CD2+ lymphocytes in the T cell enriched fraction were determined by immunofluorescence. T cells enriched populations contained > 90% CD2+, and < 5% surface Ig+ cells. The LFA-1-negative T cell clone LAD 6 was raised from a LAD patient and has been described previously (8).

Isolation of dendritic cells

DC were isolated from the peripheral blood according to Knight et al (9). In brief, mononuclear cells were separated from heparinized blood by Ficoll Hypaque density gradient centrifugation. From these mononuclear cells the adherent cells were removed by incubation on plastic dishes (Falcon) for 90 min. The non-adherent cells were isolated using a 14,5% (w/v) hypertonic metrizamide gradient. The adherent cells and the cells in the pellet of the metrizamide gradient were incubated on plastic dishes for a further 16 hr (37°C, 5% CO_2). From the newly non-adherent populations the cells were again isolated on metrizamide. All low-density cell populations were pooled and these contained 60-80% cells with a dendritic morphology.

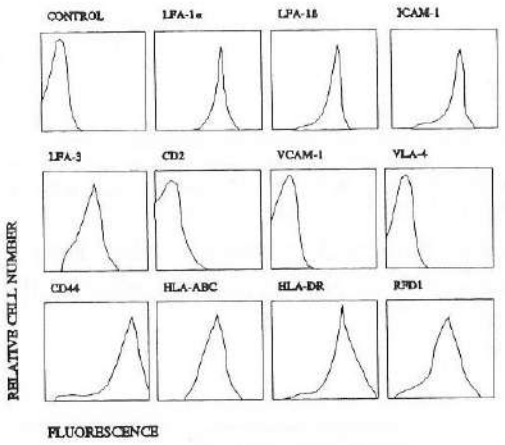

Figure 1: Expression of adhesion receptors and other cell surface antigens on blood DC. Blood DC were isolated from the peripheral blood and incubated with mAb against cell surface antigens followed by FITC conjugated goat anti-mouse Ig.

DC-T cell cluster assay

10 x 10^3 DC, labeled with the fluorescence dye PKH2-GL (Zynaxis, Malvern, PA, USA) for 15 min at room temperature, were incubated (4 hr, 37°C, 5% CO_2) with 50 x 10^3 allogeneic T cells in 250 μl flatbottom wells (Costar, Cambridge, MA, USA). Incubation of the cells was carried out in medium (RPMI 1640 with

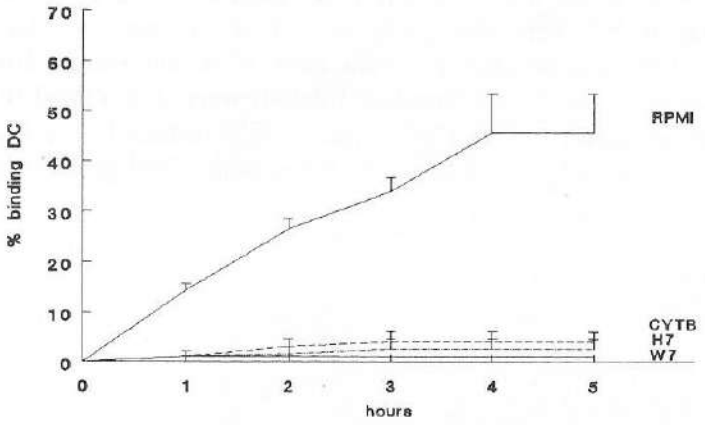

Figure 2: Kinetics of cluster formation between T cells and blood DC. Clustering of T cells and blood DC in medium alone, or in medium with Cytochalasin B (20 μM), W7 (20 μM) and H7 (20 μM). Binding was measured after 1, 2, 3, 4, and 5 hours (mean $\pm$ sd).

25 mM HEPES buffer (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories Inc.,Logan, UT), 100 U/ml of natrium penicillin G (Gist Brocades NV, Delft, The Netherlands), and 100 U/ml of streptomycin sulfate (Pharmachemie BV, Haarlem, the Netherlands) in the presence or absence of monoclonal antibodies (mAb) or inhibitors of cell metabolism. The requirement for bivalent cations was studied by washing and subsequently incubating the cells (DC and T cells) in HBSS without calcium and magnesium (Gibco), complemented with 1mM EDTA and 2 mg/ml D-glucose. Clusters were defined as DC binding 3 or more T cells. They were counted using an inverted fluorescence microscope (Zeiss) (magnification X 200) and expressed as the percentage binding DC.

$$\% \text{ binding DC} = \frac{\text{number of DC binding with 3 or more T cells}}{\text{total number of DC}} \text{ X } 100$$

Statistical analysis was performed by the student's T test.

Monoclonal antibodies and reagents

The mAb used were: CLB-LFA1/2 (IgG1) specific for the α subunit of LFA-1 (CD11a) (10); CLB-LFA1/1 (IgG1) specific for the ß subunit of LFA-1 (CD18) (11); R.R1/1 (IgG1) specific for ICAM-1 (CD54) (12); TS2/9 (IgG1) specific for LFA-3 (13); 6G4 (IgG1) and 4B2H4 (IgG1) specific for CD2; NKI-P1 (IgG1), NKI-P2 (IgG1), Hermes 3 (IgG2a) all reactive with CD44 (14,15); HP2/1 (IgG1) binds to an epitope on the α4 polypeptide (CD49d) of integrin receptor VLA-4 (CD49d/CD29) (16); 4B9 (IgG1) (17) and 2G7 (IgG1) (18) bind to a functional epitope on VCAM-1 ; W6/32 (IgG2a) specific for a nonpolymorphic determinant

determinant of HLA-ABC (19); OKIa (IgG2a) specific for HLA-DR (Becton Dickinson, CA, USA) ; RFD1 reacts with dendritic cells and activated B cells (20) and 15D9 is specific for T200 (CD45) (dr. R.A.W. van Lier, C.L.B., Amsterdam, The Netherlands).

The reagents used were: H7 (1-(5 isoquinolinyl sulfonyl-2 methyl piperazide, 20 μM, Sigma, St.Louis, MO), which prevents activation of protein kinase A and protein kinase C ; W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide, 20 μM, Sigma, St.Louis, MO) an inhibitor of calmoduline dependent protein kinase and Cytochalasin B (20 μM, Sigma, St.Louis, MO), which inhibits microfilament formation.

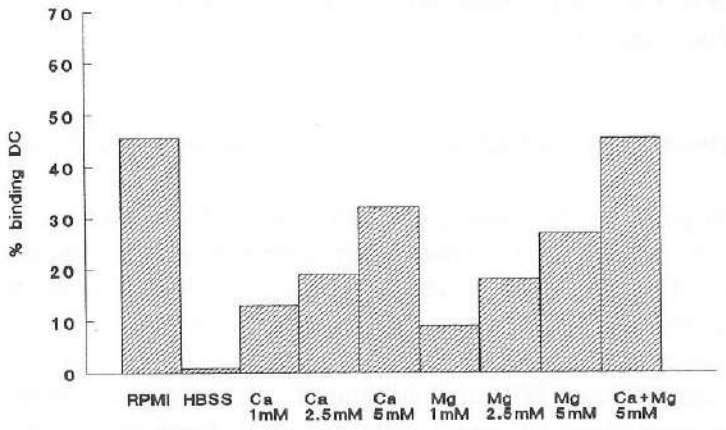

Figure 3: Cation requirement of binding between T cells and blood DC. Binding of T cells to blood DC was determined in medium, in HBSS free of Calcium and Magnesium, or in HBSS with: 1mM Calcium; 2.5 mM Calcium; 5 mM Calcium; 1 mM Magnesium; 2.5 mM Magnesium; 5mM Magnesium; or 5mM Magnesium and 5 mM Calcium. Binding was measured after 4 hours.

Immunofluorescence

Cells were sequentially incubated (PBS containing 1% BSA and 0,01 % sodium azide) with appropriate dilution of the different mAb and fluorescein isothiocyanate (FITC)-conjugated Rabbit anti-Mouse Immunoglobulins (Dakopatts, Denmark) for 30 min. at 0°C. Fluorescence intensity was measured by FACSTAR (Becton Dickinson, Mountain View, CA).

Results

Expression of adhesion receptors and other cell surface antigens on blood DC

Expression of adhesion receptors on blood DC was measured by FACS-analysis. Molecules clearly expressed at the cell surface of isolated blood DC were: LFA-1α (CD11a) and LFA-1ß (CD18); ICAM-1 (CD54);

LFA-3 (CD58); CD44; HLA-ABC; HLA-DR and RFD1. In contrast, VCAM-1 and VLA-4 were not detectable (figure 1).

Blood DC-T cell adhesion is energy dependent and requires an intact cytoskeleton

Progressive spontaneous binding between blood DC and T cells was observed upon incubation mixtures of these cells at 37°C. This cluster formation reached its maximum after 4 hours, when approximately 50 % of DC were found in clusters (figure 2). We assessed the metabolic requirements for blood DC-T cell binding. Addition of cytochalasin B, an inhibitor of microfilament formation, completely blocked the binding between blood DC and T cells. Similarly, H7, which prevents activation of protein kinase A and protein kinase C, and W7, an inhibitor of calmoduline dependent protein kinase were found to prevent binding between blood DC and T cells (figure 2). Furthermore, cluster formation was not observed at 4°C (data not shown). Hence, blood DC-T cell binding requires an intact cytoskeleton and is dependent on intracellular protein kinase activity.

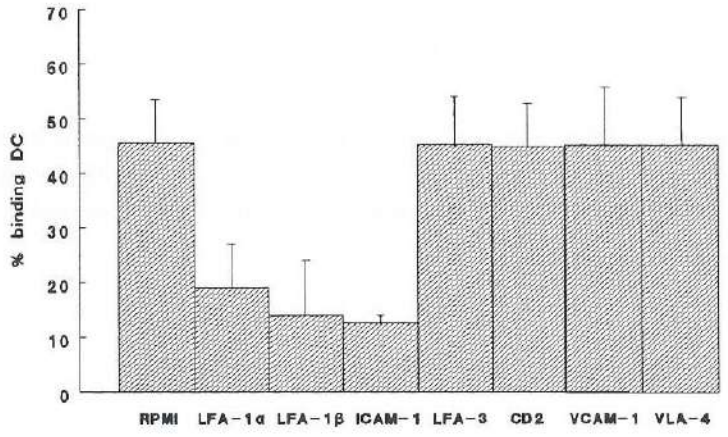

Figure 4: mAb inhibition studies of T cell adhesion to blood DC. Binding of T cells with blood DC in medium alone or in medium with anti-LFA-1α (CD11a), anti-LFA-1ß (CD18), anti-ICAM 1 (CD54), anti-LFA-3 (CD58), anti-CD2, anti-VCAM 1, and anti-VLA 4 was studied. Binding was measured after 4 hours (mean ± sd).

Blood DC-T cell adhesion requires bivalent cations

To study the cation requirement of blood DC-T cell adhesion, cells were washed in HBSS without calcium or magnesium but with 1 mM EDTA to remove all remaining bivalent cations. No binding was observed in bivalent cation-depleted medium (figure 3). The addition of 5mM calcium or 5mM magnesium only partially restored DC-T cell binding, whereas binding was completely restored by simultaneous addition of 5 mM magnesium and 5 mM calcium.

