THE PATHOGENESIS OF ALLERGIC RHINITIS, CELLULAR ASPECTS WITH SPECIAL EMPHASIS ON LANGERHANS CELLS

Cover illustration: Light microscopical section of the nasal mucosa of a patients with an isolated grass-pollen allergy, after immuno-double staining. The same cell is positive for: (front) IgE labelled with FITC (green); (back) CD1 followed by incubation with GaMIg-TRITC (red)(x 7500).

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DE PATHOGENESE VAN ALLERGISCHE RHINITIS

CELLULAIRE ASPECTEN MET SPECIALE NADRUK OP LANGERHANS CELLEN

PROEFSCHRIFT

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Dit proefschrift werd bewerkt binnen de afdelingen Keel-neus-oorheelkunde van het Leyenburg Ziekenhuis, 's Gravenhage en van het Academisch Ziekenhuis Dijkzigt, Rotterdam, de afdelingen Pathologie van het Slotervaart Ziekenhuis, Amsterdam en van de Daniel den Hoed Kliniek, Rotterdam, de afdeling Immunologie van het Academisch Ziekenhuis Dijkzigt, Rotterdam, en de afdeling Electronen-microscopie van de faculteit geneeskunde van de Vrije Universiteit, Amsterdam.

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Het proefschrift werd gedrukt door Haveka B.V. te Alblasserdam.

Aan Casper
en mijn ouders

CONTENTS

Aucun vent ne peut, nez magristral,
t'enrhumer tout entier, excepté le mistral!

Edmond Rostrand, Cyrano de Bergerac

GENERAL INTRODUCTION

DEFINITION AND PREVALENCE OF ALLERGIC RHINITIS

The term allergy was introduced by von Pirquet in 1906 to describe the phenomenon of animals and humans developing an altered response to foreign substances after repeated exposure. This term did not take into account the result of the contact i.e. "supersensitivity" or "immunity" (1). Subsequently the sense of the term allergy became restricted to immune responses which induced harmful reactions to non-harmful substances. Nowadays, allergy has been defined as "untoward physiologic events mediated by a variety of different immunologic reaction" (2).

Atopy is here defined as allergy that is IgE dependent; it is characterized by development of specific IgE antibodies after exposure to even small amounts of allergen in mostly genetically predisposed individuals. The term allergen is used for an antigen (non-self macromolecule), which initiates and elicits an IgE-mediated allergic reaction.

The most important clinical manifestations of atopic disease are allergic rhinitis, allergic conjuctivitis, allergic asthma, and atopic dermatitis. Allergic rhinitis is almost exclusively IgE dependent and consequently atopic. In this thesis, the term allergic rhinitis will be used throughout.

Allergic rhinitis is an IgE-mediated hypersensitivity disease of the mucous membranes of the upper airways characterized by itching, sneezing, nasal blockage and discharge. The primary complaints in allergic rhinitis are associated with exposure to aeroallergens. Allergic rhinitis can be divided in seasonal or perennial. However, in perennial rhinitis seasonal exacerbations usually are found as well. Perennial non-allergic rhinitis, also called vasomotor rhinitis, is a chronic, non-purulent allergy-like disease of the nasal mucosa of unknown origin (3).

Allergic rhinitis is common and has a cumulative prevalence rate of approximately 5 – 15% in the population of Western Europe (4). Although positive skin tests or specific IgE to allergens in serum have been reported to occur in as much as 25 – 40% of the population of Western Europe (5,6), only 10 – 25% show clinically manifest allergic disease, due to degree of exposure, hyperreactivity of the shock organ and age of the patient (3-6). Some reports describe an increase in the incidence of atopic disease in recent years (7,8).

The number of patients with allergic and non-allergic rhinitis admitted to the ENT department of the Dijkzigt University Hospital, Rotterdam over the past 7 years ranged between 230 and 500. The prevalence of non-allergic and allergic rhinitis is indicated in figure 1. Apart from the patients seen in colloboration with the ENT department, yearly another 400 patients with allergic rhinitis are referred directly to the department of Allergology of the Dijkzigt University Hospital, Rotterdam.

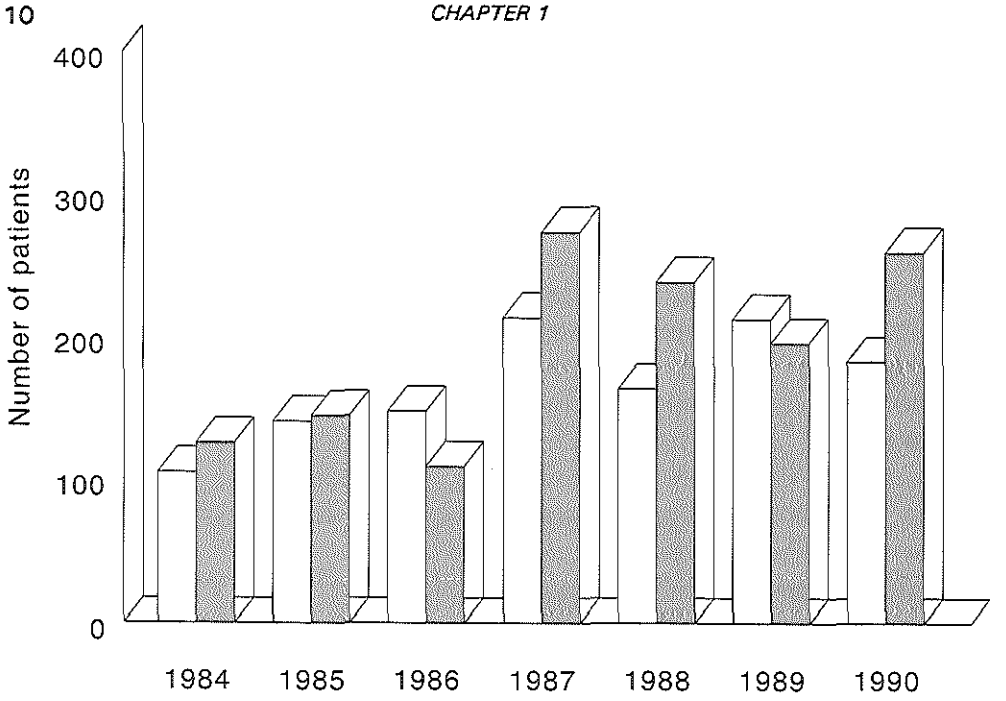

Figure 1. Prevalence of non-allergic (light columns) and allergic (dark columns) rhinitis at the ENT department of the Dijkzigt University Hospital from 1984 up until 1990.

In short, allergic rhinitis is an increasingly common disease, which warrants ample attention with regard to etiology and therapy.

HISTORIC SURVEY OF THE INSIGHT INTO THE ETIOLOGY AND PATHOGENESIS OF ALLERGIC RHINITIS

In 1873 Charles Blackley found that his complaints of sneezing, itching and a running nose in summer, were caused by grass-pollen grains (9). He performed many challenge experiments, mostly on himself, because he could only find a few other patients for miles around. Moreover, he met little cooperation from these patients, who, though interested, were not prepared to suffer for the benefit of science.

Early this century von Pirquet noted that some children showed increased reactivity after repeated vaccination with horse streptococcal antitoxin serum (1). He suggested the term allergy for the concept of such a changed reactivity. In 1923 the American researchers Coca and Cooke proposed the term atopy for clinical forms of allergy, like hay fever and asthma in 1923 (10). Two years earlier, Prausnitz and Küstner demonstrated that allergic sensitivity can be passively transferred from one individual to another (11). It was, however, not until 1966 that the serum factor involved was shown by Ishizaka (12) and independently of him at the same time also by Johansson (13) to belong to a new immunoglobulin class, IgE .

Parallel to these studies on humoral factors in allergy, cellular aspects of allergy were discovered. Paul Ehrlich described the eosinophils and mast cells in 1877 (14). Already in

1927, Eyerman described an excess of eosinophils in nasal secretion of allergic individuals manifesting nasal symptoms (15). In 1953, Riley and West demonstrated the presence of histamine in mast cells (16). The mast cell, bearing the high affinity Fc_{ϵ}-receptor for IgE, turned out to play a central role in IgE-dependent allergy (17).

In 1963 Gell and Coombs published their classification of allergic reactions (18). They subdivided the allergic reaction into 4 types. These 4 types are: the type I immediate reaction, the type II cytotoxic reaction, the type III immune complex reaction and the type IV delayed cellular immune reaction. Types II - IV are usually not considered to play a role in allergic rhinitis (3). The reaction type which constitutes the basis of allergic rhinitis is the type I, IgE-dependent, immediate reaction. When the allergen reacts with IgE bound to the surface of a mast cell, the cell degranulates releasing chemical mediators, which are responsible for the signs and symptoms that are characteristic for allergy. It is an oversimplification, however, to suppose that allergic rhinitis is only caused by mast cell degranulation.

Apart from the immediate reaction, Blackley reported already in 1873 the development of both upper and lower respiratory tract symptoms, evolving several hours after exposure to the grass-pollen he was allergic to (9). Almost a century later Dolovich et al. demonstrated an early and a late-phase reaction (LPR) occurring after skin testing. Furthermore, he found that both the early and the late-phase reaction are IgE-dependent (19). In the literature, the frequency of LPR in the nose varies considerably, which may be caused by the intensity and the methodology of the challenge procedure used in different studies (20,21). The LPR is characterized by infiltration of cells attracted to the side where mast cell degranulation takes place. During repeated provocation a steady state is reached with a complicated interplay of infiltrating cells.

Repeated exposure to an allergen has been shown to result in an increased nasal sensitivity to this allergen (22-24), called priming. When the nasal sensitivity is increased to non-specific stimuli, it is called non-specific hyperresponsiveness (24). Up till now the exact mechanisms underlying nasal priming are unknown. The allergic reaction and the consequent cascade of events constitutes such a complex process, that it is unlikely that a single cell population or mediator causes the induction of this process.

Opinions on the pathogenesis of allergic rhinitis are largely based on systemic studies and studies in other organs than the nose. Investigations on allergic rhinitis generally comprise clinical assays and/or studies of biochemical, humoral and cellular aspects of the epithelial surface and the nasal secretions.

CELLULAR ASPECTS OF ALLERGIC RHINITIS

Mast cells in nasal mucosa

Mast cells form a heterogeneous group, as assessed by morphology, fixation sensitivity, histochemical staining and intracellular content of physiological mediators. This heterogeneity imposes difficulties on the interpretation of results of different authors (25). In rodents, mast cells can be divided into mucosal mast cells and connective tissue mast

cells, which differ in appearance and behaviour. The human nasal mucosa, like the mucous membranes of rodents, contains mast cells with a different phenotypic expression compared to the mast cells of some other connective tissue sites, such as the skin (reviewed in 25,26).

The mast cell degranulation is believed to be essential for the early phase of allergic rhinitis (27,28). Quantitative studies on mast cells in nasal mucosa after allergen exposure have given widely divergent results, ranging from an overall decrease via redistribution to an overall increase (24,29,30-32). However, most likely an increase of mast cells is found in the epithelium during allergen provocation. The participation of the basophil in allergic rhinitis remains uncertain. Some groups described predominantly basophils in nasal secretion and/or nasal imprints (reviewed in 11). The relation of these cells to the mast cells in the nasal mucosa or the blood basophils remains unclear.

Mast cell mediators

During the immediate nasal response to allergen provocation an increase in histamine, prostaglandine D2 (PGD2), leucotriene C4 and D4 (LTC4,LTD4), kinines and TAME esterase has been observed (33). Most of these mediators can be secreted by various cell types, but PGD2 is considered to be produced exclusively by mast cells. Measurement of mediator release during the late nasal response demonstrated elevation in histamine, LTC4, LTD4, kinines and TAME esterase, but not in PGD2 (34). This has led to the hypothesis that the basophil and not the mast cell is important in the late nasal response. Recent studies in the lung, however, suggest that the mucosal mast cells do not produce PGD2 and hence could play a role in the late allergic reaction (25).

Eosinophils in nasal mucosa

Eosinophilia is a feature of allergic disease. The exact role of the eosinophils in allergic disease is unclear. A former hypothesis, which became popular during the 1970s, is that the eosinophils would have a role as a modulator of the allergic inflammatory reaction (35). Nowadays the most accepted hypothesis is that the eosinophil is an inflammatory cell, the degranulation of which results in tissue injury (36,37). Eosinophils are attracted among others by mediators released by mast cells and T lymphocytes (38) and eosinophils release MBP, ECP, EPO, LTC4 and PAF (33). The number of eosinophils increase in the LPR.

It goes beyond the scope of this introduction to further discuss these findings. It should be noted that, although studies have led to a wealth of information, the pathogenesis of allergic rhinitis is still not clear.

METHODS USED IN THE INVESTIGATION OF ALLERGIC RHINITIS

The nose is the part of the airway system which is best accessible for morphological and pathophysiological evaluation. Various methods have been described to obtain quantitative as well as qualitative information about the cells in the mucus covering the mucosa, in the epithelium and in the deeper layers of the nasal mucosa. Some of these methods are

subsequently described:

(a) The easiest way to obtain cells and secretions is to *blow the nose* on a plastic sheet. This method is hampered by poor reproducibility and the lack of quantitative information. Obviously, only cells which are contained in the secretion and thus dislodged from the mucosa are harvested.

(b) In the *smear technique* cells are taken from the nasal mucosa with a cotton swab and then smeared onto glass slides. This method is also easy to perform, and has the same disadvantages as the blow method (39).

(c) The *imprint technique* is used in an effort to standardize the location and the surface area from which the cells are taken. Small thin plastic strips are painted with 1% albumin to produce a sticky surface, introduced into the nose and gently pressed onto the mucosal surface. Mucus and cells adhere to the strips and can be studied after the strip has been removed from the nose. A handicap is that considerable amounts of mucus on the imprint complicate the evaluation. Moreover, only cells on the surface can be investigated.

(d) *Nasal lavage* can be performed in several ways (33,40). The advantage of this method is that a large part of the nasal mucosa can be studied, cytospins of the harvested cells can be made and the supernatant can be analysed biochemically. However, by this method only cells on the surface of the mucosa can be evaluated and quantification is difficult. The method requires active cooperation of the patient.

(e) The *nasal brush* method devised by Pipkorn et al. (41) employs a small brush of steel wire with nylon strings to harvest the cells. The cells are suspended in buffer solution so that cytospins can be made. The method takes only a few seconds, is easy to perform and reasonably well tolerated by the patient. Here, too, the evaluation is restricted to cells on the surface and some of the superficial epithelial cells.

(f) Using a small curette or Rhinoprobe (42) small *nasal scraping* specimens can easily be collected from the nasal mucosa. The procedure does not require anaesthesia, can be repeated several times and produces chunks of the superficial epithelium from a well described part of the nasal mucosa. However, evaluation of deeper layers of the epithelium and the lamina propria is not possible.

(g) *Nasal biopts* can be taken using a forceps, a punch or a small knife. A constant location where the biopt is taken, is important because of possible regional differences of the histology of the nasal mucosa (43). Usually biopts are taken from the lower edge of the inferior turbinate, 1-2 cm posterior to the anterior edge. By means of a biopsy all layers of the nasal mucosa can be studied, except the cells released in the mucus. A disadvantage is that local anaesthesia is needed. Discomfort for the patient and substantial risk of bleeding are practically eliminated by the use of a Gerritsma forceps. This forceps, designed by Victor Gerritsma in collaboration with the author, is discribed in chapter 4.

It may be concluded that: (1) biopsies are the only method to study cells in all layers of the nasal mucosa, but the procedure cannot be repeated every day; (2) the brush technique seems to be most appropriate for monitoring cellular reactions in the nasal mucosa in case of daily repetitive investigations.

HISTOLOGY OF THE NASAL MUCOSA

Most of the studies described in "cellular aspects of allergic rhinitis" are performed on superficial epithelial cell samples and nasal secretions. Only a few studies, almost all using conventional staining techniques, are performed on the full depth of the nasal mucosa.

The histological picture of the nasal mucosa was first described by Schneider (44) in 1660. The nasal mucosa consists of epithelium usually covered with mucus, the basement membrane, which separates the epithelium from the lamina propria, and the lamina propria (Fig 2).

The vestibulum of the nose and the first milimeters of the nasal cavity are lined with stratified squamous epithelium. The rest of the nasal cavity shows mainly ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium (45,46). Nasal epithelium contains a number of migratory cells. A limited number of cell populations can be distinguished without the help of special staining techniques: lymphocytes, dendritic cells and neutrophils, eosinophils and mast cells.

The lamina propria is the part of the nasal mucosa situated between the basement membrane and the underlying supportive tissue (bone, or cartilage). It is usually composed of a relatively cell-rich collagen poor, subepithelial layer with most of the mucous glands and a deeper collagen rich, cell-poor layer lying on the supporting skeleton. The normal lamina propria contains several cell populations. Cells that can be distinguished without special histochemical staining techniques are lymphocytes, neutrophils, plasma cells, dendritic cells, cells of the mono-histiocytic cell lineage, mast cells and eosinophils.

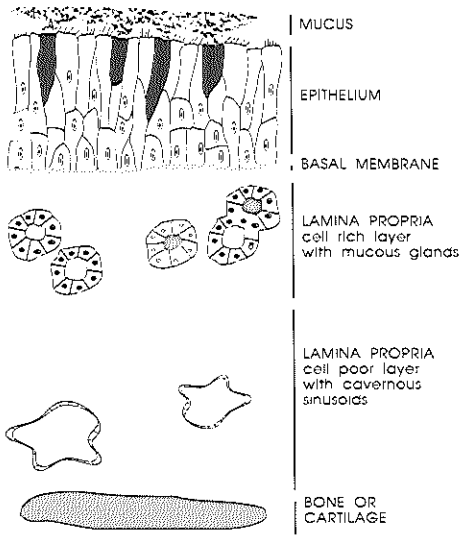

Figure 2. The nasal mucosa and its different layers

With immunohistochemical methods, employing monoclonal antibodies, it is possible to distinguish particular morphologically identical cells, when they carry different cell surface proteins. These surface marker proteins can act as antigens to which monoclonal antibodies can be generated. Differences of surface antigens can reflect different functional states of the cell.

Monocional antibodies are produced by fusion of a normal antibody-producing cell and a neoplastic cell. The resulting hybridoma cell possesses the unique combination of antibody specificity and continuous growth capability.

The surface markers of a cell, which can be receptors, ligands or enzymes, can characterize the cell and its function. Cellular receptors are not static molecules, they appear and disappear depending on the cell's function or state of maturation. The use of immunohistochemistry enables the detailed investigation of the heterogeneity and dynamics of cells in sections of the nasal mucosa.

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CHAPTER 2

LANGERHANS CELLS

Stimulation of the immune system by allergen is a necessary step in the development of atopic disease. The first and crucial step in the immune response is the binding of the allergen by antigen-presenting cells (APC) and its subsequent presentation to lymphocytes. The Langerhans cell (LC) is a potent APC. The antigen-presenting capability of LC is much greater than of blood-derived APC, like monocytes (1-3). The best-documented function of the LC is antigen presentation to T lymphocytes in contact allergic reactions in the skin (3-5). Studies performed in the last decade have shown that LC play a critical role in atopic dermatitis as well.

This chapter describes the general features of LC, their prevalence in skin, gastrointestinal mucosa and lung and the role of the LC in atopic dermatitis.

FEATURES AND FUNCTIONS OF LANGERHANS CELLS IN THE SKIN

In 1868 Paul Langerhans, a medical student at the Berlin Pathological Institute, observed a population of dendritic cells in the epidermis of human skin by using a gold chloride stain (6). The precision of his observation and description must be admired when we compare his drawings of 1868 (Fig 1) with the reproduction by enzyme-histochemistry obtained today (Fig 2). Because of their staining characteristics and morphology, he considered LC at first to be of neural origin although he later stated that: "my cells are in no way essential for nerve endings" (7).

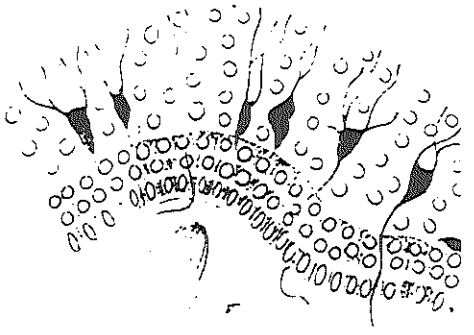

Figure 1. Original drawing of Paul Langerhans from the publication of 1868 showing a dendritic cell in the epidermis.

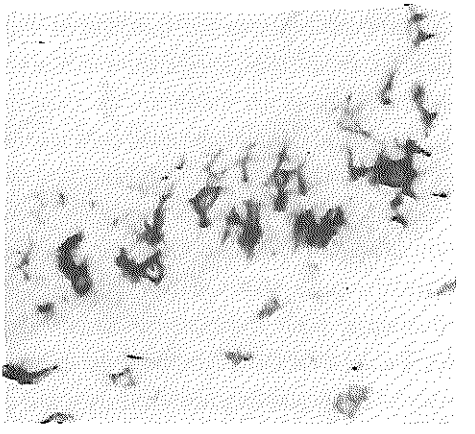

Figure 2. Langerhans cells in the epidermis displayed by enzyme histochemistry. (Courtesy of E.P. Prens)

The structure and function of these cells were not studied for nearly a century, until in 1961 Birbeck et al. observed a unique cytoplasmic granule within LC by transmission electron microscopy (8). This cytoplasmic granule is called after him, the Birbeck granule. In 1973 Silberberg et al. found clusters of LC and infiltrating lymphocytes in contact sensitivity reactions in the skin (9). They were the first to suggest a role for LC in the induction phase of delayed hypersensitivity responses. Since then, an enormous effort has been made to resolve questions concerning the function of LC.

Outside the epidermis, cells resembling LC ultrastructurally are present in a variety of tissues. These cells are called dendritic cells (DC), indeterminate cells, veiled cells or interdigitating cells, depending on their localization. LC are considered to be immature DC (10). It has been suggested that LC and interdigitating cells may originate from the same stem cell (11), but the exact phenotype of the LC precursor cell (12) and the homing mechanism of the epidermal LC are not known (13). Such LC-like cells have been demonstrated in various squamous epithelia, normal dermis, lymphatics and draining lymph nodes (14), tonsils (15), Peyer's patches of the gut (16) and blood (17).

Most of the available knowledge on the general properties of LC has been obtained from studies of the skin. LC are dendritic, bone-marrow derived cells which belong to the mononuclear phagocyte system (18,19). Immunohistochemical methods demonstrated LC to be MHC class II, S-100 (an acid calcium binding protein) positive (20,21), and to bear the CD1a (22) and CD4 antigens (23) as well as an Fc-receptor for IgG and a receptor for C3b (24). The expression of CD4 is on a lower level than that of CD1a. The LC is characterized by the presence of Birbeck granules, which can be visualized by electron microscopy and presumably derive from the cell membrane by endocytosis (25). The function of these Birbeck granules is still unknown. It has been suggested that activation of LC triggers both the endocytosis of T6 and the migration of LC (26).

The best-documented function of the LC so far is antigen presentation to T lymphocytes in contact allergic reactions in the skin (4,5,9). The site of antigen presentation to T cells is still unknown. Evidence has been presented that after antigen-binding, LC move to the dermis and migrate as veiled cells via the lymphatics, finally presenting antigen to T cells as interdigitating cells in the paracortical area of the draining lymph node (27). Resting LC and freshly isolated LC are well-equipped for antigen binding and processing, and for stimulation of activated T cells. However, these cells are weak in stimulating resting T cells. After 3 days of culture, LC loose their typical characteristics (CD1a, Birbeck granules, Fc-receptor) and change their morphology, phenotype and functional capacities so as to become indistinguishable from lymphoid dendritic cells (28,29). These cultured LC are extremely powerful in stimulating resting T cells but are weak in binding and processing the antigen. Moreover, freshly isolated LC can present whole antigen, contrary to cultured LC (DC) (29). Thus, resting LC are considered to be good APC which mature into DC which are powerful sensitizers of T cells. Epidermal LC produce interleukin-1 (30), TNF-α, IL-8 (Bieber, personal

communication), interferon-gamma (31), and prostaglandin D2 (32), thereby regulating the functional activity of other cell types in the skin.

These characteristics suggest that epidermal LC play an important role in the immune system of the skin, particularly with respect to antigen handling. Although most studies have been performed on the skin, up till now findings in other organs suggest that this particular function of the LC is not restricted to the skin.

LANGERHANS CELLS IN THE GASTROINTESTINAL MUCOSA

Very few studies have been done on antigen presentation in the human gastrointestinal tract. Wilders et al. (33) found few Ia^+ dendritic cells with little or no antigen-presenting activity in small lymphoid follicles in the wall of the normal colon. A great number of dendritic cells were found in chronic inflammatory bowel disease. These cells were described as resembling APC such as LC, veiled cells, or interdigitating cells without further characterization. Although ultrastructural studies were performed, no reference was made to Birbeck granules. Lennard et al. (34) described $CD1a^+$ cells in colorectal carcinoma, but did not find such cells in normal colon mucosa. Experimental animal studies show that Ia^+, non-lymphoid dendritic cells are present in the small intestinal villi, the Peyers patches and intestinal derived peripheral lymph (16,35). These cells are capable of processing and presenting antigens (36).

Thus, although evidence was found of the occurrence of dendritic $CD1a^+$ cells in human gastrointestinal mucosa, definite proof of the presence of LC is lacking. This means that there are no data available concerning the role of LC in human gastrointestinal allergy. Experimental animal studies, however, show that antigen-presenting dendritic cells are present in the intestinal mucosa.

LANGERHANS CELLS IN THE BRONCHIAL MUCOSA AND LUNG TISSUE

Many studies have dealt with histiocytosis-X (a disease characterized by a malignant proliferation of LC) and with involvement of the LC in various types of lung carcinoma (37,38). A few well-documented studies have been performed on LC in normal human lung tissue. $S\text{-}100^+$ cells containing Birbeck granules have been reported to occur in bronchial epithelium (39). Hammar et al. (38) found LC in lung tissue in a variety of pulmonary diseases including extrinsic allergic alveolitis. However, Sertl et al. (40) failed to find $CD1a^+$ cells containing Birbeck granules in bronchial epithelium, although they did see $HLA\text{-}DR^+$ dendritic cells. Using a tangential tissue-sectioning procedure, Holt et al. proved definitely that Ia^+ dendritic cells and $CD1a^+$ cells are present in bronchial and tonsillar epithelium. They further demonstrated that these cells form a tightly meshed network (41). Experimental animal studies show antigen-presenting dendritic Ia^+-cells within and below the airway epithelium (39,42). Holt et al. suggest that these cells serve as a last line of defence against allergic sensitization to aeroallergens which have evaded other levels of containment in the respiratory tract. He postulated that DC downregulate the IgE response via stimulation of suppressor T cells (43).

The reviewed observations suggest that dendritic antigen-presenting cells and possibly LC are present in normal bronchial epithelium. In a variety of benign and malignant pulmonary disorders the number of LC may be increased. Moreover, the role of antigen-presenting dendritic cells and LC in allergic asthma is not clear.

LANGERHANS CELLS IN ATOPIC DERMATITIS

The normal epidermis of the skin is characterized by keratinizing stratified epithelium (keratinocytes). LC, indeterminate cells, Merkel cells and melanocytes lie between keratinocytes, particularly in the middle of the deeper part of the epidermis; occasionally, a T lymphocyte is observed. The number of LC in the normal epidermis varies in different part of the body (ThM Vroom, personal communication) and ranges from 3 to 8% of the epidermal cell population.

The epidermis of clinically involved skin of patients with atopic dermatitis shows infiltration of predominantly $CD8^+$ cells and occasionally focal accumulation of LC (44). The total number of $CD1a^+$ cells per mm^2 epidermal section surface, however, is not increased (45,46).

The most striking abnormalities in the dermis of these patients concern infiltration of activated HLA-DR^+ T lymphocytes (with an elevated CD4/CD8 ratio) (47), $CD1a^+$ cells, interdigitating cells and macrophages (48,49). Therefore, the cellular infiltrate in atopic dermatitis resembles a type IV hypersensitivity reaction (49), characterized by infiltration of activated T lymphocytes, APC and macrophages.

Bruijnzeel-Koomen (50) showed that $CD1a^+$ dendritic cells in the epidermis bind IgE molecules. This phenomenon was observed in clinically involved skin, and less often in clinically non-involved skin of atopic dermatitis patients with elevated serum IgE levels. In clinically involved skin, the proportion of $IgE^+/CD1a^+$ cells ranged between 20 and 90% of the total $CD1a^+$ cell population in the epidermis. $IgE^+/CD1a^+$ cells were not described in epidermis of non-atopic controls, patients with allergic asthma, or patients with a high serum IgE due to schistosomiasis (50). Immuno-electron microscopic studies of epidermal cell suspensions of atopic dermatitis patients revealed that IgE molecules were present on $CD1a^+$ cells which contained Birbeck granules, and must therefore be considered LC. Occasionally, $IgE^+/CD1a^+$ cells without Birbeck granules (indeterminate cells) were observed. These findings have been confirmed by other groups (51,52). IgE was also found to be present on APC in the dermis (53,54).

Further studies of epidermal cell suspensions from atopic dermatitis patients revealed that IgE is bound to LC via an Fc-receptor (55). This $Fc_\epsilon R$ on LC is trypsin resistant, has affinity for IgG, binds with BB10 (a monoclonal antibody directed against the $Fc_\epsilon R$ on eosinophils, platelets and macrophages) and does not bind to anti-CD23 antibody directed against the $Fc_\epsilon R$ on B lymphocytes. Furthermore, binding of IgE to its receptor on the LC may be inhibited with anti-CD1a, which suggests that the IgE receptor and the CD1a antigen are linked. The significance of this putative association is, however, not clear.

INTERACTION BETWEEN LANGERHANS CELLS AND T LYMPHOCYTES IN ALLERGY

A postulated functional role for IgE^+ LC was investigated by the group of Bruijnzeel-Koomen by performing *in vitro* T lymphocyte proliferation tests using epidermal IgE^+ LC and IgE^- LC from patients with atopic dermatitis as APC (56). The T cell response was determined in tests with aeroallergens and Candida albicans as non-allergic antigen. The T cell response to Candida albicans, using IgE^+ LC, was in the same range as with IgE^- LC. In contrast, the T cell response to house dust allergen (HDA) was dependent on the type of APC used. A T cell response to HDA was only observed in the presence of IgE^+ LC. IgE^- LC from atopic dermatitis patients and LC from normal controls were unable to present HDA. Preincubation of IgE^+ LC with anti-IgE or anti-kappa/lambda antibodies inhibited the HDA-induced T cell proliferation, whereas the response to Candida was not affected. These *in vitro* results, which demonstrated the necessity of cell-bound IgE for the presentation of aeroallergens by LC, strongly correlate with the occurrence of a positive delayed patch reaction to the same allergens *in vivo*. When the LC of a patient appeared to be IgE^-, the *in vitro* proliferative response as well as, in most cases, the *in vivo* patch test reaction to the same antigens was negative (56). These experiments demonstrate that there are at least two different mechanisms by which LC capture antigens for antigen presentation, via HLA-DR and via IgE. In one of these, cell-bound IgE plays a crucial role.

Although these results strongly suggest that IgE^+ LC are necessary for presentation of aeroallergens to T cells, it does not reveal the mechanism underlying this presentation.

Recognition of allergen by sensitized T cells leads to a cascade of regulatory lymphokine-mediated events. Activated T cells stimulate other infiltrating cells by producing among others IL-3, IL-4, IL-5 and IL-6. In the mouse, IL-3, in cooperation with IL-4, stimulates the growth of mast cells (57). Although human IL-3 stimulates basophils (58), the role of IL-3 and IL-4 in supporting human mast cell growth has yet to be established.

In mice, epidermal LC were found to be capable of inducing antigen-specific T cells of the Th2 subtype, which are able to produce IL-4 (59). Both mouse and human IL-4 stimulate IgE production by B cells (60). Moreover, IL-4 induces a low affinity Fc_ε receptor (CD23), not only on B cells (61), but also on monocytes and LC (62). It induces differentiation of monocytes into dendritic cells as well as increases MHC class II expression on monocytes (63). The data obtained so far indicate that IgE production, induced by IL-4, is mediated through an increased release of soluble CD23, called IgE-binding factor. Furthermore, recent data from the Bieber group suggest that LC, which become $CD23^+$ after stimulation by IL-4, are potent producers of IgE-binding factors (64).

Th2 cells in mice also produce IL-5, which enhances the effects of IL-4. Moreover, IL-5 stimulates proliferation and differentiation of eosinophils. Finally IL-6 induces, among others, the terminal differentiation of B cells into plasma cells. It is unclear whether human T cells can be subdivided into Th1 and Th2 type cells (65). Results from studies on peripheral blood

T cells are contradictory (66,67). In the skin of patients with atopic dermatitis a bipartition was found between T cells producing IL-4 (Th2) and cells producing IL-2 (Th1) (Reysen FC v, Mudde GC, Bruynzeel-Koomen CAFM. Manuscript in preparation).

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CHAPTER 3

AIM OF THE STUDY

Present ideas concerning the pathogenesis of allergic rhinitis are largely deduced from systemic investigations and extrapolated from studies in the skin and the lung. Studies on allergic rhinitis generally comprise clinical aspects and/or biochemical, humoral and cellular features of the epithelial surface and the nasal secretions. Little is known about what happens in the nasal mucosa itself.

The aim of the investigations, described in this thesis, was to investigate the cellular aspects of the pathogenesis of allergic rhinitis using immunohistochemical staining techniques in biopsy specimens of nasal mucosa.

A biopsy method was developed, which can be repeated several times (chapter 4). Biopsy specimens of nasal mucosa of patients with allergic rhinitis and non-allergic controls were studied to broadly characterize and quantitate the cellular infiltrates (chapter 5 and 7). Mast cell dynamics and degranulation were studied in an allergen provocation study out of the grass-pollen season (chapter 6). Although mast cell degranulation has long time been considered to be the proper model to explain allergic rhinitis, the findings described in the first part of this thesis, suggest that other cell types, particularly antigen presenting cells and T cells, are involved as well.

The antigen presenting cells were further characterized and their contact with other cell types was evaluated (chapter 8). The dynamics and the behaviour of these cells in the nasal mucosa of patients with isolated grass-pollen allergy were studied in biopsy specimens taken before, during and after natural provocation (chapter 9) and during allergen provocation studies outside the grass-pollen season (chapter 10 and 11).

Finally the effect of treatment with the nasal corticosteroid spray Fluticasone on antigen-presenting cells, and especially the Langerhans cell was studied (chapter 12).

CHAPTER 4

A MILD BIOPSY METHOD TO OBTAIN HIGH-QUALITY SPECIMENS OF NASAL MUCOSA

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For a study on immunological changes in the nasal mucosa of patients with an isolated grass-pollen allergy, we needed a satisfactory method for nasal biopsy that would provide biopsy specimens of adequate size (ca. 2.5 mm in diameter), sufficient depth of the lamina propria, and with undamaged epithelium. Because the biopsies had to be repeated several times in each patient, the method had to be quick, easy to perform, and cause the patient as little stress as possible.

A review of the literature indicated either a high percentage of damaged specimens, especially with disturbance of the epithelium, or a high incidence of dropping out of patients when the biopsy was performed more than once.

In collaboration with Mr. V. Gerritsma, precision-instrumentmaker of the ENT Department of the Leiden University Hospital, a forceps fulfilling our criteria was designed. This instrument (Fig 1), called the Gerritsma biopsy forceps, makes it possible to take a specimen without visible damaging the epithelium. The cutting mechanism of the instrument is particularly important. The design, providing a kind of quillotine effect, gives specimens with smooth edges (Fig 2). The knife-sharp edge of the cutting cup (A) meets the sharp edge of the conical receptacle (B) squarely, without putting tangential pressure on the specimen.
The conical shape of the receptacle is intended to reduce friction on the sample to a minimum. The circular opening in the bottom of the receptacle permits passage of the specimen without squeezing of the tissue (Fig 2). The angle formed by the open forceps is so large that even a thick hypertrophic turbinate can be biopsied without difficulty.

Biopsy specimens were routinely taken from the lower edge of the inferior turbinate, about 2 cm posterior to the anterior edge. Local anaesthesia was obtained by placing a cotton-wool carrier holding 100 mg cocaine and 3 drops of adrenalin (1:1000) under the inferior turbinate without touching the site chosen for the biopsy. The specimens were frozen immediately after being embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule, in which they were given a standardized orientation.

