Allergic Rhinitis is a Local Disease; the Role of Local IgE Production, Basophils and Mast Cells

Allergische rhinitis is een lokale aandoening; de rol van lokale IgE productie, basofielen en mestcellen

Cover photo: Photomicrographs of nasal mucosa biopsy sections obtained from allergic patients and double stained immunohistochemically with antibodies directed against (left) immunoglobulin (Ig) E in blue and plasma cells in red; and double staining of grass pollen specific IgE in red and plasma cells in blue (right) (chapter 5.3).

Omslagfoto: Immunohistochemische dubbelkleuringen van het neusslijmvlies van een allergische rhinitis patiënt. De linkerfoto toont Immunoglobuline (Ig) E positieve cellen (blauw) en plasmacellen (rood). Enkele cellen zijn zowel rood als blauw gekleurd, dit zijn IgE producerende plasmacellen. De rechterfoto toont specifiek IgE voor graspollen (rood) en plasmacellen (blauw). Specifiek IgE voor graspollen producerende plasmacellen zijn te herkennen aan de rode rand om het blauw. De kleursterkte van de rode rand neemt naar de buitenkant toe af. Dit betekent dat de plasmacel specifiek IgE aan de omgeving afgeeft. De immunohistochemische methode is beschreven in hoofdstuk 5.3.

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1. Introduction

1.1 Allergy

Von Pirquet introduced the term allergy for the first time in 1906 (1). Nowadays, allergy can be defined as hypersensitivity found after repeated exposure to foreign substances which generates clinical symptoms. Antigen-specific IgE plays a key role. Allergic diseases result from an exaggerated response of the immune system to external substances. Allergy to environmental agents can affect almost every organ of the body. The commonest manifestation is allergy of the upper airways (allergic rhinitis). However, the lower respiratory tract, the conjunctiva, the skin and the gastrointestinal tract are also frequently affected by allergic disease. The diseases are common and their prevalence is increasing (2-7). They currently affect 10 to 25% of the population worldwide (8).

1.1.1 *Historical overview*

In 1921, it was confirmed that an allergy could be transferred by serum. Dr. Prausnitz injected serum obtained from the fish-allergic Dr. Kustner in his own arm. The next day, Dr. Prausnitz injected a fish extract in the same place and he exhibited a positive skin reaction to fish for the first time in his life (9). This experiment demonstrated that the serum of Prausnitz contained a factor that could mediate a positive skin test. In humans, the Praunsnitz-Kustner (P-K) test is not used. However, this test is still used in veterinary medicine (10).

In the sixties, acting independently of each other, Kimishige and Teruko Ishizaka (11) and Johansson and Bennich described Immunoglobulin E (12, 13). Immunoglobulin E is widely recognised as the major molecular component of atopy and the allergic reaction starts when IgE on mast cells binds allergens. This leads primarily to the release of various mediators (histamine, leukotrienes and prostaglandin) and to the production and release of cytokines from these cells. The action of the mediators on downstream targets leads to the clinical allergic symptoms and the action of the cytokines to allergic inflammation. The release of histamine has direct tissue effects such as blood vessel vasodilation and subsequent plasma leakage and swelling of the mucosa tissue resulting in nasal blockage. Cytokines then induce upregulation of adhesion molecules (ICAM1 and VCAM1) on the endothelial cells possibly leading to the influx of eosinophils and basophils during the early and acute phase after allergen exposure. In the chronic state of allergic inflammation, the allergic inflammation is supported by the production of prostaglandines and leukotrienes. Allergic inflammation results in a generalised cellular infiltration characterised by T cells, dendritic cells, eosinophils, basophils, mast cells and in the production of IgE by

B cells. Moreover, epithelial cells start, as a result of the exposure to 'allergic' mediators, to produce cytokines and growth factors which enhance and prolong allergic inflammation in allergic rhinitis patients.

1.2 Allergic rhinitis: epidemiology, physiological, clinical and histological aspects

Allergic rhinitis is clinically defined as a symptomatic disorder of the nose induced by an IgE-mediated inflammation after allergen exposure of the membranes lining the nose (14). The hallmark of allergic rhinitis is the temporal sequence of symptoms upon exposure to an allergen. Symptoms of rhinitis include rhinorrhea, nasal obstruction, nasal itching and sneezing, which are reversible spontaneously or by treatment (15-17). Mast cell mediators play an important role in the induction of symptoms (15, 18, 19).

1.2.1 *Epidemiology*

Sensitivity to allergens is usually diagnosed either *in vivo* by the skin prick test, where local exposure to the allergen leads to a localised response, or *in vitro* through the detection of allergen-specific IgE in serum. Positive skin tests or the detection of allergen-specific IgE in serum has been reported in up to 30-40 per cent of the population in Western Europe (8). Fortunately, only half of the sensitised population present the clinical manifestations of allergic airway disease.

1.2.2 *Nasal mucosa physiology*

The nose acts as an air-conditioning system of the airways. It is an efficient organ in terms of warming and humidifying inspired air (20). The nose generates airflow resistance because of the bony and cartilaginous pyramid and the state of congestion of the venous sinusoids in the mucosa of the inferior turbinate and anterior nasal septum (21, 22). The changes in vascular activity are cyclical. The cycle varies in duration between 4 and 12 hours but is constant within one person. This has been called the nasal cycle (23). Changes in nasal resistance modify the airflow in each nasal cavity. Moreover, turbulence encountered in the airflow will increase the deposition of particles in the inspired air on the mucosa of the nose and act as a good filtration system for the inspired air (24-26). This filtration system of the nose also means that pathogens will be filtrated continuously by the nasal mucosa (27-31). This nasal filtration is a first line of respiratory defence (21, 22).

The particles filtrated by the nose are trapped by the mucus, which moves continuously in the direction of the nasopharynx during synchronic movement of the ciliated epithelial cells (mucociliary clearance) (32). The mucus is produced mainly by seromucus glands and by goblet cells and consists of mucopolysaccharides,

bacteriostatic substances such as lysozyme and lactoferrin (24, 33, 34) and large amounts of secretory IgA antibodies for the neutralization of antigens (35).
The nasal sinus mucosa and bronchial mucosa are not normally exposed to particles in the air due to the filtration function of the nose. These epithelial surfaces contain a large number of ciliated epithelial cells but fewer glands.

1.2.3 *Pathophysiology of nasal symptoms during the allergic nasal reaction*
Sneezing, itching, rhinorrhea and nasal blockage are four common nasal symptoms of allergic rhinitis patients (fig. 1). *Sneezing* and *itching* are primarily the result of histamine. They are caused by stimulation of the histamine H1 receptor on sensory nerve endings. In addition to histamine, itching of the skin is also caused by prostaglandins. The contribution of prostaglandins to nasal itching is not clear yet.
Rhinorrhea is the discharge of excessive watery mucus from the nasal mucosa membrane. The discharge is a result of glandular activation by the parasympathetic nerves and the transudation of plasma and high-molecular-weight proteins from capillaries of the nasal vessels. After allergen exposure, nasal discharge starts within a few minutes and persists for at least half an hour or for longer periods.
Nasal blockage is a result of vasodilation and soft tissue swelling, resulting in a reduction of internal nasal diameter and an increase in airflow resistance. Nasal blockage starts within a few minutes after allergen exposure and continues until the late phase. Histamine causes direct congestion while leukotrienes account for prolonging effects. Neuropeptides like substance P and CGRP are also capable of inducing vasodilation and contributing to congestion (16, 18, 36, 37).

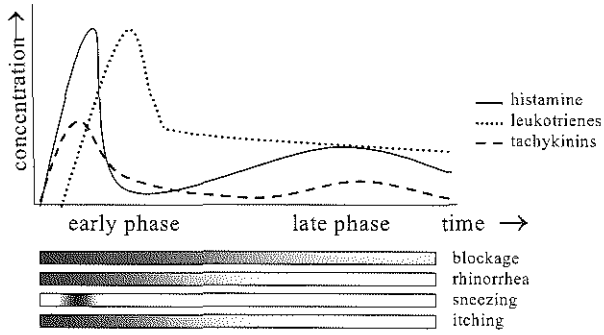


Figure 1. Nasal allergen provocation results in a significant increase in mediators (histamine, leukotrienes and tachykinins) and immediate nasal symptoms (blockage, rhinorrhea, sneezing and itching) in allergic rhinitis patients. Between 2 and 24 hours after allergen provocation, late-phase nasal symptoms, especially blockage and itching, were observed in allergic rhinitis patients.

1.2.4 *Mediators and physiology in nasal allergic reaction*

Histamine is the most important mediator in allergic rhinitis but not the sole contributor (19, 37). Alongside histamine, leukotrienes and prostaglandins are common mediators of allergic inflammation and both can be measured in nasal lavage during allergic reactions. The exact role of both mediators in the upper airways is not clear. Due to the predominant role of histamine in the upper airway allergic symptoms, it is difficult to identify the exact role of leukotrienes and prostaglandins. In the lower airway, leukotrienes cause airway obstruction and inflammation in asthmatics. Recent data support the idea that the inhibition of leukotrienes by leukotriene receptor antagonists also improves symptoms and cellular aspects of asthmatic airway inflammation. Studies using receptor antagonists for leukotrienes and prostaglandins show a reduction in allergic rhinitis symptomatology, suggesting that leukotrienes and prostaglandins contribute to allergic symptomatology, mainly in the nasal vasculature (38-40).

Recent findings provide evidence for the involvement of kinins (41) and the bradykinin B2 receptor in the development of antigen-induced airway hyperreactivity and the associated eosinophilia in the human nasal airway (42). The activation of the sensory nerves in allergic and in viral infection is accompanied by the release of tachykinins such as the neuropeptides: substance P, calcitonin gene-related peptide and neurokinins (43-45). These neuropeptides can stimulate sensory nerves and regulate glandular secretion and nasal vascular volume. Moreover, neuropeptides can induce mast cells to release their mediators. The severity of symptoms is dependent on the sensitivity and duration of the exposure to the allergen and nasal hyperreactivity (37, 46).

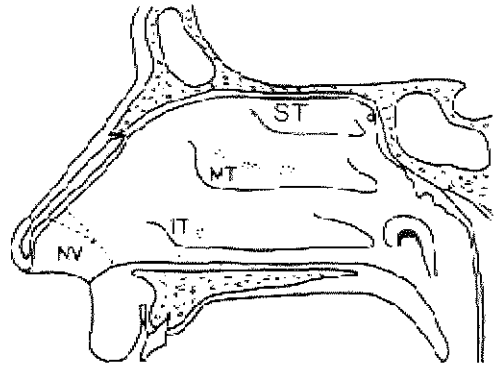


Figure 2. Lateral wall of the nasal cavity, and cross-section through the internal ostium. NV = nasal vestibule; IT = inferior turbinate; MT = middle turbinate, ST = superior turbinate.

1.2.5 *Histology*

The nasal cavities (fig. 2) are lined with mucosa. The mucosa consist of 5 different layers (fig. 3). The upper layer, which is in contact with the air, consists of mucus. The second layer is the epithelial layer, which is approximately 4 cell layers thick. The epithelial cells include basal cells, goblet cells, columnar cells (ciliated and non-ciliated) or pseudostratified cuboical epithelial cells. In the epithelium, a number of inflammatory cells such as lymphocytes, dendritic cells, macrophages, neutrophils, mast cells, basophils and eosinophils can be observed. The epithelium rests on a continuous lamina basalis. The lamina basalis forms, together with the lamina reticularis, the basement membrane, which is the third layer. The fourth layer, the lamina propria, is located between the basement membrane and the underlying supportive bone (fig. 3). The lamina propria contains several cell populations:

1) fibroblasts are part of the lamina propria and responsible for the matrix;
2) several vessels formed by endothelial cells. These can be divided into resistance and capacitance vessels;
3) glandular cells form the glands which produce the mucus;
4) plasma cells in the vicinity of the glands contribute immunoglobulins to the mucus;
5) inflammatory cells such as lymphocytes, dendritic cells, monocytes and macrophages, eosinophils, mast cells, basophils and neutrophils (47-54).

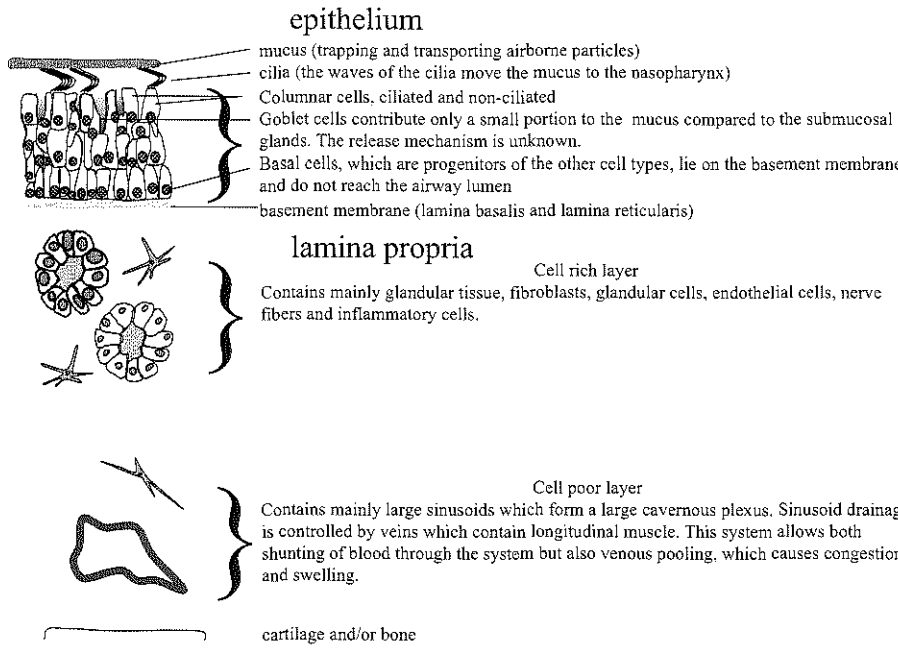


Figure 3. Nasal mucosa layers

1.3 Allergy diagnosis

1.3.1 *Clinical history of allergic rhinitis*

History is the keystone of allergic diagnosis. It should preferably be taken using structured questionnaires. Questions should cover time-related or season-related symptoms, severity, frequency, duration, triggering factors, work-related symptoms, life-threatening events and effects of avoidance. Environmental factors like the presence of pets, kind of house and workplace are also important items of information that can help to complete the history. There should be a particular emphasis on questions about previous diseases such as childhood eczema, asthma and conjunctivitis. In general, it is easier to diagnose seasonal allergic rhinitis than perennial allergic rhinitis. Patients suffering from seasonal allergic rhinitis only have symptoms in the period that a certain allergen is in the air. Seasonal allergic rhinitis is caused by outdoor allergens such as pollen. Diagnostic tests can be used to confirm the diagnosis based on history. For perennial allergic rhinitis, it is generally difficult to make a clear diagnosis based solely on history. Diagnostic testing for allergy is usually necessary to determine whether an allergen causes the disease. The most frequent causative allergens are indoor allergens like house-dust-mite allergen and animal danders. Recently, a new classification of allergic rhinitis has been proposed by the World Health Organization, subdividing the disease into 'intermittent' or 'persistent' disease and classifying allergic rhinitis into the severity categories 'mild' or 'moderate-severe' (8).

Sensitisation and clinically-relevant disease

Diagnostic tests generally measure the presence of specific IgE for a certain allergen (sensitisation). Sensitisation is found in 30 - 40 per cent of the population of Western Europe. It is very common to observe sensitisation for a certain allergen without any clinical disease. It is important to realise that sensitisation is not the same as the clinical manifestation of disease.

Very often, in the case of perennial allergens for house dust mite, it is difficult to get a clear diagnosis. There may not always be an association between sensitisation and symptoms, resulting in a false positive test result in the context of a negative history. In the case of rhinitis, anatomic disorders such as septum deviation can be the major cause of nasal obstruction in a mite-sensitised person. Because of the high sensitivity of the skin prick test, the test outcome is not always disease-specific. Results of sensitisation measurements must always be interpreted in the light of the clinical history (8).

Numerous patients have localised allergies. In these patients, sensitisation cannot be measured using a skin test or serum IgE test. However, nasal allergen provocation

tests are positive in these patients. Local sensitisation of the nasal mucosa is a phenomenon in which only the nasal mucosa and not the whole body, shows signs of allergic disease (55-57).

1.3.2 *In vivo tests*

Skin test

The skin test is one of the most commonly-used allergy tests and can be performed during an initial consultation. It will help to explain the cause of the disease to the patient. A variety of allergens can be used in skin-prick testing. They include inhalant allergens (house dust mite, grass pollen, tree, cat dander, dog hair etc.). The test should be performed with a positive control (histamine) a negative control (diluent) and standardised allergen solutions. The relation between a positive test result and overt clinical disease caused by that allergen is not absolute.

The skin test is a very sensitive test. Unfortunately, it is not always disease-specific. Other disadvantages are that medication must be stopped for periods ranging from 3 days for antihistamines to 4 weeks for local skin steroids. It is not possible to test diseased atopic dermatitis skin and the reactivity of the skin can be decreased in infants and elderly patients.

Nasal provocation test (NPT) in allergy

The nasal provocation test can be used as a non-specific test or as an allergen-specific test. The NPT is widely used to test for non-specific hyperreactivity by spraying with histamine or methacholine (58). The NPT with allergens is the ultimate test for diagnosing allergic rhinitis *in vivo*. The guidelines for the NPT indicate that it can be helpful in diagnosing allergic rhinitis when there are discrepancies between the history of allergic rhinitis and the test, or between tests (58). When this is the case, the NPT can be used to diagnose occupational allergic rhinitis before immunotherapy, particularly in the case of perennial allergic rhinitis. The NPT with allergens may occasionally be useful in determining 'local sensitisation' (8). The disadvantages are that, when it is necessary to test for more allergens, at least a one-week interval is required between each allergen provocation test and medication must be stopped for periods ranging from 3 days for antihistamines to 4 weeks for local steroids.

1.3.3 *In vitro tests*

Radio allergo sorbent test (RAST)

Since Wide and Johannson developed the RAST method for measuring allergen-specific IgE, this test has become widely recognised as an acceptable test for the routine detection of specific allergens responsible for IgE-mediated disease (12, 13).

Widely-used versions of the test are AlaSTAT from DPC and RAST from Phamacia. Both *in vitro* tests are specific and sensitive (59). For sensitisation screening, mixtures of several allergens are used in a single assay or test. The clinical relevance of these tests in allergic diagnosis is often over 85% (60). The advantage of the *in vitro* tests is that they can be used independently of patient medication or symptomatic disease status. The disadvantage is that patients do not experience the test results themselves directly. There is also a delay between the consultation and the test result.
Several other diagnostic tests - such as histamine release tests (HRT) and leukotriene release tests (LRT) - have been developed but are mainly used for research purposes. The advantages of HRT and LRT are the sensitivity of the tests and the fact that they can be used to determine whether an allergen causes allergic and/or anaphylactic reactions.

Histamine release test
Histamine is stored in large amounts in metachromatic granules of mast cells and basophils. It is actively released from these cells when challenged with allergen (to which the allergic patient is sensitised) or with 'non-specific' histamine releasers such as pharmacological agents. Sensibilisation to a particular allergen can be identified by measuring the histamine released *in vitro* by the basophils in response to an allergen (61).

Leukotriene release test or Cellular Antigen Stimulation Test (CAST)
In the CAST, sedimented leukocytes from patient blood are simultaneously primed with Interleukin-3 and stimulated with allergen. The basophils and eosinophils produce the sulphidoleukotrienes, LTC4 and its metabolites, LTD4 and LTE4. De novo formation of LTC4 can be both IgE-dependent or non-IgE-dependent in events usually described as pseudo-allergy. These newly-formed sulphidoleukotrienes (sLTs) are subsequently measured in an ELISA (62).

1.4 Allergic inflammation

Allergic rhinitis inflammation can be characterised by the presence of IgE-bearing mast cells and by the presence and influx of inflammatory cells such as T cells, dendritic cells, eosinophils and basophils. Each cell makes its own specific contribution to the allergic reaction (19, 49, 52, 63-70).

1.4.1 *Dendritic Cells (DC) and Macrophages*

Macrophages have a variety of receptors that recognise microbiological surface components, including the mannose receptor, scavenger receptor, complement

receptors and Toll-like receptors. These receptors are involved in the uptake of microorganisms by phagocytosis and in signalling for the secretion of proinflammatory cytokines that recruit and activate more phagocytes. Once bound, microorganisms are degraded in the endosomes and lysosomes, finally producing small peptides presented by MHC class II molecules. The expression of costimulatory molecules, like B7, takes place at the same time due to the binding of a microorganism to the receptors (71). Macrophages do not seem to play a crucial role in allergy.

DCs in the peripheral tissues and organs are highly phagocytotic and are actively macropinocytotic and, at this stage of maturation, they do not express costimulatory molecules. DCs are not only phagocytotic cells; they are also excellent antigen-presenting cells. DCs, after taking up a pathogen by phagocytosis, migrate to the lymphoid tissue. During this time window, they rapidly lose the ability to take up and process antigen, but synthesise new MHC class II molecules that present high levels of peptides of phagocyted pathogens. On arriving in the lymphoid tissue, they express costimulatory molecules like B7.1 (CD80) and B7.2 (CD86), which can co-stimulate naive T cells and also large numbers of adhesion molecules like ICAM-1, ICAM-2 and receptors like CCR7 which enable them to interact with antigen-specific T cells (72).

DC polarisation

In vitro data suggest that DCs derived from blood monocytes can orchestrate (fig. 4) and induce naive T cells in either T-helper type-1 (Th1) response or Th2 response, depending on their origin. Human monocyte-derived DCs are initially divided into myeloid cells (CD34+, $CD123^{hi}$ cell), the origin of DC1 (73) and a plasmacytoid cell (CD4+CD11c-CD3-cell), the origin of DC2 (74). DC1 derived from blood monocytes induces Th1 responses, whereas DC2 generated from plasmacytoid blood monocytes cultured *in vitro* induces Th2 responses (75). The origin for DC2 is not fixed and is not only linked to the plasmacytoid monocyte-derived DCs. Myeloid DCs can give rise to either DC1 or DC2 depending on the nature of the stimulus influencing the production of IL-12, indicating that IL-12 is the critical Th1-polarizing cytokine (76, 77). DCs incubated with bacterial components such as lipopolysaccharide (LPS) or CD40L can produce IL-12 in large amounts (78-80). IL-10 is able to down-regulate DC-derived IL-12 production, leading to a type-2 response (81).

Dendritic cells in allergies

Peripheral blood DCs from allergic patients with FcεRI-mediated allergen presentation function better in terms of FcεRI expression because they control the

ability to bind IgE better than non-allergic controls (82). Moreover, blood-derived DCs from patients allergic to house dust mite (HDM) who are exposed to HDM (Der-pt-1 with proteolytic activity) play a pivotal role in the amplification and maintenance of a Th2 response, whereas blood-derived DCs from healthy donors did not (83).
Chemokines play a central role in the recruitment of DCs (84-89). DC recruitment to the nasal mucosa has been observed during natural seasonal and artificial allergen exposure (64, 90). Recent findings by Jahnsen et al. suggested that plasmacytoid DCs (the DC2s) are involved in the triggering of nasal airway allergy and that they target allergic lesions via adhesion molecules that normally mediate leukocyte extravasation in organised lymphoid tissue (54). This suggests that the nasal mucosa may be a site which is well organised for allergen presentation and T cell stimulation.

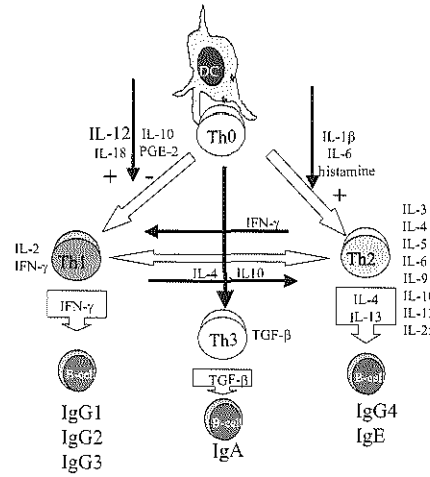


Figure 4. Dendritic cell-induced differentiation of Th0 cells into Th1, Th2 and Th3 cells, depending on the cytokine microenvironment and the time of interaction. Each T-cell subtype can induce B-cells to produce immunoglobulin.

1.4.2 *T-lymphocytes (T cells)*

Lymphocytes can be classified, on the basis of the presence of different cell membrane molecules, into T-lymphocytes, B-lymphocytes and Natural Killer (NK) cells. The NK cells do not seem to play an important role in allergy.
T lymphocytes first develop in the bone marrow and than migrate to the thymus (T cell) and then move through the blood into the peripheral lymphoid tissue until they encounter their specific antigen. Mature circulating T cells are naive T cells until they encounter a presented antigen. When a dendritic cell or another APC has picked up an antigen, they will present the antigen peptide through the MHC complex. When this activated APC encounters a naive T cell, the latter will be differentiated into an effector T cell. This activation and clonal expansion of a naive T cell by an APC is

called priming. A naive T cell can differentiate upon activation into effector T cells and memory T cells. T cells can be divided into either Th1 or Th2 or Th3 (regulatory T cells). These T helper cells differ in terms of the cytokines they produce and therefore in their function. Dendritic cells play an important role in switching on the immune response. As subtypes of the APCs, the DCs appear to influence the character of T cell differentiation, i.e. the Th1/Th2 balance and the differentiation of the T cell into Th1 or Th2 types (91, 92).

Th1 cells produce IL-2 and IFN-γ and support isotype switching to IgG1, IgG2 and IgG3; disregulation is associated with autoimmune diseases. Th2 cells produce IL-3,IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 (93) and IL-25 (94) supports isotype switching to IgG4 and IgE; disregulation is associated with allergy.

Tr (CD4+ regulatory) cells or Th3 cells were identified while investigating mechanisms associated with oral tolerance. Th3 cells which produce TGF-ß play an important role in disease prevention or cure (95) and support isotype switching to IgA (96). Another Tr cytokine is IL-10, which plays a key role in specific unresponsiveness (anergy) in peripheral T cells. The anergy is directed against the T cell epitopes of the respective antigen and characterised by suppressed proliferative and cytokine responses. Anergic T cells can be reactivated by different cytokines. Whereas IL-15 and IL-2 generate a Th1 cytokine profile and an IgG4 antibody response, IL-4 reactivates a Th2 cytokine pattern and IgE antibodies. Increased IL-10 suppresses IgE and enhances IgG4 synthesis, resulting in a decreased antigen-specific IgE:IgG4 ratio, as observed normally in patients after specific allergen immunotherapy (SIT) or antigenic T cell peptide immunotherapy (PIT) (97-99). Grass pollen immunotherapy increases IL-10 and TGF-ß mRNA expression in the nasal mucosa during the pollen season (100).

1.4.3 *B lymphocytes and IgE production*

A lymphocyte differentiated from the bone marrow (B cell) is programmed to make one single specific antibody type. This antibody is located on the surface of the cell and acts as a receptor for the antigen targeted by the antibody. The antibodies are primarily of the IgM type. In general, immunoglobulin (Ig) molecules are made up of two identical heavy and two identical light chains (H-chains and L-chains), each with a variable (V) and constant (C) region. The tips of the Fab fragments carry the idiotype-specific site. This site on the Ig molecule is the specific antigen-recognizing site. Site variation changes Ig specificity. In addition to the Fab fragment and the idiotype site, the Ig has an Fc domain, through which the molecule attaches to cell surface receptors. The specificity of a particular antibody is determined by the intrachromosomal recombination of a variable V region (V, D and J genes) and a

constant (C) region. The sequence of the CH genes in humans is μ, δ, γ1, γ2, γ3, γ4, ε, α1 and α2. Human antibodies can be differentiated into nine (sub) classes (IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE). The difference between the molecules is based on the heavy chain. The heavy chains (constant region) are differentially distributed and differentially regulated across the broad range of cell types that mediate different effector functions. The specificity of a B cell is randomly defined in the bone marrow. After leaving the bone marrow, the B cells express IgM and IgD on their cell membranes and migrate to the secondary lymphoid organs, where negative selection take place. Unsuccessful B cells (B cells with no antigen binding) undergo apoptosis. The following step is antigen-specific clonal expansion, when a mature B cell cognate interacts with an antigen-specific T-helper cell which recognises the presented antigen peptide in the B cells. MHC Class II. Cytokines produced by the T cell stimulate the B cell to produce antibodies. It is at this stage of B cell differentiation that the antibody class is determined. Cytokines in the microenvironment influence the antibody class that will be produced. The presence of TGF-β is associated with IgA1 and IgA2 (96). The Th2 cytokines IL4 and IL-13 (101) are associated with IgG4 and IgE and the Th1 cytokine IFN-γ is associated with IgG1, IgG2 and IgG3 (102).
The nasal mucosa contains B cells which can interact with T cells (103). Several reports show that there may be isotype switching into IgE transcripts in the nasal mucosa of allergic rhinitis patients (104, 105).

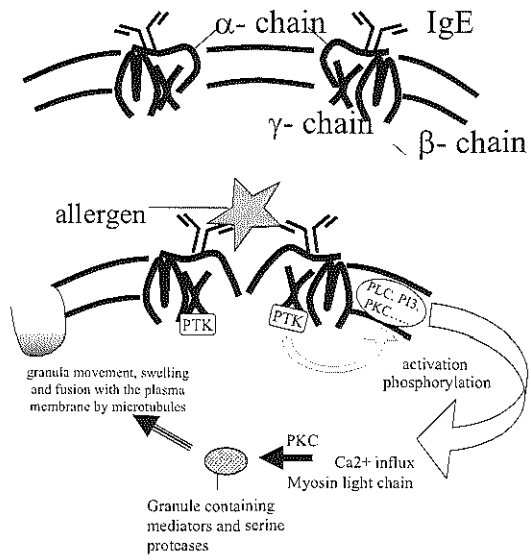
1.4.4 *Eosinophils*

Eosinophils are granulated effector cells which play a role in the allergic reactions that originate in the bone marrow. The most distinctive feature of the eosinophilic leukocyte is its granule, which stains avidly with acidic dyes such as eosin. Major basic protein (MBP) is localised in the core of the granules while eosinophilic cationic protein (ECP), together with eosinophil peroxidase (EPO), has been identified in the granule matrix. These proteins are thought to play a role in tissue damage and remodelling in allergic disease (106-115). Allergen-induced rhinitis is accompanied by an eosinophil infiltrate (17, 52). The first infiltrating eosinophils can be observed within half an hour after allergen provocation (65).