Effect of mAb on blood DC-T cell adhesion

To identify the surface molecules involved in the adhesion of blood DC and T cells, mAb inhibition studies were performed. Antibodies against both the LFA-1α chain (CD11a) and LFA-1ß chain (CD18) inhibited the adhesion up to 70% (figure 4). Antibody against ICAM-1 (CD54), a known ligand of LFA-1, inhibited the adhesion to the same extent, showing that ICAM-1 also is involved in this process. Thus, the LFA-1/ICAM-1 pathway plays a central role in blood DC-T cell clustering. In contrast to mAb against LFA-1 and ICAM-1, mAb directed against LFA-3 (CD58), CD2, VCAM-1 and VLA-4 did not affect the clustering between blood DC and T cells indicating that these adhesion receptors are not involved in the early clustering between DC and T lymphocytes. Control mAb recognizing the cell-surface antigens HLA-DR, HLA-ABC, RFD1 and CD45 also did not influence binding between DC and T cells (data not shown). MAb against CD44, however, significantly accelerated binding of T cells to DC (figure 5). This acceleration of binding was most evident during the first two hour of the clustering process.

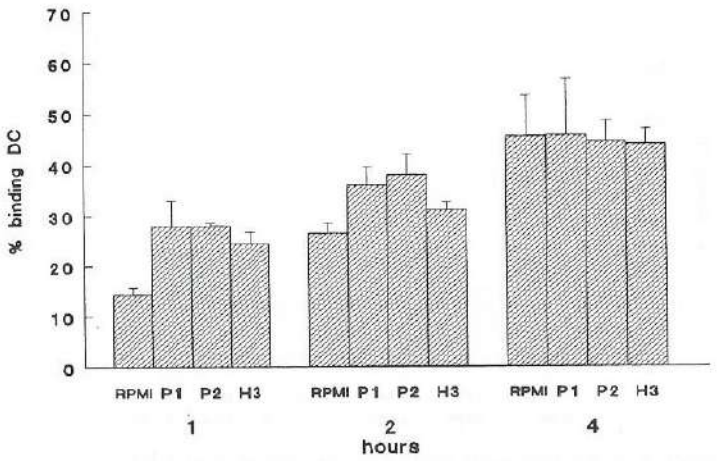

Figure 5: CD44 induced acceleration of the binding of T cells to blood DC. Blood DC and T cells were allowed to cluster in medium alone or in medium with three different mAb against CD44; NKI-P1 (P1), NKI-P2 (P2), and Hermes 3 (H3). Binding was measured after 1, 2, and 4h (mean $\pm$ sd). Enhancement of binding after 1 and 2 hours was significant ($p < 0{,}01$) for all three mAb against CD44.

Binding between blood DC and LFA-1-negative T cells

Binding experiments were performed using blood DC and a T cell clone derived from a patient with the leucocyte adhesion deficiency (LAD) syndrome. T cells from these patients, which lack the presence of CD11 and CD18 molecules on their surface, bind only to a small extent to blood DC (figure 6). As expected, this binding was not affected by mAb to LFA-1 or ICAM-1. Furthermore, like with normal T cells, it was also not influenced by mAb directed against LFA-3 (CD58), CD2, VCAM-1 and VLA-4, thus indicating that the LFA-3/CD2 and VCAM-1/VLA-4 pathways do not play a role in T cell adhesion to blood DC.

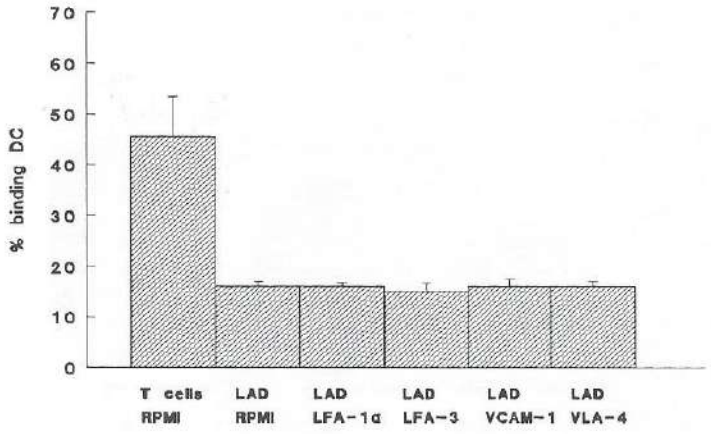

Figure 6: mAb inhibition studies of the adhesion of LFA-1-negative T cells (LAD 6 clone) to blood DC. Clustering of LAD cells with blood DC in medium alone or in medium with anti-LFA-1α, anti-LFA-3, anti-VCAM 1 or anti-VLA-4. Binding was measured after 4 hours (mean ± sd).

Discussion

Several studies have shown that DC isolated from lymphoid tissues efficiently cluster T cells during the initiation of the immune response (3,21-25). This physical association does not only depend on antigen-specific interactions but depends primarily on engagement of adhesion receptors. In our present study, we have explored the molecular basis of adhesion of T cells to DC derived from the peripheral blood. Analysis of the expression of adhesion receptors on the cell surface of blood DC (figure 1) revealed that these cells express the integrin LFA-1 and the immunoglobuline (Ig) superfamily members ICAM-1 and LFA-3. Furthermore, expression of CD44, a molecule with proteoglycan link protein homology (26,27), was found. However, blood DC did not express VLA-4 and VCAM-1. VCAM-1, like ICAM-1, belongs to the Ig-superfamily and is induced on endothelium at sites of inflammation (28-30). It plays a role in mediating lymphocyte and monocyte adhesion to activated endothelium through interaction with the integrin VLA-4 on the lymphocyte and monocyte cell surface (28,30). Furthermore, the VLA-4/VCAM-1 pathway was recently shown to be involved in adhesion of B cells to follicular dendritic cells (FDC) (31,32). The lack of expression of VCAM-1 on blood DC indicates that the VLA-4/ VCAM-1 adhesion pathway plays no part in T cell adhesion to blood DC. Moreover, the fact that blood DC, which are believed to be the precursors of VCAM-1 positive (28) lymphoid DC, lack VCAM-1 suggests that VCAM-1 expression is acquired during DC maturation and/or activation in lymphoid tissue. Although cytokines presumably play a role in this process, efforts to induce VCAM-1 expression on blood DC in vitro by a panel of cytokines, including IL-1, TNF and GM-CSF have so far been unsuccesfull (R.S., unpublished results).

Like in many other adhesive interactions of lymphocytes (33-35), the LFA-1 pathway was found to play a keyrole in the early binding between T cells and blood DC. This can be concluded from the inhibition of T cell-blood DC binding obtained with mAb against LFA-1 and ICAM-1 (figure 4) as well as from the low binding of LAD T cells to blood DC (figure 6). The important role of integrins, viz. LFA-1, in T cell DC binding was further strengthened by the metabolic energy and divalent cation dependence of the interaction (figure 2,3). Since anti-LFA-1 antibodies did not completely prevent binding of T cells to blood DC and

since LAD cells clustered with DC, although at low rates, at least one additional pathways must be involved in T cell-blood DC binding. Apparently, this pathway involves neither LFA-3/CD2 nor VLA-4/VCAM-1 since binding of T cells to blood DC was not blocked by mAb against these molecules (figure 4). The finding that T cell-blood DC binding is independent of the LFA-3/CD2 pathway contrasts the situation of T cell adhesion to tonsillar DC where the CD2/LFA-3 pathway has been found to play a role (5). In addition to the lack of VCAM-1 expression on blood DC, it presents a second difference between blood and lymphoid DC, which conceivably is related to differences in maturation/activation. This may have important functional consequences for the strength of T cell- DC binding and hence for the efficacy of antigen presentation to T cells.

Interestingly, the anti-CD44 mAb enhanced rather than inhibited conjugate formation between T cells and blood DC (figure 5). This anti-CD44 effect required an intact cell metabolism and could not be blocked by anti-LFA-1 mAb (R.S., unpublished observation). Adhesion promoting effects of anti-CD44 mAbs have also been observed in other systems (36,37). These effects as well as direct binding of CD44 to ligands on high endothelium (38-40) and in the extracellular matrix (41), such as hyaluronic acid (40,42) and collagen (41), may underly the correlation between CD44 expression and lymphoma metastasis that has been observed in studies by us and others (39,43-45).

In conclusion, our results show that the LFA-1/ICAM-1 pathway plays a central role in T cell-blood DC adhesion, an interaction pathway that also dominates T cell adhesion to lymphoid DC (5). However, on the other hand, blood DC differ from lymphoid DC since they do not express VCAM-1 nor use LFA-3 for T-cell binding. Apparently, during maturation/activation in lymphoid tissues DC acquire additional adhesive options, which may be required for efficient antigen presentation to T cells.

Acknowledgements

We thank C.G.Figdor for the LFA-1-negative T cell clone LAD 6 and for mAb NKI-P1 and NKI-P2; R.A.W. van Lier for mAb CLB-LFA-1/2, 6G4, 4B2H4 and 15D9; F.Miedema for mAb CLB-LFA-1/1; T.A.Springer for mAb RR1/1 and TS2/9; E.C.Butcher for mAb Hermes-3; F.Sanchez-Madrid for mAb HP2/1; W.Newman for mAb 2G7; J.M.Harlan for mAb 4B9 and L.W.Poulter for mAb RFD1 and R. Keehnen for expert technical assistance.

Chapter 5

Decreased function of blood dendritic cells in patients with chronic infections of the upper respiratory tract. Effect of retroviral p15E-related serum proteins

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Submitted for publication

Summary

Defects in cellular immunity, i.e. diminished chemotactic responsiveness of monocytes and impaired delayed type hypersensitivity (DTH) skin reactions upon commensal bacterial antigens have been found in patients with chronic recurrent infections of the upper respiratory tract. These defects are associated with the presence of immunosuppressive retrovial p15E-like proteins in the serum of these patients.
In the present study, we analyzed whether defects could also be found in an earlier stage of the immune response. In the induction of the immune response dendritic cells (DC) play an important role. They form clusters with T lymphocytes and stimulates them. Therefore, we tested the DC cluster capability in three different groups of patients all characterized by chronic upper airway infections: (1) patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; (2) patients with chronic rhinosinusitis,

with normal functioning cilia and with nasal polyps (PPN); (3) patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR). In addition, morphological analysis of dendritic cells by immunocytochemistry and electron microscopy was performed.
Our results show that most patients characterized by chronic infections of the upper respiratory tract had a decreased DC cluster capability associated
with the presence of immunosuppressive retroviral p15E-like serum proteins. A morphological explanation for the impaired DC function could not be found.

Introduction

A series of defects in cell mediated immunity, viz. diminished chemotactic responsiveness of monocytes and impaired DTH skin reactions upon commensal bacterial antigens, have been found in patients with chronic upper airway infections (1-5). These defects proved to be related to the presence of retroviral p15E-like serum proteins (4,5). The retroviral transmembrane envelope protein p15E, and transmembrane proteins related to p15E are assumed to play a role in the suppression of the immune response as seen in retroviral infection (6-8). However, defects in an earlier stage of the cellular immune response are not reported yet.
DC play an important role in the induction of the immune response. They act as antigen presenting cell (APC) for the initiation of primary T cell responses (9-12). These responses occur in cell aggregates or clusters of DC and T cells. In the initial phase, this process is antigen-independent (13) and adhesion receptors play a critical role (13-16). The number of formed clusters correlates well with lymphocyte stimulation (17).
In this study, we analyzed the cluster capability of blood DC in three different patient groups, all characterized by chronic recurrent upper airway infections: patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; patients with chronic rhinosinusitis, with normal functioning cilia and with nasal polyps
(PPN); patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR). Since our results showed a decreased cluster capability of patients' DC, we performed ultrastructural and immunocytochemical analysis of DC from patients and healthy controls to see whether impaired DC function is related to morphological changes. In addition, the results of the cluster capability of DC in these patients were related to the presence of immunosuppressive p15E-like proteins in patient's serum.