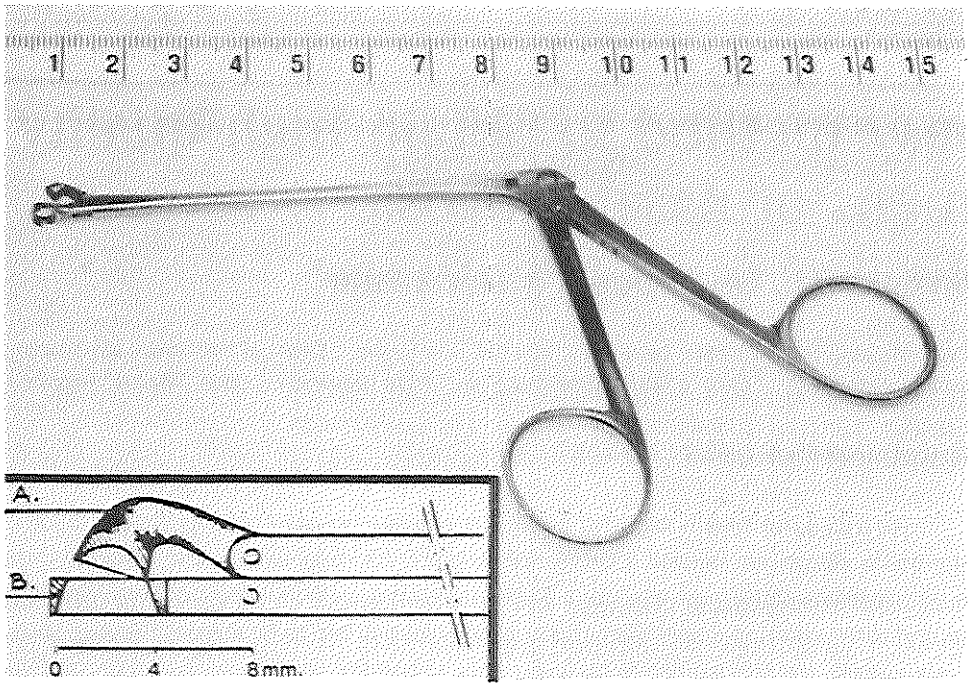

Figure 1. Gerritsma forceps, showing (inset) the knife-sharp edge of the cutting cup (A) that meets the sharp edge of the conical receptacle (B) squarely, without putting tangential pressure on the specimen.

After excision of the specimen, a small piece of gauze with vaseline was placed in the nose. The patient was expected to remove this tampon between 2 and 12 hours after the biopsy. Some patients preferred not to have the gauze. The patients were advised to use xylomethazoline nosedrops at 6-hour intervals during the first 24 hours after the biopsy.

To date, we have performed 135 biopsies in 63 patients with this Gerritsma forceps. Twenty-seven of these patients had more than one biopsy and some of them as many as five.

As a rule, the quality of the specimens was excellent (Fig 2). The samples, which had been taken sufficiently deep to permit evaluation of the epithelium as well as all of the layers of the lamina propria, showed no signs of compression. Damage to the mucosal epithelium only occurred in eight of the 135 biopts. The lamina propria could always be judged reliably. The patients underwent the biopsy procedure without problems. One patient had a significant epistaxis. Of the 29 patients in whom a second biopsy was needed, only two refused.

Our experience has led us to conclude that the Gerritsma forceps enables us to perform biopsies of nasal mucosa that provide specimens of high quality and cause minimal stress for the patient. This forceps is not only an invaluable instrument for scientific research on nasal mucosa but will certainly prove useful for the biopsy of pathological tissue in the nose and in other mucosa, especially at sites where access is difficult. Use of this Gerritsma forceps for

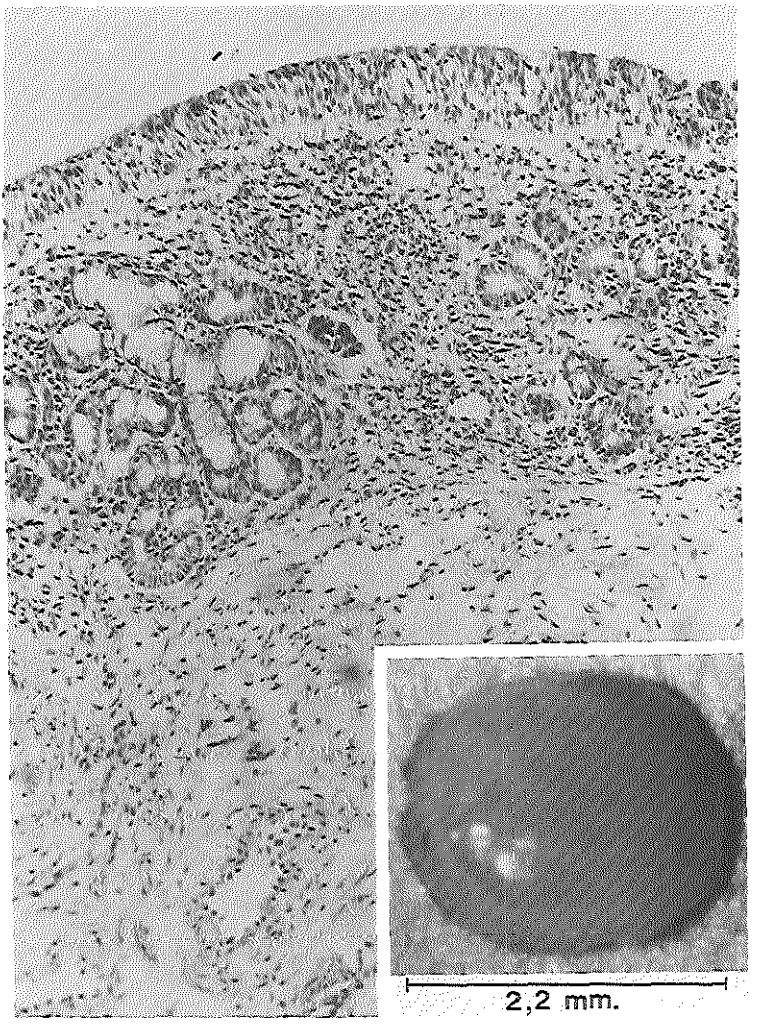

Figure 2. Light micrograph of a section of nasal mucosa biopsied with the Gerritsma forceps, showing intact epithelium and lamina propria. The specimen itself (inset) has smooth edges and no signs of compression.
HE x 70.

mucosal biopsies will enable the pathologist to make more accurate histologic diagnoses, and the all too often encountered comment "squeezed rags of tissue; definite diagnosis not possible" will no longer be appear on his reports.

Chapter 5

CHARACTERIZATION AND QUANTIFICATION OF CELLULAR INFILTRATES IN NASAL MUCOSA OF PATIENTS WITH GRASS-POLLEN ALLERGY, NON-ALLERGIC PATIENTS WITH NASAL POLYPS AND CONTROLS.

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ABSTRACT

Little is known about cellular infiltrates in nasal mucosa and the differences between these infiltrates in allergic and non-allergic patients. A reproducible and objective method making use of monoclonal antibodies for the quantification and characterization of cellular infiltrates in biopsy specimens of nasal mucosa is described. This method was used to study quantitative differences in cellular infiltrates in the epithelium and lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy, non-allergic patients with nasal polyps, and controls. A surprisingly wide variation was found in all groups. In all groups, the T lymphocytes were much more numerous than the B lymfocytes. The number of $CD8^+$ cells exceeded the number of $CD4^+$ cells in the epithelium but in the lamina propria the numbers were approximately equal. Significant differences between the three groups were found with respect to the number of $CD1a^+$, IgE^+, cytoplasmic $IgG4^+$ cells and neutrophils. No significant differences were found in the numbers of $CD4^+$, $CD8^+$, $CD14^+$, $CD22^+$, HLA-DR^+, $IgG1\text{-}3^+$ cells or eosinophils.

The use of biopsy in combination with monoclonal antibodies is an easy and well-tolerated method to study immunological reactions in the nasal mucosa. The results of this study indicate a possible role for a T cell mediated response in allergic rhinitis.

INTRODUCTION

There is only little quantitative information on the cellular infiltrates in normal and diseased nasal mucosa. Few studies have been performed to identify and quantify the cell populations occurring in nasal mucosa with the use of monoclonal antibodies against surface antigens. The lymphocyte subsets were investigated in normal human nasal mucosa by Winther et al. (1) and in diseased nasal mucosa by Hameleers et al. (2). Brandtzaeg

performed extensive studies in cells producing immunoglobulins in the nasal mucosa (3,4). Recently, immunohistochemical studies were done in chronically inflamed maxillary mucosa by Nishimoto (5). However, in all but the last of these studies use was made of semiquantitative methods which do not seem to be adequate for comparison of quantitative differences between patient groups or for longitudinal studies on effects of therapy.

The present method is suitable for quantitative studies of the cellular infiltrates in the epithelium and lamina propria of nasal mucosa, and employs monoclonal antibodies against surface antigens characteristic for various cell types. Alkaline phosphatase immunohistochemical staining of frozen sections was used to study T and B lymphocytes, CD14-, CD1a- and HLA-DR-positive cells, membranous and cytoplasmic IgE-positive cells, mast cells, eosinophils, and the plasma cells producing IgG subclasses. The differences between patients with isolated grass-pollen allergy, non-allergic patients with nasal polyps, who could be expected to show increased signs of inflammation on a non-allergic basis, and normal controls, are discussed.

Materials and methods

Patients and controls

Forty three patients and controls, 21 ♂ and 22 ♀, were selected for this study and were divided into three groups. The first group (mean age 28 yr, 3 ♂, 9 ♀) comprised 12 controls (volunteers and patients visiting the ear, nose and throat (ENT) department with an ear, or throat disease not related to the nose and nasal sinuses) without nasal complaints, no abnormalities in the nose at ENT examination, a negative radio-allergosorbent test score (RAST), and a median (range) total IgE value of 33 (4-90) IU/ml. The second group (mean age 36, 7♂, 4 ♀) comprised 11 patients with nasal polyps, a negative RAST score and a median (range) total IgE value of 30 (6-470) IU/ml, who underwent an operation to remove polyps from the nose and/or sinuses. All these patients had nasal complaints (e.g. nasal blockage, rhinorrhoea) at the moment of the biopsy. The third group (mean age 34, 11 ♂, 9 ♀) comprised 20 patients with an isolated grass-pollen allergy confirmed by a positive skin prick test reaction with Alutard Soluprick extract of 1 HEP, a median (range) RAST score of 4+ (4+-5+) and a median (range) total IgE value of 156 (15-500). All these patients had complaints of allergic rhinitis (e.g. nasal blockage, rhinorrhoea and sneezing) at the moment of the biopsy. None of the patients or controls in this study used any medication which could influence the results of this study, with the exception of the antihistamine drug terfenadine which was used by the patients with grass-pollen allergy. Terfenadine tablets were always discontinued at least 48 hours before a biopsy was performed.

Nasal biopsies

All patients were biopted once. In the non-allergic groups nasal biopsy specimens were taken between July and November in 1986. Patients and controls were biopted under informed consent conditions. In the patients with isolated grass-pollen allergy, the biopsies were performed in July of 1986, 1987, or 1988, i.e. during the grass-pollen season. Biopsy specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, with a Gerritsma forceps with a cup diameter of 2.5 mm (6). Local anaesthesia was obtained by placing a cotton-wool carrier with 100 mg cocaine and 3 drops of adrenalin (1:1000) under the inferior turbinate without touching the site of the biopsy (6). The specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and frozen immediately (7).

Table 1. Monoclonal antibodies used in the present study.

Antibody	Type	Specificity	Source
Anti-CD4-pool	M	CD4	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands (CLB),
Anti-CD8-pool	M	CD8	pooled with National Institute for Public Health and environmental Protection, Bilthoven, The Netherlands (RIVM)
66IIC7	M	CD1	Monosan, Sanbio, Uden, The Netherlands
Anti-B lymphocytes	M	CD22	Dakopatts, Copenhagen (DAKO)
Anti-monocytes	M	CD14	Bethesda Research Laboratories, Bethesda, Md., USA (BRL)
Anti-HLA-DR	M	MHC class II	CLB
Anti-IgE	M	IgE	CLB
HP6070	M	IgG1	Zymed Laboratories, San Francisco, Calif.,USA
HP6014	M	IgG2	Bio-Yeda, Israel
HP6050	M	IgG3	Bio-Yeda, Israel
HP6025	M	IgG4	Bio-Yeda, Israel

M = Mouse monoclonal; HP = IUSI/WHO study code.

Antibodies

The antibodies used are listed in Table 1. IgG subclasses are indicated by the HP numbers advised by the IUIS/WHO committee (8).

Staining procedure

The immuno alkaline phosphatase (AP) method used has been described elsewhere (9). Briefly, 6 μm-thick sections of nasal mucosa were cut on a Reichert-Jung 2800 Frigocut cryostat, transferred to gelatin-coated microscope slides, dried, and fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2), and incubated with the monoclonal antibody for 30 min at 20°C. The sections were then rinsed again in PBS for 5 min and incubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase (Dako-Immunoglobulin A/S, D314), rinsed successively in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a New Fuchsin solution (Chroma 1B467, Stuttgart, FRG). Finally, the sections were rinsed with distilled water, counterstained with Mayer's haematoxylin, and mounted in glycerin gelatin (Chroma). Control staining was performed by substitution with PBS and incubation with an irrelevant monoclonal antibody of the same subclass. For the general description and counting of eosinophils HE and Giemsa staining were performed.

Cell quantification

Surface area: The total surface area of a section and its main parts, i.e. the epithelium, lamina propria, and mucous glands (in the lamina propria), was estimated by superimposing a grid of 100 points (intersection of crosses, covering a surface area of 0.5 mm^2) on the section and using an eyepiece graticule at a magnification of 100x. The minimum number of fields required to assure

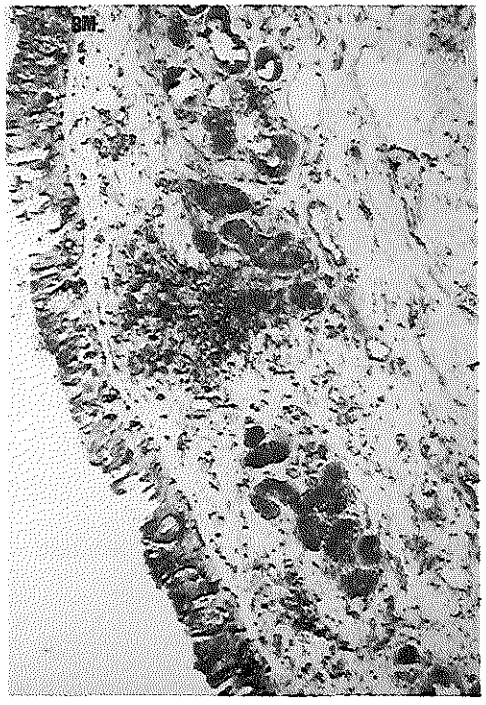

Figure 1. Cryostat section of the nasal mucosa of a patient with an isolated grass-pollen allergy, stained with monoclonal anti-HLA-DR and RaMIg-alkaline phosphatase. Many HLA-DR$^+$ dendritic cells are present in the nasal epithelium and in the lamina propria. Some of these cells are seen in the basement membrane. Epithelial cells and cells of the mucous glands show cytoplasmic staining. x 80.

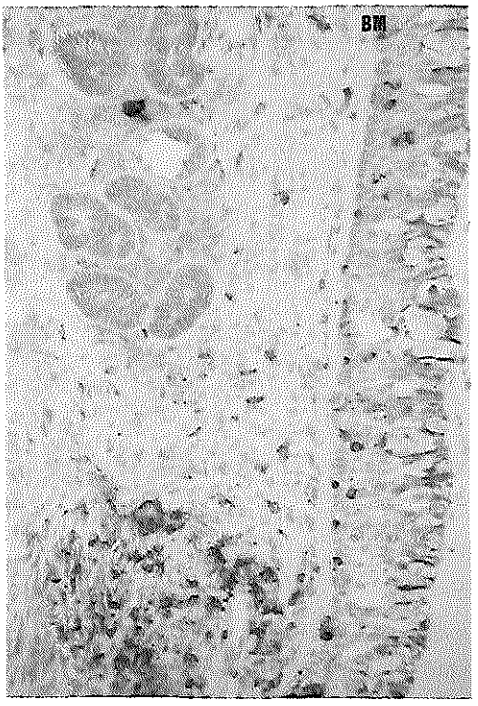

Figure 2. Cryostat section of the nasal mucosa of a patient with an isolated grass-pollen allergy, stained with monoclonal anti-CD4 and RaMIg-alkaline phosphatase. CD4$^+$ cells are more abundant in the lamina propria than in the epithelium. x 160.
BM = basement membrane.

acceptable accuracy (± 5%) was determined on the basis of a summation average graph (10). Usually two sections provided this number. All cells above the basement membrane and the ducts of mucous glands continuous with the epithelium were counted as epithelium. Cells belonging to acini and small ducts were counted as mucous glands. The total surface of the layer below the basement membrane, including the mucous glands, was considered to belong to the lamina propria. If the lamina propria was more than 1.5 mm thick (2 grids), the remainder, which always consisted of collagenous, cell-poor stroma, was not included in the assessment.

Number of cells per section

Usually, two sections were placed on each slide and the one judged to be the technically best was chosen for counting. Cells binding the monoclonal antibody used, had bright red surface membranes, red stained cytoplasm, or both, depending on the cell type or cell in question. Cells were counted if they stained red and contained a nucleus. In doubtful cases, e.g. when a group of

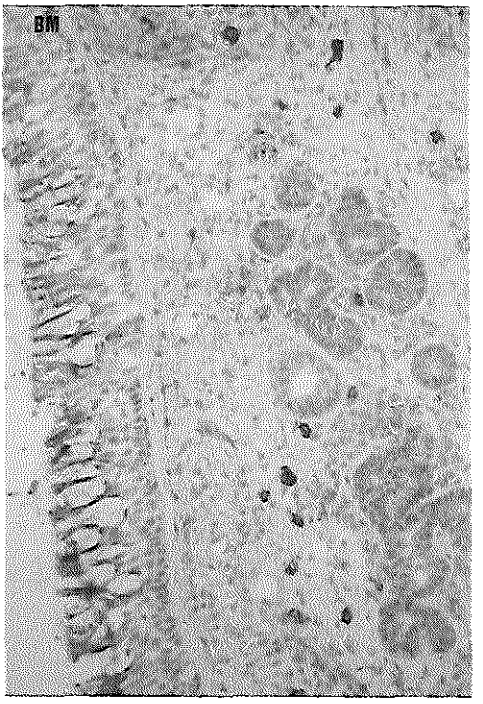

Figure 3. Cryostat section of the nasal mucosa of a patient with an isolated grass-pollen allergy, stained with monoclonal anti-IgE and RaMIg-alkaline phosphatase. IgE^+ cells are present in epithelium and lamina propria. x 160. BM = basement membrane.

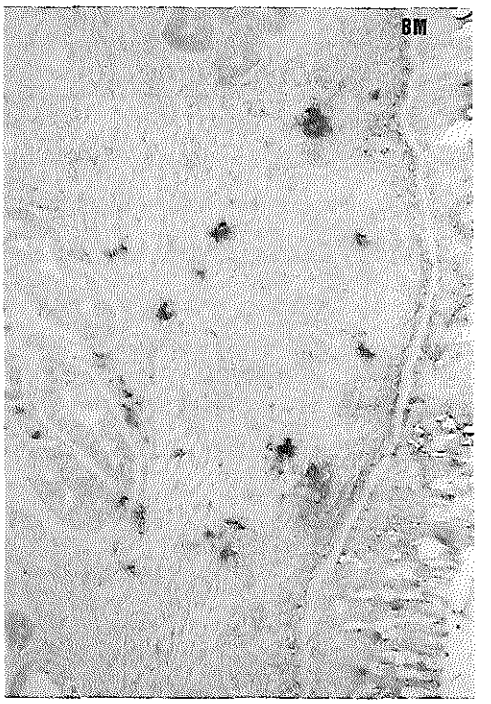

Figure 4. Cryostat section of the nasal mucosa of a patient with an isolated grass-pollen allergy, stained with monoclonal anti-IgG3 and RaMIg-alkaline phosphatase. Cytoplasmic staining $IgG3^+$ cells can be easily distinguished from the background staining. x 160.

positive cells lay very close together or projected on each other, only the definitely positive cells were counted. Some items, such as the number of HLA-DR^+ cells, could not be determined reliably either, because many positive cells lay close together or separate dendritic cells could not be readily distinguished. These items were assessed semi-quantitatively by two independent investigators.

With most of the immunohistochemical staining methods the cells could be counted easily. Separate counts were performed in the epithelium and the lamina propria at a magnification of 250 x. In the epithelium, all positive cells were counted and in the lamina propria the number of positive cells was determined by superimposing a grid of 5 by 5 squares on the section with an eyepiece graticule. Per grid-field, the cells were counted in randomly chosen squares and the grid was superimposed successively on the entire section along parallel lines. The number of squares required to achieve acceptable accuracy (± 5%) was estimated with a summation-average graph. In general two squares per grid-field leading to at least 15 squares of the entire section were determined. The total number of positive cells in the lamina propria was determined when there were only a few positive cells (fewer than one cell per two squares). Control sections with PBS or an irrelevant monoclonal antibody were negative.

Table 2. Semi-quantitative determinations

Number of biopts showing:∞	I*	II	III	Total
Thin basement membrane	1	2	1	4
Partial thick basement membrane	4	3	9	16
Overall thick basement membrane	5	3	8	16
Very thick basement membrane	2	3	2	7

	Epithelium				Lamina propria			
	I	II	III	Total	I	II	III	Total
Number of biopts showing:								
No neutrophils	6	2	6	14	2	0	0	2
Few neutrophils	5	6	10	21	7	7	11	25
Moderate neutrophils	1	3	3	7	3	2	7	12
Many neutrophils	0	0	0	0	0	2	2	4
None/very few HLA-DR$^+$ cells	6	6	13	25	0	0	0	0
Few HLA-DR$^+$ cells	4	4	3	11	4	0	8	12
Moderate HLA-DR$^+$cells	1	1	3	5	4	7	7	18
Many HLA-DR$^+$ cells	1	0	0	1	4	4	5	13
None/very few CD14$^+$ cells	3	5	2	10	1	4	3	8
Few CD14$^+$ cells	8	5	11	24	6	2	8	16
Moderate CD14$^+$ cells	1	1	5	7	4	4	6	14
Many CD14$^+$ cells	0	0	1	1	1	1	3	5

*: I = controls without nasal complaints, II = patients with nasal and/or sinusal polyps, and III = patients with isolated grass-pollen allergy.

∞: Number of biopts per group in epithelium and lamina propria.

Statistical analysis

Since the frequency distribution of the number of cells per mm^2 in the epithelium and lamina propria was not symmetrical and the variances were unequal, the Kruskal-Wallis one way analysis of variances was used to calculate the overall p value. A p value < 0.05 was considered to indicate a significant difference between groups.

RESULTS

General description

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. All but one section (excluded from the study)

showed intact or virtually intact epithelium. The lamina propria was usually composed of a relatively loose cell-rich subepithelial layer with most of the mucous glands and a deeper collagenous cell-poor layer lying on bone. The sections were all thick enough to allow assessment of both layers. Some sections did not have a clear division between the layers. In general, no significant morphological differences were found between the groups. The surface area of epithelium in the sections of nasal mucosa of the group with nasal polyps was significantly larger than that of the other groups. No significant difference was found between the groups in the amount of glandular tissue.

Detailed description

The results of semi-quantitative determinations are given in table 2, those of quantitative assessments in table 3.

The thickness of the basement membrane was assessed semi-quantitatively. No significant difference was found between the groups.

The number of neutrophils was determined semi-quantitatively. Most of the cells in the lamina propria were found in the cell-rich subepithelial layer. The normal controls showed fewer neutrophils than either the patients with grass-pollen allergy or the non-allergic patients with nasal polyps.

The number of HLA-DR$^+$ cells was determined semi-quantitatively. The degree of positivity varied considerably. In most of the biopsy specimens cells of the mono-histiocytic lineage and endothelium were positive. When more positivity occurred, mononuclear cells, i.e. activated T and B cells, dendritic cells and finally the cytoplasm of the epithelium (including that the mucous glands) became positive successively (Fig 1). No significant differences were found between the three groups.

The number of CD14$^+$ cells was determined semi-quantitatively. The results show quite some variance. The epithelium showed less positivity than the lamina propria. No significant difference was found between the groups.

The number of lymphocytes varied considerably between biopsy specimens; for example the number of CD4$^+$ lymphocytes per mm^2 section ranged between 2 and 1400 in the epithelium and 16 and 3660 in the lamina propria. The median and range of the numbers of positive cells in the epithelium and lamina propria of the separate groups are shown in table 3. The majority of the lymphocytes occurred as single cells; small aggregates were seen relatively often and large aggregates rarely (Fig 2). The occurrence of aggregates did not differ between the groups. More lymphocytes occurred in the lamina propria than in the epithelium, and T lymphocytes were much more numerous than B lymphocytes; virtually no B lymphocytes were found in the epithelium, in the lamina propria the T/B ratio was 14. The ratio of CD4$^+$ to CD8$^+$ cells was about 0.5 in the epithelium and approximately 1 in the lamina propria; around the glands most of the lymphocytes were CD8$^+$.

Table 3. Number of positive cells/mm^2 (median(range)) in epithelium and lamina propria of the nasal mucosa.

Cell type	Group I	Group II	Group III*	p-value
Epithelium:				
CD4	113(7-574)	54(2-180)	132(13-1405)	0.08
CD8	249(44-1222)	213(1-689)	252(16-662)	0.47
CD1a	15(0-43)	30(0-69)	44(6-167)	0.01
CD22	12(0-79)	2(0-66)	3(0-77)	0.09
Eosinophils	0(0-16)	0(0-37)	3(0-50)	0.65
IgE	0(0-50)	0(0-30)	21(0-354)	0.0001
Lamina propria:				
CD4	262(16-2284)	161(46-1702)	235(27-3657)	0.95
CD8	215(17-1099)	509(12-1676)	192(29-1367)	0.15
CD1a	3(0-14)	9(2-39)	5(1-74)	0.03
CD22	49(1-385)	28(1-211)	25(1-331)	0.85
Eosinophils	3(0-27)	1(0-19)	3(0-50)	0.90
IgE	10(0-83)	10(0-377)	53(0-189)	0.005
IgG1	1.0(0-7)	1.0(0-5)	1.5(0-5)	0.30
IgG2	2.0(1-11)	2.0(0-7)	1.0(0-7)	0.33
IgG3	1.5(0-9)	2.0(0-12)	3.0(0-12)	0.79
IgG4	1.5(0-12)	4.0(0-18)	1.0(0-7)	0.09

*: Group I = patients without nasal complaints; group II = patients with nasal and/or sinusal polyps; and group III = patients with isolated grass-pollen allergy.

The differences in lymphocyte subpopulations between the three groups were not significant. The non-allergic polyposis group had a tendency to have fewer $CD4^+$ cells in epithelium and more $CD8^+$ cells in the lamina propria and the control group had a tendency to have more B cells in the epithelium.

The numbers of $CD1a^+$ cells per mm^2 section are given in Table 3. The epithelium of the group with grass-pollen allergy showed significantly more such cells than the non-allergic groups did. In the lamina propria the numbers were small, and the control group had fewer than the allergic and the non-allergic polyposis groups.

The number of IgE^+ cells differed significantly between the groups. There were many more such cells in the epithelium and lamina propria of the allergic group (I) than in the non-allergic groups (II and III). These cells may be considered to be predominantly mast cells (Fig 3). Some cells showed positive cytoplasm and the morphological appearance of plasma cells. Double staining with IgE and CD38,

proved these cells to be plasma cells indeed (Fig 3).

The number of eosinophils in the epithelium and the lamina propria did not differ significantly between the groups, as can be seen in Table 3. Fourteen out of 20 in the allergic group, nine out of 11 in the polyposis group, and seven of the 12 normal controls showed eosinophils in the epithelium and/or lamina propria.

The number of cytoplasmic IgG$^+$ cells in these cryostat sections were easily distinguished in the diffusely stained connective tissue staining (Fig 4). Because not all cells with membrane staining could be distinguished with this method, only the *cytoplasmic IgG$^+$ cells*, considered to be plasma cells, were counted. The number of plasma cells was small. For IgG1, IgG2, and IgG3, no significant differences were found between the groups. IgG4-producing plasma cells seemed to be more numerous in the non-allergic groups than in the allergic group ($p = 0.09$).

Discussion

Little is known about the immunological processes occurring in the nasal mucosa, certainly compared with other epithelial structures like the skin or the mucosa of the digestive tract. Much of the available information about allergic mechanisms in the nasal mucosa is based on findings in smears, scrapings, and secretions. Knowledge about the immunological pathogenesis of the diseases of the nasal mucosa and the efficacy of treatment on it is equally limited.

Immunohistochemical studies employing monoclonal antibodies to immunological relevant cells can be expected to shed light on these aspects.The present study was designed to permit the detection of differences between cell populations in patients with an allergic disease, non-allergic patients with nasal polyps, and normal controls. The proportion of the surface area of sections representing e.g. glandular tissue varied from 0 to 42%. There were no differences between the groups with respect to the amount of glandular tissue. These results are similar to those reported by Pech (11) and Thaete (12). The appearance of the basement membrane was also variable and no significant differences were found between the groups.

Neutrophils play a role in allergic and non-allergic inflammation. Takasaka found more neutrophils in polyps of patients with chronic sinusitis than in those of allergic patients (13). Our findings suggest that the number of neutrophils is smaller in normal than in diseased mucosa, whether in allergic patients or in those with nasal polyps. No difference was seen between the latter two groups. Further quantitative studies are in progress, because the semi-quantitative assessment used for the estimation of the number of neutrophils requires careful interpretation.

The predominance of CD8$^+$ cells over CD4$^+$ is in agreement with the findings of Nishimoto et al. (5) in the lamina propria of the maxillary mucosa and of Hameleers et al. (2) in the nasal epithelium. Our results do not agree well with

the results of Winther et all. (1). They found more $T4^+$ than $T8^+$ cells in the epithelium, and an T4/T8 ratio of 2.5 and an overall T/B ratio of 3 in the lamina propria. However, Winther et al. only studied "a representative part of the section", which probably means that the method is less suitable for the detection of differences between groups.

The significantly greater number of $CD1a^+$ cells in the epithelium of the nasal mucosa of allergic patients versus non-allergic controls has been discussed elsewhere (9) as has the finding that $CD1a^+$ cells, which were proved to be Langerhans cells, express surface IgE and change in number during natural allergen provocation (14). It should be kept in mind that the number of dendritic HLA-DR^+ cells is much greater than the number of $CD1a^+$ cells in both the epithelium and the lamina propria. HLA-DR^+ dendritic cells in human nasal mucosa cannot be considered to be LC. Other HLA-DR^+ dendritic cells belong to the mono-histiocytic lineage.

The number of IgE^+ cells was, as could be expected, significantly higher in the allergic group than in the non-allergic groups. The finding of IgE^+ cells in the epithelium of 18 out of 20 patients supports the findings of Enerbäck et al. (15) and Viegas et al. (16), who used toluidine blue staining and chloracetate esterase respectively, and found mast cells in the epithelium during natural provocation. Recently, however, Enerbäck et al. were unable to reproduce their results (17). Our findings led us to conclude that cells staining with toluidine blue and IgE^+ cells did not belong to the same population. It seems possible that not all mast cells stain with toluidine blue in nasal epithelium. Further studies to investigate these differences are in progress.

Surprisingly, no significant differences were found between the number of eosinophils in the three groups. A predominance of eosinophils in the diseased mucosa was expected, because the frequent occurrence of eosinophils in nasal smears has been described in patients with allergic or non-allergic rhinitis and patients with nasal polyps but not in controls (18). Several explanations are possible. In the first place, regional differences in the number of eosinophils have been found; in patients with allergic rhinitis or polyps, eosinophils occurred in 80% of the specimens originating from the middle turbinate as against only 40% for the inferior turbinate (19). Second, in allergic disease eosinophilia has been linked to a late-phase allergic reaction in the bronchi (20,21) and the nose (22). The frequency of late phase reactions in our patient population is not known.

We found a considerable degree of eosinophilia in 6 of the 11 normal controls without any nasal symptoms, which corresponds with the findings in the only other published biopsy study concerning eosinophils in the normal human nasal mucosa (12). We therefore conclude that eosinophils occur in normal as well as diseased nasal mucosa. Possibly the difference should be sought in functional heterogeneity rather than in numerical differences (23).

Few IgG-producing plasma cells were found in the lamina propria.

Brandtzaeg pointed out in his reports of his extensive studies that the cryostat method is not ideal for the counting of IgG^+ cells because background staining can be quite extensive in inflamed tissue (3,4). However, in our material, counting of cytoplasmic IgG^+ plasma cells was always easy to perform. This divergence from previous studies might be due to methodological differences between the alkaline phosphatase method and immunofluorescence staining. The finding suggesting that IgG4-producing plasma cells are more numerous in the non-allergic groups than in the allergic group is interesting, but its exact meaning is uncertain and requires investigation.

Studies are in progress to establish the effects of different forms of treatment on the nasal mucosa. The use of biopsy in studies on allergic reactions makes it possible to evaluate the nasal mucosa as a whole instead of only the upper part of the epithelium. Furthermore, this easy and well-tolerated method provides quantitative and reproducible data on the cellular infiltrates in the nasal mucosa, which should be valuable for follow up in studies on the effect of therapy.

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CHAPTER 6

DYNAMICS OF MAST CELLS IN THE NASAL MUCOSA OF PATIENTS WITH ALLERGIC RHINITIS AND NON-ALLERGIC CONTROLS
A BIOPSY STUDY

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ABSTRACT

Mast cell degranulation is thought to be an important component of the pathogenesis of allergic rhinitis. Quantitative studies on mast cells in nasal mucosa after allergen exposure have given widely divergent results, ranging from an overall decrease via redistribution to an overall increase. We investigated this problem by employing a combination of anti-IgE and toluidine blue staining of biopsy specimens. Anti-IgE was used to identify all mast cells and toluidine blue to detect mast cells that were not (totally) degranulated.

The study was composed of two parts done in different patient groups. In the first part of the study, 12 controls and 23 patients with isolated grass-pollen allergy were studied. Biopsies were performed twice in each patient, once during the summer in the grass-pollen season and once in the winter when the patients were asymptomatic. A biopsy was performed in the controls once, i.e., between July and November. The results of this part of the study showed that non-allergic controls have the same number of mast cells in the lamina propria as found in asymptomatic allergic patients and that the controls seldom have mast cells in the epithelium. The patients with isolated grass-pollen allergy showed an increase in the numbers of mast cells in the lamina propria during natural provocation and the same seemed to occur in the epithelium as well. During natural provocation almost all of the mast cells in the epithelium and half of those in the lamina propria were degranulated.

In the second part of the study 17 patients with isolated grass-pollen allergy and four controls were challenged daily with allergen extract during a two-week period in the winter. For statistical reasons, the patients were randomly divided into two groups and three or four times biopsies were performed, i.e., once before, six moments during and once after the provocation period. The results of this part of the study showed that during provocation mast cells migrate to the surface of the nasal mucosa, where they become degranulated, and that the pool of mast cells in the lamina propria was apparently replenished by migration

of mast cells from the vessels in the lamina propria. Thus the total number of mast cells in the lamina propria remained approximately the same while the mast cells residing in an increasingly thick layer measured from the basal membrane into the lamina propria became degranulated. After two weeks, 82% of the mast cells in the lamina propria was degranulated and it was only in the deepest layers that some toluidine blue positive cells were found.

This study, where anti-IgE was used to detect all mast cells and toluidine blue to identify mast cells that are not (totally) degranulated, can explain the seemingly conflicting reports in the literature on mast cell dynamics and degranulation and shows that the reported differences are due to differences in the techniques used and the time of evaluation.