1.5 FcεRI activation and downstream effects in mast cells and basophils

The high-affinity receptor for IgE is a tetrameric receptor. It consists of the basophils and mast cells of an α subunit, a β subunit and two γ subunits(fig.5). In dendritic cells or in monocyte macrophages, the FcεRI consists of only an α subunit and two γ subunits. The α chain contains the extra cellular IgE-binding domain.

Stimulation takes place by cross-linking of two FcεRI molecules in mast cells and basophils. FcεRIs are then dimerised. Crosslinking FcεRI in the cell membrane activates two cytoplasmic protein tyrosine kinases (PTK), Lyn and Syk, that in turn phosphorylate ITAM (immunoreceptor tyrosine-based activation motifs) and activate enzymes (phospholipase C γ1 PLC γ isoforms, PI 3-kinase isoforms, PKC isoforms, Akt, BTK, MEK, ERK1/ERK2, JNK and others), adaptors (Cbl, Grb2, SLP-76, LAT and others) and GTP exchange factors/GTPases (Ras, Rho, Vav, Sos and others) and induce the mobilisation of stored and extra cellular Ca2+. ITAMs are found on both the β and γ chains and these two subunits are phosphorylated within seconds after receptor cross-linking and dephosphorylated almost immediately upon disruption of the receptor aggregates (116, 117). The PTK lyn associates with the β and γ chains when the cells are at rest and initiates phosphorylation of these chains after receptor aggregation (118). These and other biochemical and ionic events lead within seconds or minutes to the secretion of inflammatory mediators, such as histamine, that induce airway constriction, mucous production and other acute allergic symptoms (119, 120).



The FcεRI consists of an alpha subunit, a beta subunit and two gamma subunits in mast cells and basophilic cells

IgE is bound by the extracellular part of the FcεRI (a-chain). Binding of a multivalent allergen by IgE induces cross-linkage of the α-chains, followed by phosphorylation and activation of enzymes.

Myosin light chain phosphorylation is necessary for microtubular assembly and fusion of the granule with the plasma membrane.

Figure 5. The role of the FcεRI in mast cell degranulation

1.6 Mast cells and basophils

Mast cells (MC) and basophils are generally recognised as the principal cell types in the initiation of IgE-dependent immediate hypersensitivity reactions. They may also contribute to innate and acquired immunity and to tissue remodelling. Mast cells accumulate during many pathologic conditions, including parasitic infections, growth

of some solid tumors and chronic inflammatory conditions such as renal fibrosis, interstitial cystitis, rheumatoid arthritis and heart failure (121-126).

MCs are tissue-related cells; basophils can normally only be observed in the blood. MC nuclei are rounded whereas blood basophils have deeply divided lobes (47, 63, 120, 127-130).

In terms of granule constituents, human MCs have been divided into three phenotypes: those only positive for tryptase (MCT), those positive for tryptase and chymase (MCTC) and those only positive for chymase (MCC) (63, 131-136).

Basophils represent less than 1% of the leukocytes in the blood of healthy persons. By contrast with MCs, basophils isolated from normal individuals have negligible amounts of tryptase and undetectable amounts of chymase and carboxypeptidase protein.

Mast cells and basophils have several important similarities. Both have the high-affinity receptor for IgE (FcεRI) on their cell surface and contain histamine. Stimulation and regulated secretion from mast cells and basophils is induced by the crosslinking their FcεRI.

Histamine-releasing factors (HRFs) are a heterogeneous group of cytokines that cause histamine release from basophils and mast cells. About 75% of HRF activity can be neutralised by antibodies against IL-1, IL-3, IL-8, GM-CSF and TNF-α (137). Stem Cell Factor (SCF) influences HRF effects on mast cells (138).

There are also non-immunologic agonists which stimulate mast cell secretion: I) multivalent lectins like bivalent concanavalin-A cross-link membrane FcεRI or IgE, II) calcium ionophores activated by translocating calcium III) Biomolecules such as compound 48/80, complement factors C3a and C5a, morphine, codeine, mellitin, eosinophil-derived major basic protein and IV) various neuropeptides such as substance P, vasoactive intestinal peptide (VIP), somatostatin and calcitonin gene-related protein (CGRP) activate mast cells derived from skin.

Basophils respond to C3a and C5a as mast cells do. By contrast with mast cells, basophils do not respond to compound 48/80, morphine, codeine and neuropeptides (120). IL-3 activates basophils but it has only a limited effect on human mast cells (137-139). Mast cell and basophil degranulation is associated with the activation of small G proteins that cause actin polymerisation and the relocalisation of actin (140).

The major mediators, alongside histamine from mast cells, are leukotriene (LTB4, LTC4, LTD4 and LTE4) and prostaglandin (PGD2). Leukotrienes are products of arachidonic acid transformed by 5-lipoxygenase and glutathione-s-transferase and prostaglandins of arachidonic acid transformed by cyclooxygenase. Previously referred to as 'slow-reacting substances of anaphylaxis' (SRS-A), they are synthesised by many cell types such as eosinophils, mast cells, basophils and macrophages.

Leukotrienes cause enhanced vasodilation and increased permeability in vascular endothelium and play an important role in the late phase of allergic reaction by causing nasal blockage and maintaining the eosinophilic inflammation (16, 36).
The metabolising of lipids (containing arachidonic acid) occurs early during the secretory response. The generation of IP3 and diacylglycerol after FcεRI aggregation results in the release of calcium from the endoplasmatic reticulum. The generated calcium stimulates PKC and may directly facilitate the fusion of lipid bilayers as exocytosis proceeds. Arachidonic acid oxidation occurs through the cyclooxygenase (COX) pathway to prostaglandins and thromboxanes, or lopoxygenase pathways to leukotrienes and lipoxins (120).
After allergenic stimulation, mast cells initially release histamine and then leukotriene and prostaglandine. Histamine is preformed in available form and can be released immediately. Leukotriene and prostaglandine need to be metabolised and are released about 10 minutes later.
Human mast cells and basophils produce a diverse array of cytokines and chemokines. TNF-α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13 and IL-16 lymphotactin, MCP-1 TGF-ß and MIP1-α could be produced by mast cells and IL-4 and IL-13 could be produced by basophils (51, 119, 136, 141-143).

1.6.1 *Histamine*

Histamine (ß-imidazole-ethylamine, MW = 111) is an intercellular mediator with potent effects on various target tissues. It is produced by the decarboxylation of histidine. There is less than 1-3 pg of histamine per histamine-containing cell (61, 120).
Histamine is the most important mediator of allergy in the nose. It acts directly on cellular histamine receptors and has either direct or indirect effects. Histamine causes oedema formation directly and some vasodilation by a direct effect on vascular histamine receptors H1 and H2. Glands probably contain histamine h2 receptors, but their significance in the airways is unknown. Indirectly, histamine stimulates sensory nerves, probably H1 receptors and initiates a parasympathetic reflex which results in hypersecretion and transient vasodilation, this being the main cause of oedema and persistent blockage. Histamine may play a role via its H1, H2 or H3 receptors in the central nervous system (144).
Histamine has also immunomodulatory and cellular effects. Histamine alters the repertory of cytokines and chemokines secreted by mature DCs and causes DCs to increase IL-10 production and reduce IL-12 secretion. Moreover, in the presence of histamine, DCs skew naive CD4-positive T cell development towards a Th2 phenotype (145, 146). Nasal epithelial cells cultured in the presence of histamine up-

regulated IL-8 mRNA and down-regulated tight junction ZO-1 mRNA (147) cause increases in epithelial permeability (148).

1.6.2 *Tryptase and Chymase*

Tryptase is a tetrameric serine proteinase with a molecular weight of 134 kDa (149, 150). It is present in mast cells and constitutes more than 20% of total cell protein by weight (151). There is 10-35 pg of tryptase per cell (152). Chymase is a chymotrypsin-like protease expressed by a subset of human mast cells that typically predominate in connective tissues. It is stored in large amounts in mast cell secretory granules (4.5 pg/ mast cell) (152). Tryptase is stored in the secretory granule of mast cells in a fully active form.

Following exposure to allergen or other physiologically relevant and irrelevant stimuli such as neuropeptides, anaphylotoxins and opioids, tryptase and chymase levels increase in the nasal lavage fluid of allergic rhinitis patients and in the bronchoalveolar lavage fluid of asthmatics. Due to cell degranulation, tryptase and chymase are released together with other preformed mediators in a complex of 200-250 KDa with proteoglycans (153, 154). Mast cell degranulation caused by allergen exposure may result in the release of granule contents, including chymase. However, tryptase and chymase can also be released separately from each other (135).

Tryptase has been found to cleave the neuropeptides CGRP and VIP efficiently (155, 156). Tryptase has profound effects on cell behaviour, acting as a growth factor for fibroblast and epithelial cells. It stimulates IL-8 release and the up-regulating expression of ICAM-1 and PAR-2 (126, 157, 158). Chymase preferentially cleaves proteins at sites with aromatic residues in the P1 position. It may alter cytokine bioavailability by activating the interleukin-1ß (Il-1ß) precursor and degrading IL-4 (159, 160) and through the cleavage of stem cell factor (SCF) from the matrix, generating a bioactive soluble product (161). Chymase can participate in matrix remodelling by activating procollagenase. It can also control blood flow by generating angiotensin II (162, 163).

Injecting human tryptase into guinea pig skin suggests that it is a potent stimulus of microvascular leakage (124). Chymase may provide a potent stimulus for inflammatory cell recruitment following mast cell activation (164).

1.7 Mast cells and basophils: growth and differentiation

Until now, it was not possible to observe mast cell precursors *in vivo* in blood. *In vitro* mast cells and basophils can be derived from CD34-positive hematopoietic progenitors obtained from blood, cord blood and bone marrow (165-167). Differences between basophils and mast cells are based on morphology and immunological

markers which probably only show differences in maturation and differences caused by the microenvironment of the tissue or blood. In general, basophils are more primitive and have a narrower spectrum of possibilities than mast cells (120). It has been suggested that basophilopoiesis is a default differentiation pathway (168).

Data obtained from skin mast cell cultures indicated that these mast cells obtained from skin can only proliferate in the absence of serum, or fetal calf serum. This observation strongly suggests that components in serum prevent mast cell proliferation (169, 170).

Levels of mast cell chemo-attracting factors like SCF, IL-6, IL-8, TNF-α and RANTES have been found to be elevated in the nasal mucosa of allergic rhinitis patients compared to healthy controls (51, 171-173). Moreover, nasal epithelium obtained from allergic rhinitis patients produced more IL-8 and RANTES than that from non-allergic controls (51, 174). Nasal allergen provocation induces elevated levels of these mast cell chemo-attracting factors in allergic rhinitis patients (171, 175, 176). The *in vitro* observations of mast cell chemotaxis induced by RANTES and IL-8 are, interestingly, in line with the *in vivo* data (177, 178). The functional presence of chemokine receptors on mast cells are an interesting therapeutic target for these chemokines.

IL-3 selectively induces from bone marrow and peripheral blood cells with characteristics of human basophils (179, 180). Stem Cell Factor (SCF) in a serum-free medium has been identified as a critical cytokine in inducing immature mast cell growth and proliferation and differentiation *in vitro* (166, 169, 181). Several cytokines (IL-4, IL-5, GM-CSF, NGF and TGF-ß) are known to be regulators of human basophilic cell differentiation. The Th2 cytokines IL-3, IL-4, IL-5 and IL-6 promote the *in vitro* survival of mast cells but fail to induce growth (139).

In the nasal mucosa of allergic rhinitis patients, the mast cell is the cell which can release mediators and induce symptoms and complaints in allergic rhinitis patients. It is unclear where mast cells come from and where they acquire IgE.

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2. Aim of the study

The introduction to this thesis summarizes the literature which indicates that there is a discrepancy between sensitisation and allergic disease. Two aspects which might play a role in this discrepancy are the differences between production and funtion of local versus systemic IgE and the differences in mast cell and basophil function in the blood compared to in the tissues.
Mast cells, basophils and IgE are key players in the allergic inflammation. The aim of the studies described in this thesis was to focus on these differences between local and systemic function of these key factors.

The research questions addressed in this thesis are:

- Mast cells and basophils seem to play an important role in the pathogenesis of allergic rhinitis. Do the phenotypes of mast cells and basophilic cells changed by allergen provocation in the nasal mucosa of allergic rhinitis patients (Chapter 3)?

- The developmental relationship between mast cells and basophils has not yet been totally resolved. What is the relation between basophil progenitors, mast cell progenitors, basophils and mast cells in the circulation and in the nasal mucosa (Chapter 3)?

- Allergic mucosa inflammation is regulated by the local production and release of several Th2 cytokines. Which increase in cytokines and chemokines is correlated to inflammatory cells and symptomatology of the patient? What is the time line of the various cytokines and chemokines after allergen provocation (Chapter 4)?

- Is it possible to develop a method to detect specific IgE in tissues. Does production of specific IgE take place locally in the nasal mucosa (Chapter 5)?

- Where do basophils and mast-cell of allergic rhinitis patients acquire IgE (Chapter 5)?

To adress these questions multiple blood samples and biopsies of the nasal mucosa of allergic patients were taken before, during and after allergen provocation. Cellular infiltrates in these biopsies were compared to those in biopsies of normal controls. Cell phenotypes, production and release of mediators and cytokines were studied using immunohistochemical techniques and in situ hybridisation.

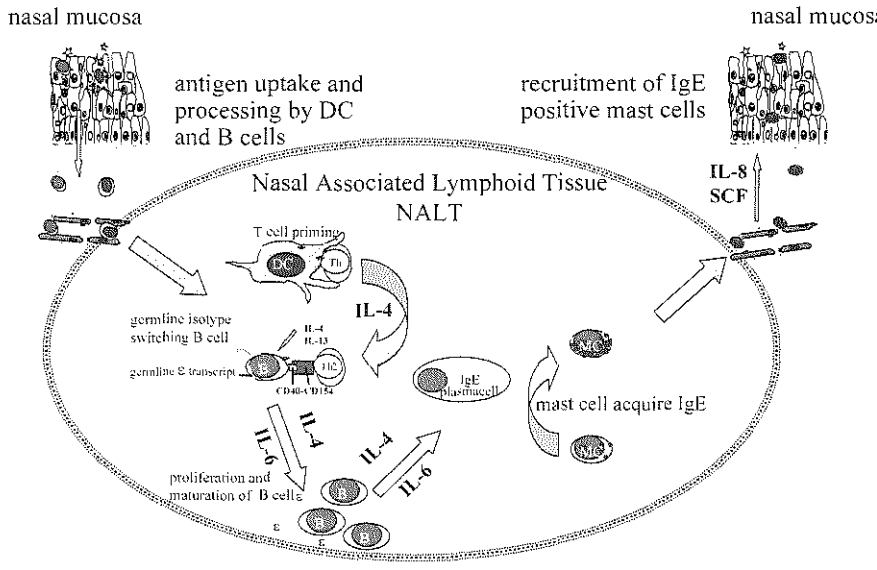

Figure 1. Mucosa sensitization in allergic rhinitis patients. Does IgE production and mast cell loading took place in the nasal mucosa itself?

3. Mast cells in the nasal mucosa

3.1 Fixation with Carnoy's fluid reduces the number of chymase positive mast cells. Not all chymase positive mast cells are also positive for tryptase

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Abstract

Mast cells in the nasal mucosa can be studied by means of monoclonal antibodies (mAb) against tryptase (T^+MC) and chymase (C^+MC). Fixation with acetone gives more positive cells than does fixation with Carnoy's fluid. In frozen biopsy specimens of allergic nasal mucosa fixed with acetone, the number of T^+MC equals that of C^+MC. When fixed with Carnoy's fluid, however, the number of T^+MC is larger than the number of C^+MC. The decrease in both T^+MC and C^+MC resulting from fixation with Carnoy's fluid is time-related and depends on the type of mAb used. Carnoy fixation time gives a decrease in the number of C^+MC within 1 min, whereas the number of T^+MC only decreases after 10 min. Within 1 minute the number of C^+MC decreases to a level where continued fixation no longer gives further decreases in the number of cells. Two populations of mast cells can be distinguished here: one sensitive and the other insensitive to Carnoy's fluid. When double-staining is used, fixation with acetone gives three populations of mast cells: one positive for tryptase (T^+C^-MC), another positive for tryptase and chymase (T^+C^+MC) and a third positive for chymase (T^-C^+MC). These three populations were found in lymfe node, spleen, thymus, dermis, lung parenchyme, small intestinal submucosa and nasal mucosa.

Introduction

Human mast cells may be classified on the basis of their neutralprotease composition. Schwartz has developed highly effective monoclonal antibodies (mAb) against tryptase (G3) and chymase (B7) (1). On the basis of double-staining with these mAb, mast cells are subdivided into tryptase-positive and chymase-negative mast cells (T^+C^-MC) and tryptase-positive and chymase-positive mast cells (T^+C^+MC). These two types of mast cells are found in the dermis, lung, small intestine, nasal mucosa and conjunctiva (1-7). The ratio of T^+C^-MC mast cells to T^+C^+MC mast cells is tissue-related (1). Enerback suggests that the ratio of T^+MC to T^+C^+MC reflects a functional state of the MC (8).

Weidner & Austen, who recently discussed the tryptase-negative and chymase-positive mast cell (T^-C^+MC), could not demonstrate immunologically detectable tryptase. They did find these mast cells in lung alveoli, bronchi, bowel mucosa and submucosa, axillary nodes and breast skin (9).

Before the introduction of mAb against proteases, mast cells were stained by means of metachromatic stains, such as toluidine blue and alcian blue. In our laboratory mast cells were stained by means of anti-IgE in combination with toluidine blue (10).

In metachromatic staining of mast cells, fixation is effected by means of Carnoy's fluid (11). In immunohistochemical staining of frozen biopsy specimens, use is generally made of mild fixatives, like acetone. Schwartz and many others after him, however, used Carnoy's fluid when staining with monoclonal antibodies against tryptase and

chymase instead of the more widely used acetone. In our laboratory we found that Carnoy's fluid had a negative effect on the number of mast cells.
To assess the effect of fixation with Carnoy's fluid on immunohistochemical single mast cell staining, we performed the following comparative studies:

- The number of T^+MC was compared to the number of C^+MC after fixation with Carnoy's fluid for 15 min as compared to fixation with acetone for 10 min.
- The number of T^+MC was compared to the number of C^+MC after fixation with Carnoy's fluid for 0, 0.01 (dip), 1, 5, 10, 15, 30, 60 min and 24 hrs.
- Furthermore, we performed doublestaining comparing fixation with Carnoy's fluid to acetone fixation.

Material and Methods

Patients

In this study, biopsy specimens of the nasal mucosa of ten grass-pollen allergic patients were studied. The patients had a history of an isolated grass-pollen allergy for a period of 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). Biopsies of nasal mucosa were taken when patients had symptoms of allergic disease.

Tissue

Biopsies of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the 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 biopsy site (12). The biopsy specimens were embedded in Tissue-Tek II O.C.T. compound (Bayer, Mijdrecht, the Netherlands) in a gelatin capsule and immediately frozen.
Furthermore normal tissue collected at obductions like submucosa of the small intestine, skin (dermis), lung parenchym, lymph node, thymus (3 months) and spleen was used. Nasal polyp collected during endoscopic surgery was used as well.

Monoclonal antibodies (mAb)

Use was made of antibody B7 (Chemicon, Brunschwig, Amsterdam, the Netherlands) against chymase and G3 (Chemicon, Brunschwig, Amsterdam, the Netherlands) against tryptase. The antibody AA1 (Dako, ITK, Uithoorn, the Netherlands), also against tryptase, was tested as well (fixed with 10 min acetone at room temperature and optimal

diluted concentration of the antibodies). We found in this study that AA1 gave a weaker signal than G3.

Fixation

The nasal biopsy specimens were fixed with Carnoy's fluid (60% ethanol/30% chloroform/10% glacial acetic acid (Merck b.v. Amsterdam, the Netherlands). A fixation time series was made ranging from 0, 0.01 (dip), 1, 5, 10, 15, 30, 60 min to 24 hrs in Carnoy's fluid. Biopsy specimens of other tissues were fixed in Carnoy's fluid for 15 min. Control fixation was achieved by means of 10 min fixation in acetone (Fluka, Bornem, Belgium).

Staining procedure

Staining was performed by means of the long-chain biotin streptavidine-alkaline phosphate method (supersensitive Biogenix, Klinipath, Duiven, the Netherlands). Each tissue specimen was cut into serial 6 µm thick sections on a Reichert-Jung 2800e Frigocut cryostat and transferred to poly-L-lysine-coated (Sigma, Bornem, Belgium) microscope slides, dried and stored in -80°C for a maximum of 3 months. The specimens were raised to room temperature, dried and fixed in acetone for 10 min at room temperature, or fixed in Carnoy's fluid and rinsed in phosphate buffered saline (PBS pH 7.8).

The staining procedure was continued by placing the slides in a half automatic stainer (Sequenza, Shandon Scientific, Zeist, the Netherlands). Following this, the sections were incubated for 10 min with 0.5% - 1% bovine serum album (BSA sigma, Bornem, Belgium) in phosphate buffered saline (PBS). The sections were then incubated with normal rabbit serum (CLB, Amsterdam, the Netherlands) for 10 min and subsequently for 60 min with the mAb anti-chymase (B7 1ug/ml) and anti-tryptase (G3 0.4ug/ml). After this, they were rinsed with PBS for 5 min and incubated with Link (rabbit anti-mouse long-chain biotinilated supersensitive AP, BioGenex AZ000UM, Klinipath, Duiven, the Netherlands) for 30 min, rinsed with PBS for 5 min, incubated with Label (streptavidine-alkaline phosphatase, BioGenex AZ000UM, Klinipath, Duiven, the Netherlands) for 30 min. They were then rinsed once more in PBS for 5 min and TRIS buffer (0.1 M pH 8.0) for 5 min and incubated for 30 min with a New Fuchsin substrate (Chroma, Kongen, Germany). Finally, the sections were rinsed in distilled water, couterstained with Mayer's haematoxylin and mounted in glycerin-gelatin.

Double staining

After fixation with acetone, the sections were rinsed in PBS and incubated with normal goat serum (1:10) (CLB, Amsterdam, the Netherlands) for 10 min and subsequently for

60 min with the mAb anti tryptase (G3 4ug/ml). They were then rinsed with PBS for 5 min, incubated with goat anti mouse β galatosidase (1:10, Southern Biotechnologies, ITK, Uithoorn, the Netherlands) for 30 min, rinsed with PBS for 5 min and blocked with 10% normal mouse serum (CLB, Amsterdam, the Netherlands) for 10 min. Following this, the sections were incubated with anti chymase biotin (3.3µg/ml, Chemicon, Brunschwig, Amsterdam, the Netherlands) for 60 min, rinsed with PBS for 5 min and incubated for 30 min with alkaline phosphatase conjugated goat anti biotin (1:100, Sigma, Bornem, Belgium). They were subsequently rinsed with PBS for 5 min, incubated with conjugated avidine-biotin-complex AP (ABC-AP Vector, Brunschwig Chemie, Amsterdam, the Netherlands) for 30 min, rinsed again with PBS for 5 min and incubated for 30 min with β-Gal substrate (5-Bromo-4-Chloro indolyl β-D Galactopyranoside, Sigma, Bornem, Belgium) (13). Finally, they were rinsed with TRIS buffer 0.1M pH 8.5, incubated for 30 min with a New Fuchsin substrate, rinsed in distilled water, counterstained with Gill's heamatoxylin (Polysciences, Brunschwig chemie, Amsterdam, the Netherlands) and mounted in glycerin-gelatin.

Toluidine blue staining

Toluidine blue, an aniline dye, stains mast cells metachromatically. Tissue sections were immunohistochemically stained with toluidine blue at pH 0.5 for at least 5 min and observed immediately.

Microscopic evaluation

After evaluation of the sections, the stained cells were counted under light microscopy at 250x (single staining) or 400x (double staining). The total surface area of each section was estimated by means of the Kontron Image Analysis System Videoplan.

Statistical analysis

The coefficients of the regression lines were worked out by performing a logarithmic transformation, thus aquiring a linear relation. The non parametric Mann Whitney U test was used to compare the differences in cell counts between the groups and to compare vector coefficients of the regression lines. A P value < 0.05 was considered to indicate a significant difference.

Results

General description

The sections of nasal mucosa had an average surface area of 2 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/or partially stratified cuboidal epithelium. 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. The number of mast cells in the epithelium was small; thus, only the lamina propria is used.

Acetone in comparison with Carnoy's fluid

After fixation with acetone, staining procedures with anti tryptase and anti chymase gave identical numbers of positive cells (see fig. 1 and table I). Both the number of T^+MC and the number of C^+MC had decreased significantly after fixation with Carnoy's fluid in relation to fixation with acetone (T^+MC $p = 0.01$; C^+MC $p = 0.0001$). Fixation with Carnoy's fluid, however, gave a significantly more marked decrease in the number of C^+MC than in the number of T^+MC ($p = 0.05$).

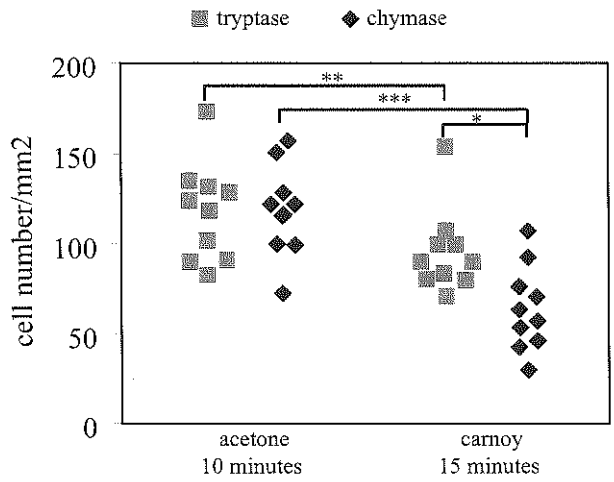

Figure 1. Score of tryptase and chymase positive mast cells/mm^2 in the lamina propria. Acetone fixation for 10 min and fixation with Carnoy's fluid for 15 min.

Table I. Number of tryptase positive and chymase positive mast cells/mm^2 in lamina propria of nasal mucosa of patients with an isolated grass pollen allergy.

Monoclonal used	No fixation (cells/mm^2)		Acetone, 10 min (cells/mm^2)		Carnoy's, 15 min (cells/mm^2)	
	median	range	median	range	median	range
anti tryptase	102	77-179	124	83-172	90	75-154
anti chymase	130	57-216	123	73-156	60	30-107

T^+MC acetone - C^+MC acetone	p = ns (statistics : ns not significant)
T^+MC acetone - T^+MC Carnoy	$p = 0.01$
C^+MC acetone - C^+MC Carnoy	$p = 0.0001$
C^+MC Carnoy - T^+MC Carnoy	$p = 0.05$
C^+MC acetone - C^+MC no fixation	p = ns
T^+MC acetone - T^+MC no fixation	p = ns

Effects of Carnoy's fluid over time

Fixation with acetone for 10 min gave no changes in the number of C^+MC and T^+MC compared to non fixation. On the other hand, fixation with Carnoy's fluid for one min showed a significant decrease in the number of C^+MC (see fig. 2): for 5 min, median (range) cel number/mm^2 74 (50-104) T^+MC and 60 (20-169) C^-MC; for 10 min, 90 (62-142)T^+MC and 54(22-102) C^-MC; and for 30 min, 106 (74-139) T^-MC and 57 (16-97) C^+MC. Fixation for more than 1 min only caused a slight further decrease in the number of C^+MC. Fixation with Carnoy's fluid gave a reduction in the number of T^+MC as well. After fixation for 10 min, a marked decrease was observed compared to the situation prior to fixation. The coefficients of the C^+MC regression lines showed a significantly larger decrease than those of the T^+MC regression lines ($p = 0.01$).

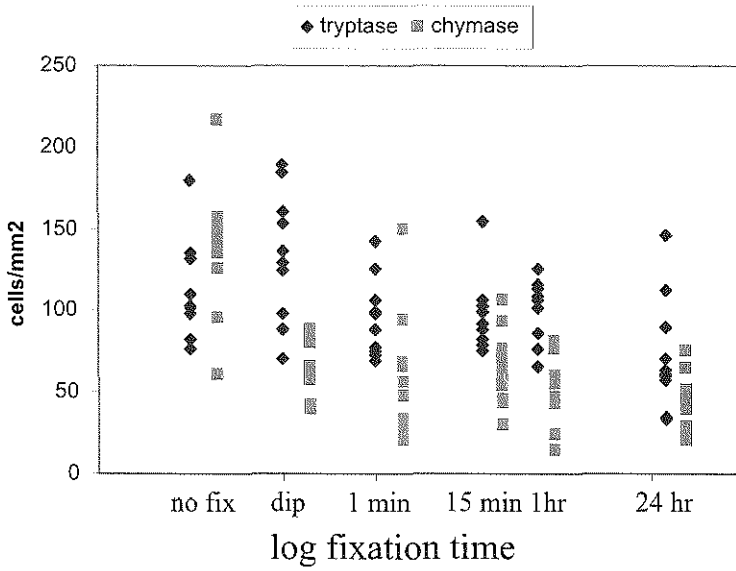

Figure 2. Score of tryptase and chymase positive mast cells/mm^2 in the laminia propria, given different (0, dip, 1 min, 15 min, 1hr and 24hr) Carnoy's fluid fixation times plotted out on a log scale.

Other tissues

Table II. indicates the number of C^+MC and T^+MC in a few other tissues, such as the submucosa of the small intestine, skin (dermis), lung parenchym, lymph node, thymus (3 months), spleen, nasal polyp, following fixation with Carnoy's fluid for 15 min and fixation with acetone for 10 min. In all tissues the number of C^-MC was found to be smaller after fixation with Carnoy's fluid than after acetone fixation. There were no consistent differences in the number of T^+MC between fixation with Carnoy's fluid and acetone.

Table II. Number of tryptase positive (T^+MC) and chymase positive (C^+MC) mast cells/mm^2 in serial biopsy samples of a number of various tissues by single staining with either anti tryptase (G3) or anti chymase (B7).

	T^+MC (cells/mm^2)		C^+MC (cells/mm^2)	
	Acetone	Carnoy	acetone	Carnoy
Spleen	0.8	1.2	0.7	0.4
Dermis	138.6	107.6	187.3	123.5
Lung parenchyma	>50	>50	4.8	0.9
Thymus (3 months)	0.9	1.1	1.8	0.9
Small Intestine Submucosa	32.2	28.8	36.4	10.4
Lymph Node	15.7	9.7	17.6	11.4
Nasal polyp	4.9	6.2	5.2	1.0

Double staining

Double staining showed three different types of mast cells. Ten nasal biopsy specimens of symptomatic allergic patients gave the following ratios: 74% (50%-88%) T^+C^+MC, 15% (6%-43%) C^+MC and 7% (1%-32%) T^+MC.