Material and methods

Patients and healthy controls

a. Healthy controls (n=20, 9 females, 11 males, ages ranging from 22-44 years, mean 33 years) without known diseases.
b.Patients with PCD, based on abnormal ciliary motility (18) and abnormal ciliary ultrastructure (19) (n=8), 4 females, 4 males, ages ranging from 6-42 years, mean 24 years).
c. Patients with PPN i.e. recurrent upper airway infections, with normal functioning cilia, and the presence of nasal polyps at the moment of surgery or endoscopic examination (n=19, 11 females, 8 males, ages ranging from 18-61 years, mean 38 years).
d. Patients with CPR i.e. recurrent upper airway infections not responding to adequate antibiotic treatment or surgical procedures, with normal functioning cilia, but without the presence of nasal polyps at the time of surgery or endoscopic examination (n=24, 14 females, 10 males, ages ranging from 7-71 years, mean 32 years).
The leucocyte counts of all patients and the healthy controls were within normal range. In addition, the serum levels of immunoglobulins were also within normal range and specific immunoglobulins to bacterial antigen (H. Influenza) were present.

T cells

Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were removed by adherence on plastic petri dishes (Falcon Plastics, Oxnard, CA) for 1h at 37°C. The non-adherent cells were depleted of B cells by a panning technique (20). In brief, plastic dishes were coated overnight with 1/100 diluted $F(ab')_2$ goat anti-human Ig (Tago, Burlingame, CA) in 0.05 M Tris-HCL buffer, pH 9.5. Per coated plate, 1 X 10^7 mononuclear cells were applied. The plates were incubated at 4°C for 1h and the nonadherent cells were harvested. The percentages of sIg^+ and $CD2^+$ lymphocytes in the T cell-enriched fraction were determined by immunofluorescence. T cell-enriched populations contained >90% $CD2^+$ and <5% surface Ig^+ cells.

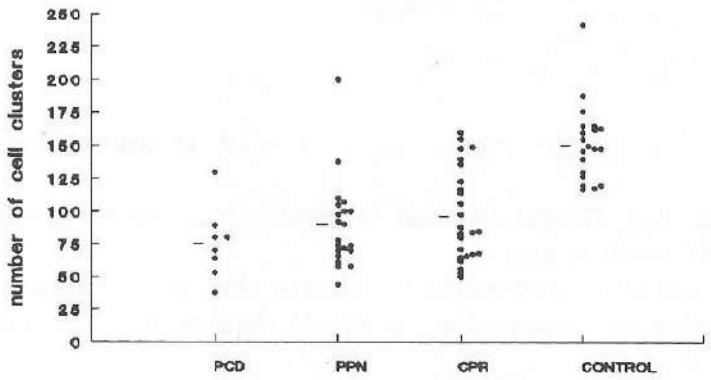

Figure 1: The clustering of blood DC of patients with PCD, PPN, or CPR and healthy controls. The clustering of patients' DC is significantly lower ($p < 0.001$) than those observed in healthy controls.

Isolation of blood dendritic cells

DC were isolated from the peripheral blood according to Knight et al (21). In brief, mononuclear cells were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. From these mononuclear cells the adherent cells were removed by incubation on plastic dishes (Falcon) for 90 min. The nonadherent cells were isolated using a 14.5% (w/v) hypertonic metrizamide gradient. The adherent cells and the cells in the pellet for the metrizamide gradient were incubated on plastic dishes for a further 16h (37°C, 5% CO_2). From the newly nonadherent population the cells were again isolated on metrizamide. All low-density cell populations were pooled and these contained 60%-80% cells with a dendritic morphology. The isolation of the cells was carried out in medium (RPMI 1640 with 25mM Hepes buffer (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories Inc., Logan, UT), 100 U/ml of sodium penicillin G (Gist Brocades NV, Delft, The Netherlands), and 100 U/ml of streptomycin sulfate (Pharmachemie BV, Haarlem, The Netherlands).

Dendritic cell clustering

The cluster assay as described by Austyn et al. (22) was performed with modifications (17). 5 X 10^4 DC were incubated (4 h, 37 °C, 5% CO_2) with 5 X 10^3 allogeneic T cells isolated from healthy donors in 250 μl flat-botommed wells (Costar, Cambridge, MA). Incubation of the cells was carried out in medium. Formed clusters (aggregates of 5 or more cells), which consist of DC and T cells, were counted by 2 persons indepently of each other using an inverted microscope (Zeiss, Oberkochen, FRG; magnification X 200) and values were expressed as the number of clusters per 6 microscopic fields.

Monoclonal antibodies

The monoclonal antibodies (mAb) used were: 4F5(IgG2a) (6) and 19F8(IgG2b) (23) both specific for p15E but recognizing different epitopes (6); W6/32 (IgG_{2a}) specific for a nonpolymorphic determinant of HLA-ABC (24); OKIa(IgG_{2a}) specific for HLA-DR (Ortho Diagnostics Systems Inc, Raritan, NJ); RFD1 reacts with DC and activated B cells (25). mAb used for recognizing adhesion receptors were: CLB-LFA-1/2 (IgG_1) specific for the α subunit of LFA-1(CD11a) (26); CLB-LFA-1/1 (IgG_1) specific for the ß subunit of LFA-1(CD18) (27); R.R1/1 (IgG_1) specific for ICAM-1 (CD54) (28); NKI-P1 (IgG_1) reactive with CD44 (29). Control mAb were an IgG2a and IgG2b, secreted by the mouse myeloma P1.17 and MPC11.OUA cell lines respectively, both obtained from the American Type Culture Collection (ATCC, Rockville, Maryland).

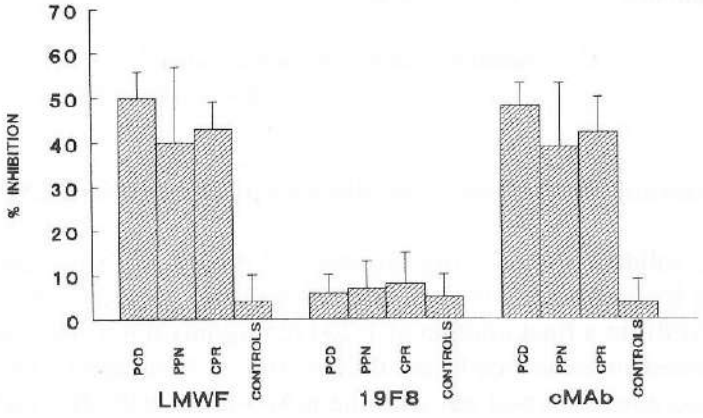

Figure 2: Inhibition of the clustering of healthy DC by LMWF's of patients with PCD, PPN, or CPR and healthy controls. Inhibition of the clustering was significant ($p < 0.001$) with LMWF's of all patients groups. The p15E-like character of the LMWF's is analyzed by performing the DC clustering with LMWF's after adsorption with anti-p15E mAb (19F8) or control mAb (cmAb) (mean ± sd).

Determination in patient's serum of low molecular weight factors inhibiting the clustering of healthy donor dendritic cells

Sera were collected from patients by venapuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, MA, USA) for 15 minutes at 700 g (molecular weight "cut off point" 25 kD). The residues, the low molecular weight factors (LMWF), were dissolved in phosphate buffered saline (PBS) and stored at -70 °C until further use.
The capability of the serum fractions to inhibit the clustering of healthy donor dendritic cells was determined by incubating the dendritic cells (5 X 10^4) and T cells (5 X10^3) in medium alone or in medium with the LMWF (final dilution 1:6, 4h, 37°C, 5% CO_2). Serum fractions were tested in triplicate. The percentage of inhibition was calculated as follows:

inhibition = $(1 - C_1/C_0) \times 100\%$

C_0 = number of cluster in medium alone
C_1 = number of clusters in medium with LMWF

Determination of the p15E-like character of patient LMWF's

To validate the p15E-like character of the LMWF's in human serum, adsorption experiments were carried out by neutralizing the serum fractions before testing in the cluster assay with a mAb directed against p15E (19F8), in a final dilution of 1:200 (25 μg/ml) at 4°C for 16h, followed by Amicon ultrafiltration to remove formed immunecomplexes: this adsorption /neutralizing procedure was carried out twice (30). Adsorption experiments carried out with the mAb 4F5 or with 4F5 and 19F8 together did not show any difference in neutralizing the serum fractions with 19F8 alone. Control experiments were carried out with the isotype matched control mAbs.

Immunofluorescence

Cells were sequentially incubated (PBS containing 1 % BSA and 0.01% sodium azide) with appropriate dilutions of the different mAb and FITC-conjugated rabbit anti-mouse Ig (Dakopatts, Copenhagen, Denmark) for 30 min at 0 °C. Fluorescence intensity was measured by FACStar (Becton Dickinson, Mountain View, CA).

Electron microscopy

Blood DC suspensions were fixed overnight in 1.5% glutaraldehyde in phosphate buffer (0.09 M, pH 7.4) at 4°C. After postfixation in 1% OsO_4 in phosphate buffer for 1h at 4°C, the cells were pelleted in 1% agar and subsequently dehydrated and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a Philips EM 301 electron microscope at 40 or 60 KV. Hereafter, the DC of patients and controls were scored for their size, outline, nucleus-cytoplasm ratio, the localization and number of different cell organelles (i.e. mitochondria, Golgi-apparatus, smooth and rough endoplasmatic reticulum, presence/absence of phagolysosomes).

Statistics

Statistical analysis was performed by the Student's t-test.

Results

Dendritic cell clustering

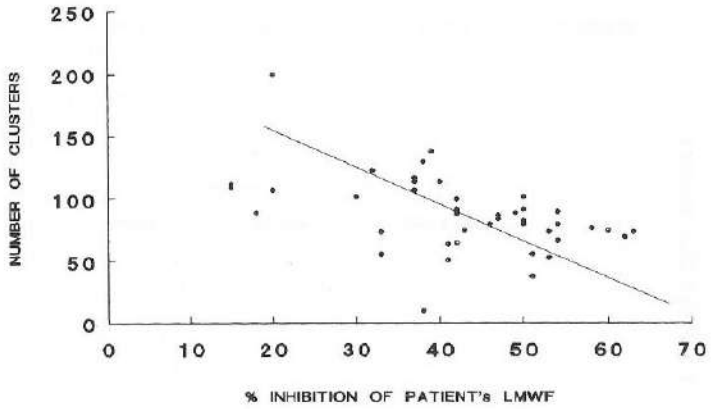

Figure 3: The inverted correlation (coefficient:-0.48, $p < 0.001$) between the clustering of patients's DC (all groups) and the inhibitory activity of patient's LMWF on clustering of DC of healthy controls. The values are given for individual patients.

The cluster capability of DC was directly assayed after isolation of the DC from the blood of patients and healthy controls. The results are shown in figure 1. In all tested patient groups, the cluster capability is significantly ($p < 0.001$) decreased compared to the values found in healthy controls; PCD: 75.5 $\pm$ 27.4 (mean $\pm$ sd), PPN: 88.8 $\pm$ 33.9, CPR: 97.0 $\pm$ 35.2, controls: 152.0 $\pm$ 29.3. Differences between the patients groups were not statistically significant.