INTRODUCTION

Mast cells appear to play an important role in allergic rhinitis. The allergic reaction of the mucosa is induced by an interaction between allergen and IgE antibodies on the surface of the mast cell. Most of the studies done on the role of metachromatically staining cells (mast cells and basophils) in the nasal mucosa have been performed in nasal smears or scraping material (1,2). Few quantitative studies have been done in biopts of nasal mucosa. We are aware of only two quantitative studies in which biopts of normal human mucosa were used (3,4). Otsuka et al. described the existence of considerable numbers of mast cells in the epithelium and lamina propria of two allergic patients and eight non-allergic controls (3). In a comparative study on allergic patients and non-allergic controls, Okuda found an increased number of mast cells in the epithelium of the allergic patients but not in the lamina propria (4).

More studies have been done on mast cells in allergic nasal mucosa and on the numerical changes shown by mast cells in epithelium and lamina propria during (natural) allergen provocation (5-10). The changes in mast cell numbers during allergen provocation have not been conclusive. Wihl and Mygind did not find any change after allergen challenge at all (5). Enerbäck et al. saw no change in the total number of mast cells but did find a redistribution of mast cells into the epithelium during natural provocation (6). The latter, however, was denied by Pipkorn and Enerbäck in 1988 (7); instead, they reported a decrease in the number of mast cells in epithelium and lamina propria. A temporary redistribution of metachromatic cells toward the mucosal surface, occurring 5-24 hr after allergen challenge, was found by Borres (8). Finally, the group of Davies found increased numbers of nasal mucosal mast cell numbers during natural provocation (9,10).

Studies on mast cells have employed a variety of different tissue fixatives and staining methods, which makes it difficult to compare the results reported by different authors. The method mentioned most often in the recent literature is toluidine blue staining at pH 0.5 after fixation with ice-cold, iso-osmotic solution of formaldehyde and acetic acid (IFAA) or ethanol. There is evidence suggesting that not all of the mast cells in the nasal mucosa are detected in this way (11,12). In a recent study we used an immuno alkaline phosphatase method with anti-IgE to study mast cells (13). Contrary to the toluidine blue staining, in this way all mast cells are discovered in biopsy specimens of allergic patients (14). A possible disadvantage may be that other cells which are known to express $Fc_{\varepsilon}II$

receptors could be positive for IgE (15-19). The anti-IgE staining showed more positive cells in the epithelium and lamina propria of allergic patients during natural provocation than in non-allergic controls. These results are in agreement with those of a study reported by Bachert (20).

To study changes in the number and degranulation of mast cells, we investigated these cells in the epithelium and lamina propria of the nasal mucosa of the lower inferior turbinate in patients with isolated grass-pollen allergy and normal controls. In this study we used toluidine blue (TB) and anti-IgE immuno alkaline phophatase methods. First, a group of patients was studied during natural provocation. Biopsy specimens of nasal mucosa were taken during the grass-pollen season and also out of the season, i.e. between October and January. These biopts were compared with those of controls. A second group of patients with isolated grass-pollen allergy and controls were studied in a provocation study done during two weeks in January. In this period biopts of nasal mucosa were taken 3-4 times in each patient. Other cells that could be IgE^+ were determined quantitatively.

MATERIALS AND METHODS

Patients and controls during natural provocation

The first part of the study on natural provocation was done in 23 patients with isolated grass-pollen allergy and 12 controls. The patients (mean age 34 yr (range 12 – 57), 14 ♂, 9 ♀) had an isolated grass-pollen allergy confirmed by a positive skin prick-test reaction to ALK Soluprick extract of 1 HEP/ml and no other positive skin prick-test reaction to 13 common allergens, a mean (range) radio-allergosorbent test (RAST) score of 5+ (3+ – 5+) and a mean (range) total IgE value of 394 (19 – 1900) IU/ml. The controls comprised 12 volunteers and patients (mean age 28 yr (range 17 – 42), 3 ♂, 9 ♀) visiting the Ear, Nose and Throat (ENT) department with an ear, or throat disease not related to the nose and nasal sinuses, without nasal complaints, no abnormalities in the nose at ENT examination, a negative RAST score, and a mean (range) total IgE value of 39 (4 – 90) IU/ml. None of the controls in this part of the study used any medication that could have influenced the results of this study. Some of the patients used the nasal corticosteroid spray budesonide and/or the antihistamine drug terfenadine. No significant differences in age or serum IgE level was found between the groups with and without medication use. Despite the treatment all patients had serious complaints. Terfenadine tablets were always discontinued at least 48 hours before a biopsy was performed. Budesonide was not discontinued during the study. The effect of budesonide could not be evaluated because patients were not randomly assigned to the use of budesonide which meant that it were the patients with more severe complaints who used the budesonide spray.

Patients and controls in the provocation study

Seventeen patients and four controls participated in the provocation part of the study. The patients (mean age 29 yr (14 – 48), 7 ♂, 10 ♀) had an isolated grass-pollen allergy for at least one year, confirmed by a positive skin prick test reaction to ALK Soluprick extract of 1 HEP/ml and no other positive skin prick test reaction to 13 common allergens, a median (range) radio-allergosorbent test (RAST) score of 4+ (3+ – 5+) and a median (range) total IgE value of 290 (22 – 1900) IU/ml. The four controls (volunteers, mean age 29 yr (24 – 44), 2 ♂, 2 ♀) without nasal complaints, had no abnormalities in the nose at ENT - examination and a negative skin prick test reaction to ALK Soluprick extract of 13 common inhalant allergens. None of the patients or controls in this part of the study used any medication. None of the patients or controls had an infection of the respiratory tract during the week preceding the challenge.

Table 1. Moment of biopsy for different patients. Patients were randomly divided in two groups (I and II). The first biopsy of the patients in group II was either taken at ½ hour after provocation or at 8 hours after provocation.

patient group	moment of biopsy before/after first provocation			
I n=7	before provocation n=8	24-48 hr n=5	8-10 days n=6	1-2 weeks after last provocation n=9
II n=10	½ hr n=5	4-6 days n=9	14 days n=9	
	8 hr n=5			

Design of the provocation study

Patients and controls were daily challenged with allergen during a two-week period. Symptoms and signs were recorded each day. All patients were biopted three or four times, all of the controls two times. To allow investigation of a time-related effect, the patients were randomly divided into two groups for statistical reasons, which meant that a sufficient number of different biopsy times could be analysed. Thus biopts were taken from the allergic patients at seven different times as shown in Table 1. Two patients in group II and one in group I were biopted only once because they withdrew from the study before the time of the second biopsy. One patient in group I was biopted only twice. All patients and controls gave their informed consent and the study was approved by the medical-ethical committees of the Leyenburg and the Dijkzigt hospitals.

Nasal biopts

Biopts of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge with use of a Gerritsma forceps with a cup diameter of 2.5 mm. Local anaesthesia was obtained by placing a cotton-wool carrier with 50 – 100 mg cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate without touching the place where the biopsy would be taken (21). The biopsy specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and frozen immediately (22).

The biopts of the patients with isolated grass-pollen allergy collected during natural provocation were taken in the first week of July at the height of the grass-pollen season and between October and January, well out of the season. The controls were biopted between July and November.

Biopts taken from the patients with isolated grass-pollen allergy during the provocation study were collected before the provocation, 1/2 hr, 8 hr and 24 hr after the first provocation, and 2, 4, 6, 8, 10, and 14 days after the first provocation and 1 and 2 weeks after the last provocation (Table I). Biopsies were always taken before another provocation took place. The biopts of the controls were collected before the first provocation, and 24 hr and 12 days after the first provocation.

Symptom scores

Patients were asked daily prior to the provocation what symptoms they had had during the preceding day. The severity of these symptoms, e.g. nasal blockage, nasal discharge, sneezing, nasal itching, and eye watering/irritation were recorded on a four point scale (0 – 3). A total symptom score was calculated as the sum of the individual symptom scores recorded. The same symptom score was used to grade symptoms occurring 30 min after the provocation test.

Nasal allergen challenge

Patients and controls were challenged daily with grass-pollen extract (1000, 3000, 10.000, 30.000, 100.000 SQ/ml; ALK obtained from Diephuis, Groningen, The Netherlands) with a pump spray delivering a fixed dose of 50 ± 2 μl. Before provocation, the subjects waited 15 min to allow the nasal mucosa to become acclimatized to room temperature. On the first day of the provocation study the patient was challenged with 1000 SQ/ml followed by increasing doses of allergen to establish a threshold dose. The duration of the interval between two doses was 15 min. Within 15 min of each challenge the number of sneezes was recorded and the amount of secretion, collected in preweighed paper tissues, was determined. The lowest concentration which gave either more than 0.5 g of secretion or more than 5 sneezes within 15 min was called the threshold dose. In the following days the same concentration was used until the patient experienced serious complaints, i.e. a score of 3 (severe symptoms) for two or more items of the symptom score of the previous day, at which point a new threshold dose was established.

Staining procedure for the biopt

Each specimen of nasal mucosa was cut into serial 6 μm-thick sections on a Reichert-Jung 2800 Frigocut cryostat and transferred to gelatin-coated microscope slides. Serial sections were stained with toluidine blue at pH 0.5 for 30 min, anti-IgE monoclonal antibody using the immuno alkaline phosphatase method, and both anti-IgE and toluidine blue in a double staining technique. The immuno alkaline phosphatase method used has been described elsewhere (23). Briefly, after being transferred to gelatin-coated microscope slides, the sections were dried and fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2), and incubated with monoclonal mouse anti-IgE (CLB, Amsterdam, The Netherlands) for 30 min at 20°C. The sections were then rinsed again in PBS for 5 min, and incubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase (DAKO D314, Dakopatts, Copenhagen), rinsed successively in PBS and TRIS buffer pH 8.0, and incubated for 30 min with a New Fuchsin solution. Finally, the sections were rinsed with distilled water, counterstained with Mayer's haematoxylin, and mounted in glycerin gelatin (Chroma, Shmid GmbH, Köngen, Germany). For double staining, after incubation with the New Fuchsin solution, the sections were rinsed with distilled water, stained with toluidine blue at pH 0.5 for 30 min and counts were performed immediately. In the provocation study, immunofluorescence double-staining was performed with monoclonal CD1a (Netherlands Cancer Institute, Amsterdam, The Netherlands) or CD38 (Ortho, Raritan, New Jersey, USA), which stain Langerhans cells and B cells/plasma cells, respectively, followed by incubation with goat anti-mouse immunoglobulin labeled with TRITC (GaMIg-TRITC, CLB, Amsterdam, The Netherlands) and reincubation with anti-IgE labeled with FITC (RaHuIgE-FITC, CLB, Amsterdam, The Netherlands). Control staining was performed by substitution with PBS and incubation with non-relevant monoclonal antibodies of the same subclass.

Light-microscopical evaluation

In two sections of each specimen, all mast cells with nuclei in the plane of the section were counted. The number of mast cells in the epithelium and lamina propria were counted separately. The total surface area of two sections and of the epithelium and lamina propria was estimated by Kontron Image Analysis System Videoplan. The number of mast cells/mm^2 section area of epithelium and lamina propria was calculated. The percentage of degranulation of the mast cells in the provocation study was calculated by dividing 100 x the number of TB$^-$IgE$^+$ cells by the number of IgE$^+$ cells.

Statistical analysis

For the first part of the study concerning natural provocation, use was made of Student's (paired) t-tests. In the provocation part of the study three repeated measurements were made for each of the two groups before and during the provocation, together constituting six points on the a time axis (see Table I).

Table 2. Number of Toluidine Blue positive (TB^+) and IgE positive (IgE^+) cells/mm^2 (mean (s.d.)) in epithelium and lamina propria of nasal mucosa of controls without nasal complaints, patients with isolated grass-pollen allergy during the grass-pollen season (symptomatic) and patients with isolated grass-pollen allergy in winter (asymptomatic).

	Epithelium		Lamina propria	
	TB^+	IgE^+	TB^+	IgE^+
Controls	3 (4)	5 (14)	83 (45)	17 (24)
Symptomatic patients with isolated grass-pollen allergy during season	6 (18)	126 (206)	68 (50)	134 (138)
Asymptomatic patients with isolated grass-pollen allergy in winter	1 (4)	67 (93)	64 (42)	80 (58)
p-value I *	#	0.01	n.s.	0.001
p-value II	#	0.005	n.s.	0.0001
p-value III	#	0.20	n.s.	0.03

*: p-value I = p-value from Student's t-test comparing controls with symptomatic patients; p-value II = p-value from Student's t-test comparing controls with asymptomatic patients; and p-value III = p-value from paired Student's t-test comparing symptomatic and asymptomatic patients.
#: Numbers too small for reliable statistics.

Because the evaluated variables were symmetrically distributed (i.e., with skewness shown to be non-significant), a repeated measurement analysis of variance was performed. In this analysis the six time effects on the dependant variable are obtained as the estimates of six coefficients in a model with three within-subject repetitions (two coefficients), two between-subject repetitions (one coefficient), the interaction between repetition and groups (two coefficients) and the constant term (one coefficient). The differences between the end of the provocation period and the period 1-2 weeks after provocation were analysed with the sign test. Rank correlation coefficients between symptoms and number of IgE^+ cells and TB^+ cells in epithelium and lamina propria were also determined.

RESULTS

General description

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. All but one section (excluded from the study) in the study on natural provocation showed intact or virtually intact epithelium. In the provocation study the epithelium could not be evaluated in three sections (before, six and eight days after provocation). The lamina propria usually consisted of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer onto the bone.

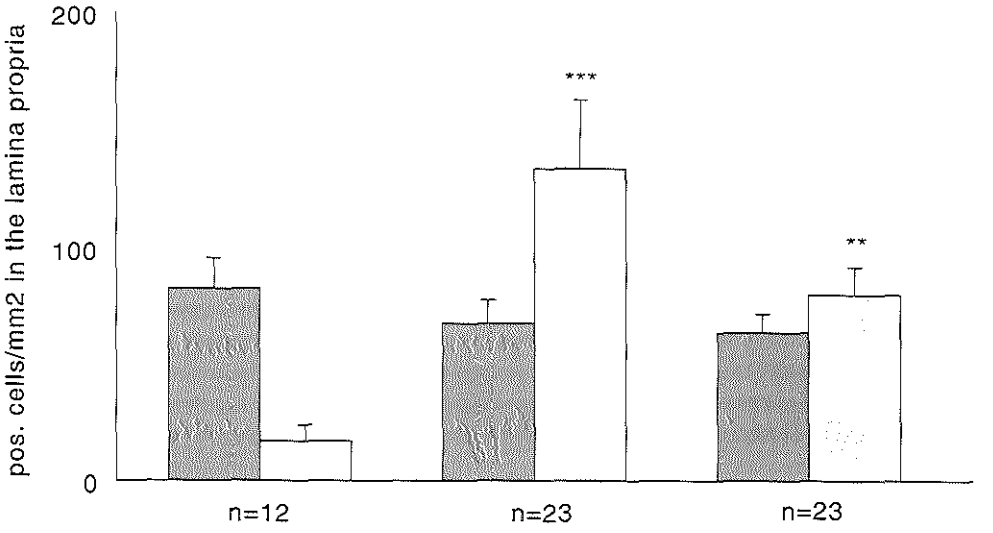

Figure 1. Mean and standard error of the number of TB^+ cells (dark columns) and IgE^+ cells (light columns) in the lamina propria of the nasal mucosa of controls (I), symptomatic patients with isolated grass-pollen allergy (II), and asymptomatic patients with isolated grass-pollen allergy (III) (biopted in the winter). The allergic group showed approximately the same number of TB^+ cells as the non-allergic group did. However, the number of IgE^+ cells in the lamina propria was significantly higher in the allergic patients than in the controls. Furthermore, a significant difference was found between numbers in the summer and winter in the allergic group.

The sections were all deep enough to allow assessment of both layers.

After toluidine-blue staining, mast cells could easily be identified by their dark-violet, metachromatic granules against a background of faintly stained tissue. The anti-IgE staining showed red cells against a blue counterstained background. In both the epithelium and the lamina propria the IgE^+ cells and TB^+ cells morphologically resembled mast cells and not basophils.

The double staining with anti-IgE and TB showed violet cells, in which usually a blue centre and a red surface could be distinguished and normal red cells as were found in the sections stained with IgE only.

Mast cells in controls and allergic patients during natural provocation

The numbers of TB^+ cells and IgE^+ cells found per mm^2 tissue section in the natural provocation part of the study are shown in table 2. The epithelium of the controls showed virtually no positive cells; in the allergic patients the number of TB^+ cells in the epithelium was low in the summer as well as in the winter. The number of IgE^+ cells in the epithelium was significantly higher in the allergic patients ($126/mm^2$ in summer, $67/mm^2$ in winter) than in the controls ($5/mm^2$). In the allergic group the difference between summer and winter did not reach significance. In the lamina propria the allergic group showed approximately the same number of TB^+ cells as the non-allergic group did. However, the

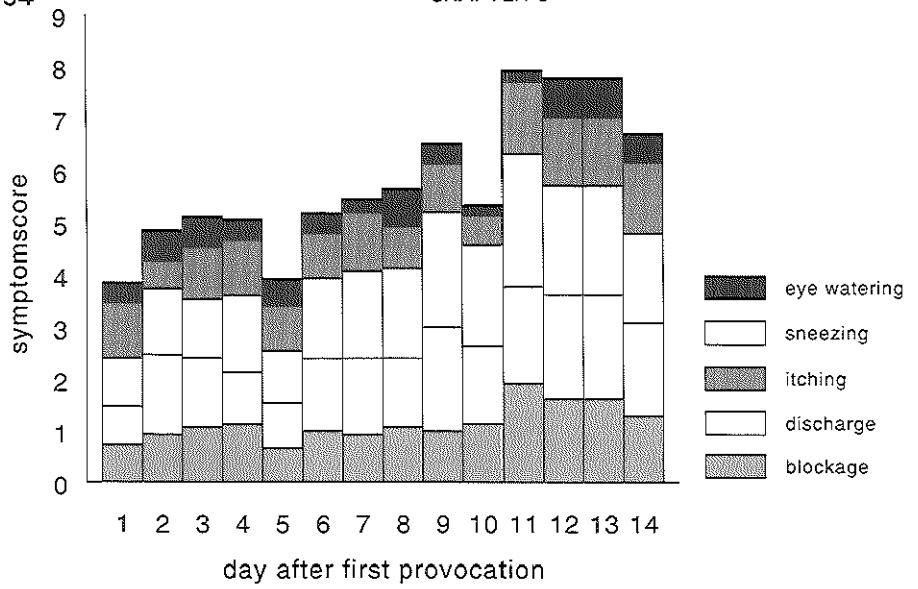

Figure 2. Mean of the symptoms nasal blockage, nasal discharge, sneezing, nasal itching and eye watering/irritation experienced in the day before challenge recorded on a four point scale (0 - 3) during the 2 week daily provocation period. The symptom score of nasal blockage, nasal discharge, sneezing, nasal itching and the total symptom score increased significantly.

number of IgE^+ cells in the lamina propria was significantly higher in the allergic patients (134/mm^2 in summer, 80/mm^2 in winter)than in the controls (17/mm^2). Furthermore, a significant difference between summer and winter was found in the allergic group (Fig 1).

Symptom score during the provocation study

During the two-week long daily provocation period the symptom score for nasal blockage, nasal discharge, sneezing, nasal itching, and the total symptom score, recorded the preceeding day, increased significantly (Fig 2). The symptom score for eye watering/ itching did not increase significantly. No significant increase in the symptom score was found within 30 min immediately after the daily provocation. No significant correlation could be found between symptom score and mast cell numbers or extent of degranulation.

Number of mast cells in the epithelium during the provocation study

Before provocation, IgE^+ cells were found in all sections analysed and TB^+ cells in four out of eight sections. The number of IgE^+ cells seemed to decrease ½ hr after provocation (from 144 cells/mm^2 before to 82 cells/mm^2 after provocation) and to increase again during the next eight hours after provocation (from 82 cells/mm^2 to 357 cells/mm^2). The number of biopts evaluated, however, was too small to allow detection of significance. During the rest of the two-week provocation period the number of IgE^+ cells decreased significantly ($p = 0.002$) (Table 3). One to two weeks after the provocation period, a second increase of the number of IgE^+ cells seemed to occur.

Table 3. Number of Toluidine Blue positive (TB^+) and IgE positive (IgE^+) cells/mm^2 (median (range)) in epithelium and lamina propria of nasal mucosa of patients with isolated grass-pollen allergy during a 2 week provocation study in winter.

Moment of biopsy	Epithelium		Lamina propria		
	TB^+	IgE^+	TB^+	IgE^+	% degranulation in the lamina propria
before provocation	2 (0-73)	144 (0-327)	34 (1-188)	61 (15-160)	22 (0-80)
½ hr after first provocation	0 (0-0)	82 (4-383)	25 (17-39)	52 (16-85)	46 (27-72)
8 hr after first provocation	0 (0-39)	357 (61-472)	26 (6-98)	55 (39-117)	62 (13-86)
24-48 hr after first provocation	0 (0-17)	91 (8-239)	60 (29-63)	65 (16-145)	34 (0-80)
4-6 days after first provocation	0 (0-9)	111 (0-364)	31 (6-96)	48 (0-120)	47 (11-86)
8-10 days after first provocation	5 (0-34)	6 (0-166)	34 (17-51)	51 (0-151)	34 (0-67)
14 days after first provocation	0 (0-0)	68 (0-279)	11 (0-51)	49 (2-197)	82 (0-100)
1-2 weeks after last provocation	0 (0-150)	189 (45-700)	23 (0-76)	83 (36-123)	67 (12-100)

The number of TB^+ cells in the epithelium was too small for statistical analysis. During provocation, 28 out of 37 sections showed no TB^+ cells in the epithelium. In the controls virtually no IgE^+ or TB^+ cells were found in the epithelium.

Number of mast cells in the lamina propria during the provocation study

The numbers of TB^+ and IgE^+ cells found per mm^2 of lamina propria in the provocation study are shown in Table 3. Throughout the provocation period the number of IgE^+ cells in the lamina propria remained about the same. The number of TB^+ cells seemed to decrease slightly ½ hr and 8 hr after provocation and to increase by a factor of two, 24 hr after provocation. During the remainder of the provocation period the number of TB^+ cells decreased significantly ($p = 0.03$).

Double-staining with anti-IgE and TB showed that early in the provocation period IgE^+TB^- cells occurred only in the subepithelial layer and that most of the cells in the deeper layers were IgE^+TB^+ cells. During the provocation period the depth of the area with mainly IgE^+TB^- cells increased steadily until at the end of the provocation period most of the cells were IgE^+TB^-. The median percentage of IgE^+TB^+ cells decreased from 78 before provocation to 18 at the end of the provocation period. One to two weeks after the

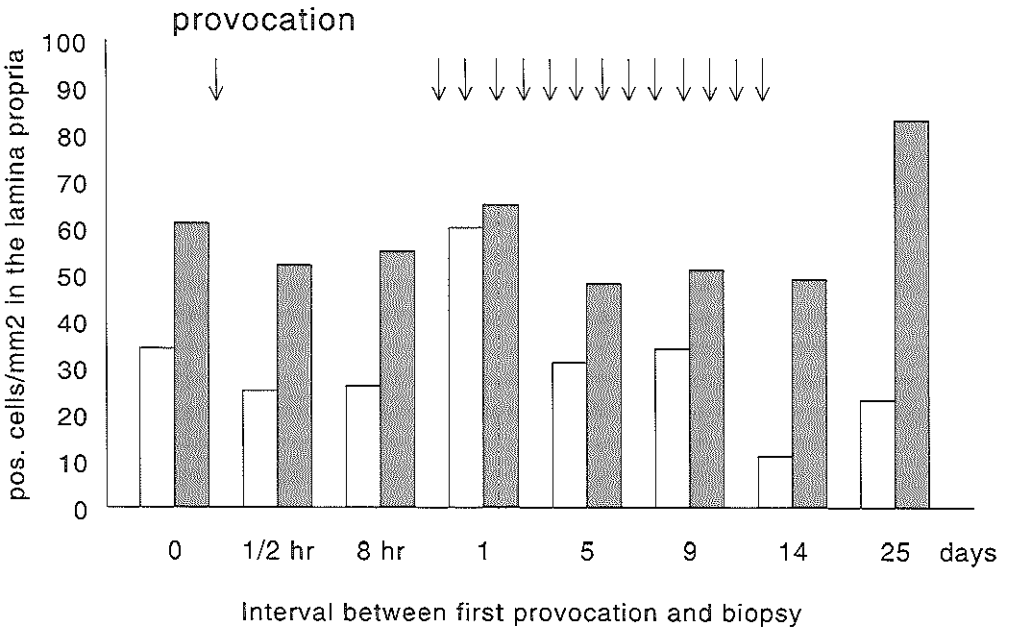

Figure 3. Median number of TB^+ cells (light columns) and IgE^+ cells (dark columns) in the lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy before, during, and after a two-week provocation study. A significant decrease in TB^+ cells occurred during the provocation period. The number of IgE^+ cells did not decrease significantly.

last provocation this percentage of IgE^+TB^+ cells had increased to 33 (Fig 3). In the controls the numbers of IgE^+ and TB^+ cells did not change during the provocation period.

IgE^+ cells not being mast cells

Occasionally, IgE^+ cells with a lymphoid morphology were seen, mainly in the lamina propria. Morphologically, such cells could easily be distinguished from mast cells and were therefore not counted. Other possibly IgE^+ cells that could be erroneously taken for mast cells are Langerhans cells and plasma cells.

Immunofluorescence double-staining for the presence of both IgE and CD1a (for Langerhans cells) or both IgE and CD38 (for plasma cells) showed that in the epithelium of the allergic patients 29% (0-100 %) of the IgE^+ cells were positive for CD1a. Of the IgE^+ cells in the epithelium, none were found to be simultaneously positive for CD38. In the lamina propria IgE^+ cells were occasionally found to be positive for CD1a or CD38 as well; these numbers always represented $<3\%$ of the IgE^+ cells. No IgE^+ cells in the biopts of the controls were found to be simultaneously positive for either CD1a or CD38.

DISCUSSION

Mast cell degranulation is an important component of the pathogenesis of allergic rhinitis. The results of the available quantitative studies on mast cells in the nasal mucosa

differ widely, ranging from an overall decrease via redistribution to an overall increase after allergen exposure (5-10). These differences could be due to the use of different staining techniques which do not always guarantee that all mast cells, including the degranulated ones, are counted. Another possible explanation of these differences is that the interpretation of the results is impeded by the lack of knowledge about the dynamics of the mast cell response in relation to allergen exposure. It is conceivable that some of the literature cited refer to different stages of an ongoing process.

In the present study we attempted to overcome both of these problems: on one hand, by using a method that always detects the mast cells in allergic patients and, on the other hand, by collecting data of the entire nasal mucosa on a large number of occasions after allergen challenge. We chose anti-IgE staining combined with toluidine blue staining of sections. After staining with anti-IgE it is certain that all mast cells in the epithelium and lamina propria of allergic patients will be detected (14), and TB staining shows the mast cells that are not (totally) degranulated. IgE^+ cells which were not mast cells were found in relatively small numbers that did not influence the results of this study, except for the LC in the epithelium of asymptomatic allergic patients. The use of seven times for biopsy in a provocation period of 2 weeks enabled us to study the dynamics of the mast cell response.

The results of this study show that mast cells are present in the lamina propria but seldom in the epithelium of non-allergic controls. This finding is in agreement with results obtained by Okuda et al.(4). Allergic patients without symptoms (isolated grass-pollen allergy in the winter) have approximately the same number of mast cells in the lamina propria as non-allergic controls do. In the winter, too, when degranulation does not occur, the number of IgE^+ cells is greater than the number of TB^+ cells; this is in all likelihood due to other IgE^+ cells that are not mast cells, such as $IgE^+ CD1a^+$ Langerhans cells (19).

During natural provocation of allergic patients the number of mast cells in both the epithelium and the lamina propria increases. This is in agreement with the findings of Lozewicz et al. who, although they did not indicate results concerning epithelium and lamina propria separately, found an increase during natural provocation (9). Although these authors used a different method (α-Naphtol chloroacetate esterase reaction), their mast cell numbers are in the same range as ours (9). These findings differ from those made in studies done by Pipkorn and Enerbäck (6,7), who found a mean mast cell number of only 1.55 (range 0-4.7) cells/mm^2 in the subepithelial layer in one study, a range of 0-30 in another study, and an increase and a redistribution to the epithelium of mast cells in these two studies, respectively. This difference might be explained by the use of the TB staining in the studies done by Pipkorn and Enerbäck (6,7), since this method detects only the mast cells that are not degranulated.

During the natural-provocation study, the allergic patients used a nasal corticosteroid spray when they had severe complaints despite the use of anti-histamines. Okuda et al. (24) and Gomez et al. (25) demonstrated a lower increase of nasal mucosal mast cells during provocation after use of nasal corticosteroid spray. This suggests that the use of nasal corticosteroids has led to an underestimation of the increase of mast cells found in the

natural-provocation study. The provocation study showed an increase in the symptom score of nasal blockage, nasal discharge, sneezing, nasal itching, and the total symptom score experienced the previous day. This indicates that the priming effect, described by others (26-28), was seen in our study, too. A possible explanation for the priming effect could be the increased release of mediators from mast cells (8). However, the absence of correlation between symptom score and number of mast cells or amount of degranulation does not support the hypothesis that the priming effect is due to increased numbers of degranulating mast cells at the mucosal surface. It seems more likely that the priming effect is due to a combination of different processes taking place after allergen provocation.

In the provocation study three periods could be distinguished. The first is the period from before provocation up to 8 hr after provocation. In this period, biopsy specimens were taken at three different times (see table 1). Thirty minutes after provocation the number of epithelial mast cells seemed to decrease, probably due to migration to the surface. Eight hours after provocation an increase in epithelial mast cells was found, probably due to migration from the subepithelial layer to the epithelium. Simultaneously a considerable number of mast cells were seen perivascularly in the lamina propria. Mast cells in the lamina propria arise from monocytic cells (29), which seem to migrate into the lamina propria from the blood. As a result, the total number of mast cells in the lamina propria remained approximately the same. The percentage of degranulated mast cells, however, increased from 22 before provocation to 62 eight hours after provocation. This degranulation is seen mainly in the subepithelial layer. These results correlate well with the findings of Borres et al. (8), who demonstrated a temporary redistribution of mast cells toward the mucosal surface 5-24 hr after provocation. Twenty-four hours after the first provocation, the number of mast cells in the epithelium had returned to a level somewhat lower than that seen before provocation. In the lamina propria the level had equalled that seen before provocation.

The second period covers the 14 days of daily provocation, each of which can be seen as a recurrence of the preceding day. Biopsies were always taken shortly before the new provocation, and thus 24 hr after the last provocation. In this period the number of mast cells in the epithelium decreased slowly. The number of mast cells in the lamina propria remained approximately the same, but they became degranulated in an increasingly thick layer measured from the basement membrane. After two weeks 82% of the mast cells in the lamina propria had become degranulated, and only in the deepest layers were some TB^+ cells found.

In the two weeks after the last provocation, i.e., the third period, the total number of mast cells seemed to increase in both the epithelium and the lamina propria. Surprisingly, two weeks after provocation, 62% of the mast cells in the lamina propria were still degranulated. This suggests either a prolonged degranulation of the mast cells in the lamina propria or a slow elimination of degranulated mast cells.

An interesting difference between the natural provocation and the provocation studies is that in the latter a running out of mast cells in the epithelium occurred that did not occur after natural provocation and that the number of mast cells in the lamina propria, con-

trary to the situation after natural provocation, did not increase. During the provocation studies an increase in mast cells occurred in the epithelium and lamina propria after the daily provocation was terminated. During natural provocation, too, the percentage of degranulation of the mast cells in the lamina propria was only 49 (unpublished results) as compared with the 82% during the provocation study. These findings suggest that the stimulus during the daily provocation study was stronger than during natural provocation. Apparently, restoration of mast cell numbers is possible during natural provocation but not during provocation studies.

The findings in the present study have shed light on the seemingly conflicting recent results reported on mast cell dynamics and degranulation, and show that the reported differences must be due to differences in the techniques applied and the times at which the response was evaluated. The absence of a significant correlation between symptoms and mast cell numbers and degranulation indicates that mast cell degranulation is not the most important step in the priming effect.

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CHAPTER 7

CD1a(T6), HLA-DR-EXPRESSING CELLS, PRESUMABLY LANGERHANS CELLS, IN NASAL MUCOSA

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ABSTRACT

In the skin, epidermal Langerhans cells (LC) constitute a major population of antigen-presenting cells. These cells are characterized by the expression of both CD1a(T6) and HLA-DR on the cell membrane. We wanted to know whether similar CD1a/HLA-DR$^+$ cells occur in the nasal mucosa of patients with an isolated grass-pollen allergy and in non-allergic controls. CD1a/HLA-DR$^+$ dendritic cells were found in columnar and cuboidal epithelium and the lamina propria of the nasal mucosa. These CD1a/HLA-DR$^+$ cells presumably correspond with LC in the skin. We also found significantly more CD1a$^+$ cells in nasal biopsy samples of allergic patients than in those of the non-allergic controls. In the allergic patients some of the CD1a$^+$ cells were found to be surface IgE positive, possibly due to passive adherence of IgE to Fc receptors.

CLINICAL ASPECTS

A crucial aspect of allergic rhinitis, the transportation of allergens into the nasal mucosa, is virtually undocumented. Recent studies suggest that Langerhans cells (LC), known as antigen presenting cells, play a role in atopic dermatitis. This study, for the first time, shows the presence of CD1a$^+$ cells, presumably LC, in the nasal mucosa. Possibly these LC also play a role in transporting allergens into the nasal mucosa. The impression exists that LC are more abundant in the grass-pollen season than before and after it. Research is continued in order to confirm this impression and to discover more data about transport function of allergens.

INTRODUCTION

Langerhans cells (LC) are large mononuclear dendritic CD1a$^+$ cells with Birbeck granules in their cytoplasm. For long, the role and function of LC remained obscure but recent research showed them to be essential antigen-presenting cells (1-3). LC are mesenchymal cells and derive from a precursor cell in the bone marrow (4). Being mobile

cells, they are able to migrate into the epidermis from a hematogenous source and probably also, when activated, from the epidermis to the dermis and regional lymph nodes (3,5). LC express Fc-receptors, C3-receptors, and class II major histocompatibility complex (MHC) molecules, designated HLA-DR in man (6-9).

In patients with atopic dermatitis, Bruijnzeel-Koomen et al. found IgE molecules on epidermal LC (10). Outside the epidermis, cells resembling LC ultrastructurally are present in a variety of tissues. These cells are called indeterminate cells, veiled cells, or interdigitating cells, depending on their localization. Such LC-like cells have been demonstrated in various squamous epithelia, normal dermis, lymphatics and draining lymph nodes, reviewed by Braathen et al. (11), and Peyer's patches of the gut (12). In bronchial epithelium LC have been reported by Richard et al. (13), but Sertl et al. could not demonstrate $CD1a^+$ cells although they found $HLA\text{-}DR^+$ dendritic cells (14).

Similarities in the expression of cell-surface receptors and of the antigens present on these cells suggest that they belong to the same lineage of antigen-presenting cells (15,16). The precise relationship between these subpopulations is not yet fully understood, but indeterminate cells are thought to be the direct precursors of LC. The monoclonal anti-CD1a (T6) antibody provides a specific and sensitive probe for the identification of LC and indeterminate cells (17-20). Outside the thymus, other $CD1a^+$ cells beside LC and indeterminate cells are only found in neoplastic diseases, such as histiocytosis X and T-cell lymphoma (21). Unlike HLA-DR, CD1a expression by LC in the skin is not, or only slightly, reduced by local corticosteroid therapy (22).

LC-like cells have not been described in columnar epithelium of the upper airways. The present study was performed to find out whether $CD1a^+/HLA\text{-}DR^+$ cells occur in the nasal mucosa, where antigenic stimulation is so frequent.

Materials and methods

Patients and controls

The 35 patients and controls selected for this study, 15 males and 20 females ranging in age from 17 to 65 years, were divided into three groups. The first group was composed of 11 patients (mean age 30 yr) with an isolated grass-pollen allergy confirmed by skin and radio-allergosorbent tests (RAST). The second group (mean age 27 yr) consisted of 12 controls (volunteers and patients with diseases of the ear, nose, and throat (ENT) not related to the nose or nasal sinuses) without nasal complaints, no nasal abnormalities seen at ENT examination, and a negative RAST. The third group (mean age 38 yr) consisted of 12 patients with nasal and/or sinusal polyps and a negative RAST, undergoing an operation to remove the polyps.