Evaluation of the other tissues (table III) showed large ratio differences. In the dermis, for instance, T^+C^+MC predominated with 87%, whereas only small percentages of T^+C^- MC (5%) and T^-C^+MC (8%) were found. In the lung, however, T^+C^-MC (90%) was mainly found, with only a small percentage of T^-C^+MC (9%) and hardly any T^-C^+MC (1%). After fixation with Carnoy's fluid, no T^-C^+MC were demonstrated in any of the previously mentioned tissues.

Table III. Percentage of single tryptase positive(T^+C^-MC), tryptase positive/chymase positive (T^+C^+MC) and single chymase positive (T^-C^+MC) mast cells in serial biopsy samples of a number of various tissues by double staining technique.

TISSUE	T^+C^-MC (%)	T^+C^+MC (%)	T^-C^+MC (%)
Spleen	33	49	18
Dermis	5	87	8
Lung Parenchym	90	9	1
Thymus (3 months)	19	67	15
Small Intestine Submucosa	68	31	1

Toluidine Blue

Double staining with toluidine blue and chymase showed most cells to be double positive for toluidine blue and chymase and existence of weakly chymase positive, toluidine blue negative stained cells could be explained by the fact that the sensitivity of

the toluidine blue method is much lower than that of the immunohistochemical method of staining chymase. Double staining with toluidine blue and tryptase showed most cells to be double positive for toluidine blue and tryptase (.

Discussion

In the literature mast cells are divided into T^+C MC and T^+C^+MC (4, 7). Carnoy's fluid is used as fixative. In our laboratory we found that Carnoy's fluid had a negative effect on the number of mast cells.

The present study on nasal biopsies of symptomatic allergic rhinitis patients showed that fixation with acetone gives a significantly larger number of T^+MC and C^+MC than fixation with Carnoy's fluid (fig. 1). This difference may be explained by the fact that Carnoy's fluid fixation weakens the binding of the mAb to the epitope against which it is directed, as the epitope is either changed or damaged. Variations in the degree of sensitivity of the epitopes to Carnoy's fluid may explain the fact that the number of chymase positive cells shows a more marked decrease than the number of T^+MC.

This finding is true not only for the nasal mucosa, but also of the small intestine submucosa, thymus, dermis, lung parenchyma, lymph node and spleen (table II). Carnoy's fluid fixation gives a decrease in the number of C^+MC for all tissues. In relation to the number of T^+MC, the picture is not as clear. For further study of the effects of Carnoy's fluid on tryptase and chymase, different fixation time series were compared (fig. 2). Fixation with Carnoy's fluid was shown to induce a time related decrease in the number of both T^+MC and C^+MC. Acetone fixation did not give changes in the numbers of T^+MC and C^+MC after the standard 10 min fixation period in relation to nonfixation. For this reason it was decided not to use a time series for acetone fixation. Although Carnoy's fluid fixation gives a reduction in the number of both C^+MC and T^+MC, the number of C^+MC decreased much more strongly than the number of T^+MC. Within 1 min the number of C^+MC had fallen to under 50% of the number which had not been fixed. It seems reasonable, then to assume that there are two populations of C^+MC, one sensitive and the other insensitive to Carnoy's fluid. A similar difference in response to fixatives - in this case formalin - was reported by Befus for metachromatic staining of mast cells (11). A similar difference in sensitivity to formalin was also reported for the T^+MC population (2, 14).

Double staining with chymase and tryptase has shown that besides T^-C^+MC and T^+C^-MC, T^-C^+MC also occur, (15) contrary to the findings of Schwartz (4, 7) and others after him. T^-C^+MC cells were demonstrated in all tissues examined. For the different tissues, however, considerable differences were found in the ratio between the T^+C^+MC, T^+C^-MC and T^-C^+MC cells (table III). This finding agrees with a recent study by Weidner on lung, skin and bowel mucosa. It must be added, however, that Weidner

used a polyclonal antibody against chymase (developed by Schechter (9). Recently Bradding et al noted that in the normal nasal mucosa biopsies, cells occasionally expressed immunoreactivity for interleukin-4 and chymase but not tryptase (16).
To be sure that the T^-C^+MC cells are mast cells a few double stainings were done. A small number of the T^-C^+MC cells were stained with toluidine blue, only the strong positive cells stained double. This effect could be the result of the limited sensitivity of the toluidine blue staining. This staining detects only the mast cells that are not degranulated. Double staining of allergic nasal mucosa with IgE and tryptase (data not shown) shows all tryptases positive cells are double positive for IgE. Double staining IgE and chymase shows all chymase positive cells are double positive for IgE.
In our opinion Schwartz and others failed to observe the T^-C^+MC population, becaussse they used Carnoy's fluid for fixation. In this situation we did not find any T^-C^-MC in our laboratory either. Studies are in progress to further evaluate single and double tryptase and chymase positive cells during allergen provocation.

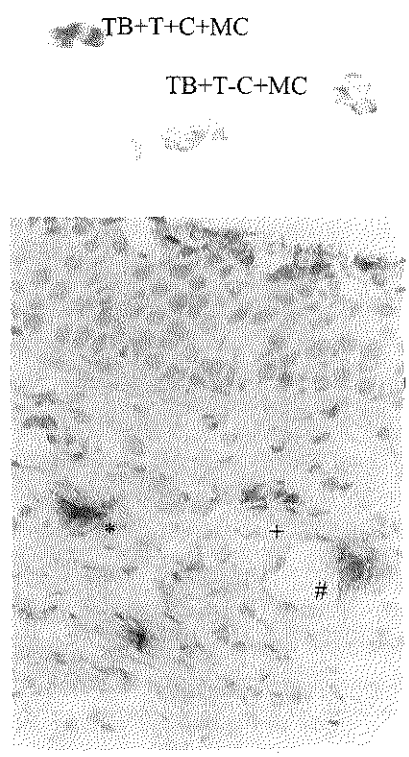

Figure 3. Double staining of human nasal mucosa with biotinylated anti chymase B7 antibodies and anti tryptase G3 antibodies. Chymase positive mast cells stained red (+), tryptase positive mast cells stained green (#), red and green stained cells are chymase and tryptase positive mast cells (*). e, epithelium; bm basal membrane; lp lamina propria (see also chapter 3.2)

Acknowledgments

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3.2 Basophil and eosinophil accumulation and mast cell degranulation in the nasal mucosa of patients with hay fever after local allergen provocation

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Abstract

Background: Basophils and mast cells have certain similarities and are believed to be important in upper and lower respiratory allergy.

Objective: We sought to apply immunohistology to investigate the distribution and numbers of mast cells and basophils in the nasal mucosa after allergen provocation.

Methods: Allergen challenge with grass pollen was performed in 9 patients with seasonal allergic rhinitis out of the hay fever season. Nasal biopsy specimens were taken before and approximately 1 hour, 24 hours and 1 week after intranasal allergen provocation. We determined relative numbers and their phenotypic characteristics by using mAbs specific for tryptase, chymase, IgE, eosinophils (BMK-13) and a new mAb against basophils (BB1) by using immunohistochemistry in frozen sections.

Results: In the nasal mucosa at baseline, practically no basophils were found in the epithelium. A significant increase in numbers was found in the epithelium and lamina propria of the nasal mucosa in the early phase as early as 1 hour after allergen provocation. At 24 hours and 1 week after allergen provocation, a significant increase in basophil numbers was found in the lamina propria only. The proportion of mast cells staining for chymase in the lamina propria decreased from a median of 38% (range, 0%-82%) to 14% (range, 0%-78%) within 1 hour of allergen provocation. The proportion of mast cells staining for chymase increased from 1% (range, 0%-86%) at baseline to 21% (range, 3%-85%) within 1 hour of allergen provocation. One week after provocation, mast cells returned to baseline numbers. A definite tissue eosinophilia was observed after allergen provocation.

Conclusion: Basophil numbers are increased in the epithelium and lamina propria of the nasal mucosa of subjects with rhinitis after allergen challenge, with influx already apparent at 1 hour. Moreover, changes in mast cell percentages and numbers were observed within 1 hour of allergen provocation.

Introduction

Mast cells and basophils are metachromatically staining cells, which are believed to be important in upper and lower respiratory allergy. These cells have certain similarities in structure and function. On the basis of conventional metachromatic staining procedures, it is not easy to discriminate between mast cells and basophil-like cells in tissues or brushings under a light microscope (1-4). Nevertheless, these cells differ in their origin and in many phenotypic characteristics. Both cell types express high-affinity receptors for IgE, which can be cross-linked by allergen binding to IgE and can induce the release of secretory granule contents. By using antibodies against the mast cell proteases tryptase and chymase, mast cells may be distinguished from basophils on the basis of their granule content (5, 6). Recent observations have

indicated that Carnoy's fluid fixation, in contrast to acetone fixation, can lead to an underestimation of the number of immunohistochemically stained chymase-positive mast cells (7, 8).

Studies measuring mediators and metachromatically staining cells in nasal lavage fluid collected from patients with rhinitis with an allergen-induced late-phase reaction have revealed high levels of histamine but relatively low levels of specific mast cell products, such as tryptase and PGD2. These findings have implicated the basophil as an important contributor to histamine release in the late phase but not in the early phase (9, 10).

The lack of a reliable means of detecting basophils in tissues has hindered understanding of the relative role of basophils and mast cells in allergic disease. Recently, however, the preparation of an mAb (BB1) specific for a unique granule constituent of basophils has opened the way for enumeration of this cell type in tissue (11).

In the present study patients allergic to grass pollen were challenged with grass pollen out of season. Biopsy specimens were taken before and 1 hour, 24 hours and 1 week after local allergen provocation and alterations in the numbers of basophils and mast cells were examined.

Material and Methods

Patients

Nine patients (median age, 22 years; range, 19-51 years) participated in this study (5 women and 4 men). All patients had a history of seasonal allergic rhinitis for grass pollen of at least 2 years. Allergy to grass pollen was confirmed by a positive skin prick test response (3+) with Alutard Soluprick extract (1 SQ/mL), with no skin prick test reactions to other relevant allergens (eg, mite, cat, or dog). Patients with a positive reaction to a tree allergen were included because this study ended long before the start of the tree pollen season in the Netherlands. The patients were free of symptoms at the start of the study. None of the patients used any medication during the study or had undergone immunotherapy in the 3 years before this study. No relevant abnormalities were found on ear, nose and throat examination. All patients gave their written informed consent and the medical ethics committee approved the study.

Study design

This study was conducted between October and December 1995, well out of 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 edge by using a Gerritsma forceps with a cup diameter of 2.5 mm. Local anesthesia was obtained by placing a cotton-

wool carrier with 50 to 100 mg of cocaine and 3 drops of adrenaline (1:1000) under the inferior turbinate without touching the biopsy site. The biopsy specimens were embedded in Tissue-TekII OCT compound in a gelatin capsule and frozen immediately. No cautery was used.12 Each patient underwent 4 nasal biopsies: at baseline, 1 hour after provocation with grass pollen, 24 hours after provocation and 1 week after provocation. It was possible to see where the previous biopsy specimen was taken and there was enough space for taking another biopsy specimen after 24 hours or 1 week (12).

Provocation

The patients were allowed to acclimatize to the room for at least 15 minutes. PBS was then administered to exclude the possibility of nonspecific hyperreactivity. After 10 minutes, patients were challenged with 50 μL of an aqueous nasal spray containing 10,000 biologic units (BU) /mL grass pollen (ALK, Groningen, The Netherlands) and another 10 minutes later with 100,000 BU/mL (ALK) to initiate a strong allergic response. After both provocations, symptoms, including sneezing, rhinorrhea, itching and nasal blockage, were recorded on a 4-point (0-3) scale. Patients were also asked to rate their symptoms during the day after provocation after 3, 6, 9, 12 and 24 hours.

Immunohistochemical staining

Staining procedures

Monoclonal antibodies directed against chymase, tryptase, IgE, eosinophil major basic protein and basophils (table I) were used together with the supersensitive immunoalkaline phosphatase method, as previously described (7).

Double-staining tryptase and chymase

Frozen sections were fixed in acetone for 10 minutes and rinsed in PBS. Endogenous peroxidase was blocked with 0.1% sodium azide and 0.01% hydrogen peroxide in PBS for 30 minutes. Sections were then rinsed in PBS for 10 minutes and incubated with 10% normal goat serum (CLB, Amsterdam, the Netherlands) and 10% normal rabbit serum (CLB). After this, the slides were incubated with mouse anti-human tryptase for 60 minutes at room temperature. The sections were then rinsed again in PBS for 5 minutes and incubated for 30 minutes with a peroxidase-conjugated rabbit anti-mouse Ig antiserum (1:200 Sigma), rinsed successively in PBS and incubated with peroxidase-conjugated mouse anti-peroxidase (1:200 Sigma). After this, the slides were incubated with the 10% normal mouse serum (CLB) for 10 minutes and incubated with biotinylated mouse anti-human chymase (B7) for 60 minutes. They were rinsed with PBS for 5 minutes and incubated with alkaline phosphatase-

conjugated goat anti-biotin (1:50 Sigma) for 30 minutes. Slides were then rinsed with PBS for 5 minutes and with TRIS buffer (0.1 mol/L, pH 8.5) for 5 minutes. This was followed by incubation for 30 minutes in Fast Blue substrate containing levamisole to block endogenous alkaline phosphatase. Finally, slides were rinsed with sodium acetate (0.1 mol/L, pH 4.6) for 5 minutes and incubated with amino-ethylcarbazole 0.05% in sodium acetate 0.1 mol/L (pH 4.6) and 0.01% peroxide substrate for 30 minutes and sections were rinsed with distilled water and mounted in glycerin-gelatin. Control staining was performed with an irrelevant mAb of the same subclass.

Table I. Antibodies used

Antibodies	Clone	Concentration or dilution	Source
Anti-chymase	B7	1 µg/ml	Chemicon (Brunschwig)
Anti-chymase^{biotinylated}	B7	1 µg/ml	Chemicon (Brunschwig)
Anti-tryptase	G3	0.7 µg/ml	Chemicon (Brunschwig)
Anti-basophils	BB1	6 µg/ml	AF Walls
Anti-IgE	HM25M	0.2 µg/ml	CLB
Anti-major-basic- protein	BMK-13	0.2 µg/ml	Sanbio
Goat-anti-mouse ^{biotinylated}		1:50	Biogenix (Klini Path)
Streptavidine alkaline phosphatase/peroxidase		1:50	Biogenix (Klini Path)
Goat-anti-biotin alkaline phosphatase		1:50	Sigma
Rabbit-anti-mouse peroxidase		1:200	Sigma
Mouse peroxidase anti peroxidase		1:200	Sigma

Light microscopic evaluation

Stained cells were counted in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (ie, the epithelium and the lamina propria) were estimated by using the Leica Image Analysis System. The number of cells per square millimeter was calculated for the epithelium and the lamina propria.

Statistical analysis

Statistical analysis was performed with the SPSS 7.5 software for Windows 95. Friedman two-Way ANOVA was used for statistical analysis. The Wilcoxon signed-rank test was used for comparing data obtained at different time points to observe degranulation or redistribution (before provocation – early phase; early phase – 1

week after provocation; before provocation – 1 week after provocation). A P value of less than 0.05 was considered to indicate a significant difference between time points. Correlation coefficients were obtained by using the Spearman rank method. Because of multiple testing, a P value of less than 0.025 was considered to indicate a significant correlation.

Results

Nasal symptoms

No symptoms were observed before allergen challenge or after challenge with saline. Nasal provocation with the allergen resulted in all patients manifesting a significant increase in immediate nasal symptoms, such as sneezing, itching, nasal blockage and rhinorrhea. Between 6 and 12 hours after the allergen provocation in all patients, late-phase nasal symptoms were observed, especially blockage and itching. Figure 1 shows total symptoms at each measuring moment before and after allergen provocation.

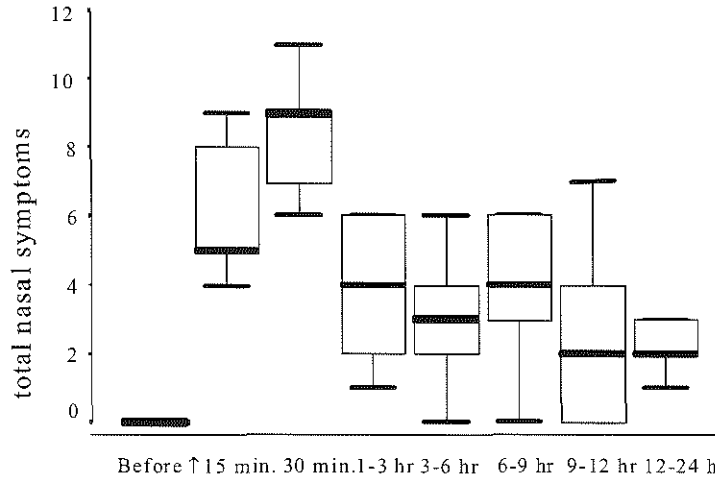

Figure 1. Total symptom score before and after allergen provocation. After both provocations (15 and 30 minutes), symptoms including sneezing, rhinorrhea, itching, and nasal blockage were recorded. Patients were also asked to rate their symptoms on a 4-point (0-3) scale during the day after provocation after 3, 6, 9, 12 and 24 hours.

Immunohistochemistry

The cryostat sections of the nasal mucosa had an average surface area of 2 mm^2 and mainly an intact epithelium. Staining with mAb for basophils, mast cells, eosinophils and IgE could easily be identified by the red cytoplasm or membrane (IgE) and dark

violet nucleus, as shown for basophils in figure 2a, before and figure 2b, after allergen provocation. For tryptase before (c) and 24 hours (d) after allergen provocation and for chymase before (e) and 24 hours (f) after allergen provocation. Counterstained with Gill's hematoxlin, magn. 1000 x.

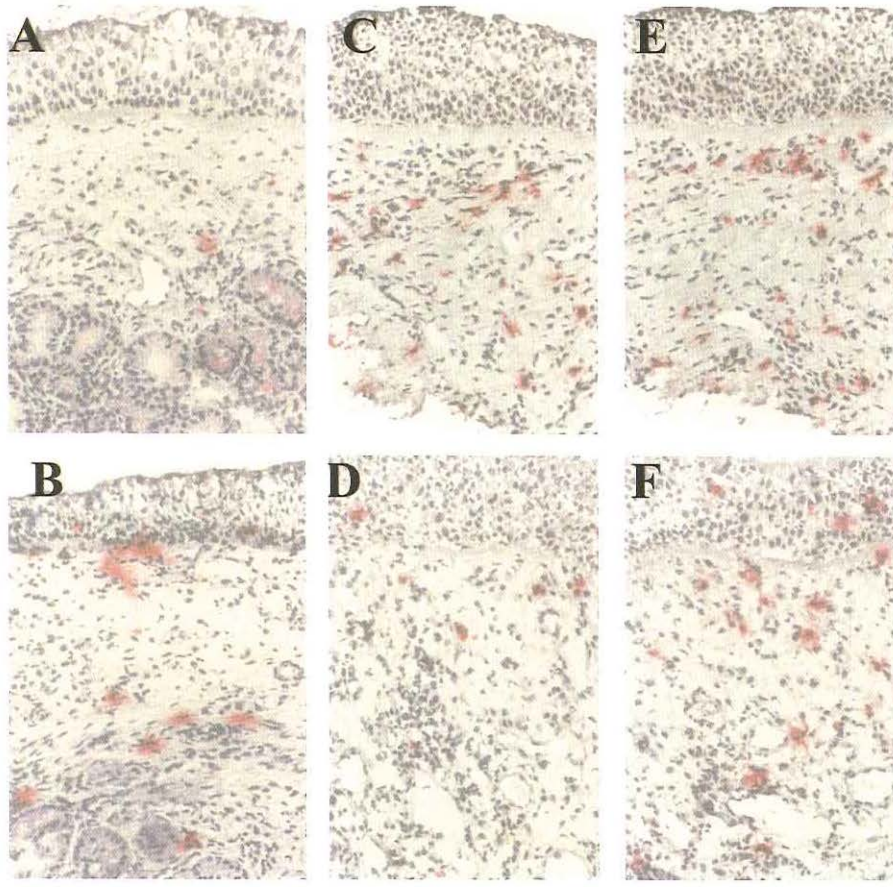

Figure 2. Photomicrograph of BB1-positive cells (red) in the epithelium and in the lamina propria of a nasal mucosa biopsy section obtained from an allergic patient before (A) and 24 hours after (B) allergen provocation. For tryptase, specimens are shown from before (C) and 24 hours after (D) allergen provocation and for chymase, specimens are shown before (E) and 24 hours after (F) allergen provocation (counterstained with Gill's hematoxylin; magnification ×1000).

Basophils

At baseline, practically no (median, 0; range, 0-33 cells/mm^2) basophils were found in the epithelium (fig. 3A). Significantly increased numbers were found in the epithelium (Friedman P = 0.04) of the nasal mucosa in the early phase after 1 hour (P

= 0.002); 24 hours after allergen provocation, the numbers of basophils were still raised, although this increase did not quite reach significance (P = 0.06). One week after provocation, the number of basophils was found to have returned to the baseline level. In the lamina propria, limited numbers of BB1-positive cells (median, 5; range, 2-27) were found mainly at baseline in the subepithelium layer and numbers increased after allergen provocation (Friedman P < 0.001). Significantly higher basophil numbers relative to baseline were observed at 1 hour (P < 0.001), 24 hours (P = 0.02) and 1 week (P = 0.02) after the allergen provocation (fig. 3B).

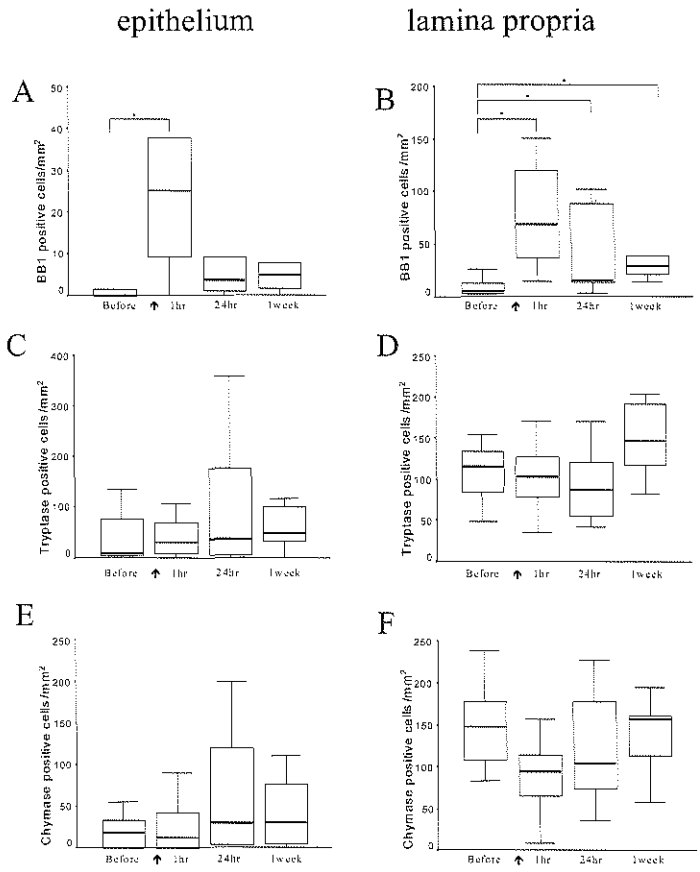

Figure 3. Numbers (in cells per square millimeter) of basophils (A and B), tryptase-positive cells (C and D) and chymase-positive cells (E and F) in the epithelium (A, C and E) and lamina propria (B, D and F) in nasal mucosa biopsy specimens. Nasal mucosa biopsy specimens were taken before provocation, 1 hour after provocation, 24 hours after provocation and 1 week after provocation. Allergen challenge (arrows) was performed with 1000 and 10,000 BU grass pollen. Box plots represent the 25th-75th percentile, the range (whiskers) and the median value.

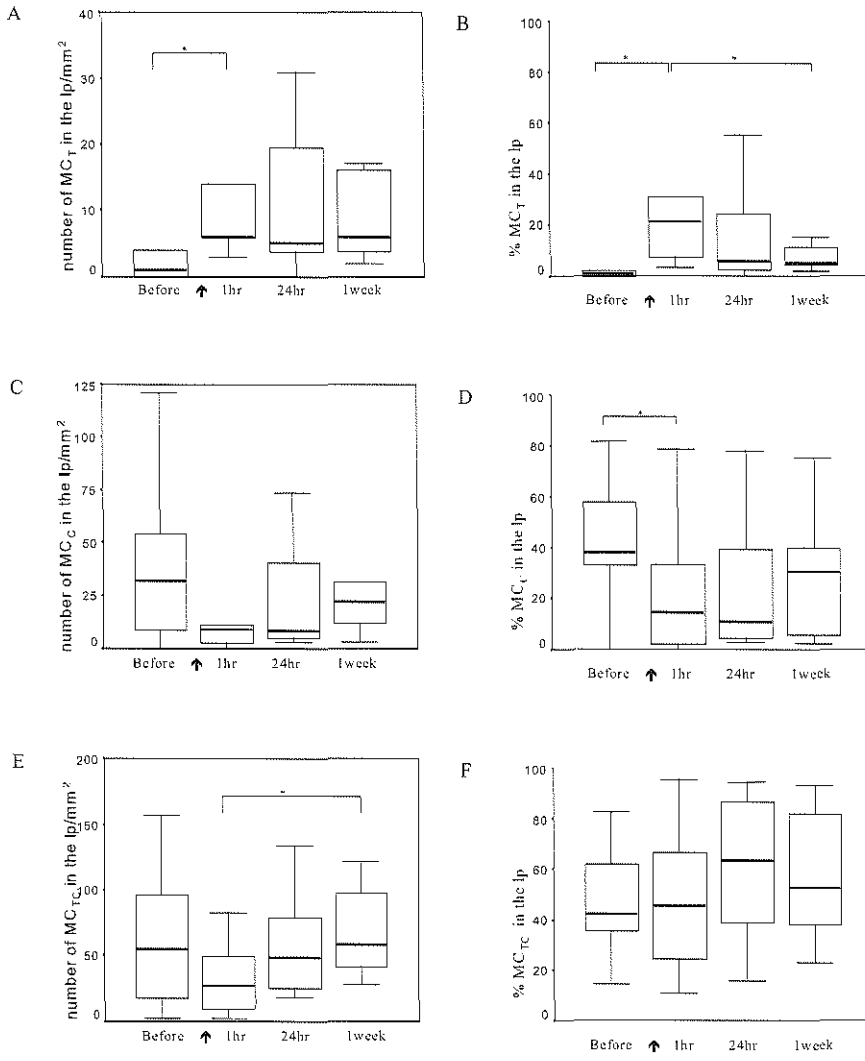

Figure 4. The numbers (A, C and E) and proportion (B, D and F) of tryptase-positive mast cell (MCT) phenotype (A and B), chymase-positive mast cell (MCC) phenotype (C and D) and tryptase-chymase double-positive mast cell (MCTC) phenotype (E and F).

Mast cell tryptase

No significant changes were found in epithelial tryptase-positive cell numbers (fig. 3C). However, in the lamina propria a trend in overall changes was observed

Friedman P = 0.06). We observed a trend of reduction in tryptase-positive cells that may indicate mast cell degranulation with regeneration of mast cell tryptase 1 week later. This was mainly caused by the higher level 1 week after provocation compared with 1 and 24 hours after provocation (Wilcoxon P = 0.03 and P = 0.07, respectively; fig. 3D).

Mast cell chymase

In the epithelium only a few chymase-positive cells were found at baseline (fig. 3E). No overall significant changes in the numbers of chymase-positive cells were observed (Friedman). In the lamina propria the numbers of chymase-positive cells tended to be lower at 1 hour (Wilcoxon P = 0.04) and to return to baseline numbers (Wilcoxon P = 0.03) at 1 week after the allergen provocation (fig. 3F).

Double staining with tryptase and chymase

Cell numbers

With a double-staining procedure, it was possible to differentiate 3 mast cell phenotypes mast cells staining for tryptase, mast cells double staining for tryptase and chymase and mast cells staining for chymase as shown in figure 6A (before allergen provocation) and figure 6B (after allergen provocation).

Numbers of mast cells staining for tryptase increased significantly (P = 0.001) from a median at baseline of 1 (range, 0-24 cells/mm^2) to 6 cells/mm^2 (range, 3-94 cells/mm^2) at 1 hour after allergen provocation. Numbers of mast cells staining for tryptase were still above baseline at 24 hours (median, 5 cells/mm^2; range, 0-58 cells/mm^2) and 1 week (median, 6 cells/mm^2; range, 2-54 cells/mm^2) after allergen provocation (fig. 4A). Numbers of mast cells staining for tryptase and chymase and mast cells staining for chymase in the lamina propria showed no overal significant changes after allergen provocation (fig. 4C and E).

Percentages

Expressed as a percentage of total mast cell numbers, the proportion of mast cells staining for tryptase phenotypes was significantly raised during the provocation period (Friedman P = 0.007). There was an increase in the proportion of mast cells staining for tryptase from 1% (range, 0%-86%) at baseline to 21% (range, 3%-85%) at 1 hour (P = 0.01) after allergen provocation and this was followed by a return to 6% (range, 0%-55%) at 24 hours and 5% (range, 2%-62%) 1 week after allergen provocation (fig. 4B).

The trend for the proportion of mast cells staining for chymase was to decrease from 38% (range, 0%-82%) to 14% (range, 0%-78%) 1 hour after allergen provocation

(Wilcoxon P = 0.04) and thereafter the proportion stayed relatively stable (fig. 4D). The percentage of mast cells staining for tryptase and chymase reflected changes in the other phenotypes: 42% (range, 14%-83%) at baseline, 45% (range, 11%-95%) after 1 hour, 70% (range, 16%-94%) after 24 hours and 53% (range, 23%-93%) 1 week after allergen provocation. However, these changes did not reach significance (fig. 4F).

Eosinophils

Eosinophil numbers in the epithelium tended to be raised after allergen challenge (P = 0.07; fig. 5A). Numbers of eosinophils in the lamina propria also increased after allergen provocation (P < 0.002). At baseline, the total numbers of eosinophils were small and they were mainly found in the deep layers of the lamina propria (fig. 5B). At 1 hour after allergen challenge, a striking increase in numbers was observed. Eosinophil numbers were still significantly higher than at baseline, 24 hours and 1 week after allergen provocation.

IgE-positive cells

No changes in the number of IgE-positive cells were observed in either the epithelium or lamina propria (fig. 5C and D).