Inhibition of the cluster capability of healthy donor DC by low molecular weight -retroviral like-proteins present in patients' serum

The results of the inhibition of the cluster capability of healthy donor DC by LMWF present in serum of patients with chronic recurrent infections of the upper airways are shown in figure 2. The LMWF of patients with PCD, PPN and CPR all showed significant ($p < 0.001$) inhibiton of the DC clustering compared to the inhibitory activity of LMWF found in healthy controls; PCD: 50.1 $\pm$ 6.0 (mean $\pm$ sd), PPN: 40.3 $\pm$ 17.2, CPR: 43.0 $\pm$ 6.1, controls: 4.3 $\pm$ 6.1.

After neutralizing the serum fractions with a p15E specific mAb (19F8) before testing in the cluster assay, no inhibition on DC cluster capability

could be detected (figure 2). Therefore, the inhibition of patients' LMWFs on DC cluster capability was caused by the presence of p15E-like proteins in the LMWFs.

Figure 3 shows the inverted correlation (-0,48, $p < 0.001$) between dendritic cell clustering and the inhibitory activity of LMWF's in patients' serum.

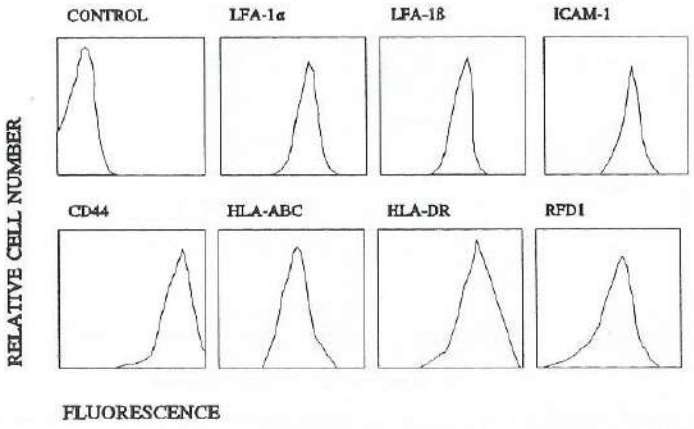

Figure 4a: Expression of surface antigens on blood DC of patients with PCD, PPN or CPR. Blood DC were isolated from the peripheral blood and incubated with mAb against cell surface antigens followed by FITC-conjugated goat anti-mouse Ig. No differences were observed between the patient groups.

There was no significant difference of this correlation between the patient groups.

Expression of surface antigens on blood DC of patients and healthy controls

In cytospin preparations, the blood DC isolated from patients and healthy controls were characterized by an irregular outline with commonly an eccentric elongated and frequently lobulated nucleus. All DC showed APh activity in a central spot of variable size and intensity near the nucleus. Figure 4a and 4b shows the reactivity of blood DC, from patients and healthy controls respectively, with different mAbs. Molecules clearly expressed at the cell surface of isolated blood DC were: LFA-1α (CD11a) and LFA-1ß (CD18); ICAM-1 (CD54); CD44; HLA-ABC; HLA-DR and RFD1. There was no difference in reactivity with the mAbs between blood DC isolated from patients (PCD, PPN, CPR) or from healthy controls.

Electron microscopy

No distinct morphological differences were observed between the blood DC isolated from patients and from healthy controls. In general, ultrastructural analysis showed an irregular plasma membrane. They sometimes possessed moderately smooth surface folds or lamellipodia, which never contained cell organelles. Other cells showed blunt pseudopodia of varying length. The nucleo-cytoplasmatic ratio varied from 1:1 to 1:3. Frequently, the nuclei were reniform with many indentations, sometimes containig nucleoli. The karyoplasm was euchromatic with some condensations against the nuclear envelope. DC also contained bundless of microfilaments near the nuclear indentations, mitochondria, polyribosomes and some strands of rough endoplasmatic reticulum (RER). The cells frequently contained vacuoles, especially near the cell surface. In some cases a cytocentre was observed surrounded by large numbers of small smooth-surface vesicles with

electron lucent contents and relatively small phagolysosomes. However, distinct signs of phagocytic activity was never observed.

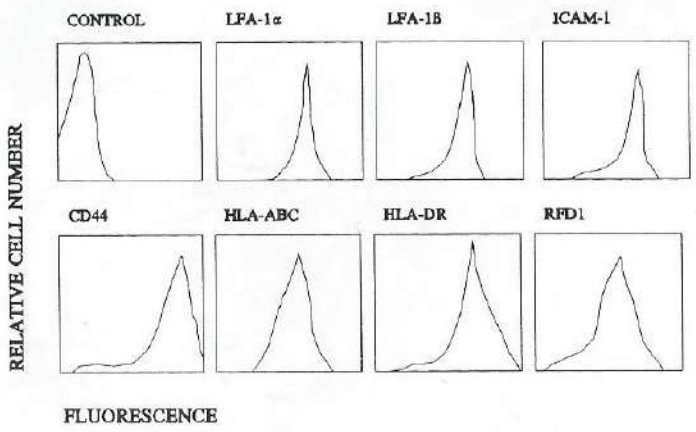

Figure 4b: Expression of surface antigens on blood DC of healthy controls. Blood DC were isolated from the peripheral blood and incubated with mAb against cell surface antigens followed by FITC-conjugated goat anti-mouse Ig.

Discussion

Defects in cellular immunity are reported in patients with chronic infections of the upper respiratory tract (1-5). These defects could be related to the presence of immunosuppressive retroviral p15E-like proteins in the serum of these patients (4,5). p15E, a transmembraneous envelope protein present in retroviruses, plays an important role in the suppression of the immune response as seen in retroviral infection (6-8).

In our present study we have examined the cluster capability of blood DC from patients with chronic infections of the upper airways. In the three patients' groups (PCD, PPN, CPR) decreased cluster capability of blood DC was detected. Furthermore, the retroviral p15E-like proteins present in the serum of these patients had inhibitory activity on the cluster capability of healthy donor DC. We could find an inverted correlation of -0.48 ($p < 0.001$) between impaired cluster capability and the presence of p15E-like serum proteins. DC play a key role in the induction of the immune response as antigen-presenting cells (9-12). They form clusters with T cells and this process is in the initial phase antigen independent (13). Since it is known that adhesion receptors play a critical role in the DC-T cell adhesion (13-16), we analyzed the expression of adhesion receptors on the surface of blood DC of these patients (Table 1). This analysis revealed that the cells express (LFA)-1 ($CD11_a$/CD18), ICAM-1 (CD54) and CD44. However, no difference in expression was detected as compared to blood DC of healthy donors.

Furthermore, incubation of healthy donor DC with patients' retroviral p15E-like LMWF did not significantly diminish the expression of the adhesion receptors (data not shown), despite significant inhibition of the dendritic cell cluster capability. The p15E-like LMWF might influence an additional adhesion pathway since it is known that anti-LFA-1 antibodies do not completely prevent binding of T cells to blood DC (16). Another mode of action can be that the p15E-like LMWF inhibit the activation status of these receptors or interact with intracellular signal pathways. Recently, it has been shown that a retroviral p15E derived

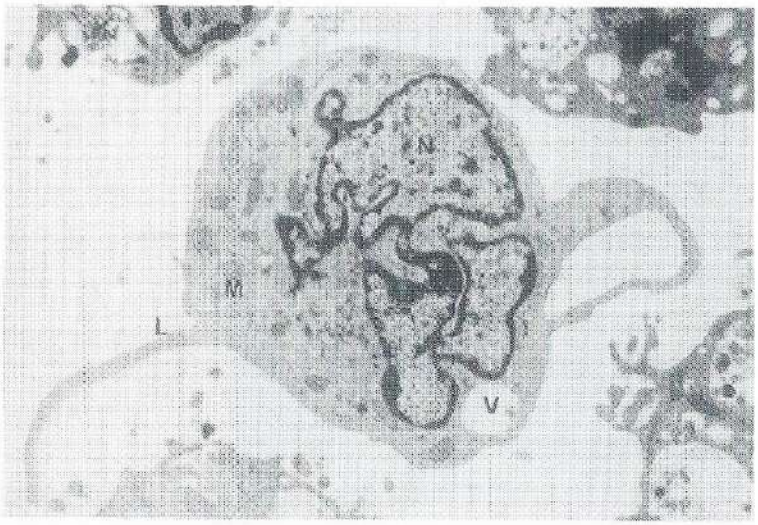

Figure 5: Electron microscopical photograph of a patients' blood DC. The cell has an irregular outline with lamellipodia (=L) and the nucleus (=N) is eccentrically localized, M = mitochondria, V = vacuole. Magnification x 4200.

hexapeptide can inhibit the intracellular Ca^{2+} changes in monocytes in response to fMLP (31).

An explanation of the diminished cluster capability could not been found in morphological changes of the patients' DC as studied by immunocytochemistry and electron microscopy. Ultrastructural analysis of patients' blood DC showed no significant difference in the presence of cell organelles as compared to the blood DC of healthy donors.

In conclusion, patients with chronic infections of the upper respiratory tract have defects in the cellular immunity. We showed that blood DC from these patients have a decreased cluster capability, and that the presence of immunosuppressive retroviral p15E-like proteins in the serum of these patients could be related to this phenomenon. These results indicate that even at the stage of in the induction of the immune response defects are present.

Acknowledgements

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Chapter 6

Improvement of cellular immune defects in patients with chronic upper airway infections. Follow-up after treatment

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Summary

Defects in cellular immunity, viz. decreased dendritic cell cluster capability, decreased monocyte polarization and defective hypersensitivity (type IV) skin tests upon commensal bacterial antigens, are found in patients with chronic upper airway infections. Low molecular weight, retroviral p15E-like proteins, present in patients' serum play a major role in the pathogenesis of these defects. Moreover, it has been shown that the expression of p15E-like proteins is high in chronic inflamed nasal mucosa.

In the present study, we show that the inhibitory effects on monocyte chemotaxis of healthy donor monocytes of retroviral p15E-like proteins in serum of patients with chronic upper airway infections (polyposis nasi, chronic purulent sinusitis) after treatment (endoscopic sinus surgery with removal of the inflamed mucosa and postoperatively topical steroids) decreased. This decrease of inhibitory activity is associated with improvement of the earlier mentioned defects in cellular immunity. Our results support the concept that the

chronically inflamed nasal mucosa is the production site of retroviral p15E-like proteins in chronic upper airway infections causing defects in cellular mediated immunity and thereby aggravating the infections.

Introduction

p15E, the retroviral transmembrane envelope protein, mediates immune dysfunction as seen in retroviral infection (1-4). This suppression involves in particular lymphocyte blastogenesis, lymphokine secretion and monocyte chemotaxis. Low molecular weight serum factors (LMWF) (Mw < 25kD) which are structurally and functionally related to p15E have been found in patients with chronic infections of the upper respiratory tract (5-8). The presence of these proteins in patients' serum could be related to the defects in cellular immunity found in these patients (5,8). These defects i.e. impaired chemotactic responsiveness of monocytes, decreased cluster capability of blood dendritic cells and impaired delayed type hypersensitivity (type IV) skin reaction upon commensal bacterial antigens, have been reported earlier (5,8-11).
Since the expression of p15E-like proteins is predominantly limited to the mucosa of the upper airways, and is significantly higher in patients with chronic infections of the upper respiratory tract than in healthy controls (7), it has been suggested that the inflamed nasal mucosa is the production site of p15E-like proteins in patients with chronic upper airway infections. Therefore, surgical removal of the inflamed mucosa should decrease the presence and bioactivity of retroviral p15E-like proteins in the serum of these patients and subsequentially restores the cellular immune defects.
The present study reports a prospective follow-up study during a period of 1 year of patients with chronic upper airway infections who underwent endoscopic sinus surgery followed by topical corticosteroids. In this period we measured the inhibitory activity on chemotaxis of healthy donor monocytes of the retroviral p15E-like serum proteins at several times postoperatively. Furthermore, 6 months after surgery, monocyte- and dendritic cell function were determined and compared with the preoperative values. In addition, control nasal mucosa biopsies were taken 6 months after surgery. The results were related to the subjective complaints of the patients and the presence of p15E-like serum proteins.