Nasal biopsy

Nasal biopsies were performed between July and November in 1986 under informed consent conditions. The biopts of the patients with isolated grass-pollen allergy were all taken in July, during the grass-pollen season. The specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, with a modified Hartmann forceps with a cup diameter of 2.5 mm.

Local anaesthesia was obtained by placing a cotton-wool carrier with 100 mg cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate without touching the place where the biopsy specimen

Table 1. Antibodies and antisera used in the present study.

Antibody	Type	Specificity	Source
66IIC7	M	CD1a	Monosan, Sanbio, Uden, The Netherlands
Anti HLA-DR	M	MHC class II antigen	Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam (CLB)
Anti-IgE-FITC	PR	epsilon heavy chain	CLB
Anti-mouse Ig-TRITC	PG	mouse immunoglobulins	Cappel, Conchranville, USA
Anti-mouse Ig-Per	PR	mouse immunoglobulins	Dakopatts, Copenhagen, Denmark
Anti-prostate antigen	M	prostate epith. cells	Eurodiagnostics, Oss, The Netherlands

M = mouse monoclonal; P = polyclonal; R = rabbit; G = goat; FITC = fluorescein-iso-thio-cyanate; Per = peroxidase; TRITC = tetramethyl-rodamine-iso-thio-cyanate.

would be taken (23). The specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and frozen immediately (24).

Antibodies

The antibodies used are listed in Table 1. The specificity of clone 66IIC7 for the CD1a(T6) marker used to label LC in the epidermis has been described elsewhere (25).

Staining procedure

The immuno alkaline phosphatase (AP) method used was a modification of the method described by Li et al. (26). Briefly, 6 μm-thick sections of nasal mucosa were cut on a Reichert-Jung 2800 Frigocut cryostat, transferred to gelatin-coated microscope slides, dried and fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline, (PBS, pH 7.2) and incubated with 66IIC7 (CD1a) monoclonal antibody for 30 min at 20°C. The sections were rinsed again in PBS for 5 min and incubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase (DAKO D314) 1:20 supplemented with 10% normal human serum (NHS) to saturate Fc-receptors and reduce background staining (DAKO D314), then rinsed successively in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a New Fuchsin solution.

This solution is made by thoroughly mixing 150 μl $NaNO_2$ 4% with 150 μl of a solution containing 1 g New Fuchsin (Chroma 1B467, Stuttgart, FRG) in 25 ml HCL 2N. Next 60 ml 0.2 M TRIS-HCl buffer (pH 8,0) containing 18 mg Naphtol-AS-MX phosphate (Sigma N-5000, St. Louis, Mo, USA) dissolved in 2 ml dimethyl formamide is added together with 15 mg levamisole (Sigma L-9756). The pH of the final mixture is adjusted to 8.0. Filter before use. Finally, the sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, and mounted in glycerin gelatin (Chroma).

Control sections incubated with monoclonal antibodies of the same subclass (IgG2a) like prostate specific antigen were negative. Immunofluorescence double-staining was performed with monoclonal CD1a followed by incubation with goat anti-mouse immunoglobulin labeled with TRITC (GaMIg-TRITC) and reincubation with anti-IgE labeled with FITC (RaHuIgE-FITC) or anti-HLA-DR labeled with FITC

(GaHuHLA-DR-FITC). In addition serial sections were incubated with anti-HLA-DR and anti-T6 alternately.

Cell quantification

The total surface area of epithelium and lamina propria in the section was estimated by superimposing a grid of 100 points (intersection of crosses, covering a surface area of 0.5 mm^2) on the section using an eyepiece graticule as described by Underwood (27). In the section all large dendritic cells containing a nucleus and staining red were considered to be $CD1a^+$ cells. The total number of T6-positive cells in epithelium and lamina propria was determined and the number of $CD1a^+$ cells per mm^2 was estimated.

Statistical analysis

Since the frequency distribution of the number of $CD1a^+$ cells per mm^2 in epithelium and lamina propria was not symmetrical and the variances were unequal, Kruskal-Wallis one way analysis of variances was used for calculating the overall p-value. For paired comparisons we used two-tailed Mann-Whitney U-test. A p-value < 0.05 was considered to indicate a significant difference between groups.

RESULTS

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed ciliated columnar epithelium and/or partially stratified cuboidal epithelium. All but one section (excluded from the study) showed intact or virtually intact epithelium.

All but two sections showed $CD1a^+$ dendritic cells in the middle and lower layers of the epithelium (Fig 1). All sections showed $CD1a^+$ cells in the lamina propria. The distribution was not always regular along the epithelium; in some cases a group of $CD1a^+$ cells was seen in one place and only few such cells in the rest of the epithelium. In the lamina propria such cells were seen mainly in the subepithelial layer and in and around the glandular tissue.

Double immunofluorescence staining with monoclonal anti-CD1a antibody and FITC labeled rabbit anti-IgE showed IgE on a few $CD1a^+$ cells in the nasal mucosa of three allergic patients investigated. Most of the $CD1a^+$ cells also expressed HLA-DR.

Table 2 gives the numbers of $CD1a^+$ cells/mm^2 surface area of the epithelium and the lamina propria in the nasal mucosa for the three groups. The number of $CD1a^+$ cells varied significantly over the three groups in the epithelium and lamina propria ($p = 0.047/p = 0.046$).

The number of $CD1a^+$ cells in the epithelium of allergic patients was significantly higher than the numbers in patients without nasal complaints and patients with nasal and/or sinusal polyps ($p = 0.018$). The numbers of $CD1a^+$ cells in the lamina propria were small. A significant difference was found between the numbers of such cells in lamina propria of patients without nasal complaints compared with allergic patients and patients with nasal and/or sinusal polyps ($p = 0.013$)

DISCUSSION

Langerhans cells are dendritic cells capable of binding and presenting potentially antigenic molecules to T lymphocytes. The finding that CD1a is a highly specific and

Table 2. Numbers of $CD1a^+$ cells/mm^2 in epithelium and lamina propria of nasal mucosa in patients with isolated grass-pollen allergy, patients without nasal complaints, and patients with nasal and/or sinusal polyps.

Group	Patients (n = 35)	$CD1a^+$ cells	
		Epithelium mean (SD)	Lamina propria mean (SD)
I	with grass-pollen allergy (n = 11)*	55 (49.5)	14 (22.0)
II	without nasal complaints (n = 12)	20 (14.1)	4 (4.0)
III	with nasal and/or sinusal polyps (n = 12)	28 (23.1)	10 (10.4)
Kruskal-Wallis overall p-value#:		0.047	0.046
Mann-Whitney: p-value I vs. II/III#		0.018	0.224
p-value II vs. I/III#		0.74	0.013

* In one of these patients the epithelium was damaged; this epithelium was not included in the study.

\# A difference between two patient groups was accepted as significant when the p-value was < 0.05.

sensitive marker for Langerhans and indeterminate cells means that LC in tissues can be detected and counted by light microscopy. Until now, epithelial LC were believed to occur only in squamous epithelium. The present results show that $CD1a^+$ cells, presumably LC, also occur in columnar epithelium and lamina propria of the nasal mucosa. Electron microscopical studies will show whether Birbeck granules - which are pathognomonic for LC - are present in these cells. We found significantly more $CD1a^+$ cells in the epithelium of nasal biopsy samples of allergic than in those of non-allergic patients.

Although the number of $CD1a^+$ cells in the lamina propria were small and results should be interpreted carefully, we found a significant difference between the number of these cells in the lamina propria of controls without nasal complaints compared to allergic patients and patients with nasal and/or sinusal polyps. So far, an explanation for this finding has not been found.

We used immunofluorescence methods and double staining with CD1a and anti-IgE, and found IgE on $CD1a^+$ cells in the nasal epithelium of patients with grass-pollen allergy. In studies on atopic dermatitis, Bruijnzeel-Koomen et al. (10) also found IgE molecules on epidermal LC and postulated that this binding occurred via the Fc-receptors on LC. It is not clear why only some of the $CD1a^+$ cells showed surface IgE. Bruijnzeel-Koomen et al. (10) also reported that some LC in the skin failed to show surface IgE, and suggested that this was to be ascribed to a variation of $Fc_\varepsilon R$ expression, as has been described for other cell

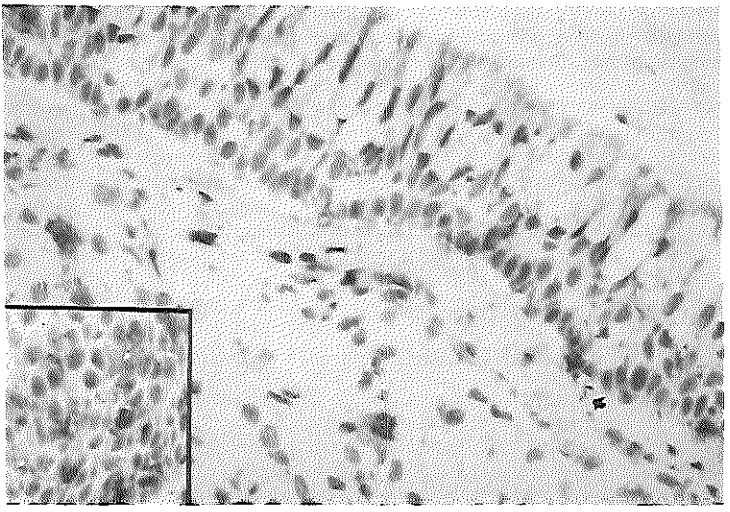

Figure 1. Light micrograph of the nasal mucosa of a patient with an isolated grass-pollen allergy incubated with AP-labeled monoclonal anti-CD1a (x320). $CD1a^+$ dendritic cells in the middle and lower layers of the epithelium, some showing dendritic processes through the basement membrane (↑). Inset: $CD1a^+$ cells in the epithelium clearly showing dendritic morphology. x 320.

populations such as eosinopils, monocytes and alveolar macrophages.

Although the possibility of an aspecific increase of $CD1a^+$ cells due to inflammation in allergic rhinitis cannot be excluded, the finding of surface IgE on these cells points towards a possible role in the allergic mechanism. If in allergic rhinitis $CD1a^+$ cells, presumably LC, do indeed play a role in allergen presentation, it remains an interesting question whether these cells are constitutionally more numerous in nasal mucosa of patients with allergic rhinitis or whether they migrate to the epithelium during the exposition. Studies in patients with grass-pollen allergy are in progress to find out whether the number of $CD1a^+$ cells in the nasal mucosa is dependent on the grass-pollen season.

In sum, the results of the present study show that:

1. $CD1a^+$ cells are present in epithelium and lamina propria of nasal mucosa;
2. the number of $CD1a^+$ cells is higher in nasal epithelium of patients with a grass-pollen allergy than in non-allergic patients without nasal complaints or with nasal and/or sinusal polyps;
3. the number of $CD1a^+$ cells is lower in nasal lamina propria of controls without nasal complaints compared with allergic patients and patients with nasal and/or sinusal polyps; and
4. IgE is present on $CD1a^+$ cells in the nasal epithelium of patients with a grass-pollen allergy.

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CHAPTER 8

LANGERHANS CELLS IN NASAL MUCOSA OF PATIENTS WITH GRASS-POLLEN ALLERGY

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ABSTRACT

Langerhans cells (LC) are known to be present in squamous epithelia of the human body. They are dendritic cells (DC) and characterized by the presence of Birbeck granules (BG). In previous studies, DC positive for CD1a and HLA-DR were found in the cylindrical epithelium and the lamina propria of the nasal mucosa. More CD1a cells occurred in the allergic patients than in the non-allergic controls.

In a combined light microscopy (LM) and electron microcopy (EM) study, biopts of nasal mucosa in allergic patients were studied. We used monoclonal antibodies against CD1a and HLA-DR, to identify DC in LM cryostat sections. The presence of BG identified most of the intra-epithelial DC as LC on the EM level, whereas a minority of DC in the lamina propria also contained BG. The ultrastructure of LC and DC in the ciliated cylindrical epithelium and the lamina propria is compared.

INTRODUCTION

Langerhans cells (LC) are dendritic epidermal cells, which are defined by the presence of Birbeck granules (BG) in the cytoplasm. Dendritic cells (DC) occur in a variety of lymphoid and non-lymphoid tissues. In the epidermis they are called indeterminate cells if BG are not detected. Both veiled cells in afferent lymph and interdigitating cells in skin draining lymph nodes, may contain BG but differ in ultrastructure (for review, see ref. 1). Dendritic cells are potent antigen presenting cells. The role and function of LC are not fully understood, but LC are related to DC and can develop the same antigen presenting capability as DC (for review, see ref 2). Both LC and DC are HLA-DR positive, but the monoclonal anti-CD1a (T6) antibody provides a more specific and sensitive probe for the identification of LC and some indeterminate cells (3-5). LC have incidentally been described in cylindrical epithelium of the respiratory tract (6-8).

In earlier studies we found HLA-DR positive and CD1a(T6) expressing DC in the epithelium and lamina propria of nasal mucosa in patients with grass-pollen allergy and controls without nasal complaints. These cells were significantly more numerous in the epithelium of nasal biopsy samples from patients with grass-pollen allergy during the grass-pollen season than in those of the non-allergic controls (9).

Electron microscopical studies were performed to find out whether BG - which characterize LC - were present in the sub-epithelial and/or epithelial DC and whether these cells form a homogeneous population in respect to the ultrastructure.

Materials and methods

In twenty patients with an isolated grass-pollen allergy (mean age 34, 11 ♂, 9 ♀) confirmed by a positive skin prick test reaction with Alutard Soluprick extract of 1 HEP, a median (range) radio-allergosorbent test (RAST) score of 4+ (4+-5+) and a median (range) total IgE value of 156 (15-500) nasal biopsies were performed during the grass-pollen season under informed consent conditions. All these patients had complaints of allergic rhinitis (e.g. nasal blockage, rhinorrhoea and sneezing) at the moment of the biopsy. The controls comprised 12 volunteers and patients (mean age 28 yr, 3 ♂, 9 ♀) visiting the ear, nose and throat (ENT) department with an ear, or throat disease not related to the nose and nasal sinuses, without nasal complaints, no abnormalities in the nose at ENT examination, a negative RAST score, and a median (range) total IgE value of 33 (4-90) IU/ml. None of the patients or controls in this study used any medication which could influence the results of this study, with the exception of the antihistamine drug terfenadine which was used by the patients with grass-pollen allergy. Terfenadine tablets were always discontinued at least 48 hours before a biopsy was performed.

Biopsy specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, with a Gerritsma forceps with a cup diameter of 2.5 mm. Local anaesthesia was obtained by placing a cotton-wool carrier with 100 mg cocaine and 3 drops of adrenalin (1:1000) under the inferior turbinate without touching the site of the biopsy (10).

For light microscopy the specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and frozen immediately. The immunolabelling was performed as described elsewhere (99). Briefly, 6 μm-thick serial sections of nasal mucosa were fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2), and incubated with anti-HLA-DR (CLB, Amsterdam, The Netherlands) or 66IIC7 (CD1a, Monosan, Uden, The Netherlands) monoclonal antibody for 30 min at 20°C. The specificity of clone 66IIC7 for the CD1a marker used to label LC in the epidermis has been discussed elsewhere (11). The sections were rinsed again in PBS for 5 min and reincubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase 1:20 (DAKO D314, Denmark) supplemented with 10% normal human serum (NHS), rinsed successively in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a New Fuchsin solution. Finally, the sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, and mounted in glycerin gelatin (Chroma, Stuttgart, FRG). Control sections incubated with irrelevant monoclonal antibodies of the same subclass (IgG2a) were negative.

For electron microscopy, the specimens were immediately immersed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer adjusted to pH 7.4 at 4°C. After 24 hours the tissue fragments were washed and postfixed in 1% OsO4 in the same buffer, dehydrated in ethanol, and embedded in Epon. Semi-thin sections were stained with toluidine blue. These sections were used for the selection of areas where pale cells with a dendritic appearance were present. The selected areas were then trimmed for ultramicrotomy and the ultrathin sections were contrasted with 5% uranyl acetate and lead citrate according to Reynolds (12) before examination in a Philips EM 300 at 60 KV with the use of an objective with a 30 μm aperture.

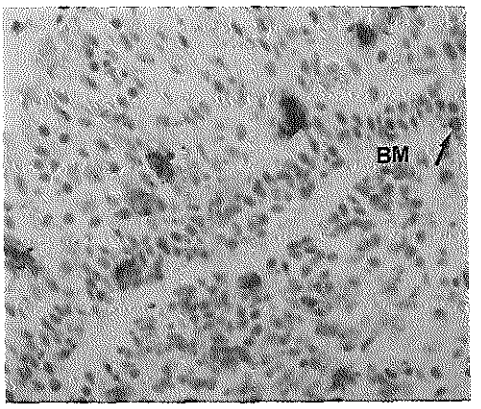

Figure 1. Cryostat section of the nasal mucosa of a patient with an isolated grass-pollen allergy, stained with 66IIC7 (anti-CD1a) and RaMIg-alkaline phosphatase. CD1a$^+$ dendritic cells are present in the middle and lower layers of the epithelium, between the cylindrical respiratory epithelial cells, and in the lamina propria. Note the CD1a $^+$ cell penetrating the basement membrane (BM) (↑). x 320.

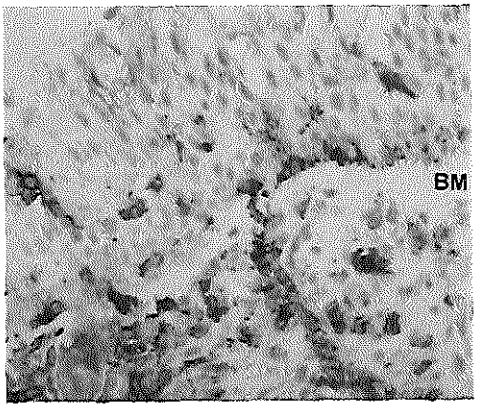

Figure 2. Serial cryostat section stained with monoclonal anti-HLA-DR and RaMIg-alkaline phosphatase. Many HLA-DR $^+$ dendritic cells are present in the nasal epithelium and in the lamina propria. Some of these cells are seen in the basement membrane. x 320.

Results

Cryostat sections and low-magnification electron microscopy gave a general view of the nasal mucosa showing the cylindrical epithelium, the basement membrane, which contained huge bundles of collagenous fibres, and the lamina propria (Fig 1-3). Between the ciliated epithelial cells large numbers of goblet cells were present. Mast cells, occasional eosinophils, and appreciable numbers of lymphocytes were scattered mainly in the basal layers of the epithelium.

In the cryostat sections of the mucosa of the allergic patients many more CD1a$^+$ cells (Fig 1) were present in the epithelium than in the controls (not shown), whereas low, but significant numbers were also present in the lamina propria. In allergic patients CD1a$^+$ dendritic cells were present in the suprabasal layer of the cylindrical epithelium and in the lamina propria (Fig 1). In the lamina propria the CD1a$^+$ cells occurred mainly in the subepithelial layer and in and around the glandular tissue.

Staining of the allergic mucosa with anti-HLA-DR showed many positive cells in the epithelium as well as in the lamina propria (Fig 2), the number varying considerably between the specimens. No significant differences could be found between the allergic patients and the controls. Small dendritic cells were present in the lamina propria and more concentrated in the vicinity of the capillaries. Cells of the monocyte-macrophage lineage, DC, and B cells were usually positive. With increasing numbers of HLA-DR positive cells, first the presumably activated T cells and the endothelial cells, and finally the cytoplasm of epithelial cells became positive.

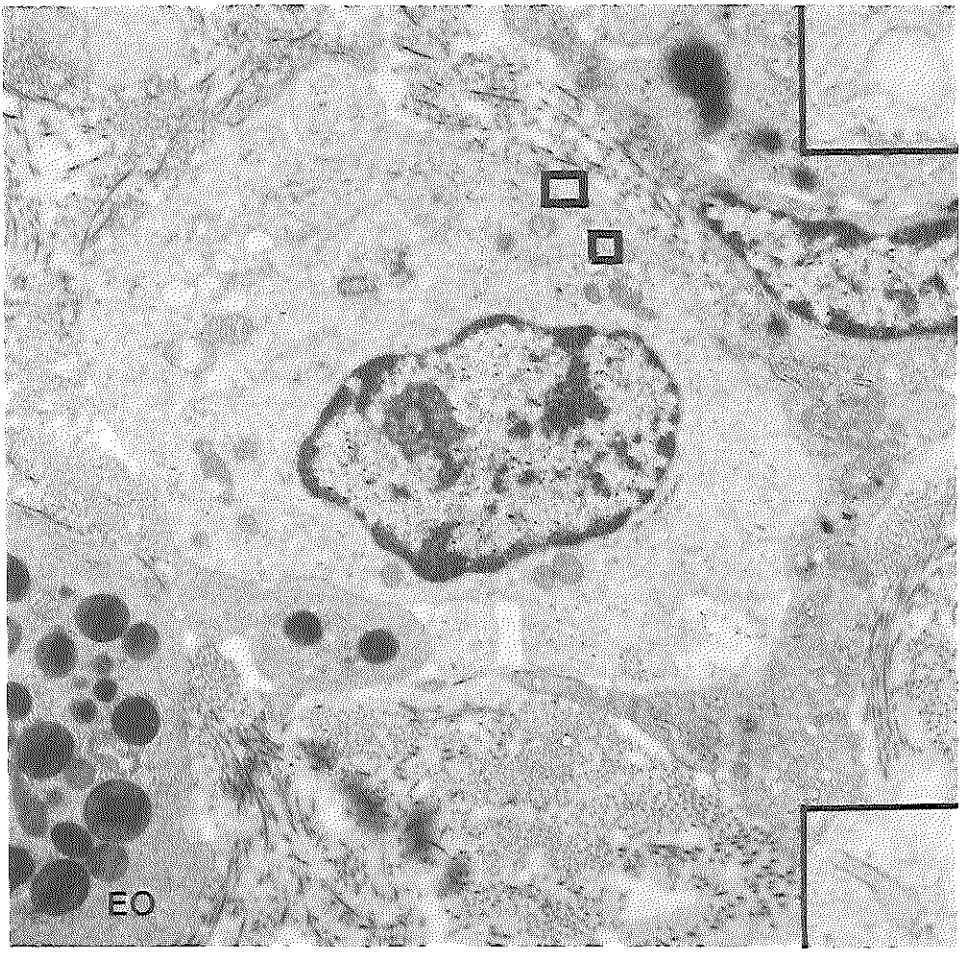

Figure 3. Electron microscopical section of the nasal mucosa showing LC in the lamina propria close to the basement membrane containing at least two Birbeck granules (insets), one with the typical racket shape. Note the collagenous fibres and part of the cytoplasm of an eosinophil (EO). (x 10.000, insets x 70.000)

Electron microscopically, DC could easily be recognized in allergic patients, by the electron-lucent cytoplasm, extending cytoplasmic processes between the epithelial cells (Fig 3). In the basal half, but suprabasal layer of the epithelium, in allergic patients many large dendritic cells with electron-lucent cytoplasm and long processes were interspersed between the adjoining epithelial cells (Fig 3a). In these epithelial DC the nucleus was slightly lobulated. The nucleo-cytoplasmic ratio was usually less than 1:5. The nucleus was euchromatic and a nucleolus was frequently seen. The cells contained small numbers of poly ribosomes and moderate numbers of mitochondria. A well-developed rough endoplasmic reticulum (RER) was present surrounding the nucleus and several well developed Golgi stacks occupied the central area of the cell (Fig 3b and c). The cells had also an abundance of small bristle-coated vesicles as well as small vesicles showing moderately electron dense

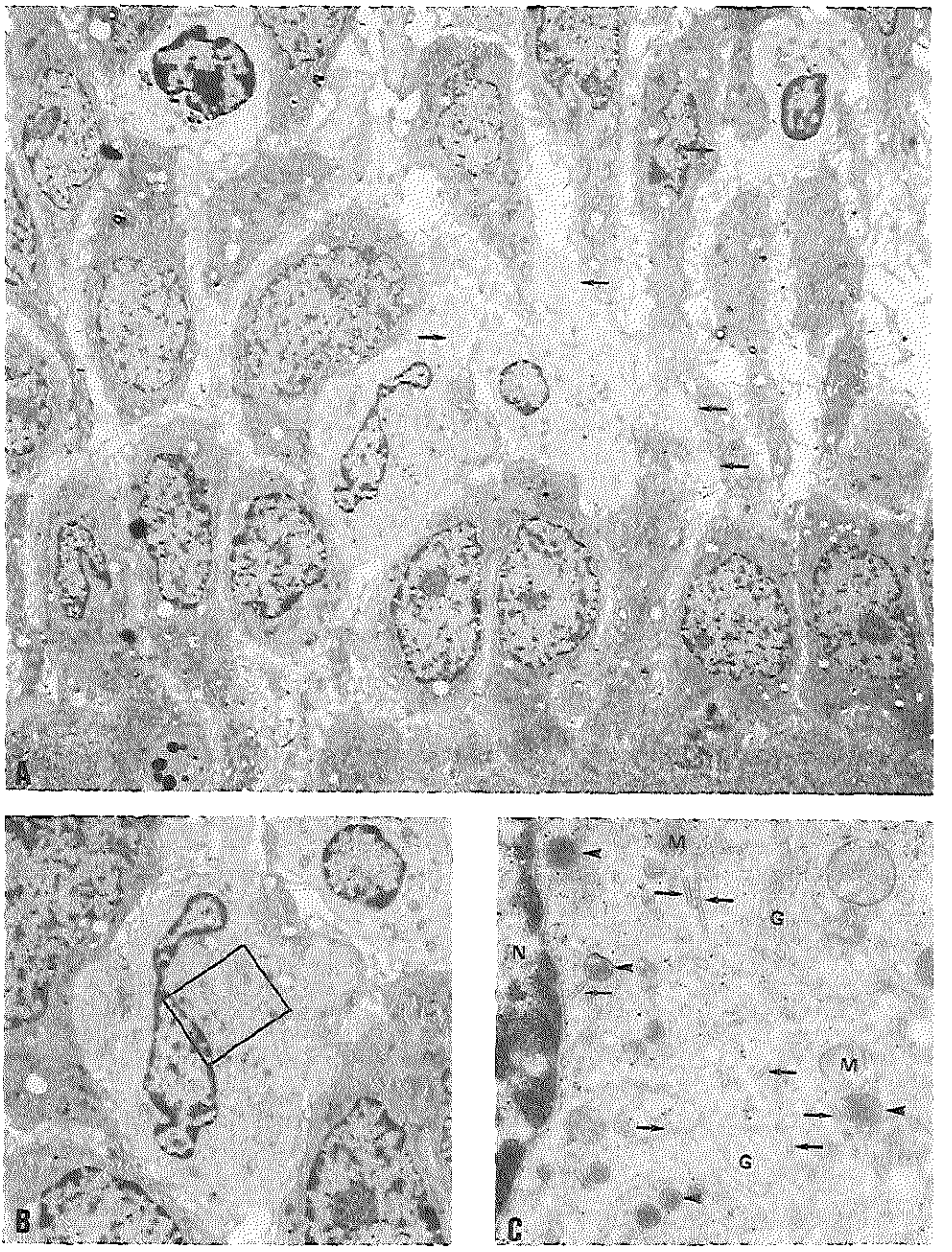

Figure 4. Electron micrographs showing Langerhans cells in suprabasal layer of respiratory epithelium. a. Langerhans cell extensions are indicated by arrows. x 2750. b. Langerhans cell at higher magnification. x 4.000. E: epithelial cell. c. Detail of central area of same LC. N: nucleus; G. Golgi stacks; M: mitochondrium. Note the large number of Birbeck granules (↑). Small electron dense vesicles are indicated by arrowheads. x 20.000.

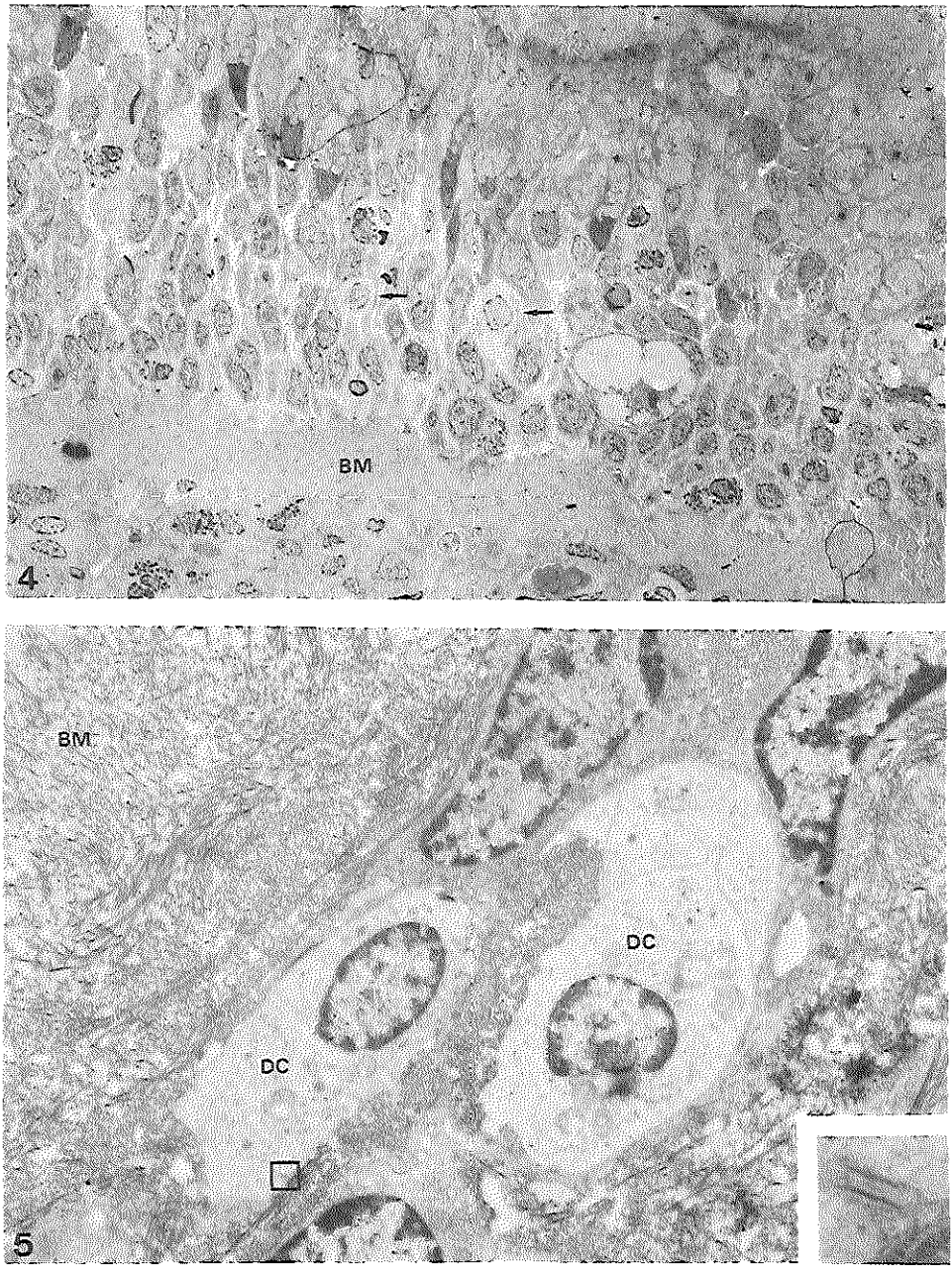

Figure 5. Electron micrograph showing basal layers of respiratory epithelial cells, basement membrane (BM), and lamina propria. Langerhans cells and/or dendritic cells are indicated by arrows. x 800.

Figure 6. Two dendritic cells (DC) in the lamina propria near the basement membrane. One contains a Birbeck granule in contact with the plasma membrane (see inset) the other not. x 85.000.

heterogeneous material, bundles of microfilaments, but no tonofilaments. Birbeck granules were observed in almost all of these epithelial dendritic cells (Fig 3c). They were present near the plasma membrane or in the center of the cell near the Golgi system, but were not seen to make contact with the plasma membrane or any other cell organelles. In the very few DC in the normal mucosa we could not catch a representative picture of an epithelial DC containing Birbeck granules.

DC in the basal layers of the epithelium and the lamina propria differed from those seen in the epithelium, in several respects. They seemed to be slightly smaller (Fig 4 and 5). The nucleus was globular, reniform, or slightly lobulated. The nucleo-cytoplasmic ratio varied from 1:2 to 1:5. The structure of the nucleus did not differ from those in the epithelial DC and a nucleolus was also frequently seen. The electron-lucent cytoplasm was relatively poor in cell organelles. A pair of centrioles was frequently present, but RER and Golgi apparatus were hardly developed. Only some polyribosomes and a moderate number of mitochondria were invariably present. Moreover, unlike the corresponding cells in the epithelium, only few of the DC showed BG. In one such a cell a BG was seen in contact with the plasma membrane (Fig 5). The ultrastructure of DC containing BG did not differ from the other DC in the lamina propria.

Discussion

In previous studies we found relatively great numbers of both CD1a-, and HLA-DR-expressing cells in columnar respiratory epithelium and the lamina propria of the nasal mucosa of patients suffering from grass-pollen allergy (9). The finding that these cells contain Birbeck granules (BG) definitely shows them to be Langerhans cells (LC). The controls showed few CD1a $^{+}$ cells and no representative picture of an epithelial DC containing BG. The LC in the nasal epithelium resemble the epidermal LC both ultrastructurally and immunocytochemically. However, only few of the DC in the lamina propria showed BG and such cells resembled the LC in the basal layers of the epithelium. No differences could be found between the DC with and without BG in the lamina propria.

The LC found in the lamina propria can be LC precursors (indeterminate cells) (3), LC which emigrated from the epithelium after stimulation (13) or dendritic cells emigrated from the blood that remain in the lamina propria (14). It is most conceivable that the cells in the lamina propria that contain BG represent DC (indeterminate cells) acquiring BG on their way to penetrate the epithelium and to become LC (3). However the possibility that they represent LC which have returned from the epithelium after acquiring BG and before full maturation as DC on their way to the regional lymph node (13) or the possibility that they represent LC, which migrate from the blood and remain in the lamina propria to form clusters with T cells directly as seen in delayed immune responses in the skin cannot be excluded (14).

However both the EM and the LM findings strongly suggest an influx of DC, into the mucosa via the capillaries of the lamina propria, which from there penetrate the epithelium, become CD1a^{+} and develop Birbeck granules, like epidermal LC (3).

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CHAPTER 9

FLUCTUATION OF THE NUMBER OF CD1a(T6)-POSITIVE DENDRITIC CELLS, PRESUMABLY LANGERHANS CELLS, IN THE NASAL MUCOSA OF PATIENTS WITH AN ISOLATED GRASS-POLLEN ALLERGY BEFORE, DURING, AND AFTER THE GRASS-POLLEN SEASON

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ABSTRACT

A monoclonal antibody against CD1a(T6) was used for studies in the nasal mucosa of patients with isolated grass-pollen allergy to find out whether the number of $CD1a^+$ cells, presumably Langerhans cells, depends on the season in which the nasal biopsy is performed. An earlier study had shown that during the grass-pollen season there are significantly more $CD1a^+$ cells in nasal mucosa of patients with isolated grass-pollen allergy than in non-allergic controls without nasal complaints. During the grass-pollen season the nasal epithelium of patients with an isolated grass-pollen allergy showed significantly more $CD1a^+$ cells than it did before and after the season. Before and after the season, the number of $CD1a^+$ cells in epithelium of the allergic patients was not significantly greater than the corresponding number in epithelium of non-allergic subjects without nasal complaints.

INTRODUCTION

Langerhans cells (LC) are large mononuclear dendritic $CD1a^+$ cells containing Birbeck granules in the cytoplasm. The role and function of LC are incompletely known, but recent research showed that they are essential antigen-presenting cells (1-4). LC are mesenchymal cells and derive from a precursor cell in the bone marrow (5). As mobile cells, they can migrate into the epidermis from a hematogenous source and, after activation, probably also from the epithelium to sub-epithelial layers and regional lymph nodes (3,6). In an earlier study we found dendritic CD1a(T6) HLA-DR expressing cells, resembling LC in the epithelium and lamina propria of nasal mucosa in patients with grass-pollen allergy and controls without nasal complaints. These cells were significantly more numerous in epithelium of nasal biopsy samples of patients with grass-pollen allergy during the grass-pollen season than in those of non-allergic controls. Using double staining methods with CD1a and anti-IgE-FITC, we also observed IgE on $CD1a^+$ cells (7,8). Because it was not yet clear

whether LC are constitutionally more numerous or their numbers increase by migration into the epithelium during the season, further investigations were performed in patients with grass-pollen allergy to determine whether changes occur in the number of $CD1a^+$ cells after the grass-pollen season.