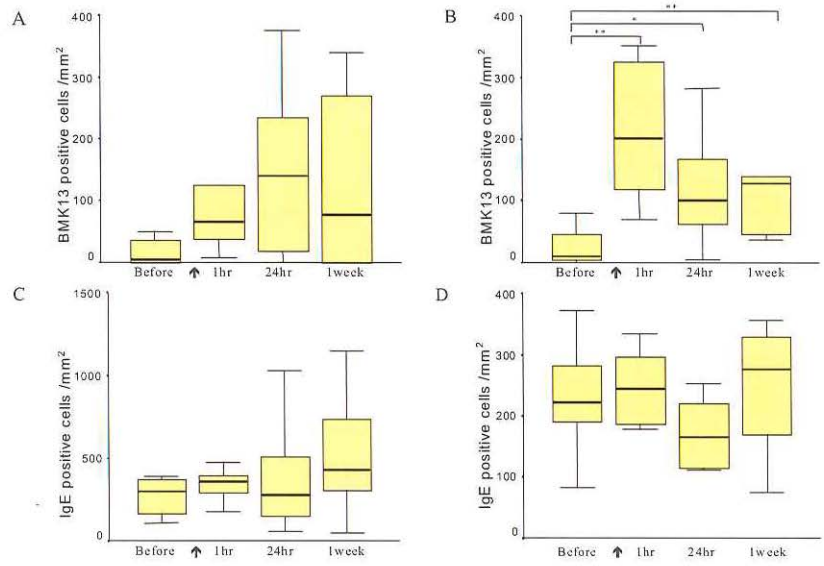

Figure 5. Numbers (in cells per square millimeter) of eosinophils (A and B) and IgE-positive cells (C and D) in the epithelium (A and C) and lamina propria (B and D) in nasal mucosa biopsy specimens. Nasal mucosa biopsy specimens were taken before provocation, 1 hour after provocation, 24 hours after provocation, and 1 week after

provocation. Allergen challenge (arrows) was performed with 1000 and 10,000 BU grass pollen. Box plots represent the 25th-75th percentile, the range (whiskers), and the median value.

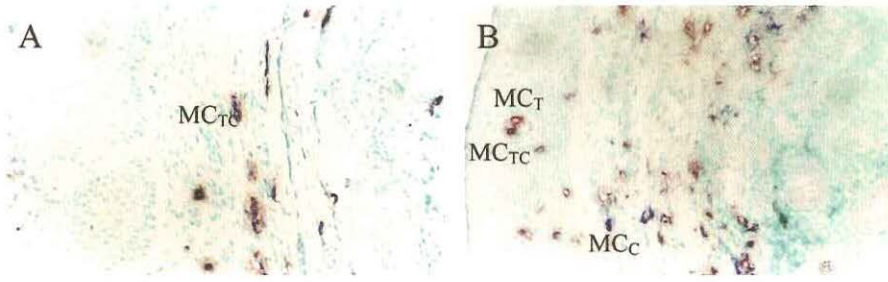

Figure 6. Mast cells. Representative sections of double staining showing tryptase (red), chymase (blue), and tryptase-chymase double-positive cells (red and blue) indicate 3 types of mast cells: tryptase-positive (MCT), tryptase-chymase positive (MCTC), and chymase-positive (MCC). A, Before provocation; B, after provocation (counterstaining with methyl-green; magnification ×1000).

Comparison of immunologically relevant cells

The numbers of all patient mast cells, eosinophils and basophils (defined as the immunologically relevant effector cells in allergy) underwent proportional changes in the lamina propria after allergen provocation. At baseline, the level of mast cells was at a median of 88% (range, 56%-97%), with the percentages of eosinophils and basophils being low at 7% (range, 0%-37%) and 3% (range, 1%-13%), respectively. After allergen provocation in the early phase, levels of mast cells diminish sharply to a median percentage of 27% (range, 12%-42%). The eosinophils and basophils increased sharply to 51% (range, 23%-80%) and 23% (range, 6%-48%). In the late phase (24 hours after provocation) all cells are present, but numbers are lower than in the early phase: mast cells, 37% (range, 1%-97%); eosinophils, 49% (range, 1%-80%); and basophils 7% (range, 0%-25%). One week after provocation, all immunologically relevant cells were still present. Levels of mast cells were highest at 46% (range, 22%-73%), followed by the eosinophils at 38% (range, 10%-75%) and then the basophils at 9% (range, 3%-45%). Figure 7 shows the median numbers of mast cells, eosinophils and basophils in the lamina propria at each time point.

Tryptase-positive basophils

Tryptase double staining was performed on biopsy specimens taken 1 hour and 1 week after provocation. Double staining showed tryptase (red) and BB1 (blue) double-positive cells (fig. 8).

The tryptase-positive BB1 cell numbers were 6.3 cells/mm^2 (range, 0-15.2 cells/mm^2) at 1 hour and 8.1 cells/mm^2 (range, 1.8-18.2 cells/mm^2) 1 week after provocation. There was only one biopsy specimen that was evaluated, taken 1 hour after allergen provocation, in which no tryptase-positive BB1 cells could be observed.

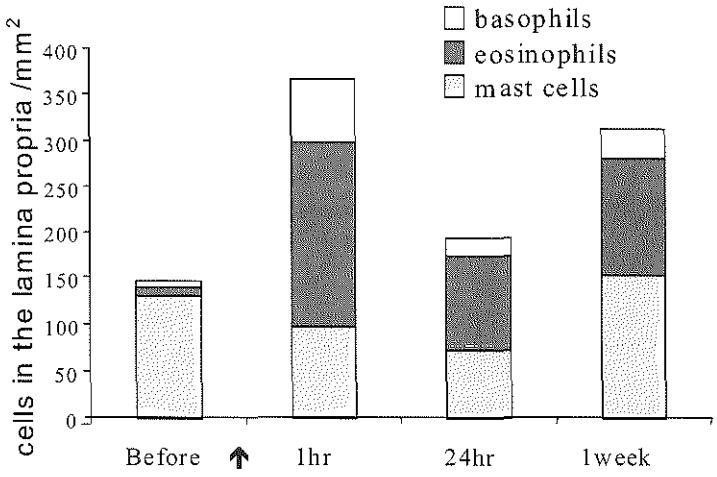

Figure 7. The inflammation of mast cells, eosinophils and basophils related to the total inflammation of these cells in the lamina propria over time.

Discussion

This study shows that allergen provocation with grass pollen results not only in an increase in eosinophils (13, 14) but also a significant increase in BB1-positive cells (basophils) in the epithelium and lamina propria of the nasal mucosa. This study has no placebo control group, but in a previous study we described the results of a placebo-controlled study in which repeated placebo spray was without observable effect in the nasal mucosa (15).

We provide evidence that there is marked basophil infiltration in the airways as early as 1 hour after allergen challenge and a prolonged increase in numbers for up to 1 week. However, the lack of association between eosinophils and basophils after allergen provocation suggests that these cell types are attracted by different factors. The dynamics of basophil infiltration after allergen provocation are quite different from those of mast cells. Although the influx of metachromatically staining cells (taken as mast cells) into the epithelium has been described by several authors (16-18), the total numbers of mast cells in the nasal mucosa are relatively constant compared with, for example, changes in eosinophil numbers (2, 13, 14, 19).

Comparing the influx of eosinophils and basophils, we noted that 1 hour after allergen provocation the influx of eosinophils in the lamina propria (20 times baseline values) is twice as large as that of basophils (10 times baseline values) and that mast cells decrease (half) as a percentage of total inflammation. In the epithelium the influx of eosinophils and basophils is comparable (15 times baseline values) and also comparable with numbers found in other studies, which show that eosinophils appeared earlier in the nasal epithelium than in the lumen after allergen provocation (13). Extravasation of eosinophils has also been shown to occur within half an hour after allergen provocation (3, 13). However, the lack of association between eosinophils and basophils after allergen provocation suggests that these cell types are attracted by different factors.

In earlier studies it has been suggested that basophils contribute mainly to the late-phase response. This had been concluded from the pattern of mediators released in the late phase (ie, histamine, leukotrienes and others, but not PGD2 (9, 10) and studies of nasal brushings and lavage fluids showing only an increase of basophils in the nasal lumen in the late phase (3, 4, 20). However, this study clearly shows a significant increase of basophils in all layers of the nasal mucosa, even in the early phase. The pattern of mediators released cannot only be explained by differences between basophils and mast cell activation but also by a different mechanism of mast cell mediator release in the early (caused by allergen/IgE cross-linking) and the late phase. This explanation is supported by *in vitro* experiments showing that neuropeptide-induced mediator release results in high levels of histamine but relatively low levels of PGD2 from mast cells, suggesting a neurogenic induction of late-phase histamine release from mast cells (21-23). In another study carried out in collaboration with Braunstahl et al (24), we observed that there was practically no eotaxin protein present in the nasal mucosa before allergen provocation.

Moreover, in that study we compared eotaxin-stained cell numbers in healthy control subjects with those in patients with seasonal allergic rhinitis and observed no differences in cell numbers before allergen provocation. Our data does not support the idea that eotaxin release is a key mechanism for early infiltration of eosinophils and basophils. It is possible that tryptase and chymase could be stimuli for the infiltration of inflammatory cells because this has been observed in animal models (25, 26). The trend toward a reduction in chymase-positive cell numbers at 1 hour after allergen provocation in the nasal mucosa suggests that degranulation may have occurred. However, in contrast to the reduction in chymase-positive cells, no fall in numbers of tryptase-positive cells could be observed. The correlation between epithelial basophils and tryptase-positive cells at 1 hour after allergen provocation ($r_s = 0.919$, $P = 0.003$) raises the possibility that these cells may, at least in part, represent the same cell.

Further double staining with CD18, tryptase and BB1 indeed showed that some of the BB1-positive cells were positive for tryptase, indicating tryptase-positive basophils (fig. 8). This concurs with the recent observation of Li et al (27), who identified basophils expressing the mast cell granule proteases chymase and tryptase in the peripheral blood of patients with asthma and allergies and drug-reactive patients. A possible interpretation could be that extravasated basophils are immature mast cell–committed progenitors that have left the blood circulation. The influx of tryptase-positive basophils could help to explain why numbers of tryptase-positive cells were not reduced, whereas chymase-positive cells were reduced. Alternatively, mast cells may be able to degranulate selectively, as has been shown by Goldstein et al (28).

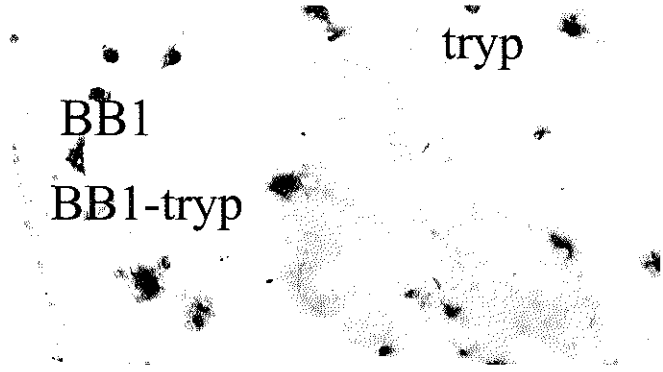

Figure 8. Tryptase-positive basophils. Double staining showed tryptase (red) and BB1 (blue) double-positive cells (counterstained methyl-green; magnification ×1000). Double staining with CD18, tryptase, and BB1 indicates that BB1-positive cells are positive for tryptase.

This study has again confirmed that the total numbers of IgE-positive cells do not increase in the nasal mucosa after allergen provocation (19). In a previous study we describe toluidine blue–positive/IgE-positive cells. In the epithelium most IgE-positive cells are not positive for toluidine blue. After allergen provocation, 29% of the IgE-positive cells were positive for CD1a. No IgE-positive plasma cells or B cells were observed in the epithelium (19). Unpublished data with C-Kit-positive cell numbers indicate that this marker detects 10 times more cells than tryptase or chymase antibodies and equal numbers of IgE-positive cells at baseline level out of season in the epithelium. This suggests that the C-KIT population probably detects totally degranulated epithelial mast cells bearing IgE on the surface.

In the lamina propria it is clear that most IgE-positive cells are granulated mast cells. In earlier studies we have shown that the population of IgE-positive cells in the nasal mucosa of patients with rhinitis who are allergic to grass pollen consists of mast cells

(confirmed by total of chymase-tryptase staining: median, 80%; range, 20%-95%; Langerhans cells: median, 10%; range, 1%-30%; B cells: median, 4%; range, 0%-14%; and plasma cells: median, 2%; range, 0%-29%), some of which may be fully degranulated mast cells containing no chymase or tryptase (29).

In conclusion, basophils, as identified by specific antibody BB1, increase in numbers in the epithelium and lamina propria of the nasal mucosa as early as 1 hour after provocation. This early influx is also observed in eosinophils.

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3.3 Mast cells and basophils seem to be two phenotypes of the same cell, maturated in different microenvironments; a nasal biopsy study

3.3 Mast cells and basophils seem to be two phenotypes of the same cell, maturated in different microenvironments; a nasal biopsy study

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Abstract

Background: Mast cells and basophils play an important role in the initiation and control of allergic inflammation in upper and lower respiratory allergy. The developmental relationship between mast cells and basophils has not yet been totally resolved. The recent development of specific monoclonal antibodies for basophils and mast cell surface markers that are independent of degranulation makes possible the further analysis of this potential lineage.

Design: In this study, basophil and mast cell dynamics and the developmental relationship were examined using nasal allergen provocation combined with repeated nasal biopsies and blood samples.

Results: In the epithelium, most mast cells (C-Kit-positive IgE-positive) are negative for tryptase and chymase and no basophils were found in the nasal mucosa at baseline. In the lamina propria, most mast cells are positive for tryptase and chymase. After allergen provocation, basophils are the only cell type of the basophil-mast cell lineage cells that flow into the nasal mucosa tissue. No increase of mast cells in the lamina propria, nor redistribution to the epithelium could be found in the (C-Kit-positive) mast cell population. A small percentages of blood basophils and nasal mucosa tissue basophils were positive for specific mast cell proteins like tryptase, chymase and C-Kit.

Conclusion: The present observations support the idea that mast cells and basophils are two fenotypes of the same cell in different micro-environments. Specific monoclonal antibodies to basophils and mast cells show that the formerly described epithelial redistribution of mast cells should be reinterpreted as an influx of basophils.

Introduction

Mast cells and basophils play an important role in the initiation and control of allergic inflammation in upper and lower respiratory allergy. Mast cells and basophils have certain similarities both in structure and function (1-4). Both basophils and mast cells are thought to arise from CD34+ progenitors in the bone marrow and peripheral blood. Basophils develop under the influence of IL-3 and SCF facilitates mast cell differentiation. SCF receptor or C-Kit (CD117) is a cell surface marker for mast cells that can also be present on hematopoetic progenitor (4).

Mast cells are found in the mucosa and play a key role in the early phase by releasing histamine. Unlike mast cells, mature basophils are normally found in the circulatory system. In the last decades appearance of tryptase positive metachromatic cells in the epithelium after allergen provocation has been interpretated as redistribution of mast cells to the epithelium (5-7).In recent years, protease content has been used to distinguish between mast cells in the airway mucosa. The three categories are:

tryptase-positive mast cells (MC_T), tryptase/chymase-positive mast cells (MC_{TC}) and chymase-positive mast cells (MC_C). Tryptase and chymase are functionally different proteases. Tryptase is mainly active in cleaving and inactivating fibrinogen, degenerating neuropeptides and stimulating epithelial cell proliferation as well as IL-8 production (8). Chymase degrades IL-4 and cleaves inactive SCF from the matrix in bioactive soluble SCF form. It also enhances the microvascular permeability and promotes the accumulation of inflammatory cells (9-11). *In vitro* mast cells have been found to be initially positive for tryptase and, during further maturation, also express FcεRI and chymase (12, 13).

Specific basophil marker BB1 (14, 15) and blood basophil marker CD203c (16, 17) have been developed recently. They make it possible to study the role of basophils in allergic inflammation directly. Moreover an influx of basophils into the nasal mucosa and especially the epithelium, has been observed after allergen provocation. These specific basophil markers have led to recent studies which show, contrary to what was believed before, that part of the basophils in the circulation of atopic patients are found to express mRNA and be positive for mast cell specific proteases (18). Moreover, basophils positive for tryptase are observed in the nasal mucosa of allergic rhinitis patients (3, 19). Also recent data have shown that mast cells in the lung can be positive for CD203c (20). This is a further indication that mast cells and basophils may be part of the same cell lineage (21).

Nasal allergen provocation combined with repeated nasal biopsies and blood samples is an effective way of studying basophil and mast-cell migration and differentiation (3, 22). One of the intriguing phenomena which may explain maturation and differentiation within the mucosa are the differences in local microenvironment between different mucosa (e.g. nose and lung) but also between different layers within the mucosa (23-27). The local microenvironment changes markedly after allergen provocation. Histamine, for example, has a number of immunomodulatory effects such as changes in the IL-10/IL-12 production in favour of IL-10 by DCs and upregulation of IL-8 mRNA (IL-8 is a inducer of chemotactic mast cell response (28, 29)).

The present allergen provocation study was performed to analyse the relationship of basophils and mast cell phenotypes in different local microenvironment.

Material and Methods

Nasal biopsies

Nasal biopsies were taken from patients in two studies. In the first study, seventeen isolated grass-pollen-allergic rhinitis patients were challenged daily with a low-threshold-dose allergen during a 2 week period. This has been described in detail

elsewhere (30). Biopsies were taken at baseline (n = 7), ½ hr (n = 5), 8 hrs (n = 5), 24-48 hrs (n = 5), 4-6 days (n = 9), 8-10 days (n = 6), 14 days (n = 9) and 1-2 weeks (n = 9) after the last provocation. To study early phase phenomena, nine patients were challenged with a very-high-dose allergen once to induce the influx of basophils described elsewhere (3).

Blood

Blood was collected from seasonal allergic rhinitis patients (n = 10). PBMCs were isolated using by polymorph prep in accordance with the manufacturer's instructions (Axis-Shield, Norway). The PBMCs were washed twice. Pellets were made and then frozen in tissue tek. Four µm-thick sections were stained immunohistochemically.

Immunostaining and antibodies

Immunohistochemical stainings were performed as previously described by our group (3, 22). The following mouse-anti–human mAbs were used: Anti-chymase (clone B7 1 µg/ml) Anti-chymase^{biotinylated} (clone B7 1 µg/ml); Anti-tryptase (clone G3) 0.7 µg/ml; Anti-basophils (clone BB1) 6 µg/ml Anti-basophils^{biotinylated} (clone-BB1) 1:50 (kindly donated by Andrew F Walls); Anti-CD203c (clone 97A6) (kindly donated by Hans-Jorg Buhringer); Anti-CD203cPE (clone 97A6) 1 µg/ml Beckman Coulter (the Netherlands); for immature cells anti-CD34 III (clone 581) 2 µg/ml Instruchemie (the Netherlands); anti-C-Kit (clone YB5.B8) 1 µg/ml Pharmingen (the Netherlands) anti-IgE (clone HM25M) 1 µg/ml CLB.

As secondary Abs: Goat-anti-mouse^{biotinylated} 1:50 Biogenix (Klini Path, the Netherlands); Rabbit-anti-PE 1:50 Biogenisis (Nuclilab, the Netherlands); Swine-anti-Rabbit peroxidase 1:50 Sigma (the Netherlands); Goat-anti-Rabbit alkaline phosphatase 1:50 Dako (the Netherlands); Streptavidine alkaline phosphatase/peroxidase 1:50 Biogenix (Klini Path, the Netherlands); Goat-anti-biotin alkaline phosphatase 1:50 Sigma (the Netherlands); Rabbit-anti-mouse peroxidase 1:200 Sigma (the Netherlands); Mouse peroxidase anti peroxidase 1:200 Sigma (the Netherlands). Rabbit-anti-PE and Goat-anti-Biotin antibodies are used against PE and biotin-labeled second primary antibodies. PE and biotin, in this application, function as a haptene for specific visualisation of the second primary antibody in double staining.

Quantification

Stained cells were counted in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (i.e. the epithelium and lamina propria) were estimated by using the

Leica Image Analysis System. The numbers of cells per square millimeter was calculated for the epithelium and the lamina propria.
The amount of tryptase per cell indicates the condition of the mast cells: a degranulated mast cell or a young mast cell has a low tryptase content. Mature mast cells have a high tryptase content. Biopsies were ranked. If the majority of cells in a section only had tryptase signal directly around the nucleus they were ranked as 0. If they had a large amount of tryptase around the cells, they were ranked as 3. Similar analyses were performed for chymase.

Statistical Analysis (SAS 6.12 for Windows and SPSS 9.0 for Windows)
Low-dose allergen provocation study: Three repeated measurements were made for all stainings in each of the groups before and during the provocation, a total of six points on the time axis. After logarithmic transformation of the measurements, a repeated measurement analysis of variance was performed. In this analysis, the six time effects on the dependent variable were obtained as the estimate 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).
In both allergen provocation studies, the differences between period points were analysed with the sign test (related samples) or with the Mann Whitney U test (unrelated samples). A p-value < 0.05 was considered to indicate a significant difference between time points. To reduce the number of data points in the figures, the sample points for 8 hrs and 24/48 hours and for 8-10 days and 14 days were combined.

Results
Mast cells have a different phenotype in epithelium and lamina propria at baseline.
First of all, we used a set of mast cell markers in serial sections to analyse the phenotype of the mast cells at baseline in relation to their microenvironment. In the epithelium, most mast cells have the C-Kit+ IgE+ tryptase- chymase- CD203c- phenotype (80 median; 0-100% range). This indicates that these cells are epithelial mast cells (fig. 1). The cells which were positive for tryptase, chymase and CD203c expressed low amounts of protein, as shown by the weak staining of these cells in the epithelium. The very small numbers of cells which are positive for chymase are always positive for tryptase (12% median; 0-68% range). No BB1-positive cells were found at baseline in the nasal mucosa. In the lamina propria, most C-Kit+ cells were also positive for tryptase, chymase and CD203c.

Epithelial mast cells mostly have the C-Kit+ IgE+ tryptase- chymase- CD203c- phenotype in the lamina propria, a C-Kit+ IgE+ tryptase+ chymase+ CD203c+ phenotype is found.

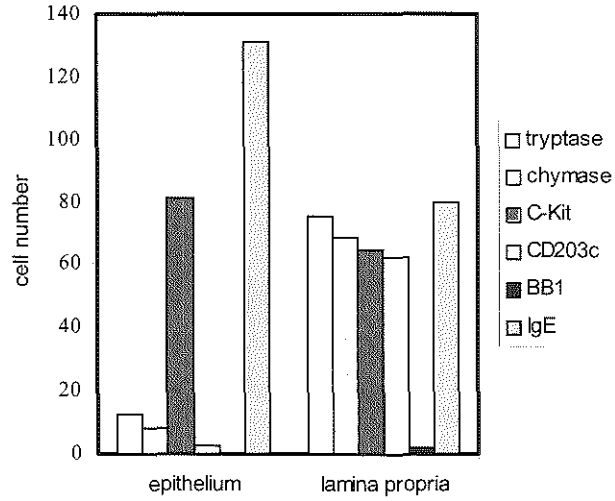

Figure 1. In the epithelium, most mast cells (C-Kit-positive IgE-positive) are negative for tryptase and chymase. In the lamina propria, most mast cells are positive for tryptase and chymase. No basophils are found in the nasal mucosa at baseline (n = 7).

Mast cells change phenotype in the lamina propria during allergen provocation

With a double-staining procedure, it is possible to differentiate between three cell phenotypes based on the mast-cell serine proteases, tryptase and chymase: mast cells staining for tryptase (MC_T), mast cells staining for both tryptase and chymase (MC_{TC}) and mast cells staining for chymase (MC_C) (31). The dynamics of mast cell protease-positive cells (fig. 2) are similar in the epithelium and lamina propria (data not shown). During the first hour, mast cell protease-positive cell numbers fall and are followed by a return to baseline levels after 8 hours - 2 days, suggesting degranulation in the lamina propria as described elsewhere (3, 22, 30). Repeated low-dose allergen challenge results in a significant reduction ($p = 0.03$) in the total number of protease-positive cells (MC_T, MC_{TC} and MC_C) in the lamina propria. This reduction persists until at least 1-2 weeks after the last provocation (end of study). When we look at the different subgroups, the numbers of MC_C and MC_T increase, with only the increase in the MC_C attaining significance ($p = 0.001$), whereas the number of MC_{TC} tended to decrease during the provocation period.

We have to consider that part of the tryptase-positive cell population is replenished by immature tryptase-positive cells that enter the mucosa. To analyse in greater detail whether the tryptase-positive cells were degranulated mast cells or had to be considered as basophils or immature mast cells entering the mucosa, we also determined the amount of proteases per cell per biopsy moment.

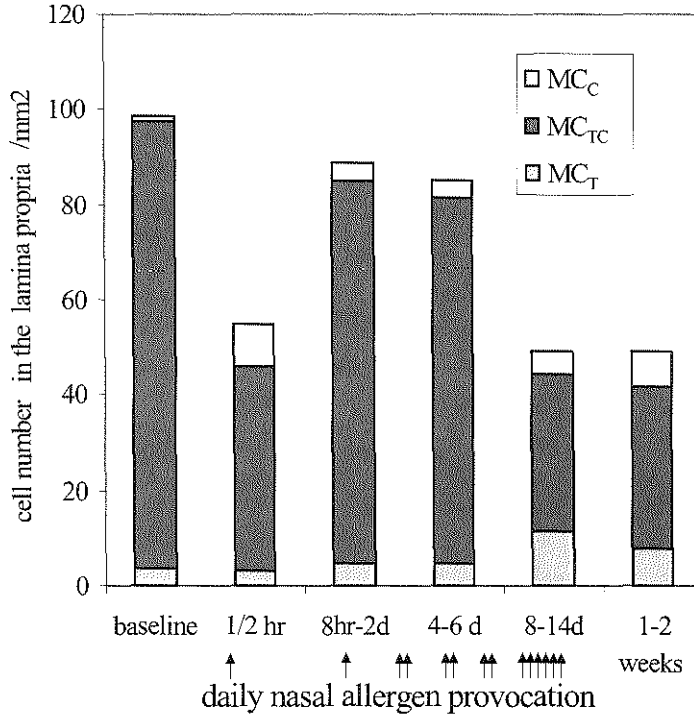

Figure 2. Mast cell serine proteases (cyto-plasmatic markers)
Mast cell median numbers from three phenotypes MC_T or MC_{TC} or MC_C tended to fall directly after allergen provocation and the total number of protease-positive cells (MC_T, MC_{TC} and MC_C) reached a minimum in the lamina propria at the end of the provocation period 8-14 days after the first provocation ($p = 0.03$). Elevated numbers of MC_C phenotype and a reduction in numbers of the MC_{TC} phenotype ($p = 0.007$) were observed when comparing baseline numbers with numbers 1-2 weeks after the last provocation (end of the study). This indicates that the change in mast cell phenotype continued for at least 1-2 weeks without provocation.

The amount of tryptase in and around cells decreases in the lamina propria during allergen provocation

Quantification of the granulation state (degranulated or granulated) of the mast cells was performed for tryptase (fig. 3) and chymase in single stainings in the lamina propria. A reduction in tryptase content per cell may be a sign of degranulation, but

minimal amounts of tryptase can also be associated with incoming basophils or immature mast cells from the circulation (3, 13, 18, 19, 32). At baseline, the tryptase content per tryptase-positive cell was high in the majority of the sections. Significantly lower amounts of tryptase were observed after 14 days of daily allergen provocations ($p = 0.0001$). There was no immunohistochemically-detectable change over time in the amount of chymase per cell (data not shown). This analysis suggests an influx of immature mast cells or basophils during repeated allergen provocation. To establish in greater detail the possible role of basophils, blood and tissue basophils were studied in more detail.

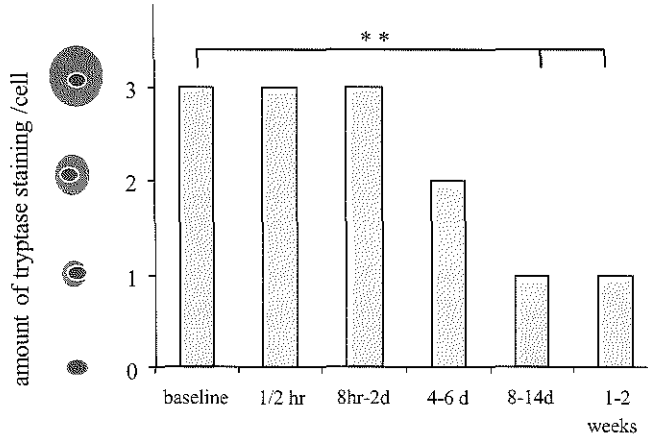

Figure 3. Amounts of serine protease in and around cells present in the lamina propria. At baseline, the levels of tryptase content per tryptase-positive cell were high in the majority of the sections. From day 4-6 onwards, a significant reduction in tryptase content could be observed ($p = 0.005$). One to two weeks after the last provocation, the tryptase content was still significantly lower than at baseline. The amount of chymase content showed no significant change over time.

Basophil influx is a reaction to allergen provocation

Basophils are the only cell type of the basophil-mast cell lineage cells that flow into the nasal mucosa tissue after allergen provocation (fig. 4). No significant alteration could be observed in the number of C-Kit-positive cells.

Presence of precursor cells of the basophil-mast cell lineage in the nasal mucosa of allergic rhinitis before, during and after allergen provocation

Cells characterised as CD34-positive and CD203c-positive can be considered to be immature cells from the mast cell/basophil lineage (16, 17). The localisation of CD34-

positive and CD203c-positive cells was confined to the lamina propria, indicating that mast cells maturate there and not in the epithelium.