Material and methods

Patients and healthy controls

Tissue	Positive/tested
nasal mucosa of healthy controls	
-concha inferior	10/12[1]
-concha media	6/12[1]

Table 1a: Frozen tissue reactivity of mAbs directed against p15E with normal nasal mucosa.
[1] Positive staining in basal cell layer of respiratory epithelium. More than 75% of the cells showed a staining intensity from +/++.

In this study, 2 different patient groups with chronic infections of the upper respiratory tract were evaluated and compared with healthy controls.
a. Patients with Polyposis Nasi (PPN) i.e. recurrent upper airway infections, with normal functioning cilia, and the presence of nasal polyps at the moment of surgery or endoscopic examination (n=22, 12 females, 10 males, ages ranging from 18-61 years, mean 42 years). Serum samples were taken preoperatively, 6 months and 12 months after endoscopic sinus surgery. From 10 patients, monocyte chemotaxis and dendritic cell clustering was performed preoperatively and 6 months after surgery. In addition, from 5 of these 10 patients biopsies of the middle turbinate and polyps were taken at the time of surgery and after 6 months. During the first three months of the follow-up period all patients were treated with topical corticosteroids (budesonide 400 μg daily). An IgE-mediated allergy for common inhalation allergens, i.e. positive skintests (diameter > 2 mm) and/or RAST class >2 (0.35 PRU/ml) was present in 20% of the patients.
b. Patients with Chronic Purulent Rhinosinusitis (CPR) i.e. recurrent upper airway infections not responding to adequate antibiotic treatment or surgical procedures, with normal functioning cilia, but without the presence of nasal polyps at the time of surgery or endoscopic examination (n=17, 9 females, 8 males, ages ranging from 15-66 years, mean 33 years). Serum samples were taken preoperatively, 6 months and 12 months after endoscopic sinus surgery. From 13 patients, monocyte chemotaxis and dendritic cell clustering was performed preoperatively and 6 months after surgery. In addition, from 5 of these 13 patients biopsies of the middle turbinate were taken at the time of surgery and after 6 months. During the first three months of the follow-up period all patients were treated with topical corticosteroids (budesonide 400 μg daily). An IgE-mediated allergy for common inhalation allergens, i.e. positive skintests (diameter > 2 mm) and/or RAST class >2 (0.35 PRU/ml) was present in 22% of the patients.
The patients groups were compared to healthy controls (n=20, 9 females, 11 males, ages ranging from 22-44 years, mean 33 years) without known diseases.
The leucocyte counts of all patients and the healthy controls were within normal range. In addition, the serum levels of immunoglobulins were also within normal range and specific immunoglobulins to bacterial antigen (H. influenza) were present.

Tissue	Positive/tested	
	0	6
inflamed mucosa of nasal polyps	22/22[1]	5/5[1]
inflamed mucosa in CPR	17/17[1]	4/5[1]

Table 1b: Frozen tissue reactivity of mAbs directed against p15E with inflamed nasal mucosa of patients with PPN and CPR at the time of surgery (t=0) (PPN: nasal polyps; CPR: middle turbinate) and 6 months postoperatively (t=6) (PPN and CPR: middle turbinate).
[1] Positive staining in basal cell layer of respiratory epithelium. More than 75% of the cells showed staining intensity from +/++.

Monoclonal antibodies

The mAb used were: 4F5(IgG2a) (12) and 19F8(IgG2b) (13) both specific for p15E but recognizing different epitopes (12); Control mAb were an IgG2a and IgG2b, secreted by the mouse myeloma P1.17 and MPC11.OUA cell lines respectively, both obtained from the American Type Culture Collection (ATCC, Rockville, Maryland).

Immunoperoxidase staining

Four micrometer thick sections of frozen tissue blocks were prepared with a cryostat (Jung-Reichert, Nussloch, FRG), mounted on poly-L-lysine coated glass slides, air dried, and acetone-fixed during ten minutes at room temperature. The sections were incubated for 60 min at room temperature with mAb, washed, and incubated for 30 min at room temperature with horseradish peroxidase conjugated Rabbit-anti-mouse IgG (Dakopatts, Denmark) in the presence of 5% normal human serum. Subsequently, the sections were weakly counterstained with hematoxylin.
Tissue reactivity was scored for the percentage of positive staining cells and the intensity of staining (-=negative, ±=very weak, +=weak, ++=strong). In the negative scored tissue samples, there was no reactivity present with the anti-p15E mAbs or there were no differences in reactivity between the anti-p15E mAbs and the respective control mAbs.

T cells

Mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were removed by adherence on plastic petri dishes (Falcon Plastics, Oxnard, CA) for 1h at 37°C. The non-adherent cells were depleted of B cells by a panning technique (14). In brief, plastic dishes were coated overnight with 1/100 diluted $F(ab')_2$ goat anti-human Ig (Tago, Burlingame, CA) in 0.05 M Tris-HCL buffer, pH 9.5. Per coated plate, 1 X 10^7 mononuclear cells were applied. The plates were incubated at 4°C for 1h and the nonadherent cells were harvested. The percentages of sIg^+ and $CD2^+$ lymphocytes in the T cell-enriched fraction were determined by immunofluorescence. T cell-enriched

populations contained >90% $CD2^+$ and <5% surface Ig^+ cells.

Complaints	0	6	12
nasal obstruction	87%	12%	14%
rhinorrhoea	73%	6%	21%
smell disturbances	67%	24%	36%
headache	47%	18%	14%
subjective improvement	-	82%	79%
recurrence	-	29%	36%

Table 2: Complaints of patients with PPN before (0) and 6 months (6) and 12 months (0) after endoscopic sinus surgery.

Isolation of blood dendritic cells

DC were isolated from the peripheral blood according to Knight et al (15). In brief, mononuclear cells were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. From these mononuclear cells the adherent cells were removed by incubation on plastic dishes (Falcon) for 90 min. The nonadherent cells were isolated using a 14.5% (w/v) hypertonic metrizamide gradient. The adherent cells and the cells in the pellet for the metrizamide gradient were incubated on plastic dishes for a further 16h (37°C, 5% CO_2). From the newly nonadherent population the cells were again isolated on metrizamide. All low-density cell populations were pooled and these contained 60%-80% cells with a dendritic morphology. The isolation of the cells was carried out in medium (RPMI 1640 with 25mM Hepes buffer (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratories Inc., Logan, UT), 100 U/ml of sodium penicillin G (Gist Brocades NV, Delft, The Netherlands), and 100 U/ml of streptomycin sulfate (Pharmachemie BV, Haarlem, The Netherlands).

Dendritic cell clustering

The cluster assay as described by Austyn et al (16) was performed with modifications (17). 5 X 10^4 DC were incubated (4 h, 37 °C, 5% CO_2) with 5 X 10^3 allogeneic T cells isolated from healthy donors in 250 μl flat-botommed wells (Costar, Cambridge, MA). Incubation of the cells was carried out in medium. Formed clusters (aggregates of 5 or more cells), which consist of DC and T cells, were counted by 2 persons indepently of each other using an inverted microscope (Zeiss, Oberkochen, FRG; magnification X 200) and values were expressed as the number of clusters per 6 microscopic fields.

Complaints	0	6	12
nasal obstruction	59%	15%	33%
rhinorrhoea	67%	15%	33%
smell disturbances	22%	15%	0%
headache	63%	31%	11%
subjective improvement	-	92%	89%
recurrence	-	23%	23%

Table 3: Complaints of patients with CPR before (0) and 6 months (6) and 12 months (12) after endoscopic sinus surgery.

Isolation of patients' blood monocytes

Peripheral blood mononuclear cells were isolated by Ficoll-Isopaque (Pharmacia, Diagnostics AC, Uppsala, Sweden) density gradients centrifugation and sequentially washed twice in phosphate buffered saline (PBS), pH= 7,4, containing 0,5% bovine serum albumin (BSA) and counted in suspension employing positive staining with non-specific esterase (NSE) (18). The percentage of NSE-positive cells varied from 5-25%. An enrichment for the monocytes in the Ficoll-Isopaque isolated fraction was obtained by Percoll (Pharmacia, Diagnotics AC, Uppsala, Sweden) gradient centrifugation (19).
After washing, the pellet containing the monocytes was resuspended in medium (RPMI 1640 supplemented with 10% fetal calf serum (Gibco, Breda, The Netherlands)) and carefully underlayed with an equal volume of Percoll 1,063. After centrifugation (40 minutes, 450 g) the cells were collected from the interface, washed twice in medium (10 minutes, 500g) and counted: the suspension now contained 70-95% NSE-positive cells.

Polarization Assay

The Cianciolo and Snyderman assay (20) was performed with slight modifications (21). Aliquots (0,2 ml) of the Percoll purified cell suspension containing 0,2 x 10^6 monocytes were added to 12-75 mm polypropylene tubes (Falcon Labware Division of Becton Dickinson Co, Oxford, CA, USA) containing 0,05 ml of either medium alone or medium with N-formyl-methionyl-leucyl-phenylalanine (fMLP) in a final concentration of 10nM. All experiments were carried out in triplicate.The tubes were incubated in 37°C in a waterbath for 15 minutes. The incubation was stopped by addition of 0,25 ml icecold 10% formaldehyde in 0,05% PBS, pH=7,2. The cell suspensions were kept at 4°C until counting in an hematocytometer using an ordinary light microscope (Zeiss, Germany magnification X 250). The test was read 'blindly' by two persons: 200 cells were counted from each tube. A cell was considered to be 'polarized' if any of the following characteristics were encountered : elongated or triangular shape, broadened lamellopodia, membrane ruffling. The percentage of polarized cells was calculated as follows:

$$\frac{\%\ \text{total cell polarized}}{\%\ \text{NSE-positive cells}} \times 100\%$$

Lymphocytes do not exhibit any polarization activity in this assay (20). The chemotactic responsiveness of a monocyte population is expressed as the percentage polarized cells in the presence of fMLP minus the percentage of polarized cells in the absence of fMLP. The assay has proven to be a rapid method to measure chemotaxis and outcomes of the assay correlate well with outcomes of the conventional Boyden chamber assay to measure chemotaxis to casein (21).

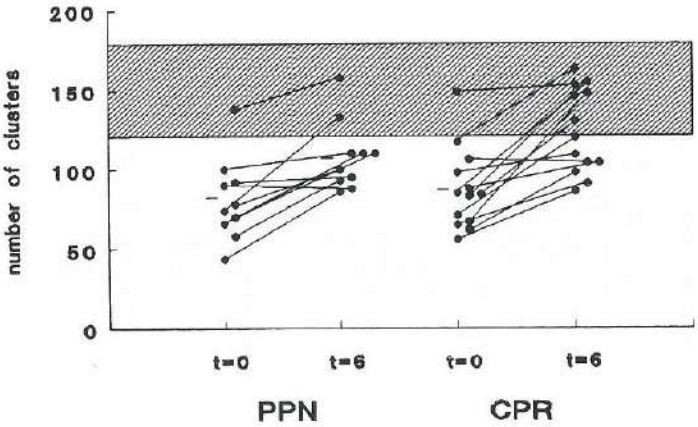

Figure 1: DC cluster assay. The clustering of blood DC of patients with PPN and CPR preoperatively (t=0) and 6 months after endoscopic sinus surgery (t=6). The values are given for individual patients. The hatched area is given for the values found in healthy controls (mean ± sd).