Materials and methods

Patients

Twelve patients with an isolated grass-pollen allergy, confirmed by skin and radio-allergosorbent tests (RAST) were selected for this study. This group consisted of 4 ♂ and 8 ♀ with a mean age of 30 years (range 17-51 yr). The control group consisted of 12 volunteers and patients with diseases of the ear, nose, and throat (ENT) not related to the nose or nasal sinuses (mean age 28 yr, range 15-42, 3 ♂, 9 ♀). These controls had no nasal complaints, no nasal abnormalities seen at ENT examination and a negative RAST.

Nasal biopsy

Nasal biopsies were performed four times in each patient, i.e., in July and October of 1986 and March and July of 1987. Biopsies of the controls were performed once in July or August 1986. Biopsies were performed under informed consent conditions. The biopsy specimens were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, with a slightly modified Hartmann forceps with a cup diameter of 2.5 mm. Local anesthesia was obtained by placing a cotton-wool carrier with 100 mg cocaine and 3 drops of adrenalin (1:1000) under the inferior turbinate without touching the place where the biopsy specimen would be taken. The specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and frozen immediately (9,10).

Antibodies

The specificity of clone 66IIC7 for the CD1a surface marker of LC has been discussed elsewhere (11).

Staining procedure

The immuno alkaline phosphatase (AP) method used was a modification of the method described by Li (12). Briefly, 6 μm-thick sections of nasal mucosa were cut on a Reichert-Jung 2800 Frigocut cryostat, transferred to gelatin-coated microscope slides, dried and fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline, (PBS, pH 7.2) and incubated with 66IIC7 (CD1a) monoclonal antibody for 30 min at 20°C. The sections were rinsed again in PBS for 5 min and incubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase (DAKO D314) 1:20 supplemented with 10% normal human serum (NHS) to saturate Fc-receptors and reduce background staining (DAKO D314), then rinsed successively in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a New Fuchsin solution.

This solution is made by thoroughly mixing 150 μl $NaNO_2$ 4% with 150 μl of a solution containing 1 g New Fuchsin (Chroma 1B467, Stuttgart, FRG) in 25 ml HCL 2N. Next 60 ml 0.2 M TRIS-HCL buffer (pH 8,0) containing 18 mg Naphthol-AS-MX phosphate (Sigma N-5000, St. Louis, Mo, USA) dissolved in 2 ml dimethyl formamide is added together with 15 mg levamisole (Sigma L-9756). The pH of the final mixture is adjusted to 8.0. Filter before use. Finally, the sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, and mounted in glycerin gelatin (Chroma).

Control sections incubated with monoclonal antibodies of the same subclass (IgG2a) like prostate specific antigen were negative. Immunofluorescence double-staining was performed with monoclonal CD1a followed by incubation with goat anti-mouse immunoglobulin labeled with TRITC (GaMIg-TRITC) and

Table 1. Numbers of CD1a$^+$ cells/mm^2 in epithelium and lamina propria of nasal mucosa in 12 patients with isolated grass-pollen allergy before, during, and after the grass-pollen season and 11 controls.

		Epithelium *		Lamina propria	
	Period of biopsy	Mean	SD	Mean	SD
Group					
I	July 1986	55	46.9	14	21.9
II	October 1986	31	22.0	7	5.3
III	March 1987	30	35.0	4	3.4
IV	July 1987	80	102.0	6	5.4
Control subjects	July-August 1986	20	14.1	4	4.0
Group comparison + (p-value)					
I/II		0.047∞		0.165	
I/III		0.019		0.082	
I/IV		0.178		0.125	
II/III		0.445		0.007	
II/IV		0.074		0.223	
III/IV		0.047		0.072	

*: In one patient in group I, another patient in group IV, and one control the epithelium was damaged, and was not included in the study.
+: Significance $p < 0.05$.
∞: p-value from paired Student's t-test comparing any 2 periods.

reincubation with anti-IgE labeled with FITC (RaHuIgE-FITC) or anti-HLA-DR labeled with FITC (GaHuHLA-DR-FITC). In addition serial sections were incubated with anti-HLA-DR and anti-T6 alternately.

Cell quantification

The total surface area of epithelium and lamina propria in the section was estimated by superimposing a grid of 100 points (intersection of crosses, covering a surface area of 0.5 mm^2) on the section using an eyepiece graticule as described by Underwood (13). In the section all large dendritic cells containing a nucleus and staining red were considered to be T6-positive cells. The total number of T6-positive cells in epithelium and lamina propria was determined and the number of T6-positive cells per mm^2 was estimated.

Statistical analysis

For the statistical analysis use was made of the paired Student's one-tailed t-test for the differences between the data for the four periods and Student's two-tailed t-test for comparison of allergic patients against controls. A p-value < 0.05 was considered to indicate a significant difference between the groups.

RESULTS

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed ciliated columnar epithelium and/or partially stratified cuboidal epithelium. All but three sections (excluded from the study) showed intact or virtually intact epithelium.

All but three sections showed $CD1a^+$ dendritic cells in the middle and lower layers of the epithelium (Fig. 1). All sections showed $CD1a^+$ cells in the lamina propria. As a rule, the $CD1a^+$ cells were distributed over the basal and middle layers of the epithelium. The distribution was not always regular along the epithelium; sometimes a group of $CD1a^+$ cells was seen in one place and only few such cells in the rest of the epithelium. In the lamina propria the cells occurred in the subepithelial layer and in and around the glandular tissue.

Double immunofluorescence staining with monoclonal anti-CD1a antibody and FITC labeled rabbit anti-IgE showed IgE on a few $CD1a^+$ cells in the nasal mucosa of three allergic patients investigated. Most of the $CD1a^+$ cells also expressed HLA-DR.

Table 1 shows the number of $CD1a^+$ cells/mm^2 in nasal mucosa of the patients with isolated grass-pollen allergy in the four periods under study and the controls. As published earlier (8) the difference between the number of $CD1a^+$ cells in the epithelium of patients with grass-pollen allergy in the grass-pollen season and non-allergic controls is significant ($p = 0.04$). The difference between these numbers before and after the grass-pollen season on one hand and controls on the other is not significant.

The numbers of $CD1a^+$ cells were compared before, during and after the grass-pollen season. In the epithelium the number of $CD1a^+$ cells in the grass-pollen season (July) was significantly higher than it was before the season (March). After the season the number of $CD1a^+$ cells was significantly lower than it had been during the preceding summer. The difference between October 1986 and July 1987 did not quite reach significance ($p = 0.074$). The lamina propria had few $CD1a^+$ cells. The differences are only significant between October 1986 and March 1987.

DISCUSSION

Langerhans cells are dendritic cells capable of binding and presenting potentially antigenic molecules to T lymphocytes and probably other cells. They are also able to secrete important immunostimulatory factors, e.g. interleukin-1, exerting an influence on T-cell proliferation (4).

The highly specific and sensitive marker for Langerhans cells and indeterminate cells, CD1a, has made it possible to detect and count LC in tissues by light microscopy. In an earlier study we found $CD1a^+$ cells in nasal mucosa of allergic patients and non-allergic controls. The finding that the number of $CD1a^+$ cells was higher in nasal mucosa of patients with grass-pollen allergy than of non-allergic controls and the demonstration of IgE on $CD1a^+$ cells, suggesting a role in IgE-mediated disease, led us to perform further investigations to determine whether the number of $CD1a^+$ cells is influenced by the exposure to grass-pollen and thus is dependent on the grass-pollen season.

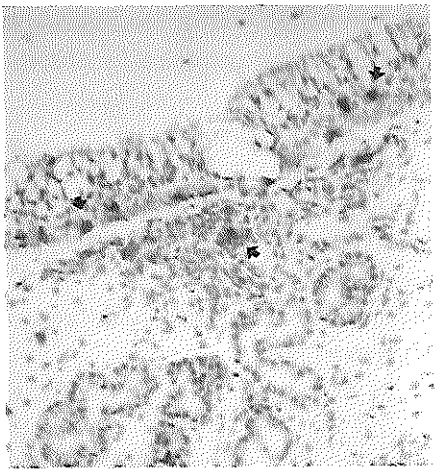

Figure 1. Light micrograph of the nasal mucosa of a patient with an isolated grass-pollen allergy incubated with AP-labeled monoclonal anti-CD1a. $CD1a^+$ dendritic cells (arrow) in the middle and lower layers of the epithelium and in the subepithelial layer of the lamina propria. x 160.

We found significantly more $CD1a^+$ cells in epithelium of nasal mucosa of patients with isolated grass-pollen allergy during the season than before or after it. It seems possible that these cells migrate into the epithelium during allergen provocation. A possible sequence has been suggested according to which morphologically less developed indeterminate precursor cells, containing fewer Birbeck granules, migrate from the dermis into the epidermis, where they reach structural and perhaps also functional maturity (6,13). It has also been suggested that LC, as antigen-presenting cells, migrate from the epidermis to lymphoid tissue (6). We found some small changes in the numbers of $CD1a^+$ cells in the lamina propria of nasal mucosa during the season. EM studies can be expected to show whether these $CD1a^+$ cells are indeterminate-like precursor cells or mature LC carrying antigen to regional lymph nodes (14). Cell division might also explain the increase of the number of $CD1a^+$ cells during allergen exposure, since there have been a limited number of observations of mitotic activity in LC (15,16).

The fact that a significant difference was found between the number of $CD1a^+$ cells in the epithelium of patients with grass-pollen allergy and non-allergic controls in the grass-pollen season but not before and after the season, suggests that LC are not constitutionally more numerous in allergic patients but that they increase after allergen provocation. An interesting question remains, i.e., whether $CD1a^+$ cells are depleted during the grass-pollen season or migrate from the epithelium after the season.

Three findings suggest a role for $CD1a^+$ cells in allergic disease: that patients with grass-pollen allergy during the grass-pollen season have a larger number of $CD1a^+$ cells than non-allergic controls do, that they have a larger number of $CD1a^+$ cells during than out of the grass-pollen season, and that such cells show IgE positivity. A role in allergen transport for these cells seems likely, although an aspecific increase due to inflammation in allergic rhinitis cannot be excluded.

It would be interesting to find out whether CD1a$^+$ cells are able to bind allergen via CD1a, Fc-receptors, or HLA-DR, and can therefore transport allergens through the basement membrane into the lamina propria and the regional lymph nodes. Further investigation is needed to evaluate the relationship between CD1a$^+$ cells, other immunological parameters in nasal mucosa, and the clinical features of allergic disease.

In sum, the results of this study show that:

1. the number of CD1a$^+$ cells in nasal epithelium of patients with grass-pollen allergy is significantly higher during the grass-pollen season but not significantly higher before and after the grass-pollen season than that in non-allergic controls; and
2. the number of CD1a$^+$ cells nasal epithelium of patients with isolated grass-pollen allergy is significantly higher in the grass-pollen season than before or after it.

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CHAPTER 10

DYNAMICS OF NASAL LANGERHANS CELLS IN PATIENTS WITH ALLERGIC RHINITIS AND NON-ALLERGIC CONTROLS DURING ALLERGEN PROVOCATION, A BIOPSY STUDY

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ABSTRACT

The incidence of Langerhans cells (LC) was studied in the epithelium and lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy during a 2 week provocation period. The study showed that the number of LC increased at these sites under these conditions. The LC seemed to migrate from the lamina propria to the epithelium. Significantly, already in the acute phase, ½ hour after the first provocation, a threefold increase in LC numbers was seen in the epithelium. The increase in the lamina propria occurred later, approximately 24 hours after the first provocation. During the provocation period a significant increase in *the number* of IgE^+ LC was also found, but *the percentage* of IgE positivity on LC remained the same.

The findings in this study support the hypothesis that IgE^+ LC are involved in allergic disease. Functional studies are necessary to further elucidate their role.

INTRODUCTION

There is an increasing interest in the role of lymphocytes in IgE mediated reactions in respiratory mucosa (reviewed in 1). However, only a few studies have been published on respiratory epithelial antigen/allergen processing and presentation, which constitutes a necessary step for T lymphocyte activation. Recent studies suggest that the principal antigen presenting cell (APC) in the lung is the dendritic cell (DC)/ Langerhans cell (LC) (2,3).

The role and function of LC are not fully understood, but LC are related to DC and can develop the same antigen presenting capacity as DC (for review, see 4). Both LC and DC are HLA-DR positive. The monoclonal anti-CD1a (T6) antibody provides a more specific and sensitive probe for the identification of LC and some precursors of LC called indeterminate cells (5-7). Moreover, LC can be distinguished by the Birbeck granule, a cell organel which is pathognomonic for LC.

LC have recently been described in cylindrical epithelium of the lung (22), the

adenoid (8) and the nose (9-11). In the epithelium of the nasal mucosa most DC contain Birbeck granules and are therefore LC (11). In the lamina propria only part of the DC are LC. Other DC may be interpreted as precursors of LC or as LC migrated from the epithelium and matured into DC, losing their Birbeck granules and CD1a positivity (12).

LC seem to play a role in allergen presentation in atopic disease (for review, see 13). Recent investigations in allergic rhinitis have shown that the number of LC in the epithelium of the nasal mucosa of symptomatic rhinitis patients with isolated grass-pollen allergy is significantly higher than in non-allergic patients with nasal polyps or normal controls (11). Futhermore, the number of LC in the epithelium of the patients with isolated grass-pollen allergy was significantly larger during the grass-pollen season (July) than before and after the season (14). Moreover in these patients 20-40% of the $CD1a^+$ cells in the epithelium and occasionally $CD1a^+$ cells in the lamina propria were found to be positive for IgE, but not for CD23 (14).

In patients with atopic dermatitis the number of LC is increased in the dermis (15,16), but not in the epidermis (17,18). IgE positivity on LC was found more often in involved skin than in uninvolved skin (19). Still, 75% of the LC in uninvolved skin was found to be positive for IgE (20). In in vitro experiments, epidermal IgE^+ LC of patients with atopic dermatitis were able to induce T cell proliferation to house dust mite allergen, contrary to IgE^- LC (21). These findings suggest that IgE positivity of LC is a necessary phenomenon of allergen presentation in allergic disease.

To further study the dynamics and IgE positivity of LC in allergic rhinitis, a provocation study was performed in patients with isolated grass-pollen allergy during 2 weeks in January. In this period, biopsies of nasal mucosa were performed 3-4 times in each patient. The number of $CD1a^+$ cells was monitored during this period and double immunofluorescence for CD1a and IgE positivity was performed.

Materials and methods

Patients and controls

Seventeen patients and four controls participated in the study. The patients (mean age 29 yr, 7 ♂, 10 ♀) had had an isolated grass-pollen allergy for at least one year, confirmed by a positive skin prick test reaction with Alutard Soluprick extract of 1 HEP/ml and no other positive skin prick test reaction with 13 common allergens, a median (range) radio-allergosorbent test (RAST) score of 4+ (3+-5+) and a median (range) total IgE value of 290 IE/ml (22-1900). The controls, four volunteers without nasal complaints (mean age 28 yr, 2 ♂, 2 ♀), had no abnormalities in the nose at ENT examination and a negative skin prick test reaction with Alutard Soluprick extract of 13 common inhalant allergens. None of the patients or controls in this part of the study used any medication, nor had anyone had an airway infection during the week preceding the challenge. Patients were randomly distributed over subgroups according to Table 1.

Design of the study

Patients and controls were daily challenged with allergen during a 2 week period. Signs and symptoms were recorded every day. All patients were biopted 3 or 4 times, the controls were biopted 3 times. In order to investigate time-related effects and for statistical reasons, the patients were randomly

Table 1. Moment of biopsy for different patients. Patients were randomly divided in two groups (I and II).

patient group	moment of biopsy after first provocation			
I n=7	before provocation n=7	24-48 hr n=5	8-10 days n=6	1-2 weeks after last provocation n=9
II n=10	½ hr n=5	4-6 days n=9	14 days n=9	
	8 hr n=5			

divided into two groups. By dividing the patients in two groups a sufficient number of different biopsy moments in time could be created. Consequently biopts of the allergic patients were taken at 7 different moments as shown in Table 1. One patient of group I and one patient of group II were only biopted once because they dropped out of the study before the second biopsy could be taken. One patient of group I was biopted only twice.

Nasal biopts

Biopts of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge using a Gerritsma forceps with a cup diameter of 2.5 mm. Local anaesthesia was obtained by placing a cotton-wool carrier with 50-100 mg cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate without touching the place where the biopsy would be taken (22). The biopsy specimens were embedded in Tissue-Tek II O.C.T. compound in a gelatin capsule and immediately frozen (23). Biopts of the allergic patients were taken before the provocation, 1/2 hr, 8 hr and 24 hr after the first provocation, 2, 4, 6, 8, 10, and 14 days after the first provocation, and 1 and 2 weeks after the last provocation (Table 1). Biopts were always taken prior to a new provocation took place. The controls were divided into two groups and were biopted twice. Two controls were biopted before provocation and 12 days after the first provocation and the other two were biopted 24 hours and 12 days after the first provocation. The study was performed under informed consent conditions.

Nasal allergen challenge

Patients and controls were daily challenged with grass-pollen extract (1000, 3000, 30.000, 100.000 SQ/ml ALK, Diephuis, Groningen) with a pump spray delivering a fixed dose of 50 ± 2 μl. Before provocation, subjects waited 15 min to allow the nasal mucosa to become acclimatized. At the first day of the provocation the patient was subjected to provocation with 1000 SQ/ml followed by increasing doses of allergen to establish a threshold dose. The interval between two doses was 15 min. Within 15 min of each challenge, the number of sneezes was counted and the amount of secretion, collected in preweighed paper tissues, was determined. The lowest concentration which resulted in either more than 0.5 g secretion or more than 5 sneezes in 15 min was called the threshold dose. In the following days the same concentration was used until the patient experienced serious complaints, i.e. a score of 3 on two or more items of the symptom score of the previous day, at which point the threshold dose was determined at new.

Staining procedure

The immuno alkaline phosphatase (AP) method used was described before (10). In short, 6 μm-thick sections of nasal mucosa were cut on a cryostat, transferred to gelatin-coated microscope slides, dried and fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS, pH 7.2) and incubated with 6BIIC7 (CD1a) monoclonal antibody (Sanbio, Monosan, Uden, The Netherlands) for 30 min at 20°C. The sections were rinsed again in PBS for 5 min and incubated for 15 min with a rabbit anti-mouse immunoglobulin antiserum conjugated to alkaline phosphatase (DAKO D314, Dakopatts, Copenhagen, Denmark) 1:20, supplemented with 10% normal human serum (NHS) to saturate Fc-receptors and reduce background staining, then rinsed successively in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a New Fuchsin solution (Chroma, Stuttgart, West-Germany). Finally, the sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, and mounted in glycerin gelatin. Control sections incubated with irrelevant monoclonal antibodies of the same subclass were negative.

Immunofluorescence double staining was performed with monoclonal CD1a followed by incubation with goat anti-mouse immunoglobulin labelled with TRITC (GaMIg-TRITC) and reincubation with anti-IgE labelled with FITC (RaHuIgE-FITC).

Light microscopic evaluation

The surface area of two entire sections and of the epithelium and lamina propria separately was estimated with the image analysis system Videoplan Kontron 2.1. In two sections from each specimen all LC with nuclei in the plane of the section were counted. The number of LC in epithelium and lamina propria were counted separately. The number of LC/mm ^{2} section area was calculated in epithelium and lamina propria.

The double staining was evaluated with immunofluorescence microscopy. All CD1a $^+$ cells, IgE$^+$ cells and cells positive for both CD1a and IgE were determined in the epithelium and the lamina propria. The percentage of IgE$^+$ CD1a$^+$ cells was calculated by dividing 100 x the number of IgE$^+$ CD1a$^+$ cells by the total number of CD1a$^+$ cells.

Statistical analysis

For CD1a staining three repeated measurements were made in each of the two groups before and during the provocation, together constituting 6 points on the time axis (see Table 1). Because the variables which were evaluated were symmetrically distributed (i.e. with skewness tested to be non-significant) a repeated measurement analysis of variance was performed. In this analysis the 6 time effects on the dependant variable are obtained as the estimates of 6 coefficients in a model with three within-subject repetitions (two coefficients), two between-subject repetitions (one coefficient), the interaction between repetition and groups (two coefficients) and the constant term (one coefficient). The differences between the end of the provocation period and 1-2 weeks later were analysed with the sign test. Differences in the number of IgE$^+$ LC and the percentage of IgE$^+$ cells LC were determined by means of a non-parametric test set against related measurements.

RESULTS

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. The epithelium could not be evaluated in three sections (one before provocation, one 6 days after and one 8 days after). The lamina propria usually consisted of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper

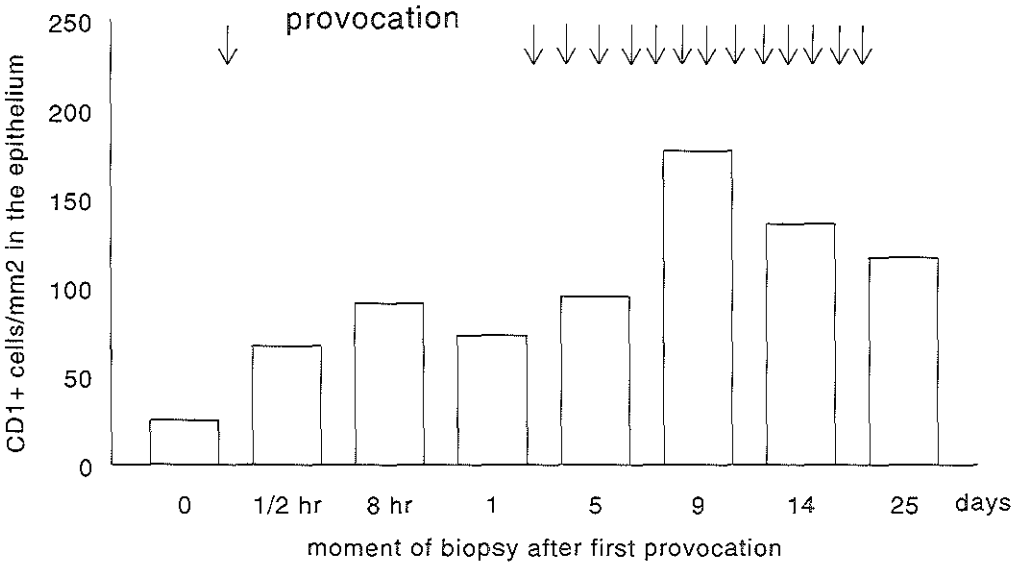

Figure 1. Median number of CD1a$^+$ cells in the epithelium of the nasal mucosa of patients with isolated grass-pollen allergy before, during and after a two-week provocation study. A significant increase in CD1a$^+$ cells was found during the provocation period.

collagenous cell-poor layer onto the bone. All sections were deep enough to assess both layers.

The number of CD1a$^+$ cells increased significantly in the epithelium (sevenfold, $p=0.008$)(Fig 1) and lamina propria (sevenfold, $p=0.01$)((Fig 2) during the provocation period. No differences were found between the two groups into which the patients had been randomly divided for statistical reasons. Already in the first 24 hr after provocation (threefold increase ½ hr after provocation) the number of CD1a$^+$ cells seemed to increase in the epithelium. However, possibly due to the small number of biopts evaluated in this period, these changes did not reach significance.

At the beginning of the provocation period, CD1a$^+$ cells were observed in the lower layers of the epithelium and in the lamina propria, predominantly in the subepithelial layer and around the vessels. After the first provocation, CD1a$^+$ cells seemed to redistribute to the epithelium.

In the second half of the provocation period, CD1a$^+$ cells were found through the whole depth of the epithelium. Occasionally, CD1a$^+$ cells reached the surface of the epithelium. The CD1a$^+$ cells in the lamina propria were found predominantly in the subepithelial layer. In this layer, clusters of CD1a$^+$ cells and activated (HLA-DR$^+$) T cells were regularly found (Fig 2). The number of CD1a$^+$ cells in the lamina propria increased at a much slower rate than in the epithelium. In the lamina propria a significant increase was found after 24 hr.

In both the epithelium and the lamina propria, the largest number of CD1a$^+$ cells was found 9 days after the first provocation. This is well before the end of the provocation period. After the 9th day a small but insignificant decrease in numbers of LC was found in

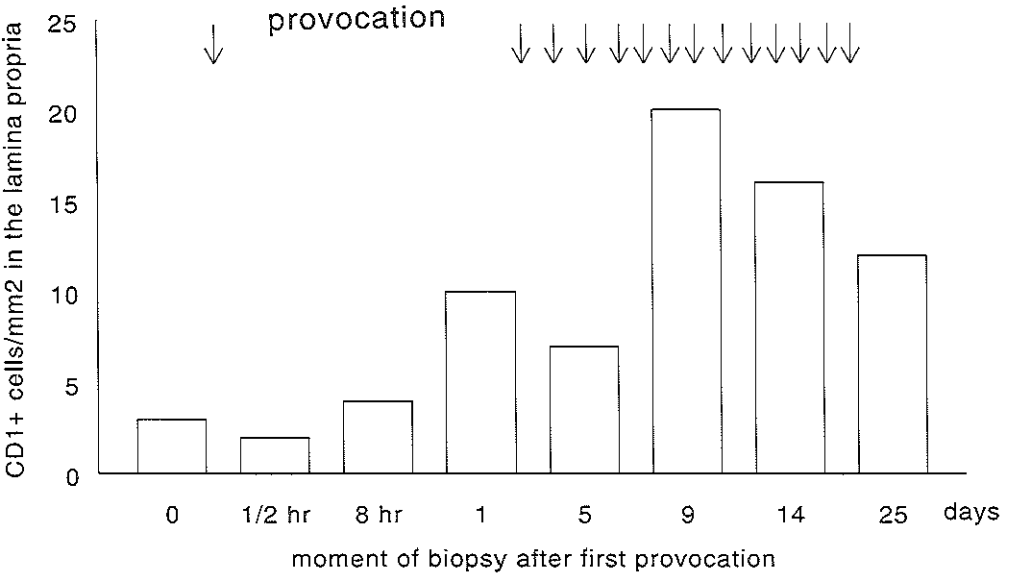

Figure 2. Median number of $CD1a^+$ cells in the lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy before, during and after a two-week provocation study. A significant increase in $CD1a^+$ cells was found during the provocation period.

the epithelium and lamina propria. This decrease seemed to persist until 1 to 2 weeks after the provocation period, although this decrease was small and not significant (Fig 1 and 2).

The number of IgE^+ $CD1a^+$ cells was determined in 8 patients (29 biopts). The mean (s.d.) percentage of IgE^+ $CD1a^+$ cells was 33 (21) in the epithelium. Occasionally IgE^+ $CD1a^+$ cells were also found in the lamina propria, mainly in the subepithelial layer. During the provocation period the *number* of IgE^+ $CD1a^+$ cells increased significantly ($p = 0.01$). The *percentage* of $CD1a^+$ cells which were positive for IgE, however, remained the same. The percentage of IgE^+ $CD1a^+$ cells did not change after the provocation period.

DISCUSSION

LC are capable of binding and presenting potentially antigenic molecules to T lymphocytes and probably to other cells as well. Furthermore, LC are able to secrete immunoregulatory factors, for example, interleukin-1 (24), interferon-gamma (25), prostaglandin D2 (26) and IgE binding factors (27). By virtue of these capabilities, LC play a role in atopic disease (14).

In respiratory allergy, part of the LC are positive for IgE. The number of LC is larger in patients with allergic rhinitis than in non-allergic controls. In such patients the number of LC is higher during natural allergen provocation than without allergen provocation. Although functional experiments in the lung show that LC/DC are the major antigen presenting cells at that site, until now no functional studies have been performed on the role of LC in respiratory allergy. The finding that in atopic dermatitis, LC are positive for IgE and that only LC positive for IgE are able to induce a T cell proliferation to house dust mite allergen in vitro, suggests that IgE positivity is necessary for the allergen presenting function of LC in

allergic disease (21).

To study the dynamics of IgE^+ LC during allergen provocation we studied patients with isolated grass-pollen allergy during a two-week daily provocation study. The number of LC increased significantly in epithelium and lamina propria during the provocation period. This finding corresponds to our previous studies during natural provocation (14). The increase in the present study is larger than that found during natural provocation, probably due to the strong allergen stimulus continuing on a daily basis. The observation of a threefold increase in LC in the first ½ hr after provocation would suggest that the increase of LC numbers is due to migration from the lamina propria and not to mitotic activity, suggested to occur in the skin (28) apart from migration (29). Moreover, LC seem to be able to migrate fast, i.e. several milimeters within 30 min. This corresponds to studies by Kamperdijk (30) in the skin, showing that LC are able to migrate from the epidermis to the local lymph node in 3 hr. Besides migration, LC may develop in the epithelium from indeterminate cells by obtaining Birbeck granules. Findings of Hanau (31), however, indicate that indeterminate cells first become CD1a positive, then obtain Birbeck granules by endocytosis, and finally migrate to the epidermis. This implies that indeterminate cells become LC before migrating to the epithelium. This is consistent with the finding in this study that in the first 24 hr after provocation the number of LC in the epithelium increases while the number of LC in the lamina propria remain the same, probably because LC migrate from the lamina propria to the epithelium and the store of LC in the lamina propria is only replenished to the same extent.

The mechanism which attracts LC to the epithelium is not exactly clear. The finding that LC increase already within half an hour after provocation makes it unlikely that LC are attracted by mast cell mediators. Other cells which are attracted by mast cell mediators, like eosinophils, can not be found in the epithelium before 8 hr after provocation (Godthelp, manuscript in prepararion). It seems more plausible for LC to be attracted independently of mast cells.

As the total number of LC in the epithelium increased while the percentage of IgE^+ LC remained the same, the actual number of IgE^+ LC in the epithelium increased. This is consitent with studies in atopic dermatitis in which the proportion of IgE^+ LC was higher in involved than in uninvolved skin and 75% of the LC in uninvolved skin was positive for IgE (20).

The findings reported in this study support the hypothesis that IgE^+ LC play a key role in allergic disease. To further study the role of LC in general and IgE^+ LC, especially in allergen provocation, functional studies are necessary. These studies should make clear whether LC in nasal mucosa are able to transport and present allergen and whether this mechanism is IgE dependent. However, as such studies require large numbers of LC (the number which can be harvested from a few cm^2 of nasal mucosa), quite a few technical problems will have to be overcome. Functional studies using LC from nasal polyps are in progress. These studies should shed light on the functional role of LC in allergic rhinitis.

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CHAPTER 11

NON-LYMPHOID CELLS IN THE NASAL MUCOSA OF PATIENTS WITH ALLERGIC RHINITIS AND NON-ALLERGIC CONTROLS DURING ALLERGEN PROVOCATION, A PRELIMINARY REPORT

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ABSTRACT

This paper reports a provocation study of 17 patients with isolated grass-pollen allergy in winter to study the dynamics of potentially antigen presenting cells in the nasal mucosa. In order to characterize different subsets of antigen presenting cells, a panel of monoclonal antibodies against dendritic cells and macrophages, namely HLA-DR, HLA-DQ, RFD1, L25, RFD9, Ki-M6 were used, as well as staining with acid phosphatase.

The data show that dendritic cells with different characteristics are present in the epithelium and lamina propria of the nasal mucosa. The incidence of these cells increased significantly during allergen provocation. No marker was found which was exclusively expressed on other dendritic cells than Langerhans cells. This study does not support the hypothesis that macrophages play a role in the pathogenesis of allergic rhinitis.

INTRODUCTION

The role and function of antigen presenting cells (APC) in allergic respiratory disease is underexposed. APC in the respiratory mucosa may present the allergen to the T cell system during the sensitization phase of allergy. Even more important, they may also contribute to the local activation of primed T cells during the effector phase of allergic reactions (1). A number of distinct cell types, including dendritic cells (DC)(2), macrophages (3), Langerhans cells (LC) (4,5), B lymphocytes (6) and epithelial cells (7) are potentially capable of presenting antigen to T lymphocytes. Although these cell types differ markedly in their effectiveness as APC, they share certain features, including the expression of MHC class II.

Recent investigations have shown that LC and HLA-DR$^+$ cells are present in nasal mucosa (8-10) and that the number of LC in the epithelium of the nasal mucosa of symptomatic rhinitis patients with isolated grass-pollen allergy is significantly higher than in normal controls (11). The number of LC in the nasal mucosa of patients with isolated grass-pollen allergy was found to increase after allergen exposure (12,13). In these patients

Table 1. Monoclonal antibodies used for immunohistochemistry.

Monoclonal antibody	Specificity	Source
Anti-HLA-DR	MHC class II (HLA-DR)	Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands (CLB)
Leu-10	MHC class II (HLA-DQ)	Beckton Dickinson, Mountain View, CA, USA
Ki-M6	Macrophages (CD 68)	Behring, Marburg, Germany
RFD9	Macrophages	L. Poulter, London, UK
L25	B cells and dendritic cells	Y. Ishii, Tokyo, Japan (18)
RFD1	Active dendritic cells and subset of B cells (class II associated)	L. Poulter, London, UK (21)
OKT6	Langerhans cells (CD1a)	Orthodiagnostic
HLA-DR-TRITC	MHC class II	Becton Dickinson,
anti-IgE-FITC	IgE	CLB

some of the LC were found to be IgE^+ (12). No IgE^+ LC were found in non-allergic controls.

The numbers of HLA-DR^+ cells did not differ between patients with isolated grass-pollen allergy and controls (8), and between patients with nasal polyps and controls (14). Bachert et al., however, described an increase in HLA-DR^+ cells during allergen provocation (15).

Cells that are positive for HLA-DR are partly DC/LC but also activated T cells, endothelial cells, macrophages and even epithelial cells. On morphological grounds we can distinguish DC/macrophages, lymphoid cells and epithelial cells and endothelial cells, positive for HLA-DR. The LC can be distinguished from other HLA-DR^+ DC by using the monoclonal antibody anti-CD1a. DC and a subpopulation of the macrophages are MHC class II positive. They can be distinguished from each other by acid phosphatase staining. Macrophages have acid phosphatase activity throughout the cytoplasm (16) while DC only show acid phosphatase in a central spot near the lobulated nucleus (17).

To further study the dynamics of APC in allergic rhinitis, a provocation study was performed in patients with isolated grass-pollen allergy during 2 weeks in January. During this period, 3 to 4 biopsies were taken from the nasal mucosa of each patient. To characterize different subsets of APC, a panel of monoclonal antibodies (mAb) against DC and macrophages was used as well as staining with acid phosphatase. The mAb used were directed to HLA-DR, HLA-DQ, RFD1, L25, RFD9, Ki-M6. Double immunofluorescence with anti-IgE was performed with some of these mAb.

Table 2. Combinations of monoclonal antibodies used in immunofluorescence double staining.

First step	Second step	Third step
OKT6	GaMIg-FITC	HLA-DR-TRITC
Ki-M6	GaMIg-TRITC	IgE-FITC
L25	GaMIg-TRITC	IgE-FITC
L25	GaMIg-FITC	HLA-DR-TRITC

Materials and methods

Patients and controls, design of the study, nasal biopsies and nasal allergen challenge were identical to those described in the study in Chapter 10.

Antibodies

The antibodies used are listed in Table 1. L25 is a mAb directed against B cells and DC (18). To our knowledge, the only other studies in the literature that used L25 are a study of Kabel et al. on intrathyroidal DC (19) and a study of Dinther-Janssen et al. on DC in the rheumatoid synovial membrane (20). The first study describes an increased number of $L25^+$ cells in Hashimoto's goiter, Graves' disease and sporadic nontoxic goiter. The second study suggests that the influx of L25 positive DC is an early event in the development of the inflammatory infiltrate found in the rheumatoid synovial membrane. Both investigators concluded the $L25^+$ cells to be activated DC.