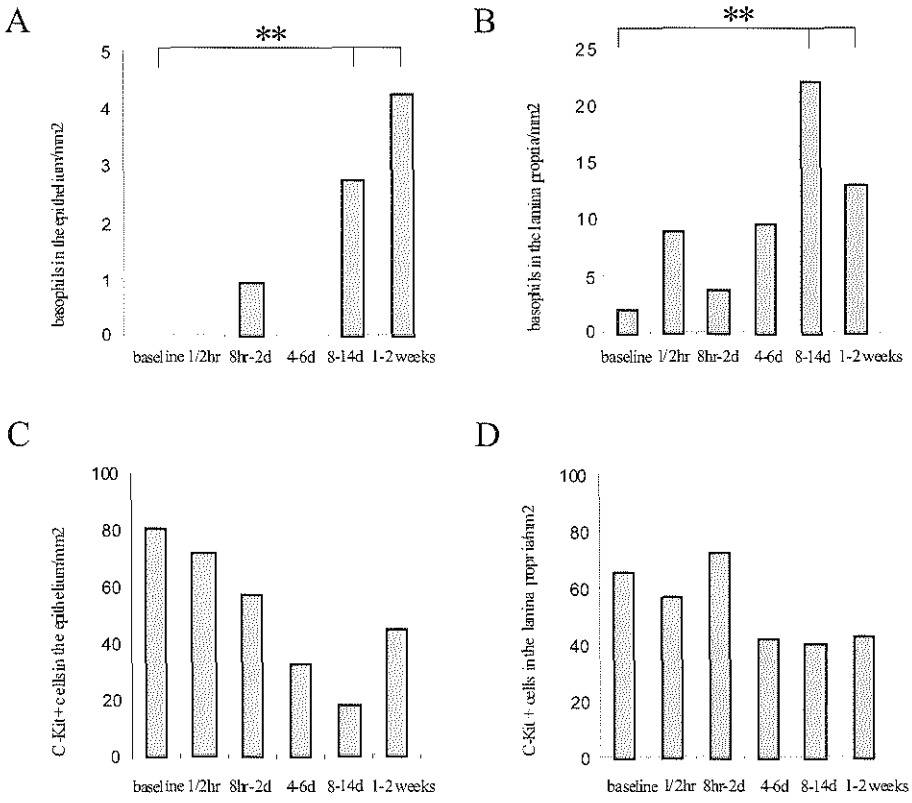

Figure 4 Basophils and mast cell (surface markers)
A) Basophil numbers (median) increased significantly (p = 0.008) and reached a maximum 8-14 days after the first provocation in the epithelium. B) Gradually, more basophils appeared subepithelially during repeated provocations in the lamina propria. The total number of basophils was increased significantly (p = 0.0008), reaching a maximum increase after 8-14 days of daily provocation. Significantly (p = 0.001) higher basophil numbers relative to baseline were observed at 1-2 weeks after the last provocation. C) C-Kit-positive cell numbers tended to be lower (p = 0.1) in the epithelium. D) C-Kit-positive cells did not change significantly in numbers during allergen provocation in the lamina propria. **<0.01

Blood basophils and nasal mucosa basophils contain mast cell proteins

Basophils are cells in the circulation and previous observations indicated an influx of basophils in both the epithelium and in the lamina propria of the nasal mucosa of allergic rhinitis patients within 1 hour after high-dose allergen provocation (3). Immunohistochemical characterisation and flow-cytometric analysis of blood

basophils of allergic rhinitis patients indicate that basophils are positive for BB-1, CD203c and IgE. Blood basophils can contain tryptase, chymase and can be positive for C-Kit (fig. 5A). Tissue basophils (BB1) identified in the early phase after allergen provocation as BB1-positive cells are positive for tryptase and even chymase and C-Kit (fig. 5B).

One hour after allergen provocation the level of basophils positive for tryptase is 27% median (range, 0-58%) and at one week after allergen provocation 41% (8-81%). Basophils positive for chymase increased in number from 3 cells/mm^2 median (range, 0-28 cells/mm^2) to 15 (1-31) cells/mm^2) and percentage from 4% (0-30%) to 18% (5-60%) and basophils positive for C-kit increased in percentage only from 9% (0-19) to 19% (4-58) between one hour and one week after allergen provocation. Basophils that can be characterised as mast cells are present in the nasal mucosa one week after allergen provocation. These data show that basophils can became C-Kit and chymase positive and they maturate into mast cells.

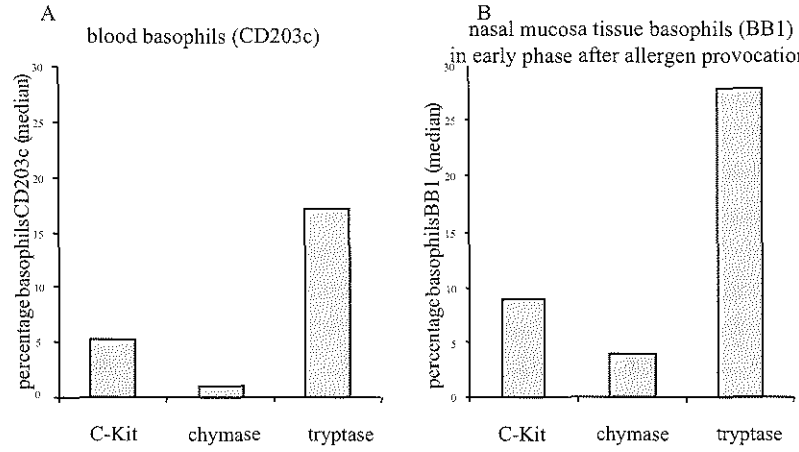

Figure 5. Blood basophils and tissue basophils are positive for specific mast cell proteins

Discussion

The present allergen provocation study was performed to analyse the relationship of basophils and mast cell phenotypes in different local microenvironment.

There is still controversy about the precise role of basophils in allergic inflammation. Studies in the eighties using morphological techniques described increased numbers of basophils during allergen provocation in the epithelium (33-35). Moreover increased basophils have been described in the circulation after allergen provocation

of nose and lungs (22). Nowadays, with the use of specific monoclonal antibodies, it is possible to differentiate between mast cells and basophils much more precisely. Based on protease stainings these early observations were rejected. These newest techniques, using specific monoclonal antibodies against basophils (14, 15) confirm the influx of basophils into the lamina propria and the epithelium and moving into the lumen (1). An explanation of the apparent toluidine blue/tryptase-positive cell movement towards the epithelium could be a combination of mast-cell degranulation in the lamina propria, resulting in a reduction of toluidine blue/tryptase-positive cells in the lamina propria and an epithelial basophil influx, resulting in a increase in toluidine blue/tryptase positive cell numbers.

Interesting is the observation that basophils can be positive for mast cell proteases and C-Kit suggesting a closer relation between mast cells and basophils (18, 19). These cells are probably from the same lineage as is suggested by Arock et al. who found that the immature mast cell line HMC-1 is positive for the specific basophil marker Bsp-1 (21). This is also in line with the unpublished observation from our group that the HMC-1 cell line is also positive for BB-1. An other interesting point is the absence of basophils at baseline and in stable disease as shown by Braunstahl (36). Apparently basophils are the mobile part of the basophil/mast cell lineage. From these combined data we can conclude that basophils play a role in allergic inflammation and that basophils can rapidly move into allergic inflamed tissue from the circulation.

In contrast to basophils, mast cells do not seem to be so mobile as was previous suggested (5). However mast cells can change from phenotype by degranulation and specific induction of the expression of serine proteases. This phenomenon has only been observed in the lamina propria. The lamina propria appears to be the main site of mast cell maturation, as is also indicated by the limitation of the presence of the CD34-positive and CD203c-positive cells to the lamina propria (37).

This change in mast cell phenotype in favour of MC_C has also been observed previously in murine intestinal mucosa. This was induced by helminthes infection, which is also a Th2-inducing microenvironment (24). Moreover, IL-10 is thought to be a critical cytokine in the induction and constitutive expression of murine chymase mRNA and protein (38, 39).

It is intriguing to see at baseline level, that only 10-20% of the C-Kit positive mast cell in the epithelium are positive for serine proteases. Contrary to the epithelium in the lamina propria were almost all C-Kit positive cells positive for serine proteases and CD203. After allergen provocation, this phenotype difference between the epithelium and the lamina propria disappears. Differences in microenvironment of the epithelium and lamina propria and differences occurring after allergen provocation could explain these changes in phenotype. For example in the lamina propria

significant higher levels of cytokines (Th1- and Th2-cytokines and IL-8 and RANTES) are observed compared to the epithelium (26). Also, allergen provocation induces stem cell factor (SCF) in general and especially in the epithelium, which results in SCF receptor (C-Kit) downregulation (40) and thus a changing mast cell phenotype after allergen provocation. An interesting observation is the remaining high number of C-Kit-positive IgE-positive cells, situated apically in the epithelium even five months after the season. This may be another sign of minimal persistent inflammation (41) and explain the fast response after renewed allergen exposure. The data described above point to a stable, sessile role for mast cells in the epithelium and lamina propria with an important role as gate keeper in the nasal mucosa which can react fast on incoming allergen but has a limited mobility.

The data described in this study support the idea that mast cells and basophils are two phenotypes of the same cell in different micro-environments. Moreover it points to basophils as the mobile and mast cells as the sessile component of the lineage.

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4. Cytokines in allergic rhinitis patients

4.1 Increase in IL-8, IL-10, IL-13 and RANTES mRNA levels (in situ hybridization) in the nasal mucosa after nasal allergen provocation.

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Abstract

Background: Allergic inflammation is regulated by the local production and release of several cytokines.

Objectives: This study was designed to assess the changes in mRNA cytokine-positive cells after allergen provocation and to compare these cytokines with tissue eosinophilia as a marker of allergic inflammation.

Methods: A grass pollen allergen provocation study was conducted in autumn, out of the hay fever season. Nasal mucosa biopsy specimens were taken before provocation and 1 hour, 24 hours and 1 week after allergen provocation. Eosinophils and mRNA-positive cells (in situ hybridization for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN-γ, RANTES and TNF-α) were assessed in the biopsy specimens.

Results: After allergen provocation, an increase in cell number was found for eosinophils and cells expressing mRNA for the chemokines IL-8 and RANTES and for the Th2 cytokines IL-10 and IL-13. Significant correlations were found between eosinophils and RANTES and eosinophils and IFN-γ in the early phase and between eosinophils and IL-5 and eosinophils and RANTES in the late phase. The increase in eosinophils and IL-10 and IL-13 mRNA–positive cells could still be observed 1 week after allergen provocation.

Conclusions: Nasal allergen provocation induced significant tissue eosinophilia and a significant increase in IL-8, IL-13 and RANTES mRNA-positive cells. A significant increase in eosinophils and IL-10 and IL-13 mRNA-positive cells compared with baseline can still be observed 1 week after a single allergen provocation.

Introduction

The pathophysiology of allergic rhinitis is based on the presence of inflammatory cells in the nasal mucosa (1). Allergen-induced rhinitis is accompanied by a cellular infiltrate in which eosinophils, mast cells, T cells and Langerhans cells are prominent (2-8). Allergic mucosa inflammation is regulated by the local production and release of several cytokines (9-13). A major role was originally assigned to the cytokines produced by Th2 lymphocytes, such as IL-3, IL-4, IL-5 and IL-13. (9, 12, 14-17). In addition to the well-known Th2 cytokines, chemokines (IL-8 and RANTES) (11, 18-20) and perhaps TNF-α are also thought to play a role in allergic inflammation (13, 21, 22). Accumulating data now points to a large network of interacting cytokines produced by a number of cells that regulate mucosa allergic inflammation. However, the existing data about cytokines is partially contradictory and sometimes confusing. This is the result of several factors, including different study designs, the limited numbers of cytokines evaluated and observations on various levels, such as the protein and mRNA levels.

Allergen challenge studies have found increases in protein and mRNA for IL-4 and IL-5 and an increase in IL-6 protein (9, 16, 23). However, in natural disease no differences were found between season and preseason IL-4, IL-5 and IL-6 protein levels (12). Rises in numbers of TNF-α mRNA-positive cells were observed after filterdisk provocation. (21). However, in a study comparing nasal polyps of allergic and non-allergic patients, more TNF-α–positive cells were found in the non-allergic group (22).

To our knowledge, no *in vivo* data is available about the dynamics of a number of cells expressing IL-6, IL-8 and IL-10 mRNA in the upper airway mucosa after allergen provocation. For other cytokines, details on time-related changes after allergen provocation are incomplete.

In this out-of-season biopsy study, we investigated, on several occasions after the provocation, the effect of a single provocation with grass pollen in patients with isolated grass pollen allergy. Eosinophils and a wide range of Th1 and Th2 cytokine– and chemokine–expressing cells (in situ hybridization for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN-γ, RANTES and TNF-α) were determined in mucosa biopsy specimens before and at various times after allergen provocation. Correlations between eosinophils (as a marker of allergic inflammation) and cytokines were evaluated.

Methods

Patients

Nine patients (5 women and 4 men; median age, 22 years; range, 19 to 51 years) participated in this study. All patients had a history of seasonal allergic rhinitis for grass pollen of at least 2 years duration. Allergy to grass pollen was confirmed by a positive skin prick test response (3+) with Alutard Soluprick extract (1 SQ/mL), with no skin prick reactions to other relevant allergens. All patients were asymptomatic at the start of the study. None of the patients were using any medication during the study or had undergone immunotherapy in the 3 years before the study. No relevant abnormalities were found on ear, nose and throat examination. All patients gave their written informed consent and the study was approved by the medical ethics committee.

Study design

This allergen provocation study was conducted between October and December 1995, well out of the hay fever season. Biopsy specimens were taken from the inferior turbinate by using a Gerritsma forceps (24). Each patient underwent 4 nasal biopsies: the first at baseline, the second 1 hour after provocation with grass pollen, the third 24

hours after provocation and the fourth 1 week after provocation. Biopsy specimens were coded and therefore the analyses were done in a blinded fashion.

Provocation

The patients were acclimatized to the room for at least 15 minutes. After that, PBS, an inert water-rich solution, was administered to rule out nonspecific hyperreactivity. After 10 minutes, patients were provoked with 50 µL of an aqueous nasal spray containing grass pollen (10,000 biologic units (BU)/mL; ALK, Groningen, the Netherlands) and 10 minutes later with another 100,000 BU/mL. After both provocations, symptoms such as sneezing, rhinorrhea, itching and nasal blockage were recorded on a 4-point (range, 0 to 3) scale. Patients were also asked to rate their symptoms during the day after provocation after 3, 6, 9, 12 and 24 hours.

Immunohistochemical staining of eosinophils

Eosinophil staining was performed by using an alkaline-phosphatase procedure with an mAb against major basic protein (BMK-13) (Sanbio, Uden, the Netherlands), as previously described by Godthelp et al (3).

Digoxigenin probe manufactured by PCR

The digoxigenin probe was manufactured by PCR with a modification of the method described by Klein (25, 26). Total RNA was isolated from stimulated (concanavalin A or LPS) blood mononuclear cells or *in vivo* allergen-stimulated nasal mucosal cells by the phenol extraction method, a modification of the protocol described by Chomczynski and Sacchi (27). One microgram of RNA was then reverse transcribed to cDNA (AMV Boehringer Mannheim Biochemica). The reverse-transcribed mix, a total volume of 20 µL, contains 50 mmol/L Tris-HCL (pH 8.3), 10 mmol/L MgCl2, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10 µg/mL BSA (Boehringer Mannheim), 1 mmol/L dNTP mix (Pharmacia), 10 µg/mL oligo(dT)12-18 (Pharmacia), 2.5 OD/mL hexanucleotide pd(N)6 (Pharmacia) and 1 mmol/L spermidine-HCl. This mixture was incubated at 42°C for 60 minutes. After this reaction, the final volume was topped up to a total volume of 200 µL with ultrapure water. Ten microliters of the cDNA was directly subjected to PCR in the presence of a master mix containing PCR buffer, 1.5 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L TRIS-HCl (pH 8.3), gelatin (0.1%), 1 U AmpliTaq polymerase, 0.025 mmol/L dNTP and 1 µL from each cytokine primer for a total volume of 50 µL (table I).

The reaction tubes were placed for 3 minutes in the PCR block (MJ Research), which was preheated to 94°C to avoid cold oligodeoxyribonuclear fusion. After preparing the PCR mixture on ice, PCR was carried out for 35 cycles under the following

conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. Final-cycle extension was at 72°C for 10 minutes.
The specificity of amplification was checked by assessing whether a fragment of the expected size had been obtained by gel (1.5%) electrophoresis or by southern blot hybridization with an internal 32P-γ ATP (Amersham)-labeled probe. The membranes were exposed to Kodak XAR films. PCR reaction products were run on gel, isolated from gel and cleaned up with the Wizard DNA Clean-Up System (Promega). The isolated DNA was used in a repeated PCR with the same cytokine-specific primers under the following conditions: 5 μL of isolated PCR product, 10 μL of 10× Taq polymerase buffer, 3 μL of 10 OD/mL sense primer and 3 μL of 10 OD/mL antisense primer (table I), 5 U Taq polymerase (Ampli Taq; Perkin Elmer Cetus, Norwalk, Conn) and 5 μL of dNTP (2 mmol/L each of dATP, dCTP, dGTP, 1.3 mmol/L dTTP and 0.7 mmol/L DIG-11-dUTP; Boehringer Mannheim) in a total volume of 100 μL.
The PCR protocol started with an adaptation of the hot start to avoid cold oligodeoxyribonuclear fusion. It was followed by 30 seconds at 94°C, 45 seconds at 55°C and 1.5 minutes at 72°C during 200 cycles. The resulting digoxigenin-labeled DNA (probe) was controlled on 1.5% agarose gel. The specificity of amplification was checked by assessing whether a fragment of the expected size (approximately 25% larger in base pairs than the normal PCR product) had been obtained.

In situ hybridization

All reactions were carried out with RNase-free materials and solutions except for the RNase treatment of the negative controls. Each tissue specimen was cut into serial 6-μm thick sections on a Reichert-Jung 2800e frigocut cryostat and transferred to poly-L-lysine (Sigma)-coated microscope slides, dried and stored at -80°C. The slides were used within 3 months of storage, heated to room temperature, dried and fixed in buffered 4% formalin for 15 minutes.
The slides were rinsed twice with PBS for 5 minutes. Proteinase K (1 μg/mL; Boehringer Mannheim) treatment was applied for 5 minutes at 37°C, followed by washing with PBS and fixing in buffered 4% formalin for 5 minutes to stop the proteinase K reaction. There then followed 2 more rinses in PBS for 5 minutes, permeabilization with 0.01% triton-X100 and a repeat washing with PBS. This was followed by dehydration by incubation, with increasing amounts of ethanol (70% for 2 minutes, 96% for 2 minutes and 100% for 5 minutes) air drying. The mixture for hybridization was pipetted onto ice and contained 30 μL 100% deionized formamide, 20 μL 20× sodium sodium citrate (SSC), 40 μL denatured salmon sperm DNA (10 μg/mL Tris-EDTA; Boehringer Mannheim), 1 μL t-RNA (100 mg/mL) and 9 μL diluted probe. The hybridization mix was incubated at 100°C for 1 minute to make the

DNA single stranded and then cooled immediately on ice for 5 minutes. The sections were incubated with the hybridization mix at 42°C for 1 hour and then for 16 hours (overnight) at 37°C in a humidity room. After hybridization, the cells were washed with 30% formamide/2× SSC at room temperature, 30% formamide/0.2× SSC, at 42°C for 15 minutes and PBS for 5 minutes.

The slides were then placed in a semiautomatic stainer (Sequenza, Shandon). After this, the sections were incubated for 10 minutes with BSA 0.5% to 1% in PBS incubated with normal sheep serum (CLB, the Netherlands) for 10 minutes and subsequently incubated with 1:500 alkaline phosphate-conjugated sheep-digoxigenin F(ab)2 fragments diluted in PBS-BSA 0.5% to 1% and normal human serum 10%. They were rinsed again with PBS for 5 minutes, rinsed with TRIS buffer (0.1 mol/L, pH 9.5) for 5 minutes and incubated with NBT-BCIP substrate (Sigma).

The incubation time is probe dependent. The substrate development was controlled with the microscope. Finally, the sections were rinsed in distilled water and mounted in glycerin-gelatine. Optimal probe concentration was obtained by dilution titration.

Table I. Primer sequence for each cytokine used for digoxigenin dUTP DNA probes

Spec	Sequence(5'- 3') primers	frag-ment	(Bp)
IL-2 sense	AAGAATCCCAAACTCACCAGGATGC	(exon 2)	200 Bp
IL-2 αsense	CCCTTTAGTTCCAGAACTATTACGT	(exon 3-4)	(51)
IL-3 sense	GCCTTTGCTGGACTTCAACA	(exon 1-2)	194 Bp
IL-3 αsense	TTGGATGTCGCGTGGGTGCG	(exon 4-5)	(52)
IL-4 sense	ACTCTGTGCACCGAGTTGACCGTAA	(exon 2)	300 Bp
IL-4 αsense	TCTCATGATCGTCTTTAGCCTTTCC	(exon 4)	(47)
IL-5 sense	AGCCAATGAGACTCTGAGGA	(exon 1-2)	319 Bp
IL-5 αsense	GGGAATAGGCACACTGGAGAGTCAA	(exon 4)	(53)
IL-6 sense	ATGAACTCCTTCTCCACAAGC	(exon 1)	610 Bp
IL-6 αsense	TGGACTGCAGGAACTCCTT	(exon 5)	(54)
IL-8 sense	CTGTGTGAAGGTGCAGTTTTGCC	(exon 1-2)	237 Bp
IL-8 αsense	CTCAGCCCTCTTCAAAAACTTCTCC	(exon 3-4)	(55)
IL-10 sense	ATGCCCCAAGCTGAGAACCAAGACCCA	(exon ?)	352 Bp
IL-10 αsense	TCTCAAGGGGCTGGGTCAGCTATCCCA	(exon ?)	(56)
IL-13 sense	CCCAGAACCAGAAGGCTCCGC	(exon 1-2)	185 Bp
IL-13 αsense	GCTGGAAAACTGCCCAGCTGAG	(exon 3-4)	(57)
IFN-γ sense	TTTAATGCAGGTCATTCAGATG	(exon 1-2)	388 Bp
IFN-γ αsense	CAGGGATGCTTCTTCGACCTCGAAAC	(exon 4)	(47)
RANTES sense	CGCTGTCATCCTCATTGCTA	(exon ?)	197 Bp
RANTES αsense	CACACACTTGGCGGTTCTT	(exon ?)	(58)
TNF-α sense	AGAGGGAAGAGTTCCCCAGGGAC	(exon 1-2)	443 Bp
TNF-α αsense	TGAGTCGGTCACCCTTCTCCAG	(exon 4)	(59)

Controlling the method

The controls used were those previously described by Bloch et al (28). To control the mRNA in situ hybridization, cryostat sections were incubated with RNase A (Boehringer Mannheim). This resulted in a significant signal reduction. Hybridization without a probe or unrelated probes gave no signal. Different cytokine probes resulted in different staining patterns (25, 26).

Quantification

The biopsy specimens were coded and 2 sections of each specimen were counted in a blinded fashion at a magnification of 400×. The surface area of the epithelium and the lamina propria of 2 sections were determined by computer image analysis (Kontrons Image Analysis System Videoplan). The number of eosinophils and the number of mRNA-positive cells per square millimeter of the section area in the lamina propria was calculated.

Statistical analysis

The statistical analysis was performed with SPSS 7.5 for Windows 95. Friedman 2-way ANOVA was used for statistical analysis. The Wilcoxon signed-rank test was used for a comparison between baseline and several points after allergen provocation. Correlation coefficients were obtained by using Spearman's rank method. Biopsy specimens taken at baseline represent the baseline level of symptoms and saline provocation (no symptoms were observed at these moments) and the biopsy specimens taken 1 hour after allergen provocation were compared with the early-phase symptomatology between 30 minutes and 1 hour; the specimens taken 24 hours after provocation were compared with the symptomatology described during 3 to 24 hours after provocation. The data of these 3 time points were correlated with each other ($n = 27$).

Correlation between eosinophils and cytokine-expressing cells were made at the time of each biopsy ($n = 9$). Because of multiple testing, a P value of less than 0.025 was considered to indicate a significant correlation.

Results

Nasal symptoms

No symptoms were observed before provocation or after provocation with saline. Nasal provocation with the allergen resulted in all patients having a significant increase in immediate nasal symptoms (eg, sneezes, itching, nasal blockage and rhinorrhea). Between 6 and 12 hours after the allergen provocation, late-phase nasal symptoms, especially blockage and itching, were observed in all patients.

Microscopic evaluation

The cryostat sections of the nasal mucosa had an average surface area of 2 mm^2 and an intact epithelium. Staining with mAb for eosinophil major basic protein (BMK-13) could easily be identified by the red cytoplasmic granules and dark violet nucleus (fig. 1a-d).

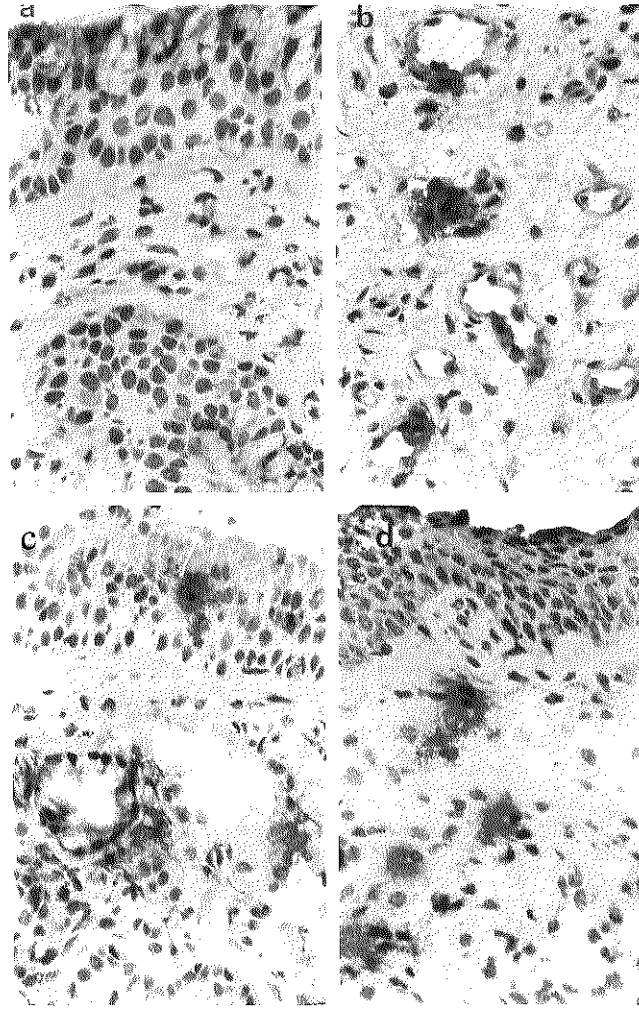


Figure 1a-d. Photomicrographs of 4 nasal mucosa biopsy sections immunohistochemically stained with BMK-13 antibodies against major basic protein. Before allergen provocation, almost no eosinophils were present (a); 1 hour after allergen provocation, eosinophil extravasation into the nasal tissue was observed (b); and 24 hours after allergen provocation in the epithelium, as well as in the lamina propria, eosinophils were present (c). d, One week after allergen provocation, the most eosinophils were present in the lamina propria (magnification ×550).

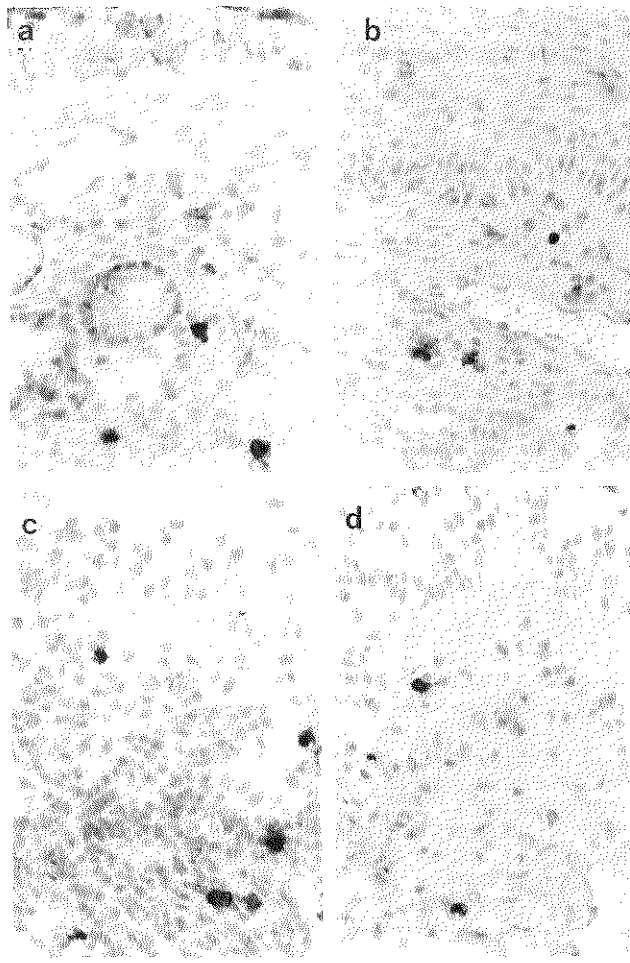


Figure 2a-d. Representative photomicrographs of in situ hybridization of nasal biopsy sections from patients with allergic rhinitis after allergen challenge with digoxigenin-labeled DNA probes coding for IL-3 (a), IL-10 (b), IL-13 (c) and RANTES (d). mRNA-positive cells exhibited a dark purple staining and displayed optimal cellular and subcellular resolution (magnification ×550).

Eosinophil numbers were determined in the lamina propria. The mRNA in situ hybridization staining patterns for cytokines varied from a dark purple circle to a large dark purple dot as shown in figure 2a-d.

Figure 2 shows representative sections from nasal mucosal biopsy specimens expressing mRNA. mRNA-positive cells were located among the inflammatory cell infiltrates within the lamina propria. The majority of cells showing hybridization signals for cytokines were present in the subepithelial layer. The number of positive

cells was only determined in the lamina propria, because the number of positive cells in the epithelium was too small for statistical analysis.