Monocyte isolation from healthy donors

From four different healthy donors monocytes were isolated from the peripheral blood. Buffy coats from 500 ml of human blood were obtained after informed consent from healthy donors. The monocytes were purified by successive isopycnic centrifugation and elutriator centrifugation as previously described (22) with minor modifications (23). These healthy monocytes were used to test the effects of patients' serum fractions.

Determination in patient serum of low molecular weight factors inhibiting the polarization of healthy donor monocytes (bioactivity of p15E-like serum proteins)

Sera were collected from patients by venapuncture and diluted 1:1 in saline. These dilutions were subjected to ultrafiltration through Amicon CF25 Centriflo cones (Amicon Corp., Danvers, USA) for 15 minutes at 700 g (molecular weight "cut off point" 25 kD). The residues, the low molecular weight factors (LMWF), were dissolved in phosphate buffered saline (PBS) and stored at -70 °C until further use.
The capability of the serum fractions to inhibit fMLP-induced polarization of healthy donor monocytes (elutriator purified) was determined by incubating the monocytes ($1X10^6$/ml) for 15 minutes at 37°C, either with fMLP alone or with fMLP in combination with a serum fraction (final dilution 1:60). The percentage of inhibition was calculated as follows:

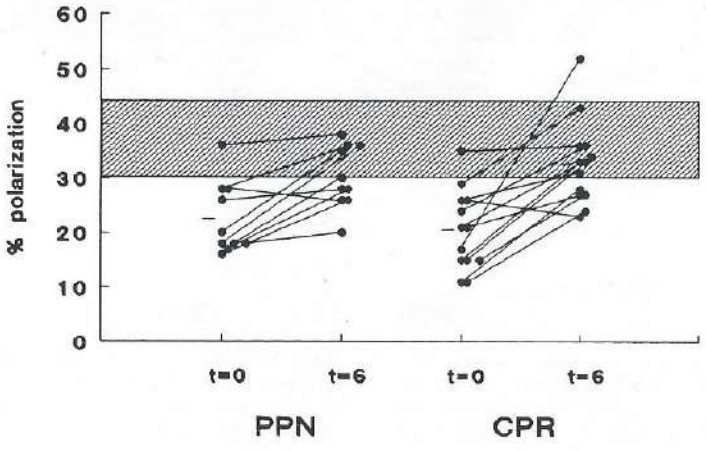

Figure 2: Monocyte polarization assay. The fMLP-induced polarization of monocytes from patients with PPN and CPR, preoperatively (t=0) and 6 months after surgery (t=6). The values are given for individual patients. The hatched area is given for the values found in healthy controls (mean ± sd).

$$\text{inhibition} = \left(1 - \frac{P_2 - P_0}{P_1 - P_0}\right) \times 100\%$$

P_0= % spontaneously polarization
P_1= % polarization after incubation with fMLP alone
P_2= % polarization after incubation with fMLP and LMWF

Serum fractions were tested in triplicate. Addition of serum fractions to non-stimulated (fMLP) donor monocytes did not affect the spontaneous polarization.

Determination of the p15E-like character of patient LMWF's

To validate the p15E-like character of the LMWF's in human serum, adsorption experiments were carried out by neutralizing the serum fractions before testing in the monocyte polarization assay with a p15E specific monoclonal antibody (19F8) in a final dilution of 1:200 (25 μg/ml) at 4°C for 16h, followed by Amicon ultrafiltration to remove formed immunecomplexes. This adsorption /neutralizing procedure was carried out twice (21). Adsorption experiments carried out with the mAb 4F5 or with 4F5 and 19F8 together did not show any difference in neutralizing the serum fractions as with 19F8 alone. Control experiments were carried out with the isotype matched control mAbs.

Statistics

Statistical analysis was performed by the Student's t-test.

Results

Reactivity of anti-P15E mAbs with nasal polyps and chronic inflamed nasal mucosa

In this study, immunohistochemical staining was performed on frozen sections. Reactivity of both anti-p15E mAbs was seen with respiratory and squamous epithelium of nasal polyps (patients with PPN) and of the middle turbinate (patients with CPR) (table 1). Reactivity with the anti-p15E mAb was also observed in the respiratory epithelium of nasal mucosa of healthy controls, however the intensity of the staining was less intense (table 2). Reactivity with the anti-p15E mAb was seen in almost all biopsies from the middle turbinate of patients with PPN and CPR, taken 6 months after surgery, and the intensity of staining could be compared with the intensity seen in healthy controls. There was no difference of staining pattern between the anti-p15E mAb 4F5 and 19F8, although the reactivity with the 4F5 mAb was more intense.

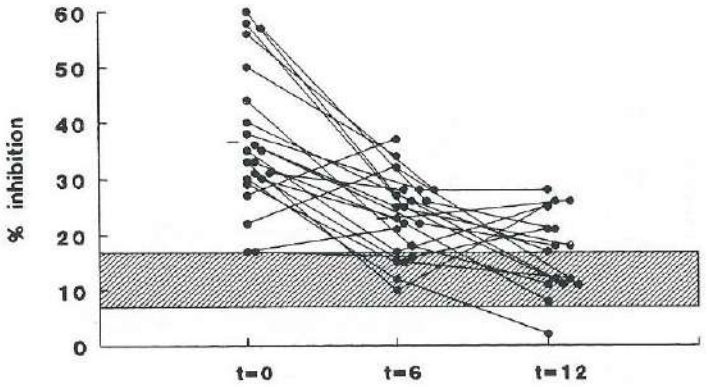

Figure 3a: Inhibition of fMLP-induced polarization of healthy donor monocytes by LMWF of patients with PPN, preoperatively (t=0), 6 months (t=6) and 12 months (t=12) after surgery respectively. The hatched area is given for the values found in healthy controls (mean ± sd).

Dendritic cell clustering

The cluster capability was assayed directly after isolation of the DC from the blood of patients and healthy controls. From 10 patients with PPN and 13 patients with CPR the DC cluster assay was performed preoperatively and 6 months after surgery. The results are shown in figure 1. The DC clustering measured 6 months after surgery was in both patient groups significantly ($p < 0,01$) higher (PPN: 108.3 ± 21.2; CPR: 123.6 ± 25.8) when compared to the values preoperatively (PPN: 81.0 ± 24.7; CPR: 87.0 ± 24.8). The increase of cluster capability was in patients with CPR higher than in patients with PPN ($p < 0,01$ vs $p < 0,05$). However, the cluster capability of DC 6 months after surgery is still significantly ($p < 0,01$) lower when compared with the values found in healthy controls (152.0 ± 29.3).

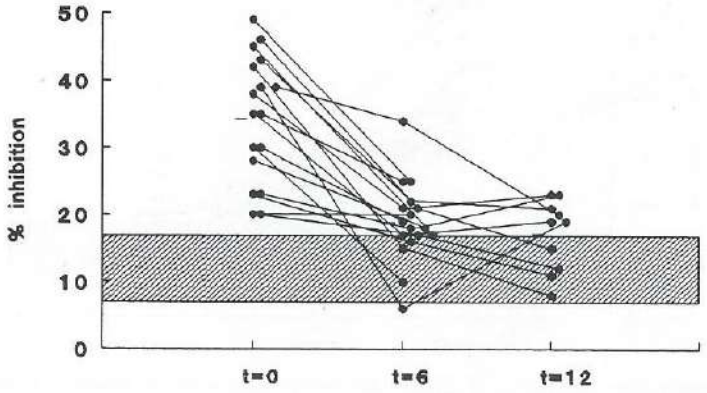

Figure 3b: Inhibition of fMLP-induced polarization of healthy donor monocytes by LMWF of patients with CPR, preoperatively (t=0), 6 months (t=6) and 12 months (t=12) after surgery respectively. The hatched area is given for the values found in healthy controls (mean ± sd).

Monocyte polarization

The monocyte polarization assay was performed directly after monocytes were isolated from the blood of patients and healthy controls. From 10 patients with PPN and 13 patients with CPR, the assay was performed preoperatively and 6 months after surgery. Figure 2 shows the results. The monocyte polarization, 6 months after surgery, was in both patient groups significantly ($p<0,01$) higher (PPN: 30.1 ± 5.2 (mean ± sd); CPR: 32.8 ± 7.7), when compared to the preoperatively values (PPN: 22.5 ± 6.3; CPR: 20.5 ± 7.0). The values found in patients with PPN, 6 months after surgery, were still significant ($p<0,01$) lower when compared to the values found in healthy controls (37.4 ± 7.0).

Influence of low molecular weight -retroviral like- serumfactors on fMLP induced monocyte polarization

The results of the inhibition of the fMLP induced polarization of healthy, elutriator purified, donor monocytes by LMWF present in serum of patients with PPN and patients with CPR are shown in figure 3a and 3b, respectively. The LMWF of patients with PPN and CPR isolated from the serum preoperatively all showed significant ($p<0,001$) inhibition of fMLP induced monocyte chemotaxis (PPN: 36.7 ± 12.4; CPR: 34.4 ± 9.4, respectively) compared to the inhibitory activity of LMWF found in the serum of healthy donors (12.0 ± 4.9). In both patient groups a clearly significant ($p<0,001$) decrease of inhibitory activity was observed in the LMWF isolated from patients' serum 6 months (PPN: 23.0 ± 7.1; CPR: 18.8 ± 6.1) and 12 months (PPN: 16.7 ± 7.5; CPR: 17.1 ± 5.3) after surgery. However, the inhibitory activity of patients' LMWF isolated 12 months after surgery still showed more activity than the activity of LMWF found in the serum of healthy donors ($p<0,05$). Three patients with PPN had an increase of p15E-like bioactivity after 6 months. Recurrence of small polyps was seen in two of these three patients. Furthermore, one patient with PPN who had an increase of p15E-like inhibitory activity in the serum 12 months after surgery, as compared

with 6 months after surgery, suffered from rhinitis for 2 weeks before testing. An increase of p15E-like inhibitory activity was accompanied with a recurrence rate of nasal polyps of 66% (2 of 3 patients), whereas a decrease of p15E-like inhibitory activity was accompanied with a recurrence rate of nasal polyps of 10% (2 of 20 patients). In patients with CPR, only one patient showed an increase of p15E-like serum activity. A clinical explanation could not be found.
The inhibition was caused by the presence of p15E-like proteins in the LMWF's, since after neutralizing the serum fractions with a p15E specific mAb (19F8) before testing in the monocyte polarization no inhibition could be detected (Fig. 4).

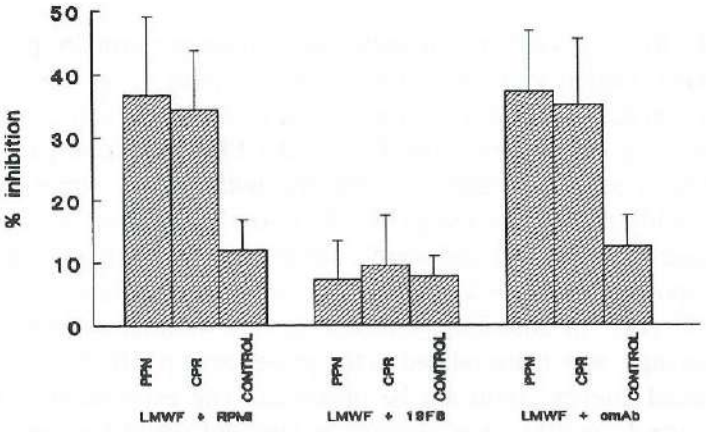

Figure 4: The p15E-like character of the inhibition of fMLP-induced polarization of healthy donor monocytes by LMWF of patients with PPN and CPR is validated by performing the polarization assy with LMWF after adsorption with anti-p15E mAb (19F8) or control mAb (cmAb) (mean ± sd).