Staining procedure of the biopsies

From each specimen serial 6 μ-thick sections of nasal mucosa were cut on a Reichert-Jung 2800 Frigocut cryostat and transferred to gelatin-coated microscope slides. The sections were stained according to a previously described immuno-alkaline phosphatase (AP) method (11): sections were air-dried, fixed in acetone for 10 min at 20°C, rinsed in phosphate-buffered saline (PBS), pH 7.2 for 5 min, rinsed in 10% normal rabbit serum for 5 min, incubated with the primary mAb for 30 min at 20°C. Sections were rinsed in PBS for 5 min and incubated for 15 min with unconjugated rabbit anti-mouse Ig serum, diluted 1:20 in PBS, supplemented with 10% normal human serum (to saturate Fc receptors and to reduce background staining), rinsed in PBS for 5 min again, followed by incubation with the AP anti-AP complex (DAKO-Immunoglobin A/S, Glostrup, Denmark D314), diluted 1:40. Subsequently, the sections were rinsed in PBS followed by TRIS buffer, pH 8.0, and incubated for 30 min with New Fuchsin staining mixture in the dark at room temperature. After staining sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, washed in running tap water and mounted in glycerin-gelatin (Chroma).

Immunofluorescence double stainings were performed by incubation with the first mAb, followed by incubation with goat anti-mouse Ig labelled with TRITC (GaMIg-TRITC) or FITC (GaMIg-FITC) and reincubation with another mAb labelled with FITC or TRITC, depending on the combination of mAb used. Each combination of mAb was used in a few representative sections. The mAb used for the double stainings are indicated in Table 2. Control staining was performed by substitution with PBS and incubation with non-relevant mAb of the same subclass.

Light microscopic evaluation

The surface area of two total sections and of the epithelium and lamina propria separately was estimated with the image analysis system Videoplan Kontron 2.1.

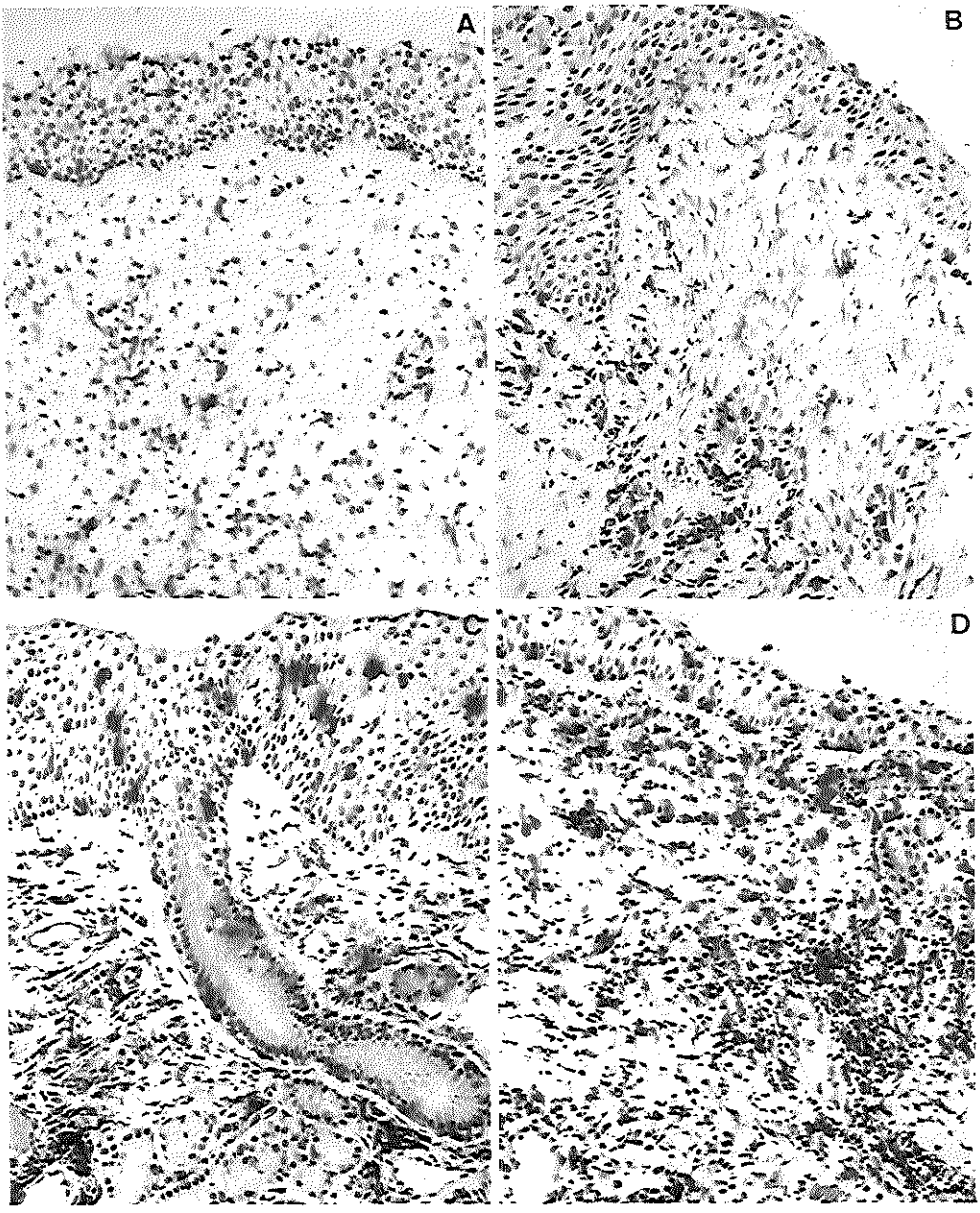

Figure 1. Examples of scores of HLA-DR$^+$ cells: (a) Nasal mucosa of patient with isolated grass-pollen allergy half an hour after first provocation: practically no HLA-DR$^+$ cells were seen in the epithelium (score 0.5) and lamina propria (score 0.5). (b) Nasal mucosa of patient with grass-pollen allergy, 4 days after first provocation: a small number of HLA-DR$^+$ cells were seen in the epithelium (score 1) and lamina propria (score 1). (c) Nasal mucosa of patient with grass-pollen allergy, 14 days after first provocation: quite some HLA-DR$^+$ cells were seen in the epithelium (score 2) and lamina propria (score 2). (d) Nasal mucosa of patient with grass-pollen allergy, 4 days after first provocation: quite some HLA-DR$^+$ cells were seen in the epithelium (score 2) and a large number in the lamina propria (score 3) (all x 160).

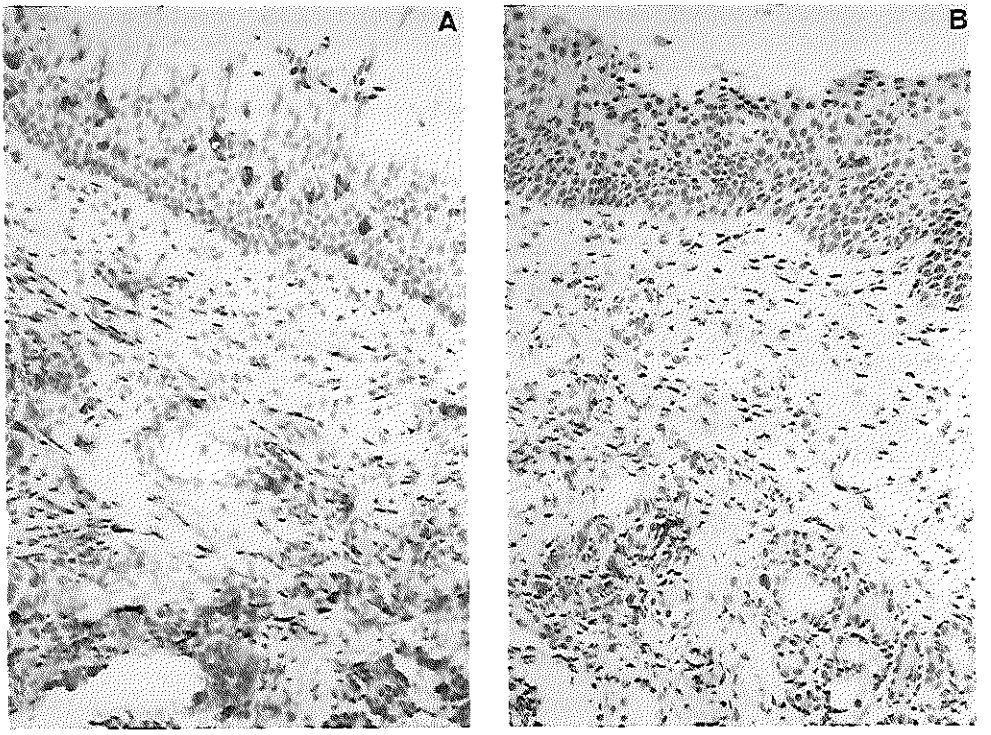

Figure 2. L25$^+$ cells in a patient with isolated grass-pollen allergy (a) half an hour after the first provocation: L25$^+$ cells with predominantly dendritic morphology were seen in epithelium and lamina propria (x 160); (b) two weeks after provocation: a very small number of weak staining L25$^+$ cells were seen (x 160).

Usually, two sections were placed on each slide and the one judged to be technically the best was picked out for counting. Cells binding the AP-conjugated mAb used had bright red surface membranes, red stained cytoplasm, or both, depending on the mAb used and/or the cell type evaluated. Cells were counted if they stained red and contained a nucleus. Separate counts were performed in the epithelium and the lamina propria at a magnification of 250. In the epithelium all positive cells were counted. In the lamina propria the number of positive cells was determined by superimposing a grid of 5 by 5 squares on the section with an eyepiece graticule. Per grid-field, the cells were counted in randomly chosen squares and the grid was successively superimposed on the entire section along parallel lines. The number of squares required to achieve acceptable accuracy (ca. 5%) was estimated by means of a summation-average graph. Two squares per grid-field were evaluated, leading to at least 15 squares of the entire section. The total number of positive cells in the lamina propria was determined when there were only a few positive cells left (less than one cell per two squares).

When groups of positive cells were in close proximity or were projected on each other, only the definitely positive cells were counted. When large numbers of positive cells lay close, as after HLA-DR staining, the number of positive cells was assessed semi-quantitatively on a score from 0 to 4 with steps of 0.5, representing a range from practically no HLA-DR$^+$ cells to large numbers of HLA-DR$^+$ cells (Fig 1).

Moreover, based on morphology and localization, semi-quantitative assessment was made as well on a 0-4 scale of the number of HLA-DR$^+$ DC/macrophages, lymphocytes and epithelial cells. Only cells which clearly resembled lymphocytes (small, round, practically no cytoplasm) were judged to be

Table 3. Number of HLA-DQ$^+$, Ki-M6$^+$, and L25$^+$ cells/mm^2 (median (range)) in epithelium and lamina propria of nasal mucosa of patients with isolated grass-pollen allergy during a two-week provocation study in winter.

Moment of biopsy	Epithelium			Lamina propria		
	HLA-DQ$^+$	Ki-M6$^+$	L25$^+$	HLA-DQ$^+$	Ki-M6$^+$	L25$^+$
before provocation	109 (0-400)	220 (74-380)	61 (11-150)	189 (0-723)	51 (28-305)	134 (4-542)
½ hr after first provocation	83 (0-265)	233 (46-423)	157 (71-300)	115 (5-554)	219 (51-353)	219 (18-270)
8 hr after first provocation	86 (22-276)	128 (80-252)	300 (78-424)	100 (3-178)	76 (9-144)	155 (20-236)
24-48 hr after first provocation	125 (0-679)	197 (136-242)	131 (0-229)	180 (0-457)	87 (18-185)	232 (28-431)
4-6 days after first provocation	108 (36-236)	185 (7-662)	92 (3-379)	183 16-370)	134 (17-384)	68 (10-246)
8-10 days after first provocation	124 (0-403)	159 (69-309)	13 (0-447)	408 (0-1206)	297 (73-363)	8 (6-1249)
14 days after first provocation	113 (36-400)	223 (71-356)	69 (1-600)	314 (46-605)	251 (45-846)	64 (30-346)
1-2 weeks after last provocation	150 (22-368)	245 (123-850)	177 (78-1250)	201 (16-849)	329 (43-567)	484 (17-643)

lymphocytes. The rest of the migratory cells were judged to be DC/macrophages. HLA-DR$^+$ epithelial cells were easily recognized by morphology and localization.

Control sections treated with PBS or an irrelevant mAb were negative.

Statistical analysis

Three repeated measurements were made for all stainings in each of the groups before and during the provocation, together constituting six points on the time axis. Because the numbers of Ki-M6$^+$ and HLA-DQ$^+$ cells were symmetrically distributed after square root transformation for the measurements in the epithelium and logarithmic transformation for the lamina propria (i.e. with skewness tested to be non-significant) a repeated measurement analysis of variance was performed. In this analysis the six time effects on the dependant variable are obtained as the estimated of six coefficients in a model with three within-subject repetitions (two coefficients), two between-subject repetitions (one coefficient), the interaction between repetition and groups (two coefficients) and the constant term (one coefficient). Differences in the score of HLA-DR$^+$ cells and L25$^+$ cells were determined by a non-parametric test against related measurements. The differences between the end of the provocation period and the period 1-2 weeks after provocation were analyzed with the sign test. Figures were considered to be significantly different when the p-value was < 0.05.

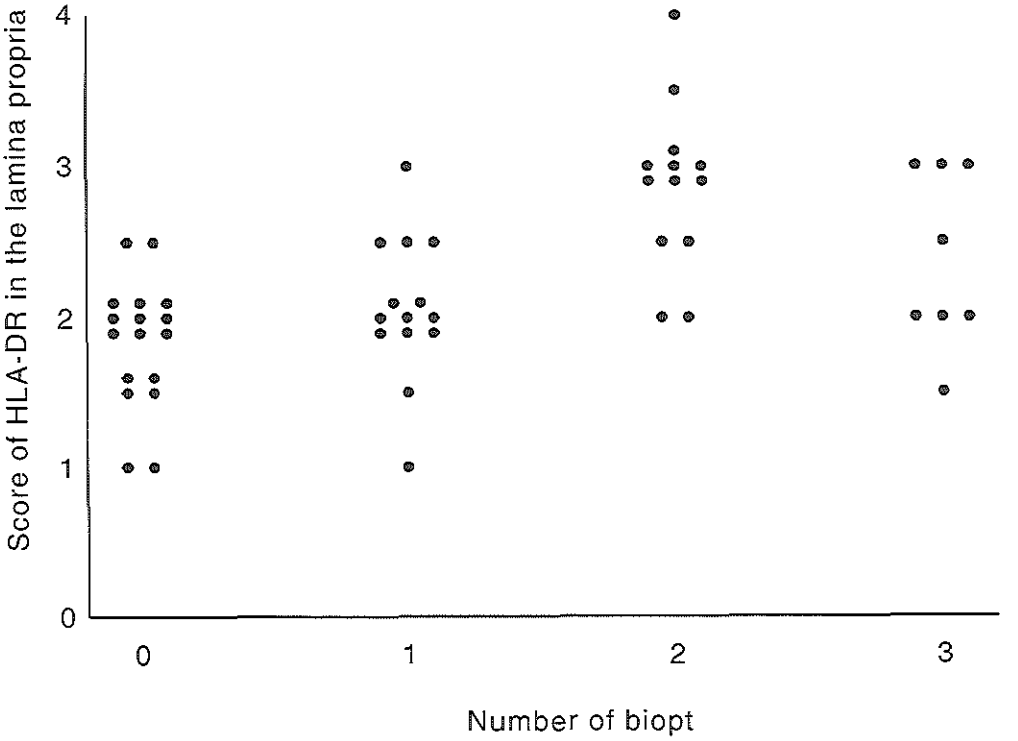

Figure 3. Score of HLA-DR$^+$ cells in the lamina propria of all patients during 3 or 4 biopsies. The null biopsy was taken before provocation, after ½ hour or after 8 hours in different patient groups. The first biopsy was taken 24-48 hours or 4-6 days after the first provocation. The second biopsy was taken 8-10 days or 14 days after the first provocation. The third biopsy was taken 1-2 weeks after the last provocation. A significant increase was found during provocation. All scores at the biopsy after provocation (biopsy 3) were lower or equal to the scores at the last biopsy during provocation.

RESULTS

General description

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. The epithelium could not be evaluated in 3 sections (before provocation, 6 and 8 days after the first provocation) which were excluded from the study. The lamina propria consisted usually of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer onto the bone. All sections were sufficiently deep to assess both layers.

HLA-DR$^+$ cells

HLA-DR$^+$ cells were found in the epithelium and, predominantly, in the subepithelial layer of the lamina propria. The HLA-DR$^+$ migratory cells showed dendritic or lymphocytic morphology. Epithelial cells in the epithelium and/or epithelial cells in the mucous glands were sometimes positive. Often large clusters (100-1000 cells) of HLA-DR$^+$ cells (with

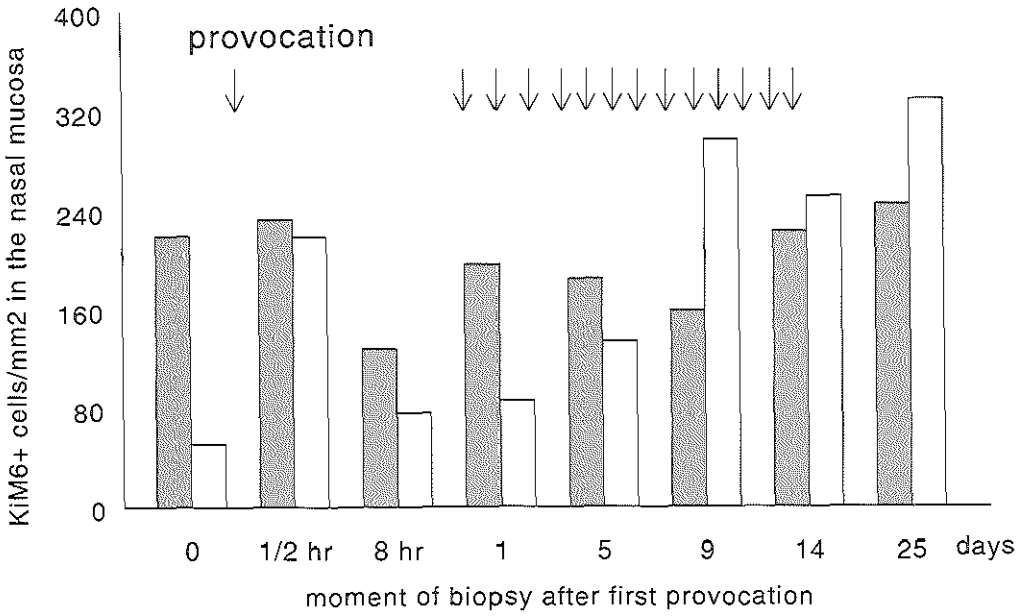

Figure 4. Median of the number of Ki-M6$^+$ cells in the epithelium (dark columns) and lamina propria (light columns) of the nasal mucosa of symptomatic patients with isolated grass-pollen allergy before, during, and after a two-week provocation study.

dendritic and/or lymphocytic morphology) were seen in the lamina propria, mainly in the subepithelial layer.

The total number of HLA-DR$^+$ cells scored on a 0-4 scale, increased significantly in epithelium ($p = 0.02$) and in the lamina propria ($p < 0.001$) during the provocation period (Fig 3). The number of HLA-DR$^+$ cells between the patients differed strongly. Some patients showed fewer HLA-DR$^+$ cells at the end of the provocation period than others at the beginning. All scores from the biopsy after provocation (biopsy 3) were lower than or equal to the scores from the last biopsy during provocation (biopsy 2). The number of biopsy specimens evaluated was, however, too small to reach significance.

The number of dendritic HLA-DR$^+$ cells as well as the HLA-DR$^+$ cells with lymphocytic morphology which were scored on a 0-4 scale, increased significantly in the epithelium (DC $p = 0.004$, lymphocytes $p = 0.06$) and the lamina propria (DC $p = 0.02$, lymphocytes $p = 0.001$. Furthermore, the number of HLA-DR$^+$ cells in these assessments differred strongly between the patients. HLA-DR$^+$ DC were already found in the epithelium and the lamina propria before provocation and their numbers increased from the beginning of the provocation period. HLA-DR$^+$ cells with lymphocytic morphology were rarely seen before provocation. They usually increased in numbers in the second half of the provocation period in the epithelium and the lamina propria. The number of HLA-DR$^+$ epithelial cells did not change during the provocation period. In the controls, the number of HLA-DR$^+$ cells did not change during the provocation.

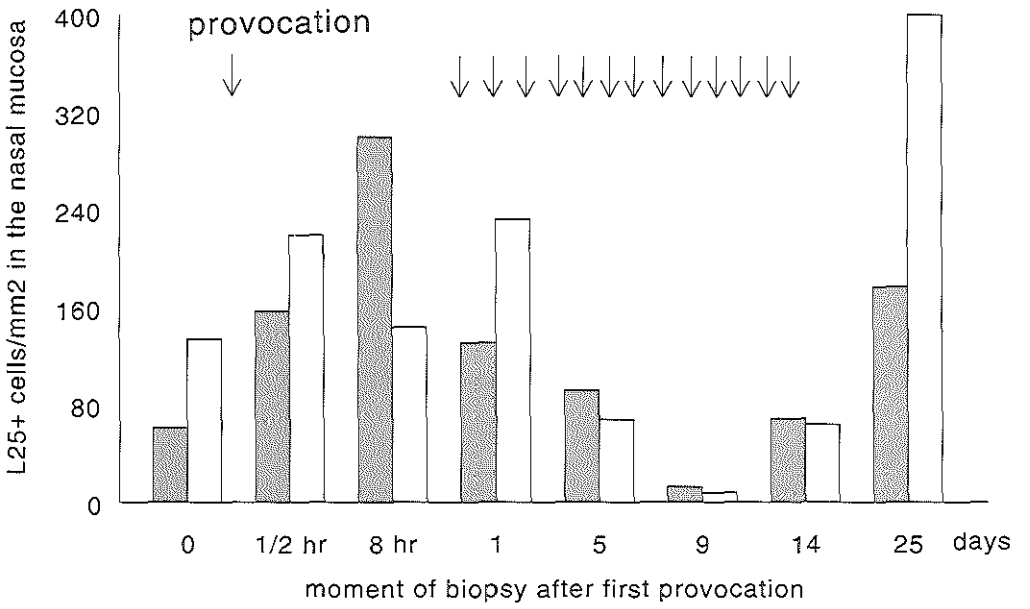

Figure 5. Median number of L25$^+$ cells in the epithelium (dark columns) and lamina propria (light columns) of the nasal mucosa of symptomatic patients with isolated grass-pollen allergy before, during, and after a two-week provocation study. A significant increase was found in the epithelium in the first 8 hours after the first provocation. During the rest of the provocation period a significant decrease was found. After the provocation period a significant increase was found in the epithelium and the lamina propria.

HLA-DQ$^+$ cells

HLA-DQ$^+$ cells were found in the epithelium and predominantly in the subepithelial layer of the lamina propria. The HLA-DQ$^+$ cells showed a dendritic morphology. HLA-DQ$^+$ cells with a lymphocytic morphology were not seen. The number of HLA-DQ$^+$ cells was always smaller than the number of HLA-DR$^+$ cells. A significant increase in the number of HLA-DQ$^+$ cells was found in the epithelium ($p = 0.03$) and lamina propria ($p < 0.001$) during the provocation period (Table 3). Clusters of HLA-DQ$^+$ cells were regularly found.

Ki-M6$^+$ cells

The cells that were positive for Ki-M6, were large cells with a bright staining cytoplasm. Ki-M6$^+$ cells were found in the epithelium and all layers of the lamina propria (Table 3). During provocation the number of Ki-M6$^+$ cells increased significantly in the lamina propria ($p = 0.003$), but not in the epithelium (Fig 4). In the lamina propria of the controls, the Ki-M6$^+$ cells increased to the same extent. However, the results of four controls cannot constitute significance.

L25$^+$ cells

The L25$^+$ cells usually showed a dendritic morphology. Lymphocytes, presumably B lymphocytes, were seldom found in the lamina propria. During the first 8 hr of provocation, the number of L25$^+$ cells increased significantly in the epithelium ($p = 0.03$) (Fig 2

and 5). In the lamina propria a small, insignificant increase was found. During the rest of the provocation period a significant decrease was found in the number of $L25^+$ cells in the epithelium ($p=0.05$) and the lamina propria ($p=0.05$) (Fig 2 and 5) (Table 3). Nine days after the first provocation, hardly any $L25^+$ cells were seen (Fig 5). Remarkably, first the $L25^+$ cells in the epithelium, then those in the subepithelial layers and finally the $L25^+$ cells in the deeper layers of the lamina propria disappeared. In the controls no changes were found in the number of $L25^+$ cells.

$RFD1^+$ cells

No $RFD1^+$ cells were found in nasal epithelium or lamina propria.

Cells staining with acid phosphatase

Cells staining with acid phosphatase were found in epithelium and lamina propria. Besides cells, mucus in goblet cells and in mucous glands were also stained with acid phosphatase. This phenomenon made a reliable count of the number of cells staining with acid phosphatase impossible.

$RFD9^+$ cells

Staining with $RFD9^+$ presented the same problems as staining with acid phosphatase.

Double staining

Double staining for CD1a and HLA-DR showed that all $CD1a^+$ cells evaluated were also positive for HLA-DR and that approximately half of the $HLA\text{-}DR^+$ cells in the epithelium and only a small proportion of the $HLA\text{-}DR^+$ cells in the lamina propria were positive for CD1a. Most of the $L25^+$ cells were also positive for HLA-DR. No $L25^+$ or $Ki\text{-}M6^+$ cells were found to be positive for IgE.

DISCUSSION

MHC class II positive APC are necessary for the presentation of antigen to T lymphocytes (22). In earlier studies, we found indications for a role for LC in allergen presentation in allergic rhinitis (11-13). As LC are not the only cells potentially capable of presenting antigen/allergen, we investigated what other potential APC are present in the nasal mucosa. Moreover, their dynamics during allergen provocation studies was determined. To find further support for their putative role in the allergic mechanism, IgE positivity was determined.

Antigen presentation to helper T cells is MHC class II dependent. We, therefore, quantitated the $HLA\text{-}DR^+$ and $HLA\text{-}DQ^+$ cells during allergen provocation. Human DC express MHC class II molecules encoded by the subloci DP, DQ and DR (22). HLA-DQ, contrary to HLA-DR, is not concentrated at points of contact between aggregating DC and

lymphocytes and more diffusely localized on the cell (23). Antigen associated with DR and DQ may be handled differently (17,24,25).

In this study we found the number of HLA-DR$^+$ cells to be larger than the number of HLA-DQ$^+$ cells, but both increased during allergen provocation. It is remarkable that the HLA-DQ$^+$ cells, as opposed to the HLA-DR$^+$ cells, all showed a dendritic morphology. The HLA-DR$^+$ DC and HLA-DQ$^+$ DC, however, are not the same population, because the number of dendritic HLA-DR$^+$ cells is larger than the number of HLA-DQ$^+$ cells. Studies are in progress to establish whether the difference in expression of HLA-DR and HLA-DQ on DC has functional relevance.

The number of activated HLA-DR$^+$ lymphocytes increased during allergen provocation. Because most of the lymphocytes in nasal mucosa are T lymphocytes (8), these HLA-DR$^+$ lymphocytes will generally be activated T cells. Whether it is a matter of the number of T cells actually increasing during allergen provocation or simply more T cells turning into HLA-DR$^+$ T cells, is yet to be evaluated.

Another matter of interest is that the extent of HLA-DR expression on epithelial cells did not increase during allergen provocation. This suggests that HLA-DR positivity of epithelial cells does not play an important role in allergen presentation.

DC are more capable of antigen presentation than other MHC class II positive cells (like macrophages and B cells) because of their ability to actively form cellular clusters with immunocompetent cells (26) and because DC have a quantitatively and qualitatively superior MHC expression (2). So far, few DC markers are available, despite the continuous efforts to make specific mAb. Alkaline phosphatase cannot be used to distinguish between DC and macrophages in sections of nasal mucosa because mucus staining makes enumeration of the cells impossible. So, in order to get an impression of which part of the HLA-DR$^+$ cells with a dendritic morphology is DC and not macrophage, we used mAb we knew to be rather specific against DC (RFD1 and L25) and macrophages (Ki-M6) in other tissues.

During provocation, the Ki-M6$^+$ cells increased in the lamina propria of allergic patients and normal controls. This is likewise caused by the daily brushing of the nasal mucosa to collect surface epithelial cells. Brushing is potentially irritative enough to attract macrophages. The lung broncho-alveolar-lavage, apparently less provocative than brushing, results in an increased number of macrophages (Thea Vroom, personal communication). Contrary to alveolar macrophages (27), no IgE positivity could be found on macrophages in the nasal mucosa.

We were unable to stain cells in the nasal mucosa with RFD1 monoclonal antibody which is shown to react with DC in other tissues (19). The reason for this failure is unclear. L25, however, also a marker of DC, was expressed by DC in nasal mucosa. L25 was thought to be a marker of activated DC. In this provocation study, however, the number of LC, after an initial significant increase in the first 8 hours after the first provocation, decreased significantly during the rest of the provocation period. During the provocation period the depth of the area in which no L25$^+$ cells could be found, increased steadily. At the end of the provocation period only a few L25$^+$ cells were left in the deepest layer of the

Table 4. Results of L25 staining in different tissues.

Tissues evaluated	Cells staining positive for L25
Dermatopathic lymphnode	- large dendritic cells in afferent lymphvessel - B cells, predominantly mantle B cells - interdigitating cells (IDC)
Liver	- Kupffer cells - fibroblast/indeterminate cells
Spleen	- IDC in T cell area and scattered through red pulp - B cells weak + in contrary to in lymphnode
Thymus	- reticulo-epithelial network
Skin	- normal skin practically negative
Lung	- interstitial macrophages strong + - alveolair macrophages weak +
Colon	- DC in inner muscular layer - macrophages high in crypts
Peripheral blood	- no positivity found

lamina propria. After the provocation period a significant increase of $L25^+$ cells was found in the lamina propria.

These findings appeared to be incompatible with the findings of Kabel et al. (19) and van Dinther-Jansen et al (20). To further specify the monoclonal, L25 staining was performed in dermatopathic lymphnode, spleen, liver, thymus, lung mucosa and colon mucosa. The results of these stainings, summarized in Table 3, show that L25 is directed to a receptor present on DC, indeterminate cells (IDC) and/or macrophages in all tissues evaluated. Together these data suggest that L25 expression on DC and/or macrophages in the nasal mucosa is inversely proportional to the activation of the cells.

In conclusion this study shows that not only LC, as described by earlier studies, but other DC as well, are found in the epithelium and lamina propria of the nasal mucosa and that the incidence of these cells increases during allergen provocation. However, no specific DC marker was found, which was expressed on DC but not on LC and/or macrophages. As a consequence it was impossible to determine more precisely the role of DC in allergen handling. Furthermore, it was impossible to detect whether other DC than LC are IgE^+. This study does not support the hypothesis that macrophages play a role in allergic rhinitis.

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CHAPTER 12

THE EFFECT OF NASAL CORTICOSTEROID SPRAY ON LANGERHANS CELLS IN THE NASAL MUCOSA

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ABSTRACT

The mode of action of nasal corticosteroid therapy on allergic rhinitis is uncertain. To investigate whether Langerhans cells ($CD1a^+$ cells) and HLA-DR^+ cells in the nasal mucosa are influenced by nasal topical corticosteroid spray, we used a new corticosteroid spray, fluticasone proprionate, for treatment of 22 patients with perennial allergic rhinitis. This treatment decreased the number of $CD1a^+$ cells and HLA-DR^+ cells in the nasal mucosa, suggesting that fluticasone proprionate decreases the antigen presentation in nasal allergy.

INTRODUCTION

Topical corticosteroids are of proven efficacy in allergic rhinitis (1-3) and have been shown to inhibit both the immediate and late response to allergen (4,5). Topical steroids inhibit the accumulation of mast cells in the nasal mucosa (4,6), the degranulation of mast cells (7) and reduce the levels of histamine, TAME-esterase activity, and kinins in the early, late and rechallenge allergic reactions in nasal secretions (5). Corticosteroids have also been found to inhibit the migration of neutrophils and eosinophils (8,9). Treatment with the corticosteroid nasal spray budesonide significantly reduces the number of eosinophils in nasal secretion of patients with perennial rhinitis (10). The mode of action of these drugs, however, remains uncertain.

In the skin, topically applied corticosteroids cause a decreased expression of MHC class II antigens (HLA-DR) on Langerhans cells (11). Also a subsequent decrease has been reported of the number of Langerhans cells in the epidermis (12). Moreover, topical corticosteroid therapy was claimed to decrease Langerhans cell-dependent T cell activation, which could not be restored by exogenous interleukin-1 (13). These findings in the skin suggest that topical steroid therapy depresses antigen presentation by a direct effect on the antigen presenting function of Langerhans cells.

In earlier studies we have identified Langerhans cells in the nasal mucosa (14). The incidence of Langerhans cells was found to increase during allergen provocation (15,16).

Table 1. RAST score of the patients.

Patient	HDM	grass	dog	cat	guinea pig	rabbit	mugwort	horse	fungi	tree
1	3+									
2	3+				1+	2+				
3	3+	4+	4+	4+	4+	2+				2+
4	4+									
5	3+									
6	3+		3+	4+						
7	4+					1+				
8	3+									
9	3+			1+						
10	4+	4+								
11	3+		1+	4+	3+	4+	1+	4+		
12	3+									
13	4+	1+	1+	4+	2+		3+	1+		
14	3+	2+								
15	4+									
16	4+									
17	3+									
18	4+	3+			4+					1+
19	4+									
20	3+									
21	4+		2+				2+			
22	4+									

To investigate whether Langerhans cell numbers in the nasal mucosa are influenced by corticosteroids, the effect of a new corticosteroid spray, fluticasone proprionate, was studied on the number of Langerhans cells in the nasal mucosa of 22 patients with perennial allergic rhinitis.

Materials and methods

Patients and controls

Twenty-two patients participated in the study. The patients (mean age 26 yr, 15 ♂, 7 ♀) had a perennial allergic rhinitis for at least one year. The diagnosis perennial allergic rhinitis was confirmed by a radio-allergosorbent test (RAST) score of at least 3^+ (3^+–5^+) for house dust mite allergen (HDM) and sometimes other allergens like dander or pollen. The RAST score of the patients are given in table 1.

Design of the study

The study was conducted in a double blind manner with patients randomized into two groups. There was a run-in period on placebo of two weeks followed by fluticasone proprionate or placebo for 12 weeks. Biopsies of nasal mucosa were taken after the run-in period and after three months use of the trial

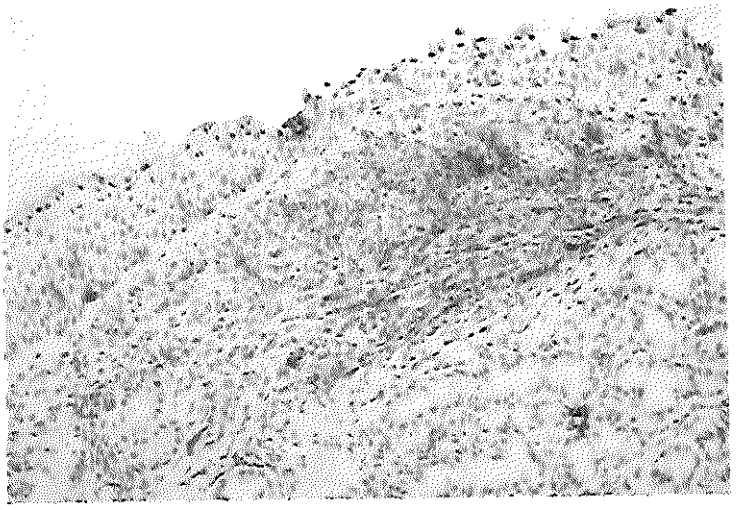

Figure 1. Light micrograph of the nasal mucosa of a patient with perennial rhinitis incubated with AP-labeled monoclonal CD1a. Subepithelial a large cluster of CD1a$^+$ cells is seen (x 160).

drug. Nasal biopts were taken from the lower edge of the inferior turbinate using a Gerritsma forceps and processed as described in chapter 4.