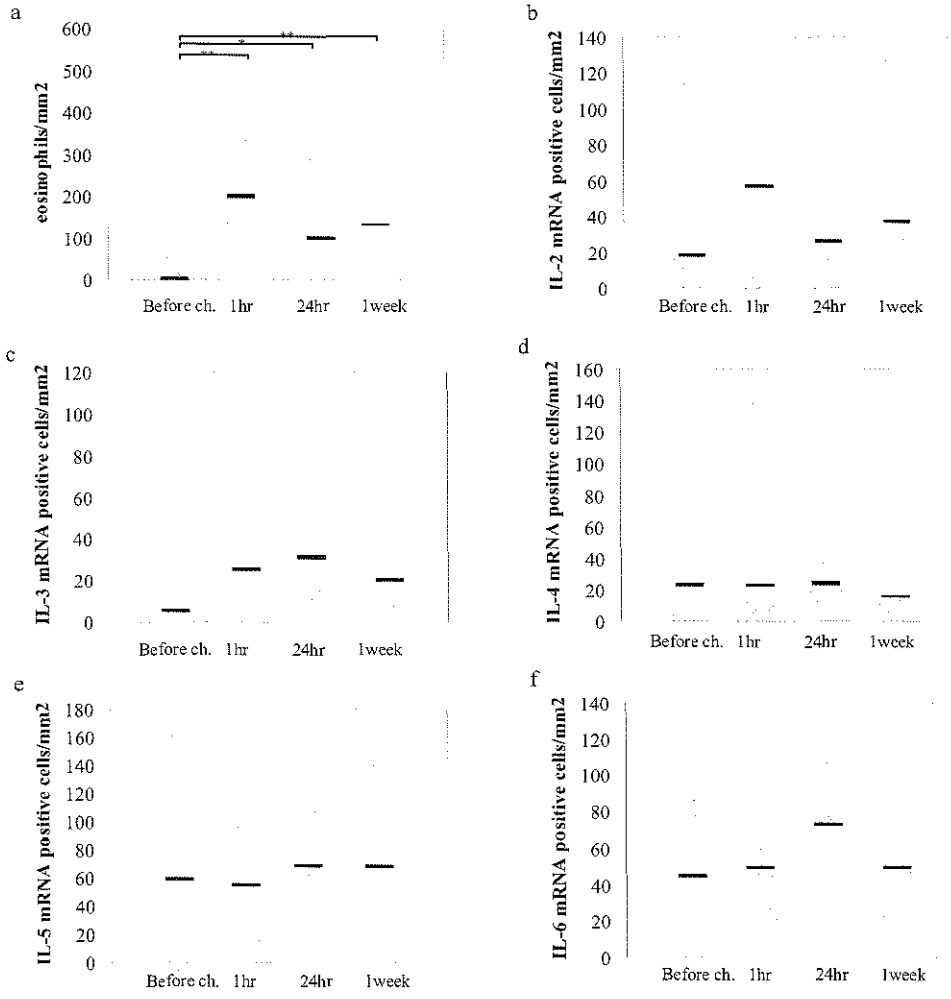
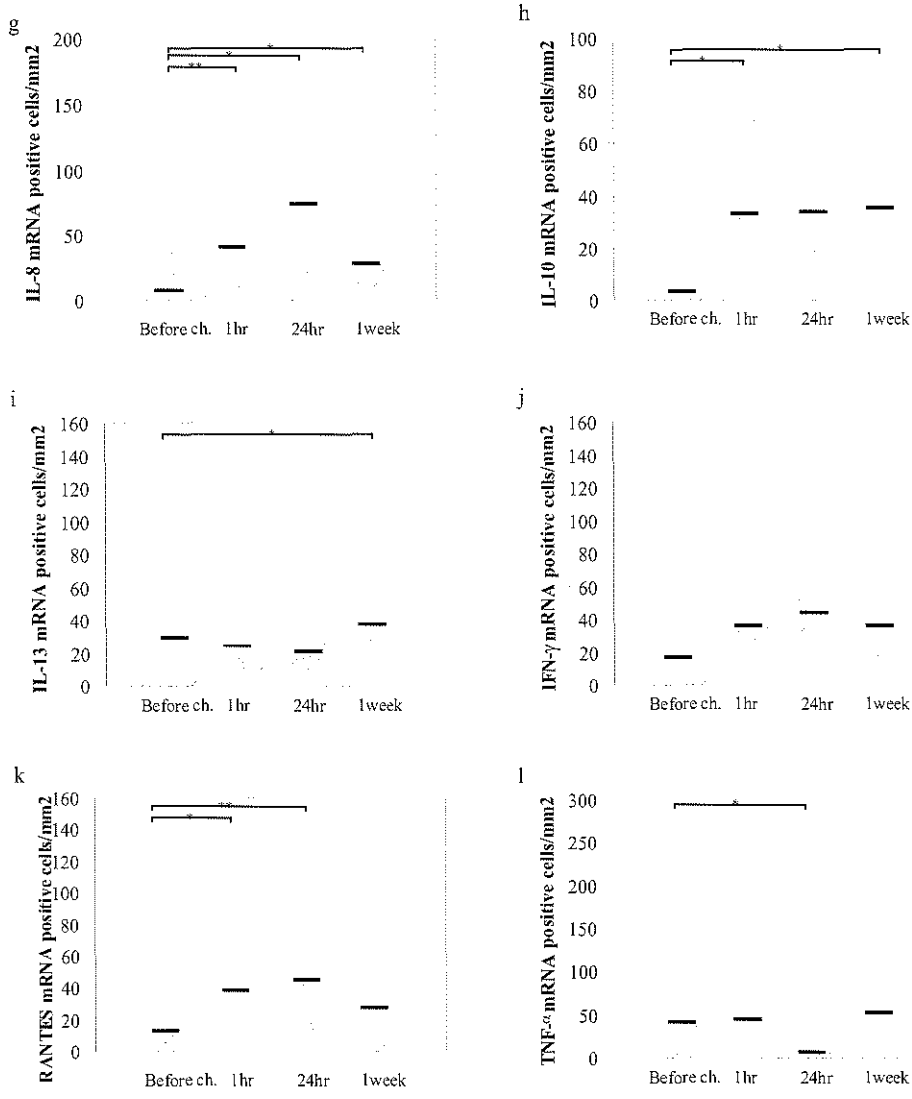


Figure 3. Number of eosinophils (a) and mRNA-positive cells (b-l) in nasal mucosa biopsy specimens. Nasal mucosa biopsy specimens were taken before provocation, 1 hour after provocation, 24 hours after provocation, and 1 week after provocation. Allergen challenge (ch) was performed with 1000 and 10,000 BU grass pollen. (fig. 3g-l see next page)

Changes after allergen provocation

Changes after allergen provocation are shown in figure 3. Eosinophils increased after allergen provocation ($P < 0.002$). Before provocation, very small numbers of eosinophils were found, mainly in the deep layers of the lamina propria. Within 1

hour after provocation, a remarkable increase in eosinophils was observed. Eosinophils were still present 1 week after the provocation. Significantly higher eosinophil numbers were observed compared with baseline 1 week after provocation ($P = 0.008$).



IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ and TNF-α mRNA–expressing cells showed no significant changes after allergen provocation. Numbers of TNF-α expressing cells decreased 24 hours after allergen provocation, but not to a significant extent. Numbers

of chemokine IL-8 ($P = 0.02$) and RANTES ($P < 0.01$) mRNA–positive cells increased significantly after allergen provocation. The dynamics for both IL-8 (fig. 2g) and RANTES (fig. 2k) were the same after allergen provocation (ie, an increase only 1 hour after allergen provocation, which remained relatively stable at 24 hours and 1 week). The Th2 cytokine IL-13 ($P < 0.04$) increased significantly and this effect remained significant until 1 week after provocation. There was a tendency toward an increase in IL-10 mRNA–positive cells ($P < 0.06$) after allergen provocation. Here also, a significant increase was found 1 week after allergen provocation compared with baseline ($P = 0.04$).

Cellular relationship to nasal symptoms

Correlations were found between numbers of eosinophils in biopsy sections and nasal symptom scores were as follows: itching, 0.654 ($P < 0.001$); discharge, 0.439 ($P = 0.025$); sneezing, 0.543 ($P < 0.001$); and total symptom score, 0.445 ($P = 0.02$).

Correlations were found between numbers of IL-8 mRNA–expressing cells in biopsy sections and nasal symptom scores were as follows: blockage, 0.468 ($P = 0.02$); discharge, 0.639 ($P = 0.001$); and total symptom score, 0.539 ($P < 0.001$). Correlations were observed between RANTES in biopsy sections and the nasal symptom score for itching was 0.580 ($P = 0.002$). Correlations were also observed for IL-10 in biopsy sections and the nasal symptom score for discharge was 0.512 ($P = 0.007$).

Table II. Eosinophil correlations with cytokines

	eosinophils correlated (1hr after provocation)	eosinophils correlated (24hr after provocation)
IL-2	0.6 (0.1)	0.2 (0.7)
IL-3	0.7 (0.04)	0.2 (0.7)
IL-4	0.7 (0.5)	0.5 (0.2)
IL-5	0.3 (0.5)	0.8 (0.009)*
IL-6	0.2 (0.6)	0.2 (0.7)
IL-8	0.6 (0.1)	-0.3 (0.5)
IL-10	0.2 (0.5)	0.3 (0.5)
IL-13	0.3 (0.4)	0.04 (0.9)
RANTES	0.8 (0.025)*	0.7 (0.007)*
TNF-α	0.7 (0.2)	0.8 (0.8)
IFN-γ	0.4 (0.02)*	0.1 (0.007)*

Correlations were between the number of BMK-13–positive cells compared with the number of cells expressing positive hybridization signals at 1 hour and 24 hours after provocation and all values are regression (P) values (* indicate: p value less than 0.025).

Cytokine mRNA relationship to tissue eosinophilia

Before allergen provocation, no significant correlations were observed between the number of cells expressing mRNA for cytokines and the number of eosinophils. In the early phase a significant correlation was observed between the number of eosinophils and the number of cells expressing mRNA for RANTES. Twenty-four hours after allergen provocation, correlations were observed between eosinophils and IL-5, RANTES and IFN-γ mRNA–positive cell numbers. A correlation between eosinophils and IL-6 ($r = 0.8$, $P = 0.007$) was observed 1 week after allergen provocation (for r [regression] and P values, see table II).

Discussion

The effect of a single allergen provocation with grass pollen on cytokine mRNA–expressing cells in nasal mucosal biopsy specimens was investigated and compared with tissue eosinophilia and symptomatology. Digoxigenin-labeled probes were used because they have almost unlimited shelf life and provide optimal cellular and subcellular resolution compared with radioactive probes (28). DNA probes were used because it is easier to avoid DNase than RNase when manufacturing these digoxigenin probes.

Single allergen provocation was used as a model for studying allergic inflammation in the upper airways. In this study a 50-μL allergen solution was sprayed into each nostril (11,000 BU in total), as described above (3, 5, 29).

To rule out the chance of nonspecific changes caused by provocation with PBS or the biopsy specimen, a second study was performed. In this study patients were sprayed with a PBS solution containing benzalkonium chloride, which was similar to the diluent of the allergen solution. Biopsy specimens were taken before and 24 hours after the last PBS provocation. Eosinophils, RANTES and IL-8 were evaluated at both time points. No significant differences (Wilcoxon signed-rank test) were found by comparing specimens taken before and after PBS provocation.

IL-2, a Th1 cytokine, plays a role in the proliferation, activation and differentiation of T cells and B cells. No changes were observed in the numbers of IL-2 mRNA-positive cells. Once again, it was shown that this Th1 cytokine was not involved in allergic inflammation during allergen provocation. These findings confirm other reports that did not find a change in IL-2 (on protein and mRNA level) in the nose (16, 30), lung (31, 32) and skin (33) after allergen challenge.

IL-4 and IL-13 have similar functions in B-cell isotype switching to IgE and also have a pivotal role in the upregulation of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on endothelial cells (34). We failed to find an increase in IL-4 during allergen provocation. This confirms data from the lower

airways, in which no differences were found between IL-4 protein-positive bronchoalveolar lavage fluid T cells in asthmatic subjects compared with non-allergic patients with asthma or control subjects (31) and also no IL-4 production/upregulation was found after allergen challenge (32). Bradding et al (12) also found no upregulation of IL-4 protein in patients with seasonal allergic rhinitis in the hay fever season compared with those patients out of the hay fever season. Those results contradict the findings of Durham et al (9, 16, 35) who used filter-disk nasal allergen provocation, which might be a stronger stimulus. The local allergen concentration in this filter-disk provocation is higher and the filter disk is held in position for 10 minutes, implying that mucociliary clearance cannot remove allergens. By contrast with IL-4 cells, we found a significant increase in the number of IL-13 mRNA–positive cells. These findings confirm data from Ghaffar (17) for the nasal mucosa and Ying (36) for the skin.

IL-3 and IL-5 are Th2 cytokines that stimulate the growth of mast cells and eosinophils. They are found to be more numerous in allergic patients compared with non-allergic control subjects and are found to be increased in allergic patients after allergen provocation (9, 33, 37-39). This study found no significant increase for IL-5 mRNA–positive cells after allergen provocation. This can probably be explained by the large range and high baseline levels in IL-5 mRNA–positive cell numbers. A clear correlation was observed between eosinophils and IL-5 24 hours after allergen provocation.

The chemokines RANTES and IL-8 are proinflammatory cytokines that are important in eosinophil chemotaxis. A significant increase of RANTES and IL-8 mRNA–expressing cells was observed after allergen challenge. Similar observations were made for protein level in nasal lavage fluid, (11, 40-42) bronchoalveolar lavage fluid (32) and for RANTES mRNA and RANTES protein-positive cells in nasal biopsy specimens of allergic patients after local allergen challenge (29).

The number of TNF-α mRNA–positive cells was unchanged after provocation, indicating that TNF-α is not involved in the allergen-induced reaction. This data confirms that TNF-α plays a role in bacterial infections but not in allergic inflammation, as is indicated by studies in nasal polyps (22) and bacterial infections (43, 44). This is despite the fact that one study found an increase in TNF-α mRNA–positive cells after filter-disk allergen provocation (21) and once an increase was found, 2 hours after allergen provocation in nasal lavage fluid on the protein level (13).

There was no significant increase in IL-6 after allergen provocation. On the protein level (protein-positive cells) in a number of studies, no differences were observed in the upper airways between allergic patients and control subjects (45). either before or

after allergen provocation (12). However, in nasal lavage fluids, IL-6 protein levels were found to be increased during the late-phase reaction (13, 41).

Numbers of IL-10 mRNA–positive cells were increased after allergen provocation. IL-10, which is considered to be a Th2 cytokine, inhibits the release of IFN-γ (46) and generally inhibits cytokine synthesis by human monocytes (47). IFN-γ, which is considered to be a Th1 cytokine, did not show a significant increase after allergen provocation, thereby confirming Durham's data (9).

The number of eosinophils correlated with nasal symptoms was previously observed by Pipkorn et al (48). Furthermore, correlation between the chemokines (IL-8 and RANTES) and nasal symptoms confirms the data from Douglass et al (49) who observed an increase in rhinitis symptomatology after IL-8 challenge in the nasal mucosa.

We used eosinophils as an indication of the amount of allergic inflammation. In the early phase only the numbers of RANTES and IFN-γ mRNA–positive cells were significantly correlated with eosinophil numbers. The symptomatology of patients in the early phase after allergen provocation is mainly caused by the release of histamine and other inflammatory mediators by mast cells (50). However, in the late phase significant correlations were found between eosinophils and IL-5 and eosinophils and RANTES.

Table III. Cytokine mRNA–positive cell fluctuation after allergen provocation in nasal mucosal biopsy specimens from patients with hay fever

Cytokine	Suspected cell target in allergic inflammation	Allergen provocation	Correlation with eosinophilia
IL-2	T-cells, B-cells (proliferation, activation, differentiation)	–	–
IL-3	Mast cells, eosinophils (colony stimulating factor)	–	–
IL-4	B-cells (IgE isotype switch), endothelium	–	–
IL-5	Eosinophils (differentiation, activation and survival)	–	+
IL-6	T, B, cells (proliferation, differentiation) and fibroblasts	–	-
IL-8	Lymphocytes, neutrophils, basophils, eosinophils (chemotaxis)	↑	–
IL-10	macrophages, T-cells (inhibitor of IFN-γ functions)	↑	–
IL-13	B-cells (IgE isotype switch)	↑	–
IFN-γ	Macrophages (activation), IL-4 antagonist (IgE isotype switch)	–	±
RANTES	Eosinophils and monocytes (chemotaxis)	↑	+
TNF-α	Fibroblast, endothelium (production of other cytokines and adhesion molecules (ELAM-1, ICAM-1))	–	–

One week after a single allergen provocation, there was still significant tissue eosinophilia and a significant increase in IL-13 and IL-10 mRNA–positive cells.
The correlation of RANTES with eosinophils in the early and late phases indicate that RANTES may be as important as IL-5 for eosinophil recruitment. In conclusion, nasal allergen provocation in patients with allergic rhinitis induced significant tissue eosinophilia and a significant increase in IL-8, IL-13 and RANTES mRNA-positive cells (table III).

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5. Intranasal detection and production of specific IgE

5.1 Allergen binding to specific IgE in the nasal mucosa of allergic patients

5.1 Allergen binding to specific IgE in the nasal mucosa of allergic patients

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Abstract

Background: Until now, it has not been possible to identify specific IgE locally in the airway mucosa. In this study we investigated the possibility of determining specific allergen binding locally in the nasal mucosa.

Methods: Nasal mucosa biopsy specimens were taken from 11 patients with symptoms of an isolated grass pollen allergy, 10 patients with symptoms of perennial allergic rhinitis in response to house dust mite allergen and 10 non-allergic control subjects. Sections of these biopsy specimens were stained by using commercially available biotinylated allergens (AlaSTAT, Diagnostic Products Corp.).

Results: Staining with biotinylated grass pollen (GP1) demonstrated positive cells only in patients with grass pollen allergy. Biotinylated Dermatophagoides pteronyssinus (D1) only stained cells in patients with perennial allergy. Specific binding of allergen to cells of patients with allergy and the blocking experiments proved the method to be highly specific. Allergen-positive cells stained double with IgE, the high-affinity receptor for IgE (FcεRI), CD1, HLA-DR, tryptase and chymase. Most allergen-positive cells proved to be mast cells.

Conclusion: This immunohistochemical study shows the presence of specific IgE against grass pollen and house dust mite allergens locally on cells in the airway mucosa.

Introduction

Allergic rhinitis is diagnosed primarily on the basis of a thorough analysis of clinical history. It may be supported by *in vivo* (e.g., skin prick test (1, 2)) and *in vitro* (e.g., serum-specific IgE as determined by RAST (3) or Alastat (4)) diagnostic tests. The presence of specific IgE on skin mast cells (skin test) and/or the existence of specific IgE in serum (RAST) indicates sensitization but not necessarily clinical allergy in the target organ. Although a positive skin test result or the presence of specific IgE to allergens in serum has been reported in 30% to 40% of the population, only 10% to 17% show symptoms of allergic disease (5-7). Moreover, the correlation between *in vivo* and *in vitro* test results varies from 10% (mite (7)) to 87% (grass pollen (8)), depending on the type of allergen and diagnostic test used (7-9).

The combination of history and diagnostic test results generally provides a reliable diagnosis of allergic rhinitis (8-10). However, considerable discrepancies between sensitization and symptomatic allergic disease are observed, particularly in children (11). Moreover, it may prove difficult to determine which allergen is the major cause of the nasal complaints, all the more so when multiple sensitizations occur.

Until now, it has not been possible to identify specific IgE locally in the airway mucosa. In this study we investigated the possibility of determining specific allergen

binding locally in the nasal mucosa of patients with seasonal allergic rhinitis, patients with perennial allergic rhinitis and non-allergic control subjects by using biotinylated allergen.

Furthermore, specificity experiments and double staining with mouse monoclonal antibodies against IgE, the high-affinity receptor for IgE (FcεRI), CD1, HLA-DR, mast cell tryptase and mast cell chymase were performed to phenotype the allergen-positive cells.

Material and Methods

Patients and control subjects

This study included 11 patients with symptoms of an isolated grass pollen allergy, 10 patients with symptoms of perennial allergic rhinitis and 10 non-allergic control subjects. Only sections of nasal mucosa from the patients with allergy were used in the double staining.

The patients with grass pollen allergy had a history of isolated grass pollen allergy for a period of at least 1 year, confirmed by a positive skin prick test reaction to Alutard Soluprick extract of 1 histamine equivalent in prick test/ml, no other positive skin prick test reactions to 13 common allergens, a median RAST score of 4+ (range, 3+ to 5+) and no other positive RAST scores.

The patients with perennial allergic rhinitis had experienced their symptoms for at least 1 year. The diagnosis of perennial allergic rhinitis was confirmed by a positive skin prick test response and a RAST score of at least 3+ (range, 3+ to 5+) for Dermatophagoides pteronyssinus (house dust mite) (HDM).

The control subjects were patients who were undergoing septorhinoplastic surgery and had no symptoms or signs of rhinitis and a negative RAST score. None of the patients or control subjects used any medication that could influence the results of this study. Biopsy specimens were obtained from the patients with perennial allergic during the HDM season (November through January).

Nasal biopsy specimens

Biopsy specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the edge, by using Gerritsma forceps with a cup diameter of 2.5 mm. Local anesthesia was induced by placing a cotton-wool carrier with 50 to 100 mg of cocaine and 3 drops of epinephrine (1:1000) under the inferior turbinate without touching the biopsy site. The biopsy specimens were embedded in Tissue-Tek II O.C.T. compound (Miles Inc.) in a gelatin capsule and frozen immediately (12). The patients with allergy underwent biopsy at the moment they were experiencing symptoms of rhinitis.

Allergens

The allergens, which are commercially available, are covalently bound to a soluble polymer-copolymer matrix labeled with biotin (Diagnostic Products Corp., Apeldoorn, the Netherlands) (4) We used GP1 (10 µg/ml), a mixture of biotinylated grass pollen (orchard, Kentucky, blue, rye and timothy grasses) and D1 (20 µg/ml), biotinylated HDM. Nonbiotinylated highly purified extracts of GP1 and D1 (1 mg/ml, Diagnostic Products Corp.) were used for the blocking experiments.

Monoclonal antibodies (table I)

Table I. Monoclonal antibodies used for allergen-positive cell characterization

Antibodies Against	Clone	Concentration	Source
CD1	okt-6	0.25 µg/ml	EUR
Tryptase	G3	1 µg/ml	Chemicon
Chymase	B7	0.12 µg/ml	Chemicon
IgE	CLB	1 µg/ml	CLB
FcεRI	15.1	2 µg/ml	JP Kinet
HLA-DR	CLB-HLA-DR(1E5)	1 µg/ml	CLB

Development of staining method

In the development of this novel approach, sections of allergic nasal mucosa were incubated with biotinylated GP1 for 30, 60 and 100 minutes and overnight. As the second step, an avidin-biotin complex conjugated with alkaline phosphatase (AP) was incubated for 30 minutes. Sections showed a light signal after a 30 minute incubation with biotinylated allergens and a stronger, specifically located signal after 60 minute incubation, but no increase in signal when this time was lengthened to overnight. In the single-staining procedure 60 minute incubation was chosen, but for the double-staining procedure the time was increased to 100 minutes to compensate for some signal loss because of the double staining. The staining method described below resulted in a strong signal for GP1 so that the concentration could be reduced to 1 µg/ml; dilution to 1 µg/ml or more resulted in staining that was too weak or negative. The concentrations eventually used in the study were 10 µg/ml for GP1 and 20 µg/ml for D1. This resulted in a strong signal without nonspecific staining. The staining procedure is outlined in table II.

Single staining

Staining was performed by using a modified AP method (15). Each tissue specimen was cut into serial 6 µm thick sections on a Reichert-Jung 2800e frigocut cryostat

(Leica) and transferred to poly-l-lysine-coated microscope slides (Sigma Chemical Co.), dried and stored at minus 70° C. The slides were used within 3 months; they were heated to room temperature, dried and fixed in acetone for 10 minutes at room temperature and rinsed in phosphate-buffered saline (PBS, pH 7.8), then placed in a semi-automatic stainer (Sequenza, Shandon).

After this, the sections were incubated for 10 minutes with bovine serum albumin, 0.5% to 1%, in PBS; incubated with normal goat serum (CLB-the Netherlands) for 10 minutes; and subsequently incubated for 60 minutes with the biotinylated allergen (D1 or GP1, Diagnostic Products Corp.).

They were then rinsed with PBS for 5 minutes, incubated with AP-goat-biotin (Sigma) for 60 minutes (to enhance staining), rinsed once more with PBS for 5 minutes, incubated with AP-avidin-biotin complex (Vector) for 30 minutes, rinsed with PBS for 5 minutes, rinsed with Tris buffer (0.2 mol/L, pH 8.5) for 5 minutes and incubated for 30 minutes with new fuchsin (Chroma-Germany) substrate (containing levamisole to block endogenous AP enzyme activity). Finally, the sections were rinsed in distilled water, counterstained with Gill's hematoxylin and mounted in glycerin gelatin (fig. 3A, B p. 113).

Table II. Outline of allergen staining method

Step	Description
1	Tissue specimens cut into 6 μm sections
2	Incubation with 10% normal goat serum
3a	In blocking experiments: preincubation with nonbiotinylated allergen
3b	Incubation with biotinylated allergen
4	Incubation with AP-goat anti-biotin
5	Incubation with AP-avidin-biotin complex
6	Substrate development with AP
7	Nucleus staining
8	Mounting in glycerin gelatin

Double-staining allergen and cell types

This double-staining method was primarily developed to characterize double-labeled cells (table III). New fuchsin substrate chromogens and β-Gal substrate chromogen result in a translucent, permeable precipitate. This enables us to detect co-localizing antibodies and allergen. After fixation with acetone, the sections were rinsed in PBS, incubated with normal goat serum (CLB-the Netherlands) for 10 minutes and subsequently incubated for 100 minutes with the biotinylated allergen (D1 or GP1); and after this, sections were incubated for 60 minutes with mouse monoclonal

antibodies (table I). They were then rinsed with PBS for 5 minutes, incubated with AP-goat-α-biotin (Sigma) and subsequently incubated with β-galactosidase-(β-Gal) goat-α-mouse for 60 minutes, rinsed with PBS for 5 minutes and incubated with AP-avidin-biotin complex (Vector) for 30 minutes.
Finally, samples were rinsed with PBS (pH 7.8) for 5 minutes, rinsed with PBS (pH 7.2) for 5 minutes to reach an optimal pH for reacting with β-Gal substrate (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [Sigma]) for 60 minutes, rinsed with Tris buffer (0.2 mol/L, pH 8.5), incubated for 30 minutes with new fuchsin substrate, rinsed in distilled water and mounted in glycerin gelatin (fig. 3C-F p. 113).

Table III. Outline of double staining of allergen and IgE

Step	Description
1	Tissue specimens cut into 6 μm sections
2	Incubation with 10% normal goat serum
3	Incubation with biotinylated allergen
4	Incubation with monoclonal mouse anti-human IgE
5	Incubation with AP-goat anti-biotin
6	Incubation with β-galactosidase-goat anti-mouse
7	Incubation with AP-avidin-biotin complex
8	Substrate development with β–galactosidase
9	Substrate development with AP
10	Nucleus staining
11	Mounting in glycerin gelatin

Testing of specificity

Sections were preincubated with nonbiotinylated allergen extract before incubation with biotinylated allergen extract to examine the specificity of allergen binding. For analysis of whether GP1 binds only to nasal mucosa from patients with grass pollen-induced allergic rhinitis and D1 only to tissue from patients with HDM-induced allergic rhinitis, the sections from patients allergic to grass pollen were incubated with biotinylated HDM extract (D1) and vice versa.

Control subjects

Biopsy specimens obtained from non-allergic patients were used to control the allergen staining. To control monoclonal antibodies, an irrelevant mouse IgG subclass was used. The sections showed no positivity, no nonspecific binding of the conjugates and no endogenous enzyme activity.

Light microscopic evaluation

The surface areas of the epithelium and lamina propria were estimated separately with the Kontron Image Analysis System Videoplan 2.1.

Results

General description

The nasal mucosal sections had a median surface area of 2.8 mm^2 (range, 1.0 to 4.3 mm^2) and usually showed a lining of ciliated columnar epithelium with or without goblet cells and of partially stratified cuboidal epithelium. The lamina propria generally consisted of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer on the bone.

Single staining

The number of GP1 or D1 cells per square millimeter section of nasal mucosa is shown in figure 1A (epithelium) and figure 1B (lamina propria). GP1+ cells were only found in the nasal mucosal sections of patients with an isolated grass pollen allergy. No GP1+ cells were found in the biopsy specimens from patients with perennial allergy or in the non-allergic control group. D1 cells were only found in the nasal mucosa of patients with perennial allergic rhinitis (allergic to HDM). No D1 cells were found in the biopsy specimens of the patients with an isolated grass pollen allergy or in the non-allergic control group.

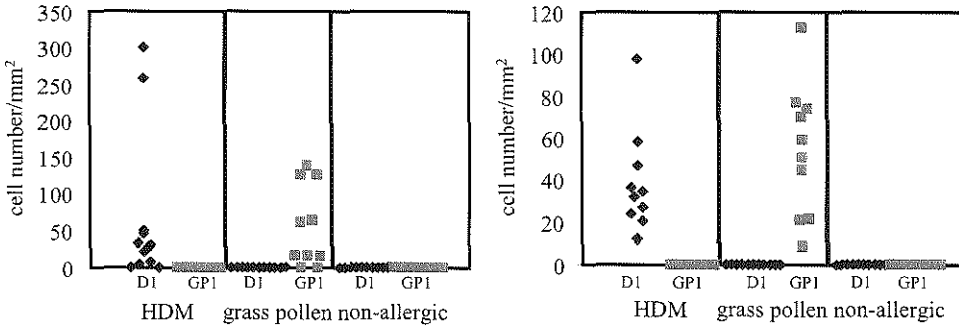

Figure 1. Number of allergen-positive cells in epithelium (A) and lamina propria (B). Staining with biotinylated grass pollen (GP1) showed positive cells in patients with grass pollen allergy only. Staining with biotinylated D. pteronyssinus (D1) only showed positive cells in patients with perennial allergy.

Blocking experiment

Blocking with grass pollen extract on the sections of patients with an isolated grass pollen allergy resulted in a median 81% (range, 50% to 86%; 25th to 75th percentile) decrease in the number of GP1+ cells compared with the number of GP1+ cells not blocked. In the nasal mucosa of the patients with perennial allergic rhinitis (all

positive for HDM), blocking resulted in a median decrease of 99% (range, 84% to 100%; 25th to 75th percentile) in the number of D1+ cells compared with the number of D1 cells not blocked (fig. 2).

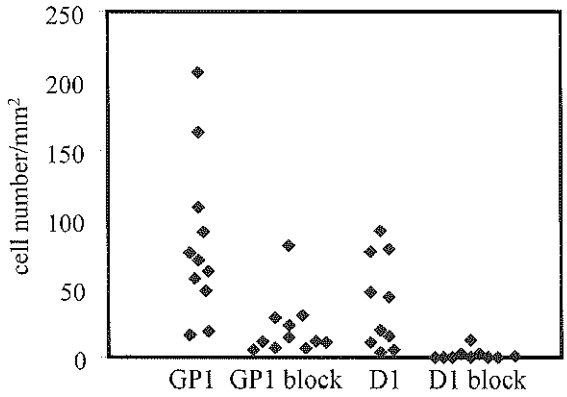

Figure 2. Blocking experiment by preincubation with nonbiotinylated allergens. Blocking experiments proved the method to be highly specific.

Double staining

Single allergen–positive (GP1 or D1) cells were sharply localized red-staining cells. The cells staining positive for a monoclonal antibody stained soft blue; double positive cells were a mixed color and dark. The phenotypes of allergen-positive cells are shown in tables IV and V in more detail.