Clinical features

The clinical data of patients with PPN and CPR are given in table 2 and table 3, respectively. In both patient groups, the majority of the patients were satisfied with the postoperative results. The recurrence rate of nasal polyps, as demonstrated with nasal endoscopy, at 6 and 12 months after endoscopic sinus surgery was 29% and 36%, respectively. 23% of the patients with CPR had recurrent inflamed mucosa.
The values of the performed assays (monocyte polarization, dendritic cell clustering, inhibitory effects of p15E-like proteins) found in patients with IgE-mediated allergy, did not show any significant differences when compared to the values found in patients without IgE-mediated allergy. Furthermore, profound differences in expression of p15E-like proteins in nasal mucosa, between patients with and without IgE-mediated allergy, could not be observed.

Discussion

P15E, the retroviral transmembraneous envelope protein, plays an important role in the suppression of the immune system as seen in retroviral infections (1-4). The expression of retroviral p15E-like proteins is demonstrated in chronic inflamed nasal mucosa, and is high enough to produce detectable serum levels. This is seen e.g. in patients with PPN and CPR (7). These p15E-like proteins are related to the presence of defects in cellular immunity in patients with chronic upper airway infections (5,8).
This study shows that the p15E-like bioactivity in patients' serum decreases after removal of the inflamed mucosa by functional endoscopic sinus surgery. Supplementary effects of the application of topical steroids is proposed, since it is known that corticosteroids reduce the inflammatory reaction by inhibition of cytokine release (24). In addition, restoration of the cellular immune defects (monocyte chemotaxis, dendritic cell clustering), which are related to the presence of p15E-like proteins, was observed. Additional effects of IgE-mediated allergy could not be observed. The expression of p15E-like proteins in biopsies of the middle turbinate 6 months after surgery is low and could be compared with the intensity seen in normal nasal mucosa. These data support the concept that the inflamed (para)nasal mucosa in chronic upper airway infections is the major production site of retroviral p15E-like proteins, which subsequently cause defects in cellular immunity. Removal of the mucosa and treatment with topical corticosteroids is followed by a decrease of p15E-like activity in patients' serum, which results in restoration of the defects in cellular immunity.

Chapter 7

General Discussion and Conclusions

In this thesis the presence of p15E-like proteins and their related defects in cellular immunity in chronic upper airway infections are described. The main conclusions are:

1. The expression of p15E-like proteins in human tissues is limited to the epithelia of the respiratory tract.
2. In chronic upper airway infections, the chronically inflamed mucosa of the upper respiratory tract is the main production site of p15E-like factors.
3. p15E-like proteins are associated with defective monocyte polarization and dendritic cell clustering, seen in patients with chronic upper airway infections.
4. Treatment of chronic upper airway infections by endoscopic removal of chronically inflamed mucosa and postoperative application of topical corticosteroids, results in reduction of the inhibitory activity of p15E-like proteins and subsequently in improvement of monocyte polarization and dendritic cell clustering.

Patients with chronic upper airway infections

In this study several patient groups, all characterized by chronic upper airway infections, were analyzed. These groups consist of: patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; patients with chronic rhinosinusitis, with normal functioning cilia and with nasal polyps (PPN); patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR). The main clinical complaints of these patients were purulent nasal discharge, nasal obstruction, impairment of smell and headache. In all patient groups, p15E-like serum proteins and defects in cellular immunity could be demonstrated. These defects were of the same magnitude. Thus, these defects are not related to one specific kind of upper airway disease (CPR), but related to chronic inflammatory reactions of the upper airways in general. The observation that p15E-like proteins are present in patients with PPN and CPR (both acquired

diseases) and also in PCD, in which the chronic upper airway infections are caused by congenital defective mucociliary clearance, suggests that the production of p15E-like proteins is a result of the inflammatory reaction in the upper airway mucosa itself. IgE-mediated allergy does not have an enhancing effect: there were no significant differences observed in patients with an IgE-mediated allergy when compared to patients without an IgE-mediated allergy.
The presence of p15E-like proteins in the serum of patients with chronic upper airway infections could be related to diminished monocyte polarization and diminished cluster capability of blood dendritic cells. The inhibition of monocyte polarization might be caused by receptor-antagonism of N-formylpeptides (1). Additional immunosuppressive sites may be present since p15E-like proteins also inhibit monocyte polarization induced by Zymosan-stimulated human serum and lymphocyte-derived chemotactic factor (2). p15E-like immunosuppressive effects are already present at the level of the induction of the immune response, since DC clustering is inhibited by these proteins. However, the exact mechanism of diminished clustering caused by p15E-like proteins is not elucidated yet. The early, antigen-independent, association of dendritic cells and T cells is mainly mediated via the LFA-1/ICAM-1 pathway. p15E-like proteins do not change the expression of adhesion receptors on the cell surface. These factors might influence additional adhesion pathways or interfere with the activation status of adhesion receptors or intracellular signal pathways. Evidence for the latter is given by experiments in which p15E-related synthetic hexapeptides showed interference with intracellular Ca^{2+} changes in monocytes in response to fMLP (1).

The role of retroviral p15E in upper airway infections

We have demonstrated that in healthy persons the expression of p15E-related proteins is limited to the epithelia of the upper respiratory tract. Increased expression was seen in inflammatory diseases of the airways and in squamous cell carcinoma of the head and neck region. The expression correlates well with the presence of p15E-like proteins in patients' serum. Removal of the inflamed upper airway mucosa and additional treatment with topical corticosteroids were associated with a decrease of P15E-like inhibitory effects in serum fractions. Furthermore, we tested in a limited number of patients with nasal polyps whether p15E-like proteins could be isolated from the polyps (Scheeren, unpublished data). From these tissues, we could isolate low molecular weight factors (LMWF's) which exerted the same inhibitory effects as the p15E-like serum proteins. The inhibition of LMWF's isolated from nasal polyps was caused by the presence of p15E-like proteins in the LMWF's, since after neutralizing the LMWF's with monoclonal antibodies directed against p15E before testing in the assays no inhibition could be detected. Thus, it appeared that the inflamed upper airway mucosa is the production site of p15E-like proteins in chronic upper airway infections.
p15E-like proteins could not be detected in the sera of patients with chronic inflammatory bowel disease or

chronic dermatitis. Furthermore, patients with squamous cell carcinoma of the lung or cervix, adenocarcinoma, melanoma or lymphoma did not express p15E-like proteins in either tissues or serum. These observations suggest that p15E-like proteins play only a significant role in (upper) airway disease. The immunosuppressive effects of p15E-like proteins and their related defects in cellular immunity can result in an inadequate immune response with the persistence of the inflammatory reaction. In malignant disease, it has been suggested that p15E-like proteins are one of the factors responsible for the diminished capacity of macrophages to invade tumors, a phenomenon assumed to represent an important mechanism in tumor rejection.
In addition, the presence of p15E-like proteins has also been demonstrated in the sera of patients with autoimmune diseases (Graves' disease, insulin-dependent diabetes mellitus). The origin and role of p15E-like proteins in these diseases are not clear yet (3).

The origin of human p15E-like factors

Several observations support the idea that human p15E-like proteins might be of endogenous origin. The expression of p15E-like proteins has been demonstrated in non-virus induced tumors and tumor cell lines (4-6) as well as in chronic inflamed tissue (7,8). During tumorigenesis and chronic inflammatory reactions, endogenous retroviral env-genes may be activated. Env-genes of human endogenous retroviruses have been well documented (9). Moreover, env-genes that share homology with retroviral p15E have been found in human DNA and the presence of mRNA derived from these genes has been detected in most normal human tissues and tumor cell lines (10-12). However, the exact source of human p15E-like proteins has not been clarified yet. For that reason, it would be helpful if these human p15E-like factors were purified to homogeneity. Several efforts, by different laboratories, have been made without any success. The major reason for these unsuccessful purification efforts might be the very small amounts of p15E-like proteins present in tissue (head and neck tumors, chronic inflamed upper airway mucosa) or serum. If sufficient amounts of tissue with p15E-like proteins can be used, purification of these proteins should be successful. Purified p15E-like proteins enable the acquisition of sequence data, which in turn facilitate cloning of p15E-related proteins. Future research on human p15E-like proteins should focuss on these aspects.

Summary

Several factors, such as anatomic abnormalities, defects in mucociliary clearance, modifications of nasal secretions, defects in humoral and cellular immunity, may contribute to the pathogenesis of chronic upper airway infections. In patients with chronic purulent rhinosinusitis, defects in cell-mediated immunity have been found. Furthermore, it has been demonstrated that these defects could be associated with the presence of proteins related to the retroviral transmembrane (TM) protein p15E.
In this thesis, we have examined the role of p15E-like proteins and defects in cell-mediated immunity in the pathogenesis of chronic upper airway infections.

In ***chapter 1***, a summary is given of the defense mechanisms of the upper respiratory tract. Furthermore, the anatomy of the nose and paranasal sinuses is described.

In ***chapter 2*** we studied the immunohistochemical distribution of p15E-related proteins in normal, inflamed and neoplastic human tissues and correlated the findings with the presence of these proteins in patients' sera. Demonstration of p15E-like proteins in sera of patients with upper airway infections and of patients with head and neck carcinomas correlated exclusively with the presence of p15E in normal and pathologic epithelium of the upper respiratory tract. p15E was not demonstrated in epithelia of other localisations. Our results suggest that chronic stimulation or neoplastic transformation of the epithelia of the upper respiratory tract stimulates the production of p15E-like proteins leading to their reported immunosuppressive actions.

Chapter 3 describes the presence of partial defects in cellular immunity, i.e. impaired delayed type hypersensitivity (type IV) skin reactions upon commensal microorganisms of the upper respiratory tract and impaired chemotactic responsiveness of monocytes in three different groups of patients, all characterized by chronic upper airway infections: 1) patients with primary ciliary dyskinesia (PCD), a congenital disorder of respiratory cilia, resulting in absence of mucociliary clearance and, as a consequence, in chronic respiratory infections; 2) patients with chronic rhinosinusitis, with normal

functioning cilia and with nasal polyps (PPN); 3) patients with chronic rhinosinusitis with normal functioning cilia but without nasal polyps (CPR).
Our results show that in all three groups the majority of the patients had defects in cellular immunity associated with the presence of p15E-like proteins in their serum. These defects were of the same magnitude. Thus, these defects are not related to one specific kind of upper airway disease but related to chronic upper airway infections in general.

Furthermore, we studied whether defects could also be found at the induction stage of the immune response in patients with chronic upper airway infections. In the induction of the immune response, dendritic cells (DC) play an important role and they initiate T cell responses which occur in cell clusters of DC and T cells. In ***chapter 4***, the molecular basis of adhesion of T cells to blood DC is described. Analysis of the expression of adhesion receptors on the cell surface of blood DC revealed that these cells express LFA-1 (CD11a/18), ICAM-1 (CD54), LFA-3 (CD58), and CD44, but are VLA-4 (CD49d) and VCAM-1 negative. The LFA-1 pathway was found to play a key role in T cells-blood DC adhesion. The analysis of the cluster capability of DC of patients with chronic upper airway infections is described in ***chapter 5***. In all three patient groups (CPR, PPN, PCD) a decreased cluster capability was found and could be associated with the presence of immunosuppressive retroviral p15E-like serum proteins. Furthermore, an morphological analysis of dendritic cells by immunocytochemistry and electron microscopy was performed but a morphological explanation for the impaired DC function could not be found.