Staining procedure

From each specimen serial 6 μm-thick sections of nasal mucosa were cut on a Reichert-Jung 2800 Frigocut cryostat and transferred to gelatin-coated microscope slides. The sections were stained according to a previously described immuno-alkaline phosphatase (AP) method (17) using the monoclonal antibodies 66IIC7 (CD1aa, Sanbio Monosan) or the anti-HLA-DR (Central laboratory of the Red Cross Blood Transfusion Service, Amsterdam).

Light microscopic evaluation

The surface area of two sections in total and of the epithelium and lamina propria seperately, was estimated with the image analysis system Videoplan Kontron 2.1. Cells binding the monoclonal antibody used had bright red surface membranes, red stained cytoplasm, or both, depending on the cell type and/or monoclonal antibody evaluated. Cells were counted if they stained red and contained a nucleus. When a group of positive cells lay very close together or projected on each other, only the definitely positive cells were counted.

The number of CD1a$^+$ cells were counted separately in the epithelium and the lamina propria of two sections at a magnification of 250x. The number of CD1a$^+$ cells/mm^2 section area was calculated in epithelium and lamina propria.

The number of HLA-DR$^+$ cells, could not be determined reliably because many positive cells lay close together and/or separate dendritic cells could not be readily distinguished. The number of HLA-DR$^+$ cells was assessed semi-quantitatively on a score from 0 to 4 with steps of 0.5, representing a range from practically no HLA-DR$^+$ cells to large numbers of HLA-DR$^+$ cells. Moreover based on morphology and localization, also on a 0to 4 scale, semi-quantitative assessment was made of the number of HLA-DR$^+$ DC/macrophages, lymphocytes and epithelial cells. Only cells which clearly resembled lymphocytes (small, round, practically no cytoplasm) were judged to be lymphocytes. The rest of the migratory cells were judged to be DC/macrophages. HLA-DR$^+$ epithelial cells were easily recognised by morphology and location.

Control sections treated with phosphate-buffered saline or an irrelevant monoclonal antibody were negative.

Statistical analysis

Since the frequency distribution of the number of CD1a $^+$ cells per mm^2 in epithelium and lamina propria was not symmetrical and the variances were unequal, the two-tailed Mann-Whitney rank sum test of the differences was used. Differences in score of HLA-DR $^+$ cells were analyzed with the sign test. A p-value < 0.05 was considered to indicate a significant difference between groups.

RESULTS

General description

The sections of nasal mucosa had an average surface area of 3 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. The epithelium could not be evaluated in 1 patient which was excluded from the study. The lamina propria consisted usually of a subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer onto the bone. All sections were deep enough to assess both layers.

CD1a$^+$ cells

CD1a$^+$ cells were found in the epithelium and, predominantly, in the subepithelial layer and in and around the glandular tissue of the lamina propria. All sections showed CD1a$^+$ dendritic cells in the epithelium and in the lamina propria before the start of the trial. Occasionally clusters (100-1000 cells) of CD1a$^+$ cells (dendritic and lymphocytic morphology) were seen in the lamina propria, mainly in the subepithelial layer (Fig 1).After three months of therapy the number of CD1a$^+$ cells in epithelium (Fig 2) and lamina propria (Fig 3) in the group receiving fluticasone proprionate was significantly lower than in the placebo group (epithelium $p=0.006$, lamina propria $p=0.008$).

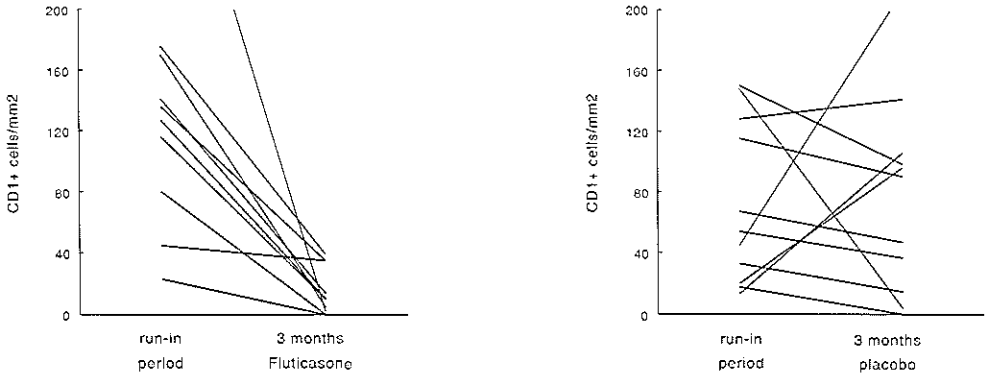

Figure 2. The number of CD1 $^+$ cells in the nasal epithelium of patients with perennial allergic rhinitis before and during fluticasone proprionate therapy.

HLA-DR$^+$ cells

HLA-DR$^+$ cells were found in the epithelium and predominantly in the subepithelial layer of the lamina propria. The HLA-DR$^+$ migratory cells showed mostly dendritic and sometimes lymphocytic morphology. Epithelial cells in the epithelium and/or epithelial cells in the mucous glands were positive in 13 of the 44 section evaluated. Occasionally clusters (100–1000 cells) of HLA-DR$^+$ cells (dendritic and lymphocytic morphology) were seen in the lamina propria, mainly in the subepithelial layer.

Before the trial, no significant difference was found in the total score of HLA-DR$^+$ cells, or the score of HLA-DR$^+$ cells with dendritic or lymphoid morphology between the placebo group and the group going to receive fluticasone proprionate (Fig 4,5). However, the score of HLA-DR$^+$ cells differed largely between the patients. After three months of therapy a significant decrease was found in the score of the HLA-DR$^+$ cells with a dendritic morphology (epithelium $p=0.008$, lamina propria $p=0.006$), the score of the HLA-DR$^+$ cells with a lymphocytic morphology (epithelium $p=0.03$, lamina propria $p=0.004$) and the total number of HLA-DR$^+$ cells in epithelium ($p<0.001$) and lamina propria ($p<0.001$) of the group receiving fluticasone proprionate and not of the placebo group (Fig 3,4).

Discussion

Nasal corticosteroid therapy has become an established therapy in hay fever. Several cell types in the nasal mucosa are influenced by nasal corticosteroid therapy. The exact mode of action, however, is unclear. So far, in studies on biopts of nasal mucosa, contrary to in the skin, no significant structural changes could be ascertained following local corticosteroid therapy (18,19).

In the skin topical corticosteroids reduce the number of LC and the expression of HLA-DR on these cells. Possibly due to the decreased HLA-DR expression, the antigen presenting capacity of LC was reduced in mice (20) and possibly also in man (13). These

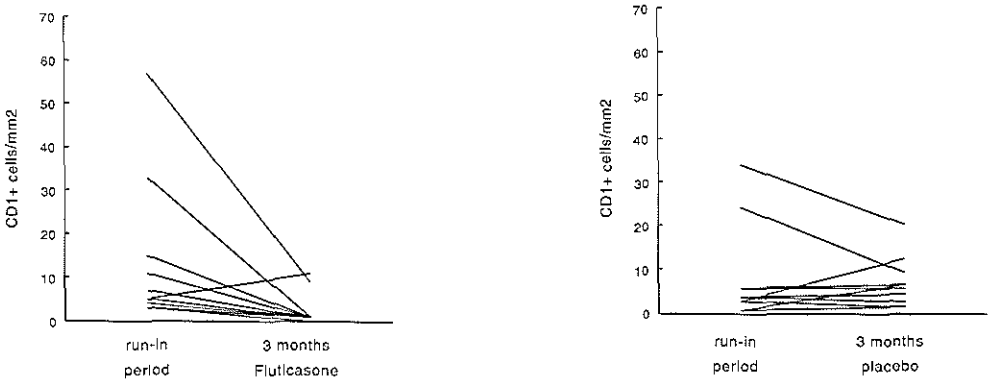

Figure 3. The number of CD1a$^+$ cells in the lamina propria of patients with perennial allergic rhinitis before and during fluticasone proprionate therapy.

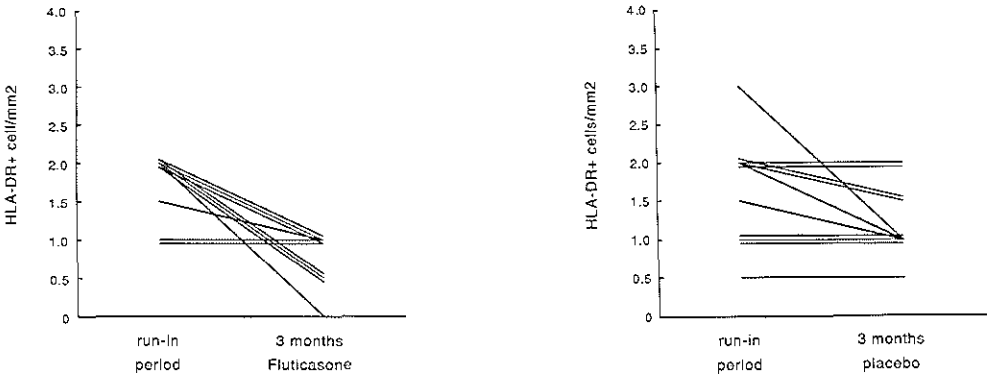

Figure 4. The score of HLA-DR$^+$ cells in the nasal epithelium of patients with perennial allergic rhinitis before and during fluticasone proprionate therapy.

findings suggest that topical steroid therapy depresses antigen presentation by a direct effect on the antigen presenting function of LC.

In this study we evaluated the number of CD1a$^+$ cells and HLA-DR$^+$ cells in the nasal mucosa of patients with perennial allergic rhinitis before and 3 months after fluticasone proprionate therapy. The score of HLA-DR$^+$ cells decreased significantly during therapy. In the skin of the guinea pig, Belsito et al. (21) described a loss of Ia (MHC class II) but no irreversible structural changes during glucocorticosteroid treatment. Also in human skin selective reduction in the expression of HLA-DR but not CD1a was found (11,12). These results suggest a change in cell surface markers rather than loss of the whole cell. However, studies in the human skin also show a decrease of CD1a$^+$ cells following more prolonged (a few days) glucocorticosteroid treatment. In literature no EM studies of human skin could be found to definitely proof that the number of CD1a$^+$ cells decreases and not only the expression of CD1a.

In this study we found a considerable decrease in numbers of CD1a$^+$ cells in the epithelium during fluticasone proprionate therapy (from 86 to 5). This decrease is much larger than the decreases found in the skin. Possible explanations are that the glucocorticosteroid therapy is more effective in the nose probably due to better penetration or that the new fluticasone proprionate is a stronger working glucocorticosteroid. In the skin the decrease in number of CD1a$^+$ cells was dependent on the potency of the topical corticosteroid used (22).

In conclusion, the present study shows a decrease in CD1a$^+$ cells and HLA-DR$^+$ cells in nasal mucosa of patients with perennial allergic rhinitis during fluticasone proprionate therapy, suggesting a decrease in antigen presentation. The following decrease in T cells stimulation may result in a reduction of the reactions that are dependent on T cell derived mediators. Further studies are necessary to determine whether LC/DC disappear from the nasal mucosa during fluticasone proprionate therapy or that these findings are a result of

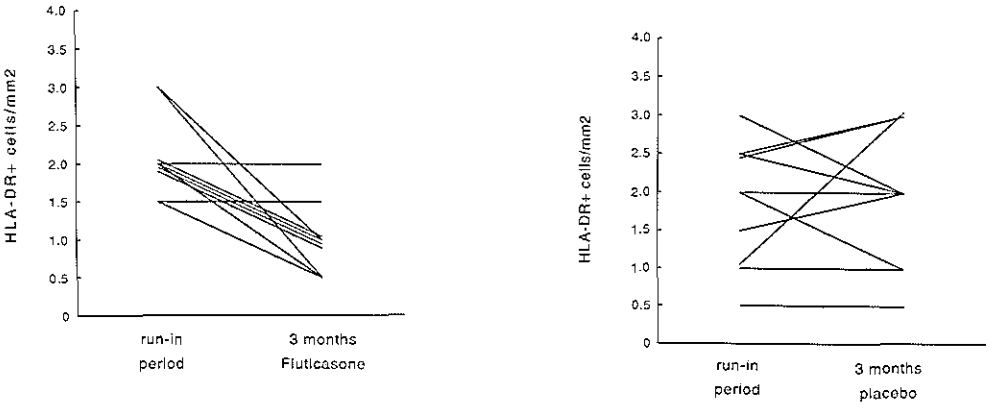

Figure 5. The score of HLA-DR$^+$ cells in the lamina propria of the nasal mucosa of patients with perennial rhinitis before and during fluticasone proprionate therapy.

a change in cell surface markers. Also the effect of fluticasone proprionate therapy on antigen presentation of LC/DC has to be evaluated.

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Chapter 13

GENERAL DISCUSSION

The aim of this study was to investigate the cellular aspects of allergic inflammation in the nose using immunohistochemical staining techniques of sections of biopsy specimens of nasal mucosa.

The results of various investigations have previously been discussed in the individual chapters. In this chapter, an attempt is made to discuss general aspects of the studies, described in this thesis and to integrate the findings into a new hypothesis concerning the pathogenesis of allergic rhinitis.

METHODS OF INVESTIGATION

Patients and controls

The investigations presented in this thesis concern (1) patients with isolated grass-pollen allergy before, during and after natural provocation, (2) patients with isolated grass-pollen allergy before, during and after allergen provocation studies, (3) non-allergic patients with nasal polyps, who could be expected to show increased signs of inflammation on a non-allergic basis, and (4) non-allergic controls without nasal complaints. Table 1 summarizes the patient groups investigated in the subsequent chapters. Patients with isolated grass-pollen allergy were studied because they exhibit allergic rhinitis only in a limited and well defined period of the year. The advantage of this approach is that these patients only have complaints in that limited period and therefore can be used as their own controls. Moreover, allergen provocation studies can be performed out of the grass-pollen season. On the other hand, it is possible that an isolated allergy, which is relatively seldom, shows another picture than allergic rhinitis caused by multiple allergens, e.g. because the priming and hyperreactivity reactions are less impressive due to the relatively limited exposition. This is suggested by differences in symptomatology between isolated pollen allergy (comparatively more sneezing and running of the nose than nasal blockage) and perennial rhinitis caused by multiple allergens (emphasis on nasal blockage)(1). However, the advantages of the well-defined groups by far outweight the drawbacks.

Technical aspects of the experiments described

The method of investigation employed in the studies described in this thesis, namely immunohistochemical evaluation of biopts of nasal mucosa, has the advantage that individual cells and their characteristics as well as their contact with other cell types and their location can be studied in all layers of the nasal mucosa . Another advantage is that conclusions can be drawn from direct observations in stead of in vitro studies or animal models.

Table 1. Patient groups investigated and methods of allergen provocation used in the different chapters.

Chapter	Groups	Allergen provocation for allergic patients
5	grass-pollen allergic non-allergic with nasal polyps non-allergic without nasal symptoms	during natural provocation
6	grass-pollen allergic	before, during and after natural provocation before, during and after provocation studies
7	grass-pollen allergic non-allergic with nasal polyps non-allergic without nasal symptoms	during natural provocation
8	grass-pollen allergic non-allergic without nasal symptoms	during natural provocation
9	grass-pollen allergic	before, during and after natural provocation
10	grass-pollen allergic	before, during and after provocation studies
11	grass-pollen allergic	before, during and after provocation studies
12	house dust mite allergic	during natural provocation

Moreover, the large number of sections which can be cut from a single biopsy specimen (50–100) allows the study of large numbers of cell populations from a single sample.

In the literature, biopsies of nasal mucosa are considered riskful (because of significant epistaxis), troublesome and unpleasant for the patient. The superiority of the cellular information obtained compared to other cell sampling methods (chapter 1), however, is seldomly discussed. Methods of obtaining material from the epithelial surface of the nasal mucosa are regarded as harmless, easy and friendly to the patient. However, the patients who participated in our studies and underwent brush and biopsy, with the Gerritsma forceps, on the same day after the tests, always indicated that the biopsy procedure is less uncomfortable than the brush. Before the tests, most patients expected the opposite. Although no attempt was made to prevent bleeding by applying a gauze, significant epistaxis was observed only in two cases in over 400 biopts performed (One of these two patients had a significant epistaxis from the brush as well). The biopsies can be performed many times in one patients without inducing scarring of the mucosa. Although in some patients biopted relatively often within a short period of time a small dent was seen at the place of the biopsy, no microscopical signs of scarring were found.

The method has shortcomings, when it comes to studying developmental stages and functional aspects of cells. Although a number of biopts can be taken from a single patient

in a comparatively short period of time, biopts from a single patient cannot be taken more frequently than once a day or 2-3 times a week.

The immunohistochemical method used, depends on the availability of specific monoclonal antibodies and the phenotype of the cell containing the surface antigen to which the monoclonal antibody is directed. Surface antigens are not static molecules but may appear and disappear depending on the cell's function and/or state of maturation. The disappearance of receptors in particular, may complicate the interpretation of the developmental state of a cell, as it is impossible then to tell whether the cell has not yet received or, on the contrary, already lost the receptor. The staining intensity of the cells in immunohistochemistry depends on the number of marker molecules per cell. Consequently, a small number of marker molecules per cell may result in an underestimation of the number of cells positive for that particular marker.

Furthermore, conclusions are yet to be drawn from a series of static pictures which occasionally leaves one in doubt about the direction (e.g. to or from the epithelium) in which the cell is moving or the maturation state of the cell. Finally, the functional activities of the cells cannot directly be evaluated by this method.

As it was not always possible to count all cells, a method of enumeration was developed, ensuring a maximum of objectivity with a minimum number of cells to be counted. In this method (chapter 5), sections are evaluated using randomly chosen squares of a grid successively superimposed on the entire section along parallel lines. Most of the cellular reactions seem to take place in the subepithelial layer of the lamina propria. As the cells, as a rule, predominate in this layer and the nasal mucosa was evaluated over the entire depth, an underestimation of (changes in) cell numbers may have occurred. Even with this disadvantage, however, the method was preferred to counting the cells in a small subjective "representative part of the section".

In vitro, functional studies require large numbers of cells derived from a specific tissue layer. Generally, cells in such numbers are not available from human nasal mucosa. Possible solutions for this problem are being evaluated at the moment. One solution involves the use of single cell suspensions of nasal polyps from allergic and non-allergic patients, which have a cell content that appears to be qualitatively and quantitatively comparable to the nasal mucosa (2). This method can yield sufficient cells to perform functional experiments like lymphocyte transformation tests. Another possibility is to study cytokine production by in situ hybridisation, polymerase chain reactions (PCR) or ELISA. Whether or not these methods can be used on small cell samples has yet to be evaluated. We regard the immunohistochemical evaluation of biopsies of nasal mucosa as a valuable method to study cell populations in their "natural environment" and in their relation to other cells. A combination of this method with functional studies will contribute towards obtaining further data on functional aspects.

Table 2. Differences in cell numbers in the nasal mucosa of patients with isolated grass-pollen allergy during the grass-pollen season as compared to non-allergic patients with nasal polyps, and normal controls.

Cells	Monoclonal antibody	Differences between groups	Remarks
helper/inducer T lymphocytes	anti-CD4	not demonstrated	macrophages/LC also weakly positive
suppressor/cytotoxic T lymphocytes	anti-CD8	not demonstrated	
mature B cells, not plasma cells	anti-CD22	not demonstrated	
Langerhans cells	66IIC7 (anti-CD1a)	allergic > non-allergic	depends on allergen provocation
monocytes	anti-CD14	not demonstrated	
DC, (activated) lymphocytes, epithelium, endothelium	anti-HLA-DR	not demonstrated	
mast cells and IgE^+ B cells/plasma cells	anti-IgE	allergic > non-allergic	
eosinophils		not demonstrated	
$IgG1^+$ plasma cells	HP6070	not demonstrated	
$IgG2^+$ plasma cells	HP6014	not demonstrated	
$IgG3^+$ plasma cells	HP6050	not demonstrated	
$IgG4^+$ plasma cells	HP6025	not demonstrated	

CELLULAR ASPECTS OF NASAL MUCOSA

To obtain a first impression of the cellular composition of the nasal mucosa in allergic rhinitis, cell populations were studied in biopsy specimens of patients with isolated grass-pollen allergy during the grass-pollen season. These patients were compared to non-allergic patients with nasal polyps and normal controls (chapter 5). The results of this study are summarized in Table 2. A surprisingly wide variation of cell numbers was found between individual members within each group. Previous studies on nasal secretions and serum suggested that certain cell populations would differ between the groups (3,4). However, such differences were not observed in the biopsy samples.

Actually, no differences were found in the number of eosinophils, T cells and HLA-DR expressing cells. These negative results may be due to an insufficient use of relevant monoclonal antibodies chosen for these studies. The use of carefully selected monoclonals against subgroups of cells or products of cells may show such differences. For example, the use of monoclonal antibodies against products of eosinophils like Eosinophil peroxidase, Eosinophil cationic protein and Major basic protein indicates that, although eosinophils them-

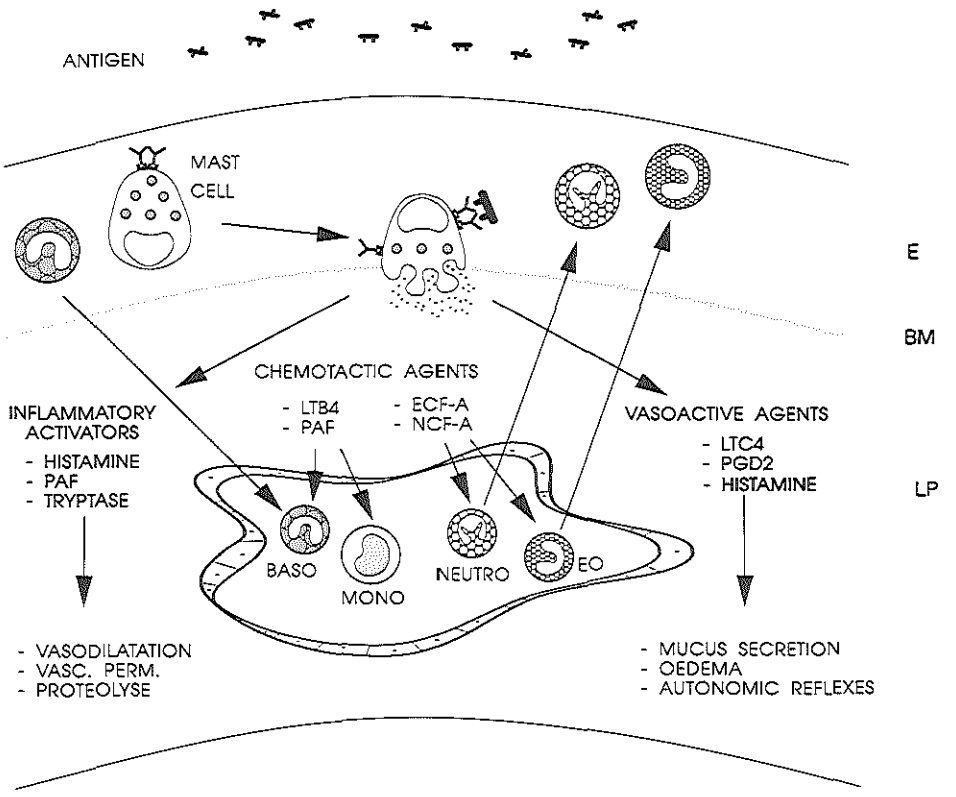

Figure 1. Mediators released by mast cells and their actions in the nose.
E = epithelium, BM = basement membrane, LP = lamina propria.

selves could not be demonstrated, the presence of their products proves that they have been active in the tissue investigated (5,6).

Furthermore, no significant differences between the groups were found in the number of $CD4^+$ and $CD8^+$ T lymphocytes. However, we did find a significantly larger number of IL-2 receptor positive T lymphocytes in the lamina propria of patients with house dust mite allergy than in controls (7). Determination of other T lymphocyte subpopulations and functional activities of T lymphocytes, e.g. the production of cytokines has not been undertaken in the context of this thesis.

It is also possible that the lack of differences in cell numbers between allergic and non-allergic patients and the wide individual variation of cell numbers within each group are of a physiological significance. This is, however, insufficiently understood at the moment. Indeed, the large numbers of cells with a "harmful" potential like eosinophils and the high expression of activation markers like HLA-DR in controls without any nasal complaints, illustrate the difficulty of drawing conclusions on functional aspects on the basis of immunohistochemical data only.

Allergic inflammation in the nasal mucosa is sustained by induction, amplification and

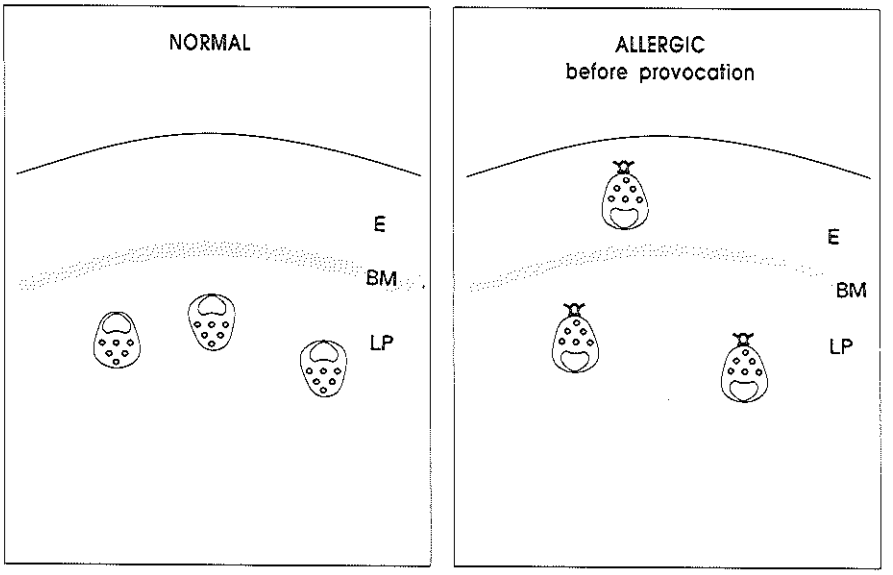

Figure 2. Mast cells in controls without nasal complaints and in allergic patients before allergen provocation. E = epithelium, BM = basement membrane, LP = lamina propria.

maintenance mechanisms which originate from complex cellular and molecular interactions. Mast cell degranulation has long been considered the only model to explain allergic rhinitis. However, the recognition that low-affinity IgE receptors ($Fc_{\varepsilon}RII$) exist on macrophages (8,9), eosinophils (10), platelets (11), T lymphocytes (12), DC (13,14) and Langerhans cells (15, chapter 9) suggests that other cell types may also be involved in the pathogenesis of allergic rhinitis. Moreover, not all drugs which are effective mast cell stabilizers, prevent the development of the late-phase reaction (LPR)(16,17) that characteristically occurs 6–12 hours after the immediate type reaction (chapter 1). On the other hand, systemic corticosteroids, which are extremely effective anti-inflammatory agents, do not influence the immediate allergic reaction when given immediately prior to allergen challenge. Hence, these agents do not seem to influence the mast cell directly. Corticosteroids are, however, very effective in blocking LPR in nose, skin and lungs (18-21).

In this thesis we focussed on those cells which seem to be able to induce allergic inflammation after binding of allergen: mast cells, which are known to play an important role in allergic rhinitis, and dendritic cells, which were demonstrated to play an important role in allergen presentation in atopic dermatits (12).

MAST CELLS

Mast cells and basophils are the only cells that express high affinity Fc_{ε} receptors. Cross-linking of mast cell bound IgE molecules by allergen and the successive degranulation of mast cells results in signs and symptoms of the immediate type allergic reaction. Mast cells are found in equal numbers in nasal lamina propria of allergic patients before and during

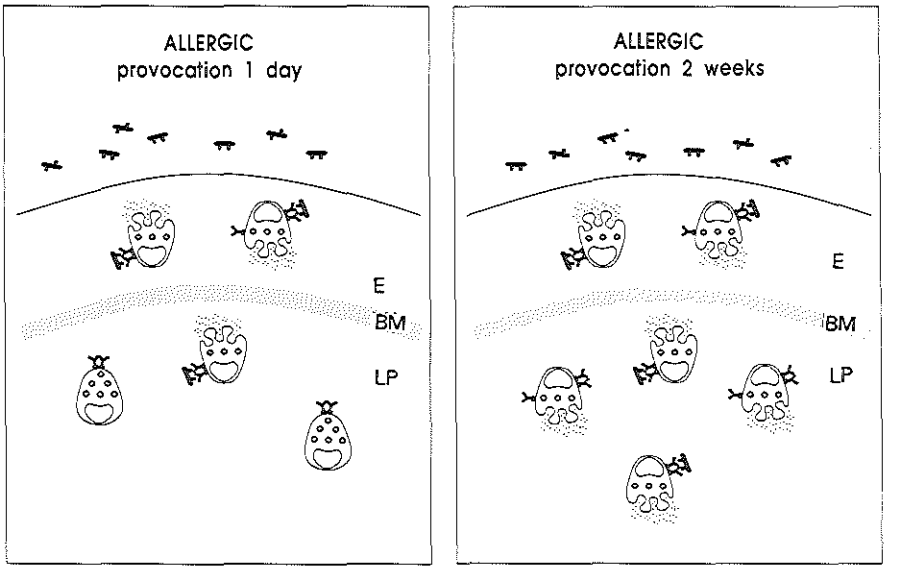

Figure 3. Mast cells in allergic patients one day after allergen provocation and after 2 weeks of daily allergen provocation. E = epithelium, BM = basement membrane, LP = lamina propria.

natural provocation and non-allergic controls (chapter 6). The controls, however, seldom have mast cells in the epithelium (chapter 6)(Fig 2). During natural allergen provocation and allergen provocation tests in allergic patients, mast cells migrate to the surface of the nasal mucosa, where they degranulate (chapter 5 and 6)(Fig 3). The store of degranulated mast cells in the lamina propria is probably replenished by immigration of mast cells from the blood or from blood derived monocytes (22). The mast cells, residing in an increasingly thick layer measured from the basement membrane into the lamina propria, degranulate during continued provocation; finally up to 50-80% of the mast cells can be found to be degranulated (chapter 6). During the degranulation, mast cells release a number of chemotatic factors (Fig 1) for the recruitment of inflammatory infiltrating cells (23-26). The release of these factors represents a pathophysiological link between immediate-type and late-phase reactions (chapter 1). According to murine studies, mast cells may release pro-inflammatory cytokines (IL-3, IL-4, IL-5, IL-6, GM-CSF) further stimulating the allergic inflammation (27,28). The latter process, however, is not established in humans. Tissue specific studies are necessary to further clarify this.

Dendritic cells

Dynamics of dendritic cells

The DC population, constituting indeterminate cells, LC, veiled cells and interdigitating cells, is another cell population able to bind allergen. The relation between LC and other DC is described in chapter 2. LC are concluded to be excellent antigen binding and proces-

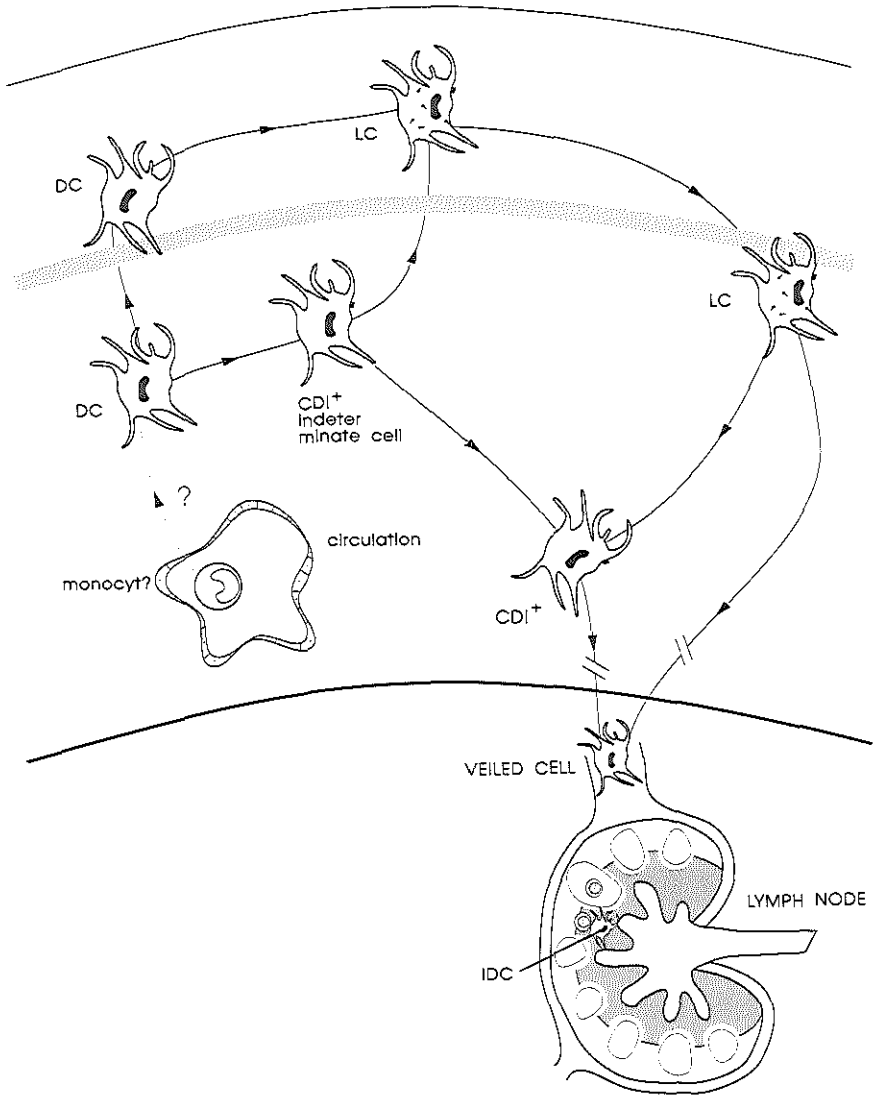

Figure 4. Different members of the DC population in the nasal mucosa and their possible relationships.

sing cells but maturation to DC is necessary to sensitize T lymphocytes (29). In this discussion, the term DC is used to refer to a member of the dendritic cell population without further differentiation.

Different members of the DC population are observed in the nasal mucosa (Fig 4). In the epithelium, CD1a$^+$ cells are found in all groups of the patients evaluated (chapter 7,9 and 10). These CD1a$^+$ cells are likely to be LC because DC containing Birbeck granules are

regularly found in the epithelium of allergic patients (chapter 8). The final proof of Birbeck granules in $CD1a^+$ cells demonstrated with immuno-electron microscopy has yet to be given. In allergic patients, over half of the DC studied in the epithelium by electron microscopy showed Birbeck granules (chapter 8).

In immunohistochemistry approximately 2-3 times as many $HLA\text{-}DR^+$ DC were found in the epithelium than $CD1a^+$ cells (chapter 5 and 11). The $HLA\text{-}DR^+$ DC in the epithelium which are not LC can be either precursor LC or matured LC (chapter 2).

In the lamina propia most $HLA\text{-}DR^+$ cells are not $CD1a^+$ (chapter 5 and 11). The $CD1a^+$ cells in the lamina propria can be indeterminate cells moving to the epithelium or LC coming from the epithelium that did not yet lose CD1a. LC with Birbeck granules were found close to the basement membrane in the lamina propria as well (chapter 8). $HLA\text{-}DR^+$ cells in the lamina propria that are not $CD1a^+$ can be precursors of LC that are not yet $CD1a^+$ or matured LC which have already lost Birbeck granules and CD1a. The finding that most $HLA\text{-}DR^+$ cells in the lamina propria are not positive for CD1a suggests that most LC mature into DC in the lamina propria.