Double staining with allergen (GP1 or D1) and antibodies showed that most of the allergen-positive cells contain tryptase or chymase (i.e., mast cells) and that CD1 (i.e., Langerhans cells) and HLA-DR–positive cells are able to bind the allergens too. More than 90% of the allergen-positive cells bear a high-affinity receptor for IgE and have IgE antibodies on their cell surfaces. Allergen-positive cells stained double with IgE, FcεRI, CD1, HLA-DR, tryptase and chymase. Most allergen-positive cells proved to be mast cells.

Table IV. Percentages of allergen-positive cells in the epithelium that are positive for IgE, FcεRI, CD1, HLA-DR, mast cell tryptase and mast cell chymase

	Double-staining cells (%)					
Allergen	IgE	FcεRI	CD1	HLA-DR	MC tryptase	MC chymase
GP1 median (range)	67 (24-100)	100 83-100)	10 (0-63)	33 (0-100)	50 (0-100)	86 (0-100)
D1 median (range)	38 (25-100)	100 (100-100)	1 (0-100)	33 (0-90)	92 (0-100)	0 (0-100)

Table V. Percentages of allergen-positive cells in the lamina propria that are positive for IgE, FcεRI, CD1, HLA-DR, mast cell tryptase and mast cell chymase

	Double-staining cells (%)					
Allergen	IgE	FcεRI	CD1	HLA-DR	MC tryptase	MC chymase
GP1 median (range)	83 (50-100)	92 (73-99)	14 (0-25)	35 (3-50)	64 (11-99)	93 (66-99)
D1 median (range)	95 (47-100)	94 (75-99)	4 (0-13)	37 (0-57)	90 (45-97)	90 (40-99)

Discussion

To our knowledge this is the first test method to detect binding of allergens to cells locally in the shock organ that is potentially suitable for routine use. The only other study of binding of HDM locally was described by Shimojo et al (13) However, in that study, Shimojo et al (13) used serum IgG from a hyposensitized patient to detect HDM in tissues. This renders the study unsuitable for routine use. Until now, only *in vitro* methods have been used to show specific IgE. The presence of specific IgE on skin mast cells (skin test) and/or the existence of specific IgE in serum (RAST) points to sensitization, but not necessarily to clinical allergy in the target organ.

In this study specificity of the allergens was tested by means of blocking experiments and incubation with a nonrelevant allergen (D1 as nonrelevant allergen for patients allergic to grass pollen and GP1 as nonrelevant allergen for patients allergic to HDM). Specific binding of the biotinylated allergen was indeed reduced sharply by preincubation with nonbiotinylated allergen. Incubation with a nonrelevant allergen showed no staining. These results prove the specificity of the binding of GP1 allergen and the binding of D1 allergen to the allergic nasal mucosal tissue.

More than 90% of the allergen-positive cells bear a high-affinity receptor for IgE and have IgE on their cell surfaces. IgE-positive-allergen-positive cells bear IgE on the cell membrane and are presumed to be able to bind the allergen against which the IgE is directed. FcεRI-positive–allergen-positive cells have free IgE receptor and specific antibodies against the allergen.

There are at present three possible explanations for our observations that single allergen-positive cells bind allergen and that no IgE is immunohistochemically detectable.

- First, this might be a result of allergens binding to different types of molecules in the immune system, such as IgG subtypes and IgA (14). This binding to immunoglobulins other than IgE has not yet been tested.
- Second, the allergen staining method used might be more sensitive than the usual two-step immunohistochemical staining method used for staining the IgE; in these

IgE-negative–allergen-positive cells, the amount of IgE is below the immunohisto-chemical detection level when the allergen can still be detected with the enhanced staining.

- Third, FcεRI–negative and allergen-positive cells could be cells without free FcεRI molecules, because the 15-1 antibodies against FcεRI only react with free receptors (J. P. Kinet, personal communication).

By double staining to co-localize the phenotype of allergen-binding cells, we have shown that the majority are mast cells and a few are CD1a-positive cells (Langerhans cells) and other HLA-DR-positive cells. In a few biopsy specimens of patients allergic to GP1, some eosinophils (BMK-13) that stained weakly positive for the allergens were found. This binding to eosinophils is specific because we did not see any binding of the other allergen; however, we could not observe co-localization with IgE. Our previous findings showed that mast cells and Langerhans cells in allergic nasal mucosa can be IgE-positive (15, 16). In other tissues, FcεRI are also found on mast cells, basophils (17, 18), monocytes (19) and eosinophils (20). These cells are all potentially able to bind IgE and can be part of the population of 'allergen and IgE double positive cells'.

The total percentages of the different allergen-characterized cells are greater than 100%. In allergic subjects 80%, of the mast cells are doubly positive for tryptase and chymase (21). Moreover, all CD1-positive cells bear the major histocompatibility complex class II (HLA-DR), as do mast cells (22, 23). Double staining with tryptase and HLA-DR or chymase and HLA-DR showed HLA-DR–positive mast cells (manuscript in preparation).

This study shows specific allergen binding to IgE cells locally in the shock organ. Additional studies focusing on other allergens are in progress to test this method in patients with discrepancies between clinical symptoms and RAST or skin test results.

Acknowledgments

We thank dr. J.P. Kinet for kindly supplying the 15-1 monoclonal antibodies against FcεRI and Diagnostic Products Corporation the Netherlands for supplying the allergens.

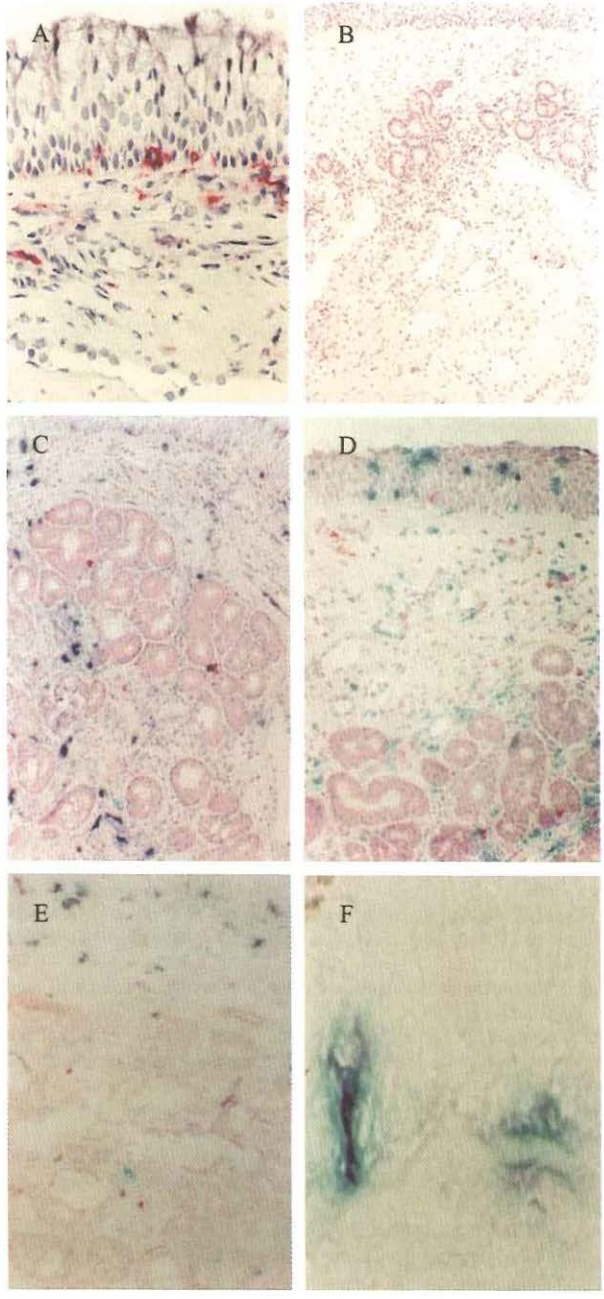

Figure 3. Cryostat section of nasal mucosa of a patient with isolated grass pollen allergy (A, C, F) and a patient with perennial allergy to HDM (B, D, E). A, Single staining with GP1 in patient allergic to grass pollen; allergen-positive cells stained red. B, Single staining with GP1 in patient allergic to HDM; no GP1-positive cells were observed. Double-staining allergen (GP1 or D1) and mouse monoclonal antibodies against: IgE (C), HLA-DR (D), mast cell chymase (E), and Langerhans cells (CD1) (F). Allergen-positive cells were sharply localized, red-staining cells. Cells staining positive for a monoclonal antibody stained soft blue; double-positive cells were a mixed color and dark.

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5.2 Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients

5.2 Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients

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Abstract

Allergic diseases are characterized by allergic complaints in the shock organ and specific immunoglobulin (Ig)E in serum. Literature data indicate that the nasal mucosa itself could produce at least a large part of the specific IgE in allergic rhinitis patients.

In order to investigate this hypothesis, nasal mucosal biopsies from the inferior turbinate were taken from symptomatic grass pollen allergic rhinitis patients, symptomatic house dust mite allergic rhinitis patients and non-allergic healthy controls, confirmed by radioallergosorbent test and skin-prick test. Immunohistochemical double-staining was performed for B-cells (CD19) with IgE, plasma cells (CD138) with IgE and plasma cells with biotinylated allergens.

Significantly more IgE-positive B-cells and IgE-positive plasma cells were found in the nasal mucosa of allergic patients than in that of non-allergic controls. Double staining with biotinylated allergens and plasma cells showed allergen-positive plasma cells in the nasal mucosa of allergic patients and no allergen-positive plasma cells in the nasal mucosa of non-allergic patients. Blocking experiments using polyclonal antibodies directed against IgE showed a significant reduction in the number of allergen-positive cells in contrast to experiments using polyclonal antibodies directed against IgG, IgA or IgM.

This study describes new evidence that specific immunoglobulin E is produced locally in the nasal mucosa in patients with seasonal allergic rhinitis and perennial allergic rhinitis, but not in non-allergic controls.

Introduction

To date, it has not been known where the production of specific immunoglobulin (Ig)E takes place in allergic rhinitis patients. Some studies have suggested that the nasal mucosa itself is able to produce at least a large part of the IgE (1-5). Huggins and Brostoff (6) reported patients who had specific IgE in their nasal secretions and no detectable levels of specific IgE in their serum.

In addition, specific IgE is present on cells in the nasal mucosa (7). The nasal mucosa itself contains all the cell types (dendritic cells, T-cells and B-cells (8)) and cytokines (interleukin (IL)-4 and IL-13 (9-12)) necessary for an IgE immunoresponse. Other studies have suggested that IgE synthesis takes place in the downstream cervical lymph nodes (13, 14) or upper respiratory and lower respiratory lymph nodes (15). However no information has been obtained as to whether the IgE comes from the circulation (transudate) or from the mucosa glands (exudatate).

Zurcher et al. (16) demonstrated that functional B-cells, isolated from the nose and cultured in a CD40-stimulating system, could synthesize IgE. Durham et al. (17)

reported that local allergen provocation induces ε germline transcripts in nasal B-cells. They were not able to detect IgE-positive B-cells. Pawankar et al. (18) suggested novel and critical roles for mast cells obtained from allergic rhinitis patients in amplifying IgE production, within the local microenvironment of the nasal mucosa. In this study, the possibility of determining the presence of IgE-positive B-cells, IgE-positive plasma cells and allergen-binding plasma cells in the nasal mucosa of seasonal allergic rhinitis patients, perennial allergic rhinitis patients and non-allergic healthy controls was investigated. Double-staining experiments were performed to verify whether all allergen-positive cells were also positive for IgE. Blocking experiments using polyclonal antibodies directed against Igs were performed to analyse the specificity of the allergen-binding cells for IgE.

Materials and Methods

Patients and controls

Nasal biopsy (2.5 mm) was performed using Gerritsma forceps (19). Twelve grass pollen allergic rhinitis patients (six male/six female), median age 30 yrs (range 15-45 yrs), with a history of clear seasonal rhinitis for 2 yrs and a skin-prick test with a reaction of 3+ for grass pollen and a radioallergosorbent test (RAST) score median of 4+ (range 3+-5+) for grass pollen.

The biopsy samples from the seasonal grass pollen allergic rhinitis patients were taken during the grass pollen season. Sixteen perennial allergic rhinitis patients (10 male/six female) median age 25 yrs range 18-51 yrs) yielded a positive skin-prick test for house dust mite (HDM), 3+ and a RAST score median of 3+ (range 3+-5+) for Dermatophagoides pteronyssinus.

Patients had to have two or more symptoms of perennial rhinitis (nasal blockage, rhinorrhoea, sneezing) and to have required medication for perennial rhinitis for 1 yr. The biopsy samples from the perennial allergic rhinitis patients were taken between October and January (inclusive) during the HDM season. The control biopsy samples were taken from twelve healthy volunteers (seven male/five female), median age 36 yrs (range 18–62 yrs) without nasal complaints or nasal abnormalities on ear, nose and throat examination and a negative RAST.

None of the patients had structural nasal abnormalities, undergone nasal surgery in the past 6 months, acute respiratory or sinus infection, serious or unstable concurrent disease, proven allergy for an other relevant inhalant allergen, undergone treatment with systemic or inhaled/intranasal corticosteroids or inhaled/intranasal sodium cromoglycate in the preceding month or used astemizole in the previous 6 weeks.

The study was approved by the Ethics Committee. All patients gave informed written consent.

Allergens

The allergens were covalently bound to a soluble polymer/copolymer matrix labelled with biotin (Diagnostic Products Corporation, Apeldoorn, the Netherlands) (7):

- GP1 (50 μg/ml), a mixture of biotinylated grass pollen (orchard, kentucky, blue, rye and timothy grasses) and
- D1 (20 μg/ml) biotinylated HDM.

Blocking experiment

In order to determine whether the allergen binding was specific to IgE, cryostat sections were preincubated with blocking polyclonal antibodies directed against IgA, IgE, IgG and IgM (5 mg/ml) central laboratory of the Netherlands Red Cross blood transfusion service (CLB), The Netherlands) and phosphate-buffered saline (PBS, pH 7.8), followed by the normal allergen staining procedure. These polyclonal antibodies blocked the cell-bound antibodies of different types. The blocking was based on spheric hindrance or the idiotypes of the antibodies were masked, so that the allergen could not bind. In order to determine the amount of blocking, the number of allergen-positive cells were counted and the signal intensities ranked in a blinded fashion.

Staining procedure

The antibodies used for the staining procedure are detailed in table I. Frozen sections (6-μm thick) were fixed in acetone for 10 min at room temperature (±22° C) and rinsed in PBS (pH 7.8). Endogenous peroxidase blocking was carried out by means of treatment for 30 min in 1% sodium azide and 0.01% hydrogen peroxide in PBS. The slides were then placed in a semi-automatic stainer (Sequenze; Shandon, Amsterdam, the Netherlands).

Double staining plasma cells or B-cells and immunoglobulin E The sections were subsequently incubated for 10 min with PBS containing bovine serum albumin (BSA, 1%), normal goat serum (10%) and normal rabbit serum (10%) (CLB, Amsterdam, the Netherlands) and then for 60 min with the antibodies to CD138 (plasma cells syndican I; Serotec (Diagnostic Products Corporation, the Netherlands). They were then rinsed with PBS for 5 min, incubated with biotinylated goat antimouse (Biogenix, Klinipath, Duiven, the Netherlands) for 30 min, rinsed once more with PBS for 5 min, incubated with alkaline phosphatase-conjugated streptavidin (Biogenix, Klinipath for 30 min, rinsed with PBS for 5 min and incubated for 60 min with FITC-conjugated rabbit antihuman IgE. After rinsing with PBS, they were incubated for 30 min with peroxidase-conjugated rabbit-anti FITC (Dako (ITK, Uithoorn, the Netherlands)). Finally, the slides were rinsed with PBS and tris-hydroxymethyl-aminomethane (Tris) (0.2 M, pH 8.5) for 5 min each, incubated for 10

min with 1.0 mM Fast Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with 1% 3-amino-9-ethylcarbazole (AEC) for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Double staining immunoglobulin E and biotinylated allergen

The sections were subsequently incubated for 10 min with PBS containing BSA (0.5-1%), normal rabbit serum (10%) and normal goat serum (10%) (CLB), followed by 100 min with biotinylated allergen (AlaSTAT, DPC) (7) and thereafter for 60 min with the polyclonal antibodies to IgE. They were then rinsed with PBS for 5 min, incubated with alkaline phosphatase-conjugated goat-antibiotin (Sigma) for 30 min. After rinsing with PBS, they were incubated for 30 min with peroxidase-conjugated rabbit anti-FITC (Dako, (ITK), Uithoorn, the Netherlands). Finally, the samples were rinsed with PBS for 5 min, rinsed with Tris (0.2 M, pH 8.5) for 5 min, incubated for 10 min with 1.0 mM Fast Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with AEC for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Double staining plasma cells and biotinylated allergen

The sections were subsequently incubated for 10 min with PBS containing BSA (0.5–1%) and normal rabbit serum (10%) (CLB), followed by 60 min with the antibodies to plasma cells and thereafter for 100 min with biotinylated allergen.

They were then rinsed with PBS for 5 min, incubated with peroxidase-conjugated Rabbit antimouse (Sigma) for 30 min, rinsed once more with PBS for 5 min and incubated with peroxidase-conjugated mouse antiperoxidase for 30 min. They were then rinsed with PBS for 5 min and incubated with alkaline phosphatase-conjugated goat antibiotin (Sigma) for 30 min. Finally, the samples were rinsed with PBS for 5 min, rinsed with Tris buffer (0.2 M, pH 8.5) for 5 min, incubated for 10 min with 1.0 mM Fast Blue substrate, rinsed with sodium acetate (0.2 M, pH 4.6) for two periods of 5 min, incubated with AEC for 30 min, rinsed in distilled water and mounted in glycerine/gelatin (1:1).

Controls

The controls used were biopsy sections from non-allergic patients and those stained immunohistochemically with irrelevant mouse IgG subclasses. In these sections, no positivity, nonspecific binding of the conjugates or endogenous enzyme activity were found.

Statistical analysis

The blocking experiments were analysed using the Friedman two-way analysis of variance (ANOVA) test. The distribution of cells in the epithelium and in the lamina propria was not symmetrical and the variances were unequal. For statistical analysis, Kruskal-Wallis one-way ANOVA was used to calculate the overall p-value. A p-value of <0.05 was considered to indicate a significant difference between groups of nasal mucosal biopsies. The nonparametric Mann-Whitney U-test was performed to analyse each group with respect to each other.

Results

Blocking experiment via preincubation with polyclonal antibodies

In nasal mucosal sections from mite allergic patients (n = 6) and grass pollen allergic patients (n = 10), the number of allergen-positive cells was always lower after preincubation with polyclonal antibodies directed against IgE ($p<0.0005$, Friedman two-way ANOVA). The intensity of the signal was also decreased by preincubation with antibodies directed against IgE. No significant effect was seen after preincubation with the other Igs or PBS on the number of allergen-positive cells or the intensity of the specifically stained cells (fig. 1).

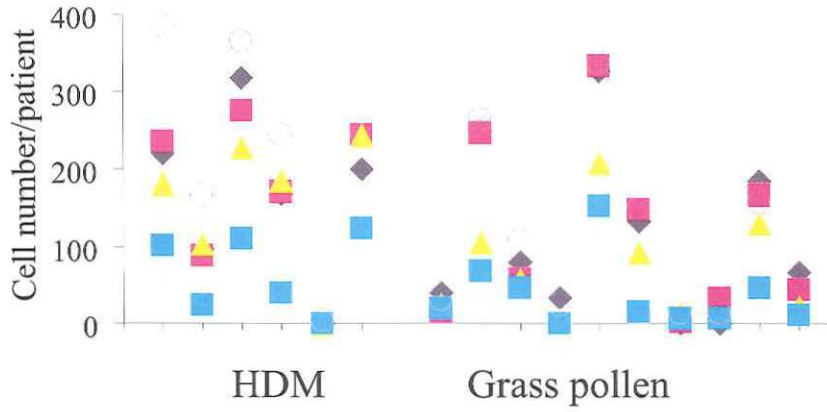

Figure 1. Blocking experiments using polyclonal antibodies directed against immunoglobulin (Ig) E (■) showed a significant reduction in allergen-positive cells in contrast to those using polyclonal antibodies directed against IgG (■), IgA (▲) or IgM (♦) and phosphate-buffered saline (○). Biopsy samples from each patient house dust mite (HDM), n = 6; grass pollen, n = 10) are plotted separately.

Immunohistochemical staining

Single staining

The number of IgE-positive cells/mm^2 section of nasal mucosa is given in table II for the epithelium and lamina propria. No IgE-positive cells were observed in the epithelium of healthy control biopsy samples. Significantly (10 fold) more IgE-positive cells were found in the nasal mucosa of allergic patients (grass pollen or mite allergic patients) than in healthy non-allergic control subjects. No differences were observed in the numbers of B-cells (CD19) or CD138-positive cells (plasma cells). Allergen-positive cells were found in allergic patients only, not in controls. GP1-positive cells were only found in patients with only grass pollen allergy. D1-positive cells were only found in the nasal mucosa of patients with perennial allergic rhinitis (allergic to HDM). No allergen-positive cells were found in non-allergic controls.

Table I. Antibodies used to stain biopsy specimens

Antibodies	Concentration or dilution (μg/ml)	Source
Anti CD19	1.3	Immunotech (Coulter, Netherlands)
Anti CD138 (Plasma cells-syndican I)	1	Serotec (DPC, Netherlands)
Anti-IgE^{FITC}	50	CLB (Netherlands)
Biotinylated grass pollen	50	DPC (Netherlands)
Biotinylated Der PI	20	DPC (Netherlands)
Goat-anti-mouse biotin	1:50	Biogenix (Klini Path, Netherlands)
Streptavidine Alkaline Phosphatase/Peroxidase	1:50	Biogenix (Klini Path, Netherlands)
Goat-anti-biotin Alkaline Phosphatase	1:50	Sigma (Netherlands)
StreptAvidineBiotin Complex conjugated Alkaline Phosphatase	1:50	Vector (Brunschwig Chemie, Netherlands)
Rabbit-anti-FITC peroxidase	1:50	Dako (ITK, Netherlands)
Rabbit-anti-mouse peroxidase	1:100	Sigma (Netherlands)
Mouse peroxidase anti peroxidase	1:100	Sigma (Netherlands)

Double staining

Immunoglobulin E-positive B-cells

Double staining with polyclonal antibodies directed against IgE and monoclonal antibodies directed against B-cells resulted in the easy identification of red IgE-positive cells, blue B-cells and mixed-colour (red and blue) IgE-positive B-cells (fig. 3A p. 128). In two of the controls, no IgE-positive B-cells were found in the lamina propria. Allergic patients were found to have four times as many IgE-positive B-cells in the lamina propria ($p<0.02$) than non-allergic controls.

Grass pollen allergic patients had significantly more IgE-positive B-cells in the epithelium ($p<0.02$) and in the lamina propria ($p<0.05$) than non-allergic controls. Perennial (mite) allergic patients were found to have more IgE-positive B-cells ($p<0.05$) in the lamina propria than non-allergic controls. No significant differences were observed between hay fever patients and perennial mite allergic patients (fig. 2A).

Immunoglobulin E-positive plasma cells

Double staining with polyclonal antibodies directed against IgE and monoclonal antibodies directed against plasma cells resulted in the easy identification of red IgE positive cells, blue plasma cells and mixed-colour (red and blue) IgE-positive plasma cells (fig. 3B p. 128). Plasma cells were only evaluated in the lamina propria because CD138 is also expressed weakly in epithelial cells (fig. 3B p. 128).

Cells positive for IgE alone, IgE positive plasma cells and non-IgE-positive plasma cells were found in allergic patients as well as in non-allergic controls. However, in seven of the controls, no IgE-positive plasma cells were found. In the lamina propria of the nasal mucosal biopsies from allergic patients, significantly eight-fold ($p<0.0007$) more IgE-positive plasma cells were present than in biopsy samples from non-allergic controls (fig. 2B).

Immunoglobulin E-positive allergen positive cells

IgE-positive cells stained red, the allergen-positive IgE-positive cells stained mixed red/blue and hardly any cells positive for allergen alone (blue, if present) were found. All allergen-positive cells were also positive for IgE, except in one of the grass pollen allergic patients (median 100% (range 71–100%) and one of the mite allergic patients (median 100% (range 96–100%)).

Allergen positive plasma cells

Double staining was performed using biotinylated allergens (blue) and antibodies directed against plasma cells red. The allergen-positive cells generally showed a

membrane-staining pattern, with the allergen bound on the surface of the cell. However, another staining pattern was found for the allergen-positive plasma cells, which also showed blue staining in and around the cell (fig. 3C p. 128). This pattern of staining was observed in all grass pollen allergic patients. However, in two of the mite allergic patients, no allergen-positive plasma cells were found.

Table II. Number of cells found in the nasal mucosa of grass pollen allergic rhinitis patients, perennial allergic rhinitis patients and healthy controls

cell type	seasonal allergics	perennial allergics	controls	ANOVA p-value*
epithelium				
IgE	4 (1-41)	14 (2-32)	0 (0-0)	0.0003
CD19	0 (0-0)	0 (0-6)	0 (0-3)	0.2
IgE-positive B-cells	0 (0-1)	0 (0-0)	0 (0-0)	0.05
lamina propria				
IgE	417 (273-590)	209 (97-330)	22 (7-37)	0.0001
CD19	225 (144-293)	152 (81-233)	142 (114-203)	0.2
Plasma cells	43 (18-100)	78 (60-113)	122 (46-151)	0.06
Allergen-positive cells	45 (32-85)	30 (16-57)	0 (0-0)	0.0000
IgE-positive B-cells	8 (4-16)	6 (3-13)	1 (1-5)	0.02
IgE-positive plasma cells	8 (13-18)	9 (6-16)	0 (0-6)	0.0007
Allergen-positive plasma cells	5 (3-8)	2 (2-7)	0 (0-0)	0.0000

*: Kruskal-Wallis one way analysis of variance. Data are presented as median (interquartile range).

Discussion

Local production of specific IgE in the nasal mucosa has been hypothesized by several authors (1-5). Durham et al (20) recently described the expression of ε germ line gene transcripts indicating isotype-switching B-cells and an increase in the level of messenger ribonucleic acid (mRNA) encoding the heavy chain of IgE in nasal B-cells in the nasal mucosa after local allergen provocation. Zurcher et al (16) demonstrated the possibility of IgE synthesis by nasal epithelial B-cells; functional B-cells can be isolated from the nose and, after stimulation with IL-4 and antibodies directed against CD40, IgE-protein synthesis could be induced. Diaz-Sanchez et al (21) also found IgE-producing B-cells and mRNA encoding IgE in nasal lavages fluid from allergic rhinitis patients.

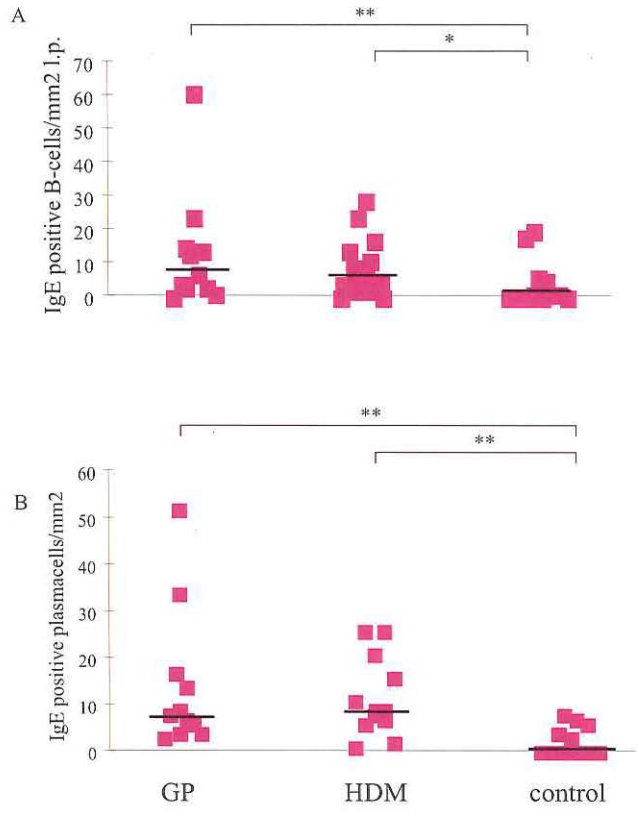

Figure 2. Density of: A. immunoglobulin (Ig) E-positive B-cells; and B. IgE-positive plasma cells in the nasal mucosa (lamina propria) of grass pollen allergic patients (GP, n = 11), perennial allergic patients (HDM; a) n = 16, b) n = 12) and healthy controls (n = 12). *: $p<0.05$; **: $p<0.01$

In this biopsy study, the production of IgE by B-cells and plasma cells *in vivo* was evaluated in naturally provoked allergic rhinitis patients. This study showed significantly more IgE-positive B-cells and IgE-positive plasma cells in allergic patients than in non-allergic controls. IgE-positive B-cells and plasma cells were also observed in healthy controls. An explanation for these IgE-positive cells could be the smoking of some of the patients, (environmental) tobacco smoke increases the number of IgE-positive cells (22, 23). The allergen-positive plasma cells (specific IgE) were observed only in allergic patients, in accordance with previous observations of allergen-positive cells occurring only in the nasal mucosa of allergic rhinitis patient and not in non-allergic controls (7).

The observation of the presence of allergen-positive plasma cells and the finding that all allergen-positive cells were also IgE-positive indicate that the local production of allergen-specific IgE takes place in the nasal mucosa. Blocking experiments showed that the binding was specific for IgE but not for IgG, IgA or IgM, meaning that specific IgE is the critical factor for allergen binding to cells and that this binding was not based on the allergen binding to IgG, IgA or IgM.

IgE antibodies remain firmly fixed to mast cells for an extended period. IgA antibodies function by inhibiting the adherence of coated micro-organisms to the surface of mucosa cells, thereby preventing entry into the body tissues. IgG has properties with respect to the neutralization of bacterial toxins and binding to microorganisms to enhance their phagocytosis. The locally-produced IgA or IgG levels were not high enough to neutralize the allergen binding to specific IgE-positive cells. In a previous study, it was observed that monoclonal antibodies directed against IgE recognize >80% of all allergen-positive cells (7); the present study used rabbit polyclonal antibodies directed against IgE and showed that all allergen-positive cells were also IgE-positive.

The significantly higher number of IgE-positive B-cells in the nasal mucosa of allergic patients compared to the nasal mucosa of non-allergic controls and the observation of no difference in numbers of B-cells expressing CD40 (data not shown) are an indication that, the ε germ line switches to IgE-positive B-cell early in life. These switched B-cells require only IL-4 to respond with persistent IgE formation (24). The significantly higher numbers of IgE-positive plasma cells in the allergic nasal mucosa compared to the controls suggests that the maturation of IgE-positive B-cells to IgE-producing plasma cells takes place locally in the nasal mucosa. B-cells express CD40 during the mature/activated stage (25). It is not possible to differentiate between IgE-positive B-cells if they are IgE-positive memory B-cells or at least activated B-cells (26). Once formed, the switched IgE-positive memory B-cells are

long-lived cells, but not all IgE responses lead to the formation of IgE-positive memory B-cells (27).