In ***chapter 6***, the improvement of dendritic cell cluster capability and monocyte chemotaxis after treatment (endoscopic removal of the inflamed mucosa followed by the application of topical corticosteroids) in patients with nasal polyps (PPN) and in patients with chronic purulent rhinosinusitis (CPR) is described. This improvement is related to the decrease of inhibitory activity of p15E-like serum proteins. These results support the concept that chronically inflamed (para)nasal mucosa is the main production site of immunosuppressive retroviral p15E-like proteins in chronic upper airway infections causing defects in cell-mediated immunity and thereby aggravating the infections. Therefore, adequate treatment of chronic upper airway infections is important.

Samenvatting

Meerdere factoren, zoals anatomische afwijkingen, verminderd mucociliair transport, veranderingen in de samenstelling van neussecreet, defecten in de humorale en cellulaire immuniteit, kunnen een rol spelen bij het ontstaan van chronische bovenste luchtweginfecties. Bij patienten met chronische purulente rhinosinusitis zijn afwijkingen in de cellulaire immuniteit aangetoond die konden worden geassocieerd met de aanwezigheid van eiwitten die gerelateerd zijn aan het retrovirale, transmembraneuze, eiwit p15E.
In dit proefschrift wordt de rol van p15E-achtige eiwitten en afwijkingen in de cellulaire immuniteit bij het ontstaan van chronische bovenste luchtweginfecties besproken.

In ***hoofdstuk 1*** wordt een overzicht gegeven van de afweermechanismen die aanwezig zijn in de bovenste luchtwegen. Tevens wordt de anatomie van de neus en neusbijholten beschreven.

In ***Hoofdstuk 2*** wordt de distributie van p15E-achtige eiwitten in normaal, ontstoken en neoplastisch humaan weefsel door middel van immuunhistochemische technieken onderzocht. De resultaten werden gecorreleerd aan de aanwezigheid van deze eiwitten in het serum van de patienten. De aanwezigheid van p15E-achtige eiwitten in het serum van patienten met chronische bovenste luchtweginfecties en van patienten met carcinomen in het hoofd/hals gebied bleek uitsluitend overeen te komen met de aanwezigheid van p15E in normaal en pathologisch epitheel van de bovenste luchtwegen. p15E kon niet worden aangetoond in normaal, ontstoken of neoplastisch epitheel elders. Deze resultaten suggeren dat chronische stimulatie en/of neoplastische veranderingen van het epitheel van de bovenste luchtwegen de locale productie en uitscheiding van p15E-achtige immuunsuppressieve eiwitten stimuleren.

Hoofdstuk 3 beschrijft de aanwezigheid van enkele afwijkingen in de cellulaire immuniteit, n.l. verminderde huidreacties volgens het vertraagde overgevoeligheidstype (type IV) ten aanzien van commensale micro-organismen van de bovenste luchtwegen en verminderde monocytpolarisatie, bij 3 verschillende patienten groepen, allen met chronische bovenste luchtweginfecties: 1) patienten met primaire ciliaire dyskinesie (PCD), een aangeboren afwijking van de trilharen op het epitheel, resulterend in afwezigheid van mucociliair transport en daardoor in chronische luchtweginfecties (PCD); 2) patienten met chronische rhinosinusitis met normaal functionerende trilharen en de aanwezigheid van neuspoliepen (PPN); 3) patienten met chronische

rhinosinusitis met normaal functionerende trilharen en zonder de aanwezigheid van neuspoliepen (CPR). De resultaten laten zien dat in alle patienten groepen de meerderheid van de patienten afwijkingen hebben in de cellulaire immuniteit die gerelateerd zijn aan de aanwezigheid van p15E-achtige eiwitten in het serum van deze patienten. De afwijkingen waren van dezelfde grootte en kwaliteit. Defecten in de cellulaire immuniteit zijn dus niet gerelateerd aan een specifieke aandoening van de bovenste luchtwegen, maar gerelateerd aan chronische bovenste luchtweginfecties in het algemeen.

Naar aanleiding van deze resultaten hebben we onderzocht of er al afwijkingen aanwezig waren op het niveau van de inductie van de immuunrespons bij patienten met chronische bovenste luchtweginfecties. In de inductiefase van de immuunrespons spelen dendritische cellen een belangrijke rol door het initieren van T cel reacties, hetgeen gebeurt in celclusters van dendritische cellen en T cellen. In ***hoofdstuk 4*** worden de adhesiereceptoren beschreven die een rol spelen bij de adhesie van dendritische cellen en T cellen. De expressie van adhesiereceptoren op het celoppervlak van dendritische cellen werd geanalyseerd. Dendritische cellen brengen tot expressie LFA-1 (CD11a/18), ICAM-1 (CD54), LFA-3 (CD58), en CD44 maar zijn negatief voor VLA-4 (CD49d) en VCAM-1. LFA-1 bleek een grote rol te spelen in de adhesie tusen dendritische cellen en T cellen.
De analyse van de cluster capacitieit van dendritische cellen van patienten met chronische bovenste luchtweginfecties wordt beschreven in ***hoofdstuk 5***. In alle patienten groepen (PCD, PPN, CPR) werd een verminderde cluster capaciteit aangetoond die wederom kon worden gerelateerd aan de aanwezigheid van immuunsuppressieve p15E-achtige eiwitten in het serum van deze patienten. Tevens werd door middel van electronen microscopisch en immuncytochemisch onderzoek gekeken naar de aanwezigheid van eventuele morfologische veranderingen van de dendritische cellen van patienten met chronische bovenste luchtweginfecties. Een morfologische verklaring voor het verminderd functioneren van de dendritische cellen kon niet worden gevonden.

Hoofdstuk 6 beschrijft de verbetering van de cluster capaciteit van dendritische cellen en het chemotactisch vermogen van monocyten na behandeling (endoscopische verwijdering van ontstoken slijmvlies gevolgd door postoperatief gebruik van locale corticosteroiden) van patienten met neuspoliepen (PPN) en patienten met chronische purulente rhinosinusitis (CPR). Deze verbetering gaat samen met een vermindering van remmende activiteit van p15E-achtige eiwitten in het serum van deze patienten. Deze gegevens ondersteunen de hypothese dat het chronisch ontstoken slijmvlies van de neus en neusbijholten de voornaamste bron is van immuunsuppressieve retroviraal p15E-achtige eiwitten die voorkomen bij patienten met chronische bovenste luchtweginfecties. Deze eiwitten veroorzaken partiële afwijkingen in de cellulaire immuniteit en kunnen de infecties verergeren. Deze bevindingen benadrukken nogmaals dat adequate therapie van chronische bovenste luchtweginfecties noodzakelijk is.

Abbreviations

AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
APh	acid phosphatase
BSA	bovine serum albumin
C3	complement factor 3
CD	cluster of differentiation
cmAb	control monoclonal antibody
CPR	patients with chronic purulent rhinosinusitis
DC	dendritic cell
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FDC	follicular dendritic cell
FeLV	feline leukemia virus
FITC	fluorescein isothiocyanate
fMLP	formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte macrophage-colony stimulating factor
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
H/N CA	carcinoma of the head and neck region
H7	1-(5 isoquinolinyl sulfonyl)-2 methyl piperazide
ICAM-1	intercellular adhesion molecule-1
IFN-τ	interferon-τ
Ig	immunoglobulin
IL	interleukin

kD	kilo dalton
KV	kilo volt
LAD	leucocyte adhesion deficiency
LC	Langerhans cell
LFA	lymphocyte function-associated antigen
LMWF	low molecular weight factor
mAb	monoclonal antibody
MHC	major histocompatibility complex
MIF	migration inhibition factor
MLR	mixed leucocyte reaction
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus
MW	molecular weight
NK	natural killer cell
NSE	non-specific esterase
OsO_4	osmium tetra-oxide
PAF	platelet activating factor
PBS	phosphate buffered saline
PCD	patients with primary ciliary dyskinesia
PKC	protein kinase C
PPN	patients with nasal polyps
RER	rough endoplasmatic reticulum
sIgA	secretory immunoglobulin A
SLE	systemic lupus erythematosus
TCR	T cell receptor
TGF-ß	transforming growth factor-ß
Th1	T helper cell 1
Th2	T helper cell 2
TNF-α	tumor necrosis factor-α
TM	transmembrane envelope protein(s)
TP-1	thymostimulin
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
W7	N-(6 aminohexyl)-5-chloro-1-naphthalene sulfonamide

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Chapter 1

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Chapter 7

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Curriculum vitae

Robert Scheeren werd op 10 februari 1962 geboren te Leiden. Na het behalen van het Atheneum examen aan het St. Paulus Lyceum te Tilburg in 1980, werd de studie geneeskunde aan de Rijksuniversiteit van Leiden begonnen. Het artsexamen werd behaald op 23 september 1988, waarna hij als assistent-in-opleiding (AIO) 2 jaar (tot 1 oktober 1990) werkzaam was op de afdelingen Keel-, Neus- en Oorheelkunde en Pathologie van het Academisch Ziekenhuis van de Vrije Universiteit te Amsterdam. Op 1 oktober 1990 werd een aanvang gemaakt met de opleiding tot Keel-, Neus- en Oorarts in hetzelfde ziekenhuis (opleider: Prof.dr. G.B. Snow).

CHRONIC UPPER AIRWAY INFECTIONS

The role of p15E-like proteins and defects in cell-mediated immunity

STELLINGEN

1. Humane p15E-achtige eiwitten komen voornamelijk tot expressie in het epitheel van de bovenste luchtwegen.

2. De p15E-achtige serumeiwitten die bij patienten met chronische bovenste luchtweginfecties voorkomen, worden vooral in het chronisch ontstoken slijmvlies geproduceerd.

3. Partiële defecten in de cellulaire immuniteit komen voor bij patienten met chronische bovenste luchtweginfecties en zijn al aanwezig op het niveau van de inductie van de immuunrespons.

4. Endoscopische verwijdering van chronisch ontstoken neus(bijholten)slijmvlies, gecombineerd met postoperatieve locale corticosteroidtherapie, resulteert in verminderde aanwezigheid van p15E-achtige serumeiwitten en daaraan gerelateerd, verbetering van de partiële defecten in de cellulaire immuniteit.

5. Het meten van de bioactiviteit van p15E-achtige serumeiwitten kan van ondersteuning zijn bij de follow-up van patienten met chronische bovenste luchtweginfecties.

6. De clustering tussen bloed dendritische cellen en T-lymfocyten verloopt voornamelijk via LFA-1/ICAM-1.

7. Postoperatieve controle na endoscopische neusbijholtenchirurgie dient endoscopisch te geschieden.

8. Preparaten welke naast een corticosteroid een antibioticum bevatten, zijn voor de locale behandeling van ooraandoeningen irrationeel.

9. Het feit dat verzekeraars vervanging van hoorapparaten financieren indien deze ouder zijn dan 5 jaar, moet niet per se inhouden dat elk hoorapparaat na 5 jaar vervangen dient te worden.

10. Een deel van de kritiek op het werk van de justitiële kinderbescherming komt voort uit het geprojecteerde onvermogen van de samenleving zichzelf daadwerkelijk en effectief in te zetten voor de belangen van het kind.

11. Als partners kan men scheiden, als ouders nooit.

12. De kans dat een boterham met pindakaas met de besmeerde zijde op de grond belandt is exponentieel evenredig aan de prijs van het tapijt.

13. ***Medische risico's*** in de sportwereld, is een eufemisme voor het gebruik van stimulerende middelen.

14. No man's opinions are better than his information. *Paul Getty (1960).*

Robert Scheeren