Dendritic cells during allergen provocation

The number of $CD1a^+$ cells (LC) and $HLA\text{-}DR^+$ DC increases during allergen provocation in both the epithelium and the lamina propria of allergic patients (chapter 9-11)(Fig 5). The increasing LC/DC numbers at the site of the allergic reaction might cause the process to be perpetuated. The stimulus which attracts DC or their precursors to the nasal mucosa is not known. The increase in the numbers of LC/DC may be due to increased local differentiation into dendritic cells, or to increased migration of precursor DC in the lamina propria maturing into LC there. The mobilization of LC/DC in tissues seems to be in response to a specific cytokine, GM-CSF, which might be released as an early event after deposition of antigen by epithelial cells (30,31). As a result, the production of DC or their precursors in the bone marrow might be stepped up. These cells localize to sites where 'homing receptors' for dendritic cells occur. Although tissue-specific homing mechanisms have only been demonstrated for lymphocytes (32), transfer experiments with intravenous injection of DC suggest that such homing patterns also exist for DC (33). The human gp90 HERMES-1 homing receptor has recently been demonstrated on interdigitating cells (34). Thus, an overall generalized increase in circulating dendritic (precursor) cells is likely to be associated with a local increase of DC, resulting in a consequent increase of local T cell stimulation and thus a locally increased inflammatory reaction by inflammatory cells attracted by T cell derived cytokines. Dendritic cells rather than T cells may act as 'primed' migratory presenters. It is tempting to suggest that this local "memory" of dendritic cells could explain different modes of expression of allergic disease. An additional argument is the remaining of IgE positivity on LC long after the termination of the allergen provocation (chapter 9 and 10).

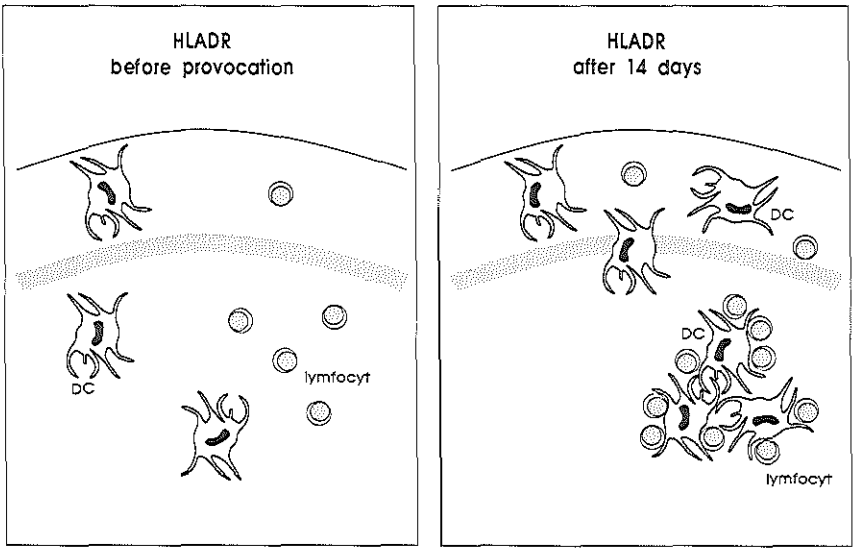

Figure 5. LC and DC in the nasal mucosa of allergic patients increase during allergen provocation.

Allergen binding of dendritic cells

LC and DC in the skin and LC in the nasal mucosa can bind IgE (35). In atopic dermatitis, IgE is bound to a functional FcR (36). LC from atopic dermatitis patients depend on such surface-bound IgE to mount T-cell proliferation to house dust allergen (an allergen) but not to Candida albicans (an antigen)(37, chapter 1). The exact nature of the LC FçR remains controversial, because LC which are positive for IgE do not express the low affinity receptor for IgE (CD23). However, normal LC can be induced to express CD23 after incubation with IL-4 and IFN-γ (38). These findings strongly suggest that selective binding of allergen by IgE increases their presentation to T lymphocytes.

Further evidence indicating a role for IgE in antigen capture by APC is derived from studies using CD23-expressing B-cell lines. IgE bound to CD23, in contrast to IgG1 bound to FcγR, allows non-specific B cells to specifically bind and present antigen to antigen-specific T cells.

B cells express CD23 in association with HLA-DR (39). Follicular dendritic cells in lymphoid tissue also strongly express CD23 (40). These findings suggest that IgE can bind via $Fc_\epsilon RII$ to a variety of APC, not only in allergic disease but also in the normal immune responses (41). Whether LC/DC in the nasal mucosa actually bind allergen and need IgE for allergen binding and presentation has yet to be determined, but the findings in atopic dermatitis stimulates the idea that similar mechanisms may occur in allergic rhinitis.

T lymphocyte sensitization in the nasal mucosa

Although the literature suggests that sensitization of T lymphocytes occurs in lymph nodes (42), the finding of clusters of HLA-DR^+ cells and $CD1a^+$ cells with T cells (chapter

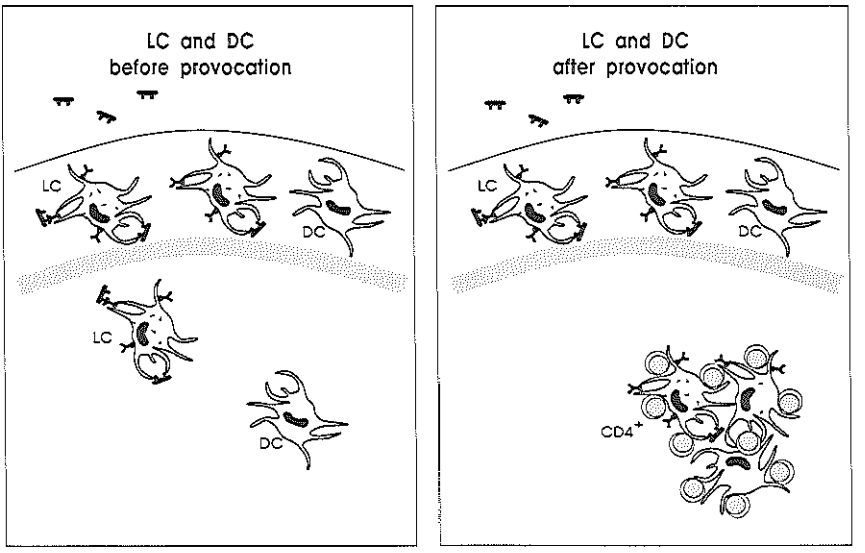

Figure 6. LC and DC in the lamina propria of the nasal mucosa of allergic patients cluster with $CD4^+$ lymphocytes during allergen provocation.

10,11) (Fig 6) suggests that stimulation can also take place in the lamina propria itself. This hypothesis is supported by the fact that, contrary to the lungs and the intestines, it is not clear which lymph nodes are draining the nasal mucosa.

The ring of Waldeyer, consisting of tonsils, adenoids, palatine and lingual tonsils, and lymphoid nodules on the posterior pharyngeal wall, is usually considered to be the major mucosa-associated lymphoid tissue of the nose. Most of this lymphoid tissue encounters alimentary antigens as well. In the rat a more specific nasal-associated lymphoid tissue is found (43). However, the ring of Waldeyer does not contain afferent lymph vessels and metastases of malignant tumors in the nasal cavity are never found in this ring of lymphoid tissue (44). This militates against a role for the ring of Waldeyer as draining lymph node of the nose.

In the clusters found in the lamina propria usually all DC are $HLA\text{-}DR^+$, but only a part of them are $CD1a^+$. IL-2 positive T cells, which are found in the nasal mucosa of allergic patients (7), can be stimulated by antigen presenting LC, in contrast to resting T cells. The $CD1a^+$ DC found in the clusters in the lamina propria are likely to be LC or relatively young matured DC which did not yet lose their CD1a expression.

Helper T cells in nasal mucosa

What happens after the binding of allergen to the LC/DC in the nasal mucosa is largely speculative. Immunohistochemical studies on T cells in nasal mucosa do not reveal differences between total T cell numbers, numbers of $CD4^+$ cells (putatively called 'helper T cells') and $CD8^+$ cells ('cytotixic T cells') between allergic patients and controls

Table 3. T cell derived cytokines and their cellular source and cellular targets and effects in atopic diseases.

Cytokine	Cellular source	Cellular target(s)	Effects
Interleukin 1	LC, DC, macrophages, natural killer (NK) cells B cells, epithelial cells, fibroblasts	Neutrophils, macrophages, T and B cells, endothelial and epithelial cells	Cytokine release from activated T cells
Interleukin 2	Activated $CD4^+$ cells	macrophages, T and B cells	Growth and proliferation of T and B cells, lymphokine production by T cells.
Interleukin 3	Activated $CD4^+$ cells	multipotential stem cells, mast cells	Differentiation and maturation of haemopoietic stem cells
Interleukin 4	Activated $CD4^+$ cells, mast cells (murine)	T and B cells, macrophages, mast cells	Proliferation of activated T and B cells, increased expression of HLA-DR, induction of expression of CD23
Interleukin 5	T and B cells, macrophages, endothelial cells	eosinophils	Stimulates bone marrow to form eosinophils
Interleukin 6	Activated $CD4^+$ cells, monocytes, fibroblasts, endothelial cells	B cell, heamopoietic stemm cells	Growth and differentiation of B cells, synergistic with Il-3
Interleukin 8	Activated $CD4^+$ cells, DC, monocytes, fibroblasts	T cells, neutrophils, basophils	Chemotaxis of neutrophils and T cells
Interleukin 10	Activated $CD4^+$ cells	T cells, neutrophils, basophils	Down regulation of IFN-γ by activated $CD4^+$ cells
Interferon-γ	Activated T cells, NK cells	Macrophages, T cells, B cells, NK cells	MHC class I and II induction. Down-regulation of IL-4 induced IgE production

(chapter 5) (7,45). However, an increased number of $CD4^+$ cells was found in the blood in allergic rhinitis and asthma (46,47), in the bronchial alveolar lavage recovered after bronchial allergen challenge (48) and intradermal infiltrate of the late-phase allergic skin reaction (49). During a prolonged allergen provocation test of patients with isolated grass pollen allergy, an increase in $CD4^+$ T cell numbers was observed (Holm, personal communication).

From a functional point of view the expression of activation markers (IL-2R, HLA-DR, VLA-1) on these T cells is more important (50). In the epithelium and lamina propria of the nasal mucosa the expression of HLA-DR on T cells increased during allergen provocation (chapter 11). Moreover, the number of $Il\text{-}2R^+$ T cells, too, was larger in the lamina propria

of patients with perennial allergic rhinitis than in non-allergic controls (7). In the blood of patients with a severe asthma increased expression of T cell activation markers was also found (50). In other words, activated $CD4^+$ appear to play a role in allergic rhinitis.

In the mouse two different types of helper T cells are designated (51): those able to produce IL-2 and/or IFN-γ (defined as Th1) and those producing IL-4, IL-5 and IL-6 (defined as Th2). Some lymphokines, like IL-3 and GM-CSF, can be produced by both Th1 and Th2 cells. Th2 cells play a central role in IgE mediated allergic reactions because of their enhancing effect on IgE production.

Recent studies are not conclusive as to whether the division in Th1 and Th2 cells in man is as clear as in mice. Some authors described blood-derived T-cell clones which could not produce IL-2, this considered to be Th2 (52). T-cell clones, which could not produce IL-2, were also established in the skin of patients with atopic dermatitis (Reysen FC v, Mudde GC, Bruynzeel-Koomen CAFM. Manuscript in preparation). Other investigators claim that the division into Th1 and Th2 cells cannot be made in human blood when higher numbers of T cells are tested (53).

A possible explanation for these discrepancies might be that the differentiation into Th1/Th2 cells is a local phenomenon which can only be found in the tissue itself. It could be speculated that IgE^+ APC are necessary to induce differentiation into Th2 cells. Technical differences, e.g. differences in the way of stimulation of the clones, or the number of clones tested, may also account for these different opinions. It seems likely, however, that helper T cells do exist which either exclusively or predominantly produce the lymphokines that are produced by Th2 cells in mice.

The functions of T cell-derived cytokines which could be produced by Th2 cells after stimulation with APC are listed in Table 1. The T cell derived cytokines have a variety of effects on mast cells (growth, differentiation, activation), eosinophils (growth and differentiation) and B cells (differentiation, production of specific IgE). As stated earlier, none of these T cell-derived cytokines has been studied in the nasal mucosa and further study is necessary to help determine their function in the nasal mucosa and their putative role in allergic rhinitis.

CONCLUSIONS

In the studies described in this thesis a number of observations concerning the cellular aspects of the pathogenesis of allergic rhinitis is reported. On the one hand, these conclusions elaborate upon the conventional understanding of the pathogenesis of allergic rhinitis. These conclusions are discussed in the relevant chapters. On the other hand the findings gave rise to new ideas on the pathogenesis of allergic rhinits, which are discussed below.

Reviewing the results of the studies, reported in this thesis, combined with findings from other authors in human and murine models, the following hypothesis for allergen binding and presentation can be presented (Fig 7):

- aeroallergens are able to bind to IgE on LC in the nasal epithelium;
- IgE^+ LC with allergen bound on the IgE migrate into the lamina propria;

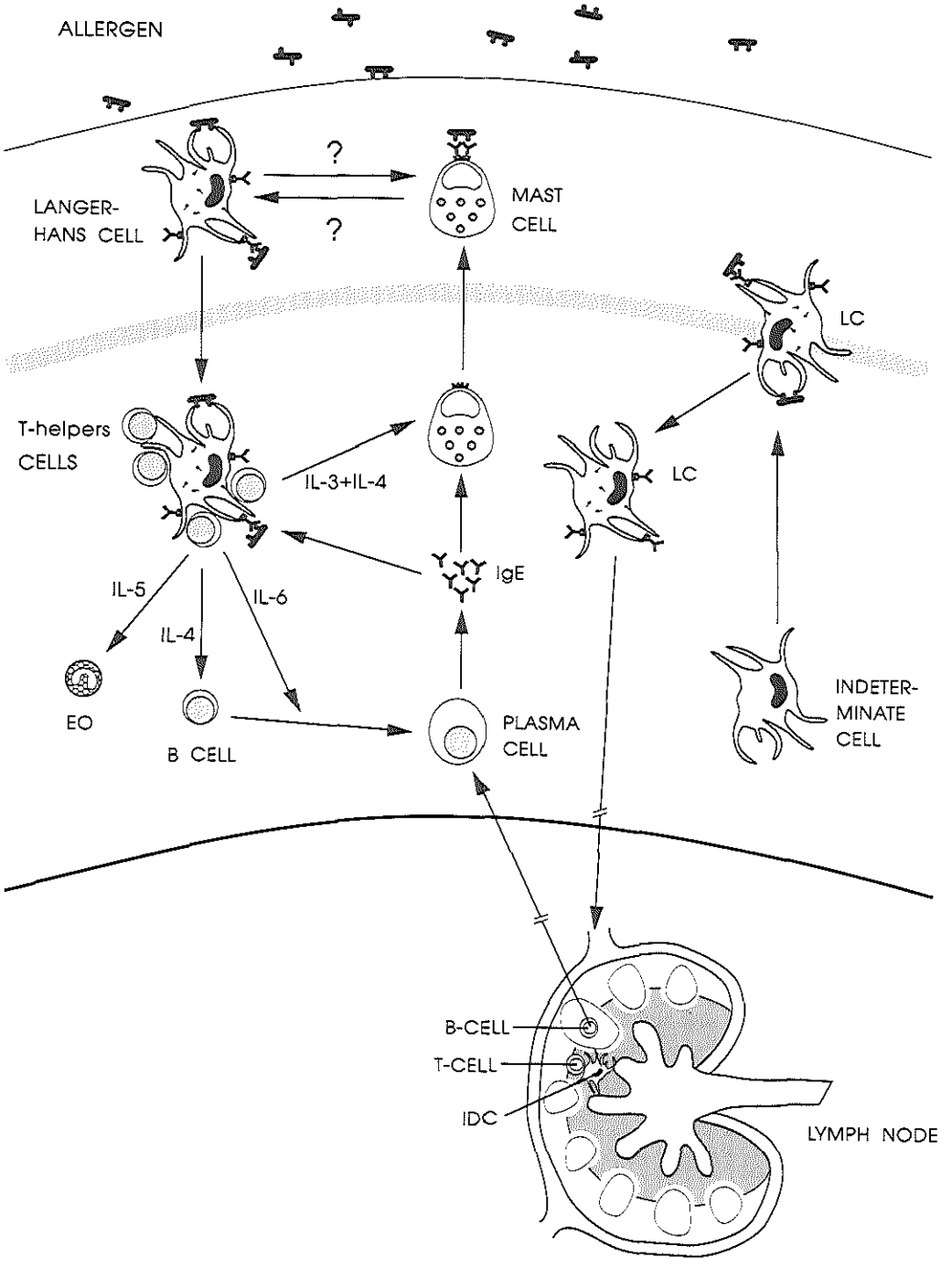

Figure 7. A model for the pathogenesis of ongoing allergic rhinitis with special emphasis on allergen uptake, processing and presentation.

- in the lamina propria the IgE^+ LC successively lose Birbeck granules and CD1a (and possibly also IgE);
- LC/DC are able to form clusters with T cells in the lamina propria where they can stimulate activated Th2 cells;
- allergen bearing LC/DC possibly migrate, as veiled cells through the lymph vessels, to the regional lymph node, where they transform into interdigatating cells and stimulate activated or non-activated T cells;
- Th2 cells (in the lamina propria and/or in the lymph node) produce a number of T cell-derived cytokines which stimulate the IgE production as well as inflammatory reactions;

We suggest that simultaneous with mast cell degranulation T cell dependent reaction mechanisms initated by IgE^+ LC play a role in allergic rhinitis. This T cell dependent reaction might be involved in the maintenance of the allergic rhinitis.

Clinical aspects

The results of this study point to a new pathogenetic mechanism in the allergic rhinitis. Beside mast cell degranulation, T cell-dependent mechanisms seem to play a role in allergic rhinitis. Although this thesis constitutes only a first exploration, which is broad and superficial, it may open a new field of research in allergic rhinitis.

This thesis demonstrates that the nasal mucosa is easily accessible for taking biopts and relatively safe to challenge. The nasal mucosa could thus be a good model to study airway allergic disease.

The notion that APC and T cells play a role in allergic rhinitis opens new ways to develop medication directed at keypoints in this system. The medication now used in allergic rhinitis, either directed at preventing mast cell degranulation, at the effects of mast cell degranulation or is found "empirically". The finding that corticosteroid nasal spray has an effect on LC/DC in nasal mucosa (chapter 13) could be a first indication of these possibilities. New medication which acts either on binding of the allergen on the APC or on APC-T cell interaction may be developed. More speculatively even, but not impossible regarding the results in oncology, one could arque that interleukins or anti-interleukins too can be used block effector T cells.

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SUMMARY

Present ideas concerning the pathogenesis of allergic rhinitis are largely deduced from systemic investigations and extrapolated from studies in the skin and the lung. Studies on allergic rhinitis generally comprise clinical aspects and/or biochemical, humoral and cellular features of the epithelial surface and the nasal secretions. Little is known about what happens in the nasal mucosa itself.

The aim of the investigations described in this thesis was to study the pathogenesis of allergic rhinitis using immunohistochemical staining techniques on biopsy specimens of nasal mucosa.

In **chapter 1** a short historic overview is given. A summary of the current knowledge on cellular aspects of allergic rhinitis is given. Moreover, as relevant data are mostly derived from cell samples of the superficial epithelium and of nasal secretions, the various methods of cell sampling are discussed. Finally, the histology of the nasal mucosa, and the use of immunohistochemical staining techniques are described.

Chapter 2 deals with features and functions of Langerhans cells (LC) in the skin. Epidermal LC play an important role in the immune system of the skin, particularly with respect to antigen handling. The prevalence of LC in other organs is described. Definite proof of the presence of LC in the human gastrointestinal mucosa is yet to be found. Experimental animal studies, however, show that antigen-presenting cells (APC) are present in the intestinal mucosa. Dendritic APC and presumably LC are present in the human lung and bronchial epithelium. The role of APC and LC in allergic asthma is not clear. In atopic dermatitis IgE^+ LC play an important role in allergen presentation to T lymphocytes. These (activated) T lymphocytes produce a number of cytokines which stimulate the IgE production as well as the local inflammatory reaction.

Chapter 3 summarizes the aim of the study.

In **chapter 4** a new instrument is introduced. These Gerritsma forceps allow biopsies of nasal mucosa to be performed, providing specimens of high quality and causing minimal stress to the patient.

In **chapter 5** cellular infiltrates have been studied in biopsy specimens of nasal mucosa of patients with isolated grass-pollen allergy during the grass-pollen season, non-allergic patients with nasal polyps and normal controls. A surprisingly wide variation has been found within all groups. Significant differences between the three groups were found with respect to the number of $CD1a^+$, IgE^+ and cytoplasmic $IgG4^+$ cells and neutrophils. No significant differences has been found in the numbers of $CD4^+$, $CD8^+$, $CD1a4^+$, $CD22^+$, HLA-DR^+, $IgG1\text{-}3^+$ cells or eosinophils.

In **chapter 6** the role of mast cells in the nasal mucosa has been investigated further during allergen provocation. In the literature, quantitative studies on mast cells in the nasal

mucosa after allergen exposure had yielded widely divergent results, ranging from an overall decrease, to redistribution to the epithelium, to an overall increase. In biopts of the nasal mucosa of patients with allergic rhinitis, the monoclonal antibody anti-IgE is used to identify all mast cells. Toluidine blue is used to detect mast cells that were not (totally) degranulated. The study was composed of two parts. In the first part, 12 controls and 23 patients with isolated grass-pollen allergy have been studied. The data from this part of the study led to the conclusion that patients with isolated grass-pollen allergy without complaints (in the winter) have the same number of mast cells as the controls do. IgE^+ mast cells are only demonstrated in allergic patients. During allergen provocation in summer, the number of mast cells increases in the lamina propria and possibly in the epithelium as well. During natural provocation virtually all mast cells in the epithelium and half of the mast cells in the lamina propria are degranulated.

In the second part of the study 17 patients with isolated grass-pollen allergy and 4 controls were daily challenged with allergen extract during a two-week period in winter. The results of this part of the study show that during provocation mast cells migrate to the surface of the nasal mucosa, where they become degranulated. The pool of mast cells in the lamina propria apparently is replenished by migration of (precursor) mast cells from the vessels in the lamina propria. During the provocation period most of the mast cells in the lamina propria degranulate as well.

Chapter 7 reports the occurence of CD1a/$HLA\text{-}DR^+$ dendritic cells (DC) in the epithelium and the lamina propria of the nasal mucosa. A significantly higher number of CD1a/$HLA\text{-}DR^+$ cells, presumably LC, is found in nasal biopsy samples of allergic patients than in those of the non-allergic controls. In the allergic patients some of the $CD1a^+$ cells are found to be surface IgE-positive.

Chapter 8 deals with electron microscopy studies in biopts of the nasal mucosa of allergic patients. The presence of Birbeck granules (BG) in most of the intra-epithelial DC indicates that these must be LC. A minority of the DC in the lamina propria contains BG as well. The ultrastructure of LC and DC in the ciliated cylindrical epithelium and in the lamina propria is discussed.

In **chapter 9** biopts of nasal mucosa of patients with isolated grass-pollen allergy are analysed to find out whether the number of LC depends on the season in which the nasal biopsy is performed (allergen exposition or not). During the grass-pollen season the nasal epithelium of patients with an isolated grass-pollen allergy shows significantly larger number of $CD1a^+$ cells than before and after the season. The number of $CD1a^+$ cells in the epithelium of the allergic patients before and after the season is not significantly larger than the corresponding number in the epithelium of non-allergic subjects without nasal complaints.

In **chapter 10** the dynamics of LC has been further studied in the epithelium and lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy during a two-week provocation period in winter. The numbers of LC are shown to increase at these sites and under these conditions. Remarkably, already in the acute phase, ½ hour after the

first provocation, a threefold increase in LC numbers was found in the epithelium. The increase in the lamina propria occurrs at a later stage, approximately 24 hours after the first provocation. During the provocation period a significant increase in *the number* of IgE^+ LC has also been found. *The percentage* of IgE^+ LC, however, remained the same.

Chapter 11 discusses DC in the epithelium and the lamina propria of the nasal mucosa of patients with isolated grass-pollen allergy during a two-week provocation period. The data show that DC with various characteristics are present in the epithelium and lamina propria of the nasal mucosa. The incidence of $HLA\text{-}DR^+$ DC increased significantly during allergen provocation. No marker has been found which was exclusively expressed on other DC than LC. This study does not support the hypothesis that macrophages play a role in the pathogenesis of allergic diseases.

In **chapter 12** is investigated whether LC and $HLA\text{-}DR^+$ cells in the nasal mucosa of 22 patients with perennial allergic rhinitis are influenced by a new nasal topical corticosteroid spray, Fluticasone. This treatment decreases the number of $CD1a^+$ cells and $HLA\text{-}DR^+$ cells in the nasal mucosa, suggesting that fluticasone proprionate decreases the antigen presentation in nasal allergy.

In **conclusion**, the studies in this thesis show that LC and DC are present in the nasal mucosa and that their numbers increase during allergen provocation. Moreover, LC in the nasal mucosa can bind IgE. LC and DC form clusters with T cells in the lamina propria. These findings suggest that simultaneous with mast cell degranulation, T cell dependent reaction mechanisms, possibly initiated by IgE^+ LC, play a role in allergic rhinitis.
The finding that corticosteroid nasal spray affect LC/DC in nasal mucosa could be a first indication that new medication, seizing on the binding of the allergen on the APC or on APC-T cell interaction, might have a beneficial influence on the symptomatology of allergic rhinitis.

SAMENVATTING

Kennis over de pathogenese van allergische rhinitis is tot nu toe grotendeels ontleend aan systemische studies en onderzoekingen in de huid en in de long. Studies over allergische rhinitis betreffen meestal klinische aspecten en biochemische, humorale en cellulaire karakteristieken van het oppervlakkige epitheel en het neussecreet.
Het doel van het onderzoek, beschreven in dit proefschrift, was de pathogenese van allergische rhinitis te onderzoeken, door toepassing van immunohistochemische kleuringstechnieken op biopten van neusslijmvlies.

In **hoofdstuk 1** wordt een kort historisch overzicht van de kennis omtrent allergische rhinitis gegeven. De huidige kennis over cellulaire aspecten van allergische rhinitis, grotendeels ontleend aan studies van oppervlakkig epitheel en neussecreet, wordt beschreven. Bovendien worden verschillende methoden ter verkrijging van cellulair materiaal besproken. Ten slotte wordt de histologie van het neusslijmvlies en het gebruik van immunohistochemische kleuringstechnieken aan de orde gesteld.

In **hoofdstuk 2** wordt een overzicht gegeven van kenmerken en functies van Langerhans cellen (LC) in de huid. Epidermale Langerhans cellen spelen een belangrijke rol in het immuun systeem van de huid, vooral wat betreft de verwerking van antigeen. Ook het voorkomen van LC in andere organen wordt besproken. In de humane gastrointestinale mucosa zijn LC nog niet definitief aangetoond. Wel zijn in dierexperimenten antigeen-presenterende cellen (APC) aangetoond in het darmslijmvlies. Dendritische APC en waarschijnlijk ook LC zijn aanwezig in de menselijke long en in het bronchiaal epitheel. De rol van APC en LC in allergisch asthma is niet duidelijk. In atopische dermatitis spelen IgE^+ LC een belangrijke rol in antigeen presentatie aan T lymphocyten. Deze (geactiveerde) T lymphocyten produceren een aantal cytokinen die de IgE produktie en de locale inflammatoire reactie stimuleren.

In **hoofdstuk 3** wordt het doel van het onderzoek omschreven.

In **hoofdstuk 4** wordt een instrument geintroduceerd dat speciaal is ontwikkeld voor het onderzoek. Deze Gerritsma forceps maakt het mogelijk biopten van het neusslijmvlies te nemen zonder noemenswaardige beschadiging van het weefsel en met een minimale belasting voor de patiënt.

In **hoofdstuk 5** zijn cellulaire infiltraten bestudeerd in het neusslijmvlies van patiënten met een geïsoleerde graspollen allergie tijdens het graspollen seizoen, van niet-allergische patiënten met polyposis nasi en mensen zonder luchtwegproblemen. In alle groepen wordt een opvallend grote variatie gezien. Significante verschillen tussen de drie groepen zijn gevonden t.a.v. het aantal $CD1a^+$, IgE^+, $IgG4^+$ cellen en neutrofiele granulocyten. Geen significante verschillen zijn gevonden t.a.v. het aantal $CD4^+$, $CD8^+$, $CD1a4^+$, $CD22^+$, $HLA\text{-}DR^+$, $IgG1\text{-}3^+$ cellen of eosinofiele granulocyten.

In **hoofdstuk 6** wordt de rol van de mestcel in het neusslijmvlies tijdens allergeen provocatie verder geanalyseerd. Quantitatieve studies t.a.v. mestcellen in het neusslijmvlies na allergeen expositie hadden tot nu toe ver uiteenlopende resultaten opgeleverd,

uiteenlopend van een algemene afname, via een redistributie naar het epitheel, tot een algemene toename. In biopten van het neusslijmvlies van patiënten met allergische rhinitis zijn alle mestcellen aangetoond met het monoclonale antilichaam anti-IgE en de niet (geheel) gedegranuleerde mestcellen aangetoond met toluidine blauw. De studie bestaat uit 2 delen. In het eerste deel zijn 12 controles en 23 patiënten met een geïsoleerde graspollen allergie onderzocht. De resultaten van dit deel van de studie tonen aan dat patiënten met een geïsoleerde graspollen allergie zonder klachten (in de winter) en niet-allergische controles het zelfde aantal mestcellen hebben, en dat alleen de allergische patiënten IgE^+ mestcellen hebben. Tijdens allergeen provocatie in de zomer neemt het aantal mestcellen toe in de lamina propria en mogelijk ook in het epitheel. Tijdens allergeen provocatie zijn bijna alle mestcellen in het epitheel en de meeste mestcellen in de lamina propria gedegranuleerd.

In het tweede deel van de studie zijn 17 patiënten met een geïsoleerde graspollen allergie en 4 controles, gedurende 2 weken in de winter, dagelijks geprovoceerd met allergeen extract. De studie toont aan dat, tijdens allergeen provocatie, mestcellen naar het oppervlak van het neusslijmvlies migreren, waar zij degranuleren. De "pool" van mestcellen in de lamina propria wordt blijkbaar aangevuld door migratie van (voorlopers van) mestcellen vanuit de bloedvaten in de lamina propria. Tijdens de allergeen provocatie periode degranuleerden ook de meeste mestcellen in de lamina propria.

In **hoofdstuk 7** wordt het voorkomen van $CD1a^+$/HLA-DR^+ dendritische cellen (DC) in het epitheel en de lamina propria van het neusslijmvlies beschreven. Deze $CD1a^+$/HLA-DR^+ cellen, waarschijnlijk LC, komen significant vaker voor in biopten van neusslijmvlies van allergische patiënten dan van niet-allergische controles. Op sommige $CD1a^+$ cellen in het neusslijmvlies van de allergische patiënten kan IgE op het oppervlak worden aangetoond.

In **hoofdstuk 8** zijn biopten van neusslijmvlies van allergische patiënten electronenmicroscopisch bestudeerd. De aanwezigheid van Birbeck granules in de meeste DC in het epitheel toont aan dat dit LC zijn. Ook in de lamina propria zijn in een klein aantal van de DC Birbeck granules gevonden. De ultrastructuur van LC en DC wordt besproken.

In **hoofdstuk 9** zijn biopten van het neusslijmvlies van patiënten met een geïsoleerde graspollen allergie bestudeerd om te kijken of het aantal LC afhankelijk is van het seizoen waarin het biopt is genomen (wel of geen allergeen expositie). Tijdens het graspollen seizoen worden in het epitheel van patiënten met een geïsoleerde graspollen allergie significant meer LC aangetoond dan voor of na het seizoen. Buiten het seizoen is het aantal LC bij patiënten met een geïsoleerde graspollen allergie niet significant hoger dan bij niet-allergische controles zonder neusklachten.

In **hoofdstuk 10** wordt de dynamiek van LC in het epitheel en de lamina propria van het neusslijmvlies van patiënten met een geïsoleerde graspollen allergie verder bestudeerd gedurende een 2 weken durende provocatie studie in de winter. De studie toont aan dat het aantal LC toeneemt op de eerder genoemde plaatsen in de eerder genoemde condities. Het is opvallend dat reeds in de acute fase, ½ uur na de eerste provocatie, een drievoudige toename van het aantal LC in het epitheel wordt gevonden. De toename van LC in de lamina

propria wordt pas later gezien, ongeveer 24 uur na de eerste provocatie. Tijdens de provocatie periode wordt ook een significante toename van het *aantal* IgE^+ LC gezien maar het *percentage* IgE^+ LC verandert niet.

In **hoofdstuk 11** zijn DC bestudeerd in het epitheel en de lamina propria van het neusslijmvlies in een 2 weken durende provocatie studie in de winter. DC met verschillende kenmerken worden gevonden in het epitheel en de lamina propria van het neusslijmvlies. Het aantal HLA-DR^+ DC neemt significant toe tijdens de provocatie periode. Er is geen merker gevonden die exclusief aanwezig is op DC en tegelijkertijd niet op LC. De hypothese dat macrophagen een rol spelen in de pathogenese van allergische aandoeningen wordt door de bevindingen niet gesteund.

In **hoofdstuk 12** is bestudeerd of LC en HLA-DR^+ cellen in het neusslijmvlies van 22 patiënten met een perenniale allergische rhinitis beïnvloed worden door een nieuwe corticosteroid neusspray, Fluticasone. Deze behandeling vermindert het aantal $CD1a^+$ cellen en HLA-DR^+ cellen in het neusslijmvlies significant. Dit suggereert dat behandeling met fluticasone proprionate leidt tot een vermindering van antigeen stimulatie in allergische rhinitis.

Concluderend, kunnen we stellen dat de studies, beschreven in dit proefschrift, aantonen dat LC en DC aanwezig zijn in het neusslijmvlies en dat hun aantal toeneemt tijdens allergeen provocatie. Bovendien, kunnen LC in het neusslijmvlies IgE binden, en zijn LC en DC in staat clusters te vormen met T cellen in de lamina propria. Deze bevindingen suggereren dat naast mestcel degranulatie, T cel afhankelijke reactiemechanismen, geïnitieerd door IgE^+ LC, een rol spelen in allergische rhinitis. Het feit dat corticosteroid neusspray een invloed heeft op LC/DC in het neusslijmvlies kan een eerste aanwijzing zijn dat nieuwe medicamenten die aangrijpen op de binding van allergeen op de APC of op de APC-T-cel interactie, een gunstige invloed kunnen uitoefenen op de symptomatologie van allergische rhinitis.

Abbreviations

AP	Alkaline phosphatase
APC	Antigen presenting cell
BG	Birbeck granules
BM	Basement membrane
CD	Cluster of differentiation
dept.	Department
DC	Dendritic cell
E	Epithelial cell/Epithelium
ECP	Eosinophil cationic protein
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENT	Ear, nose and throat
EPO	Eosinophil peroxidase
Fc	Constant fragment of immunoglobulin
FITC	Fluorescein-iso-thio-cyanate
G	Golgi apparatus
GaMIg	Goat anti mouse immunoglobulin
GM-CSF	Granulocyte macrophage colony stimulating factor
HDA	House dust antigen
HDM	House dust mite
HE	Hematoxilin eosin
HEP	Histamine equivalent in prick test
HLA	Human leucocyte antigen
hr	hour
IFAA	Iso-osmotic solution of formaldehide and acetic acid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IU	International units
LC	Langerhans cell
LM	Light microscopy
LPR	Late phase reaction
M	Mitochondrium
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
N	Nucleus
NHS	Normal human serum
PAF	Platelet activating factor
PBS	Phosphate buffered solution

PCR	Polymerase chain reaction
PGD2	Prostaglandine D2
RaHuIg	Rabbit anti human immunoglubulin
RaMIg	Rabbit annt mouse immunoglobulin
RAST	Radio-allergosorbent Test
RER	Rough endoplasmatic reticulum
SD	Standard deviation
TB	Toluidine blue
TNF	Tumor necrosis factor
TRIS	Tris(hydroxymethyl)aminomethane
TRITC	Tetra-methyl-rodamine-isothiocyanate

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Het in dit proefschrift beschreven onderzoek werd verricht binnen de afdelingen Keel-neus-oorheelkunde van het Leyenburg Ziekenhuis, 's Gravenhage en van het Academisch Ziekenhuis Dijkzigt, Rotterdam, de afdelingen Pathologie van het Slotervaart Ziekenhuis, Amsterdam en van de Daniel den Hoed Kliniek, Rotterdam, de afdeling Immunologie van het Academisch Ziekenhuis Dijkzigt, Rotterdam, en de afdeling Electronen-microscopie van de faculteit geneeskunde van de Vrije Universiteit, Amsterdam.

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Curriculum Vitae

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