The present data suggest that the maturation of IgE-expressing B-cells (activated or memory) to IgE-producing plasma cells takes places in the nose. It is noteworthy that many of the cytokines induced by allergen provocation such as IL-4, IL-6 and IL-13 are also B-cell proliferation factors (11, 12, 28). The nasal mucosa might also be the place of maturation of B-cells. In the nasal mucosa are germinal centres containing dendritic cells, T-cells and B-cells. The availability of IL-4 and IL-13 produced in the nasal mucosa makes it possible for isotype switching of B-cells to IgE-positive B-cells and proliferation and maturation of B-cells to IgE-producing plasma cells to occur. Moreover, recent work done by the group of Durham et al. (17) reports an increase in the number of B-cells expressing ε germ line transcripts locally in the nasal mucosa of hay fever patients after allergen provocation. This increase in ε germ line transcripts could be suppressed by local steroid treatment. Similar observations of isotype switching have been made by Saxon et al. (21) in nasal mucosal cells. Not only T-cells but also mast cells and basophils have the capacity to stimulate IgE synthesis by B-cells, as producers of IL-4 and IL-13 and by the interaction of mast cells CD154 (CD40 ligand) with B-cell CD40 (18, 29, 30). The mast cell/basophilic induction and stimulation of B-cell IgE-production indicate that immunoglobulin switching, previously thought to take place only in lymph node germinal centres, may also occur in peripheral organs such as the nose. However, it is not clear what impact this mast cell/B-cell interaction has on the amount of IgE produced.

Comparing the present upper airway results with data from the lower airways, Chvatchko et al. (31) describe antigen-driven differentiation of B-cells via induction of a follicular dendritic cell network in mice, with germinal centres occurring in the parenchyma of inflamed lungs. These germinal centres would then provide a local source of immunoglobulin E-secreting plasma cells, contributing to the release of factors mediating inflammatory processes in the lung (31).

Transplantation literature case reports describe nonasthmatic recipients of asthmatic lungs who develop asthma after transplantation; however, asthmatic recipients of normal lungs do not develop asthma for up to 3 yrs after transplantation. This supports the local characteristic of lung disease in asthma (32). It is unclear which portion of specific IgE is produced in the nasal mucosa of allergic rhinitis patients because lymphoid tissues may also be involved in the production of IgE (4, 15, 33). However, this study clearly demonstrates that B-cells/plasma cells in the nasal mucosa of allergic rhinitis patients produce (specific) IgE. This concurs with the hypothesis put forward in the 1970s (1-3, 6).

Acknowledgements

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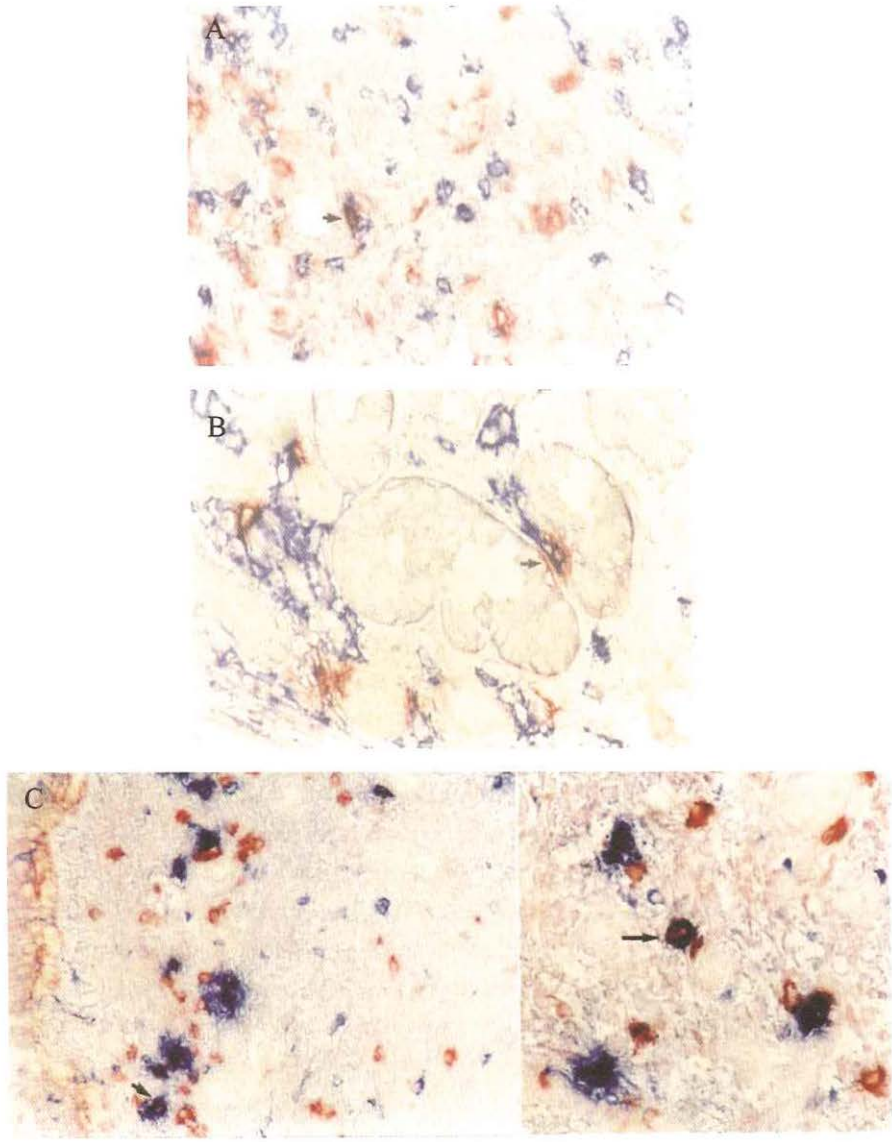

Figure 3. Photomicrographs of three nasal mucosa biopsy sections obtained from allergic patients and double stained immunohistochemically with antibodies directed against immunoglobulin (Ig) E (red) and: B-cells (blue) (upper) and; plasma cells

(blue) (middle); and double staining using biotinylated allergens (blue) and antibodies directed against plasma cells (red) (lower).

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5.3 Basophils and mast cells acquire IgE locally in the nasal mucosa of allergic rhinitis patients

5.3 Basophils and mast cells acquire IgE locally in the nasal mucosa of allergic rhinitis patients

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Submitted for publication

Abstract

Background: Previous observations by others and ourselves have provided strong indications that IgE is produced locally in the nasal mucosa of allergic rhinitis patients.

Methods: In this out-of-season biopsy study, we investigated the effect of a single provocation with grass pollen on the local production of IgE in the nasal mucosa. First we determined, at baseline and at several time points after allergen provocation, the number of IgE-producing cells in nasal mucosal biopsies on both the mRNA and protein levels and grass pollen-specific IgE-producing cells (GP-IgE) on protein level.

Results: This study indicates the presence of IgE- and GP-IgE-producing cells locally in the nasal mucosa. Allergen provocation induced a trend of higher numbers of IgE-transcript and IgE-positive B-cells but no change was observed in plasma cells producing IgE and GP-IgE.

After allergen provocation, basophils, both negative and positive for IgE, increased significantly in numbers. The proportion of IgE-positive basophils fell directly after allergen provocation and the proportion increased slightly between 1 hr and 1 week after allergen provocation, indicating IgE-loading of the basophils.

At baseline, 95% of the nasal mucosal mast cells were loaded with specific IgE for grass pollen. IgE loading of mast cells (C-kit) was observed after allergen provocation in the nasal mucosa of allergic rhinitis patients. The high percentage of specific IgE-positive mast cells out of season clarifies why allergic rhinitis patients are still very reactive to allergen challenge even when there is a long period of allergen avoidance.

Conclusion: This study supports the idea that basophils and mast cells can be loaded in the nasal mucosa with locally produced IgE.

Introduction

Allergic rhinitis is characterised by allergy-related nasal symptoms in the presence of IgE directed against a specific allergen. When IgE attached to the surface of the mast cell binds an allergen, the mast cell degranulates, releasing mediators like histamine, leukotrienes and prostaglandins. The action of these mediators on downstream targets leads to the typical clinical symptoms of sneezing, itching, rhinorrhoea and nasal blockage and to allergic inflammation (1-3). The combination of history and systemic sensibilisation tests for specific allergens (skin prick test) generally provides a reliable diagnosis of allergic rhinitis (4-7). However, considerable discrepancies between the degree of systemic sensitisation and the local symptomatic allergic disease have been observed (8, 9). This points to a role of the nasal mucosa in the etiology of allergic disease, possibly through the local production of specific IgE in the nasal mucosa (9-11).

Changes in the levels of specific IgE can be observed in blood both after allergen provocation and after the start of the pollen season (12, 13). On top of the systemic changes in specific IgE levels, local levels in the nasal secretion can also change, for instance after house dust mite allergen exposure or avoidance (14). Specific IgE is also present on cells in the nasal mucosa of allergic rhinitis patients (15). The 'old' idea of local production is supported by the observation of Durham and co-workers who showed that allergen provocation induces IgE-specific ε germ line transcription in nasal B-cells (16, 29). The presence of mast cells loaded with (specific) IgE in the nasal mucosa is the most important and specific difference that can be observed between allergic rhinitis patients and healthy controls (15). In children allergic to aeroallergens, two thirds of the nasal mast cells are positive for IgE. Children allergic to food allergens have a significantly lower percentage (11%) of IgE-positive mast cells IgE (17). This suggests that differences in local IgE concentrations and local microenvironment can be shock organ-dependent and result in differences in mast cell IgE load. Previous observations from our laboratory regarding basophils showed an influx within 1 hour after allergen provocation, in both the epithelium and in the lamina propria of the nasal mucosa of allergic rhinitis patients (18). Since microenvironment determines whether basophils are IgE-positive, it should be possible to characterise the IgE positivity of basophils present in the nasal mucosa. C-Kit (CD117) is a cell surface marker for mast cells that can also be present on hematopoetic progenitor cells and has also been observed recently on blood and tissue basophils (19-21). This marker is independent of the degranulation state of mast cells, in contrast to other mast cell markers like tryptase and chymase. Moreover, Kawabori and co-workers suggested that C-Kit could also be present on mast cell progenitors in the nasal mucosa (22).
In this out-of-season biopsy study, we investigated the effect of a single provocation with grass pollen on local production of IgE in the nasal mucosa. First we determined, at baseline and at several time points after allergen provocation, the number of IgE-producing cells in nasal mucosal biopsies on both mRNA and protein level and grass pollen-specific IgE-producing cells (GP-IgE) on protein level. Next, we studied changes in IgE and GP-IgE load on the nasal mast cells and basophils of these patients with an isolated grass-pollen allergy. This study supports the idea that mast cells can be loaded locally in the nasal mucosa with specific IgE that is produced in the mucosa tissue of allergic rhinitis patients.

Materials and Methods

Patients

Nine patients, mean age 26 (range 19-51), participated in this study (5 female and 4 male). They all had a history of seasonal allergic rhinitis for grass pollen of at least

one year. Allergy to grass pollen was confirmed by a positive skin prick test and no positive skin-prick reactions to other relevant allergens. All patients were symptom-free at the start of the study and no relevant abnormalities were found in ENT examination. None of the patients used any medication during the study or had undergone immunotherapy in the three years before this study. Biopsies were taken from the inferior turbinate using a Gerritsma forceps. Each patient underwent four nasal biopsies, one prior to and three after provocation (1 hour, 24 hours and 1 week). All patients gave their written informed consent and the medical ethics committee of the Erasmus MC approved the study.

Single high-dose provocation

The patients acclimatised to the room for at least 15 minutes. A placebo provocation with PBS (phosphate buffered saline) was used to rule out non-specific hyper-reactivity. After ten minutes, patients were provoked with 50 µl of an aqueous nasal spray containing grass pollen (10,000 BU/ml, ALK, Groningen,the Netherlands) and another ten minutes later with 100,000 BU/ml (ALK, Groningen, the Netherlands) in order to initiate a strong allergic response.

No symptoms were observed before provocation or after provocation with saline. Nasal provocation with the allergen resulted in all patients in a significant increase in immediate nasal symptoms like sneezing, itching, nasal blockage and rhinorrhoea. Between 6 and 12 hours after the allergen provocation, late phase nasal symptoms were observed in all patients, in particular nasal blockage and itching.

Immunohistochemical staining

Double staining of B-cell, plasma cells and basophils with IgE was performed in an alkaline phosphate-peroxidase procedure using monoclonal antibodies against B-cells (CD19), plasma cells (CD138), basophils (BB1) and mast cells (C-Kit) in combination with polyclonal antibody for IgE as previously described by our group (23).

Double staining using monoclonal antibodies for C-Kit and chymase (see table I) with biotinylated grass pollen differed from the double staining mentioned above due to an extra Tyramide Signal Amplification (TSA, NEN Inc., Boston, MA) step to enhance the allergen-specific signal. Tissue fixation was done with acetone for 10 minutes. This was followed by blocking of endogenous avidine and biotin with Vector Blocking Kit according to the specifications of the producer (Vector Lab, Burlingame, CA, USA) or by the use of 1% blocking reagent in PBS (Roche 10961760). Slides were rinsed in PBS (5 min) and incubated with biotinylated grass pollen (GP1) allergen (Diagnostic Products Corp, Apeldoorn, the Netherlands) for 1 hour. After rinsing with PBS the slides

were incubated (1 hour) with the antibodies. Endogenous peroxidase was blocked using azide (0.3%) and peroxidase (0.1%) and methanol (50%) in PBS. Blocking was followed by the TSA procedure to enhance the GP1 signal. This was done by incubation with streptavidine PBS/BSA solution (1:100) (NEN Inc., Boston, MA) (30 min), rinsing in PBS, tyramid-biotinyl signal amplification diluent (1:50) (NEN Inc., Boston, MA) (10 min.), rinsing in PBS and finally incubation (30 min) with HRPαbiotin (1:200, Vector, Burlingame, CA).

After PBS rinsing, the slides were incubated (30 min.) with AP-conjugated-GoatαMouse F(ab)2 fragments (Immunotech, France) followed by incubation (30 min) with APAAP (1:50, DAKO, Denmark) and an incubation for maximum 30 minutes in Fast Blue substrate, containing levamisole to block endogenous AP. Slides were rinsed with NaAc (0.1 M pH 4.6) for 5 minutes and incubated with AEC substrate for 30 minutes. Finally, sections were rinsed with distilled water and mounted in glycerin-gelatin. Control staining was performed by substitution with irrelevant antibodies of the same subclass.

Digoxigenin probe manufactured by PCR and in situ hybridisation

The digoxigenin probe specific for the Fd part of IgE was obtained by PCR and used for in situ hybridisation using a method described previously (24). The PCR primers are sense 5'-CAC ACA GAG CCC ACC CTC TTC CCC-3'and antisense 5'-CTG AAA CTA GTG TTG TCG ACC CAG TCT GTG GA-3'. The PCR product was checked for the correct size (330 bp) and the presence of the appropriate restriction sites for HaeIII and AvaI (25). The digoxigenin probe was manufactured by PCR using DIG-dUTP and dTTP (1:3), instead of dTTP that was used in the normal PCR reaction. The resulting DIG-labeled DNA probe was checked on 1.5% agarose gel, by assessing whether a fragment of the expected size had been obtained.

Controls for the in situ hybridisation were those previously described by our group (24). To check the specificity for mRNA in the situ hybridisation, cryostat sections were incubated with RNase A (Boehringer Mannheim), resulting in a significant signal reduction. Hybridisation without the specific probe or unrelated probes gave no signal.

Quantification

The biopsies were coded and two sections of each biopsy were counted in a blinded fashion at a magnification of 400 x. The surface area of the epithelium and the lamina propria of two sections were determined by computer image analysis (Leica Image Analysis System). The number of cells and mRNA-positive cells per square millimetre in the lamina propria was calculated.

Table I. Antibodies employed

Antibodies	Clone (Murine monoclonal)	concentration or dilution	Source
Anti CD19	IOB4	1.3 μg/ml	Immunotech (Coulter, Netherlands)
Anti CD138 (Plasma cells-syndican I)	BB-4	1 μg/ml	Serotec (DPC, Netherlands)
Rabbit-anti-IgE		50 μg/ml	Dako (Netherlands)
Biotinylated grass pollen		50 μg/ml	DPC (Netherlands)
Anti-chymase	B7	1 μg/ml	Chemicon (Brunschwig, Netherlands)
Anti-basophils	BB1	6 μg/ml	AF Walls
Anti-CD203c	97A6	1 μg/ml	Beckman Coulter (Netherlands)
anti-CD117 (C-Kit)	YB5.B8	1 μg/ml	Pharmingen (Netherlands)
Goat-anti-mouse biotinylated		1:50	Biogenix (Klini Path, Netherlands)
Streptavidine alkaline phosphatase or peroxidase		1:50	Biogenix (Klini Path, Netherlands)
Goat-anti-biotin alkaline phosphatase		1:50	Sigma (Netherlands)
Rabbit-anti-mouse peroxidase		1:200	Sigma (Netherlands)
Mouse peroxidase anti peroxidase		1:200	Sigma (Netherlands)

Microscopic evaluation

The cryostat sections of the nasal mucosa had a median surface area of 2.9 mm^2 (range 0.4-8.3) in the lamina propria and an intact epithelium with median surface area of 0.47 mm^2 (range 0.12-1.45). Biopsies are excluded who not met the criteria for evaluation (epithelium > 0.1 mm^2; lamina propria >0.4 mm^2).

IgE-positive B-cells had a mixed red and blue membrane staining pattern, the plasma cells producing (specific) IgE had a red staining pattern in the cytoplasm and outside just around the cell and the plasma cells themselves had a blue membrane staining pattern. The mRNA in situ hybridisation staining patterns for IgE mature transcripts varied from a dark purple circle to a large dark purple dot.

Statistical Analysis (SPSS 9.0 for Windows 98)

Friedman Two-Way ANOVA was used for statistical analysis. The Wilcoxon signed rank test was used for a comparison between baseline and several points after allergen provocation.

Correlation coefficients were obtained by using the Spearman rank method. The incoming cells are defined as number at baseline minus number at 1hour after allergen provocation.

Results

Presence of grass pollen-specific IgE-producing cells in the nasal mucosa

Mature epsilon RNA expressing cells

In the nasal mucosal biopsies, only a few epsilon RNA-expressing cells were found at baseline out of season. These cells were all located in the lamina propria with no epsilon-expressing cells present in the epithelium. In the lamina propria, the majority of patients had fewer than 4 cells/mm^2 (median: 3, range: 0-77 cells/mm^2), three patients had no detectable epsilon mRNA transcripts and a single patient had a very high baseline level (fig. 1A). The distribution of epsilon-positive cells after allergen provocation does not change, with the epithelium still devoid of epsilon expressing cells. Epsilon-expressing cell numbers in the lamina propria after allergen provocation tended to increase from 3 at baseline to 23 after one hour (range: 3-65, Wilcoxon p = 0.03), 9 after 24 hours (range: 0-40, p = 0.05) and 6 after one week (range: 0-31, Wilcoxon p = 0.05) (fig. 1A).

Local expression of IgE protein in the nasal mucosa

After having established the presence of mature IgE transcripts, we next investigated the local production of IgE protein. In the nasal mucosa of allergic rhinitis patients, we were able to observe IgE- and grass pollen-specific IgE-positive (GP-IgE) cells, B-cells and plasma cells with no changes in cell numbers after allergen provocation.

Intranasal IgE and GP-IgE producing cells were analysed for their potential to contribute to local IgE production in the nasal mucosa.

We were able to detect IgE-positive B-cells (fig. 1B), IgE-positive plasma cells (median number 18-34 cells/mm^2) and GP-IgE-positive plasma cells (median number 3-7 cells/mm^2) in the lamina propria at baseline, indicating a persistent presence of IgE-producing cells in the nasal mucosa. Twenty-four hours after allergen provocation, the number of IgE-positive B-cells tended to increase (Wilcoxon p = 0.04) (fig. 1B). No shift was observed in the fraction of IgE- or grass pollen-specific IgE-producing plasma cells in relation to the total number of plasma cells (data not shown). One third of the IgE-producing plasma cells were grass pollen allergen-specific.

Our data show that IgE-producing cells are present continuously, both out of season and after allergen provocation, indicating that free IgE is available in the nasal mucosa of allergic rhinitis.

Mast cells and basophils acquire IgE

Influx of IgE-positive and IgE-negative basophils after allergen provocation

Previous observations from our laboratory regarding basophils showed an influx within 1 hour after allergen provocation in both the epithelium and the lamina propria of the nasal mucosa (18). In the present study, we characterised the IgE positivity of basophils present in the nasal mucosa. At baseline basophils are practically absent in the epithelium. After allergen provocation, an epithelial influx of basophils could be observed being maximal 1hr after provocation (data not shown).

At baseline the basophils (median 5; range2-27 cells/mm^2) present in the lamina propria are almost all loaded with IgE (median: 100%, range: 50-100%). A significant (Wilcoxon p = 0.02 and p = 0.04) influx of both IgE-positive basophils (median: 45, range:13-121 cells/mm^2) and IgE-negative basophils (median: 12, range 0.3-63) could be observed after allergen provocation (fig. 2A). Due to this influx, the percentage of IgE-positive basophils fell significantly (Friedman p = 0.03) to 79% at 1 hour (range 58-93%, Wilcoxon p = 0.04), 86% at 24 hours (range: 46-96%, Wilcoxon p = 0.01) and 93% at one week (range: 27-97%, Wilcoxon p = 0.01) after allergen provocation relative to baseline (fig. 2A).

Although the absolute numbers of basophils did not change between the moments after allergen provocation, the percentage of IgE-positive basophils tended to return to baseline level (100%), suggesting local IgE loading of basophils (fig. 2B).

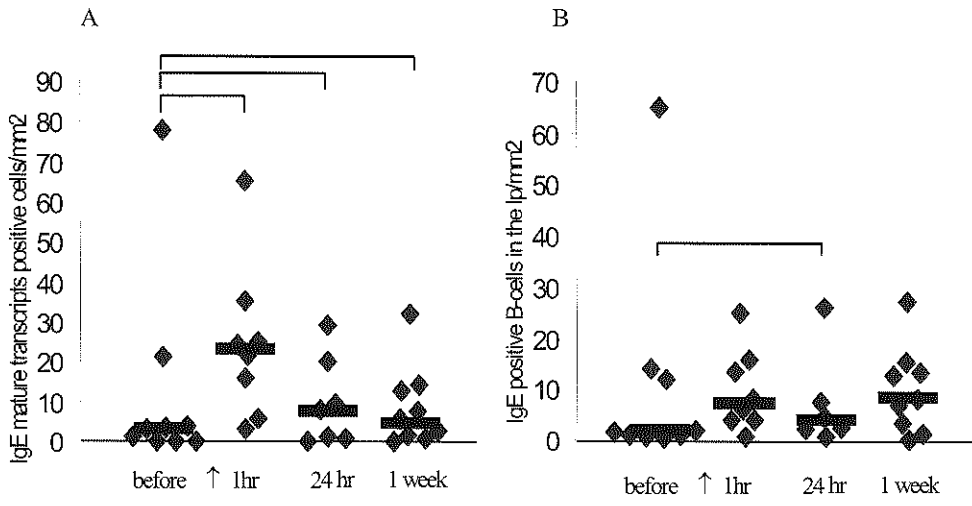

Figure 1. IgE-producing cells in the lamina propria of allergic rhinitis patients. The nasal mucosa contain mature transcript ε transcripts (A) and IgE-positive B-cells (B).

C-Kit-positive mast cells acquire IgE in the nasal mucosa

At baseline, C-Kit-positive mast cells are present in both the epithelium and the lamina propria of the nasal mucosa. In the epithelium C-kit positive cells were usually IgE positive. No changes were found after allergen provocation.

Allergen provocation also has no effect on total numbers of mast cells (C-Kit-positive) in the lamina propria (fig. 2C). The number of IgE-negative mast cells decreased 50% ($p = 0.02$) between baseline (median: 22 cells/mm^2, range: 6-101) and one week after allergen provocation (median: 12, range: 1-49), leading to a significant increase (Wilcoxon $p = 0.02$) in the percentage of IgE-positive mast cells from 82% (range: 4-96%) at baseline to 93% (range: 59-100%) at one week after allergen provocation (fig. 2D). These results suggest that mast cells acquire IgE in the nasal mucosa. Double staining of GP-IgE with the mast cells shows GP-IgE-positive mast cells (15). We were not able to show that the acquired IgE was grass pollen specific.

Basophils are the entering IgE-positive cells.

A significant correlation (Rs 0.762 ($p = 0.03$)) was observed between the incoming IgE-positive basophils (BB1-positive) and the incoming IgE-positive C-kit-negative cells. This supports the finding that there is an influx of IgE-positive basophils after allergen provocation. Basophils positive for IgE are responsible for a direct influx of IgE-positive cells. The total IgE numbers do not increase, indicating that cells from the lamina propria move away (in the lumen or blood stream).

Discussion

This study shows the presence of IgE and GP-IgE producing cells locally in the nasal mucosa. Allergen provocation induces a trend of increased numbers of IgE transcript 1 hour, 24 hours and 1 week and of IgE-positive B-cells at 24 hours only. No change was observed in the number of plasma cells producing IgE or GP-IgE during this provocation study as has earlier be observed in a study during and outside the pollen season in hay fever patients (unpublished data).

Our data suggest that IgE-producing plasma cells and B-cells are continuously present and are responsible for the persistent production of IgE in the nasal mucosa of allergic rhinitis patients.

Although we were not able to show an increase in specific IgE producing cells in the nasal mucosa, increased local production has been implied by old studies from Platts-Mills showing that specific IgE ratio versus total IgE is higher in nasal secretion than in serum (11). The possibility of IgE production by tissue explants (29) and the observation that specific IgE increased after (seasonal) allergen exposure or allergen avoidance (9-11, 26).

The increased production of specific IgE does not necessarily need to be reflected by elevated IgE-producing cell numbers. Allergen challenge, however, does lead to an increased production of specific IgE (26). In line with these observations are the observation of elevated levels of IL-6, an important growth factor for the stimulation and production of IgE by plasma cells after provocation in allergic rhinitis patients (27, 28). Our data now and our previous finding of allergen-positive plasma cells and allergen-positive cells that are all IgE-positive (23) indicate that local production of allergen-specific IgE takes place in the nasal mucosa.

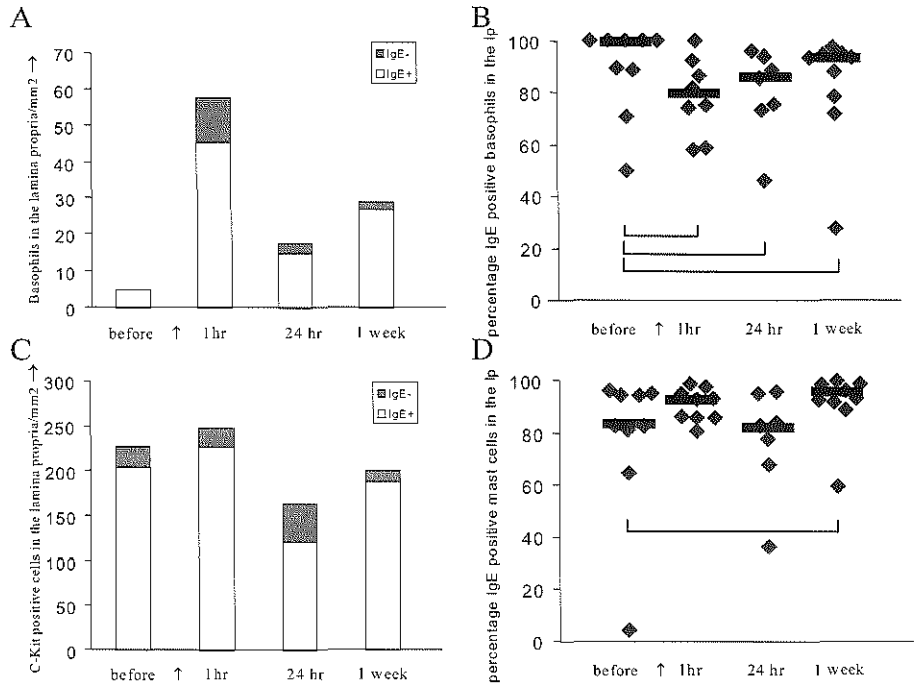

Figure 2. Median numbers (cells/mm^2) of basophils positive or negative for IgE (A) and percentages of IgE-loaded basophils in the lamina propria in nasal mucosal biopsies (B). Median numbers (cells/mm^2) of C-Kit-positive cells positive or negative for IgE (C), percentages of IgE-loaded C-Kit-positive cells (D) (↑ = nasal allergen provocation)

The second part of this study supports the idea that basophils and mast cells are loaded with IgE locally in the nasal mucosa of allergic rhinitis patients. The few basophils present at baseline are almost all loaded with IgE (100% double positive for BB1 and IgE). After allergen provocation, an influx of IgE negative basophils was observed, as shown by the measurement of an increase in numbers and a decrease in the proportion of

IgE-loaded basophils to 80%. After the influx, basophils became IgE-positive. This is shown by the return in the percentage of IgE-loaded basophils to more than 90%. Also the percentages of IgE-positive-C-kit-positive mast cells increased after allergen provocation. These combined observations indicate that IgE loading of basophils and mast cells takes place locally in the nasal mucosa.

The present findings indicate that the nasal mucosa must be seen as an important player in the manifestation of allergic disease and not just as a passive player in the allergic rhinitis patient. This has the therapeutic consequence that it is important to treat the 'allergic nose' first. Topical steroid therapy reduces the increase of IgE germline transcription (16, 35) and the serum IgE increase after allergen provocation (13).

The finding in this study - that both specific IgE production and mast cell sensitisation takes place in the nasal mucosa - might explain the discrepancies between the significant reduction in free IgE compared to the limited reduction in symptoms by the treatment of allergic rhinitis with anti IgE therapy. Although anti-IgE treatment reduces the free IgE it is not known that anti-IgE treatment is able to reduce the cell bound IgE locally in the nasal mucosa (37,38).

In conclusion, this study shows clearly that GP-IgE production and mast cell sensitisation persistently takes place locally in the nasal mucosa of allergic rhinitis patients. This phenomenon explains why allergic rhinitis patients are still very reactive to allergen challenge, even when there is long period of allergen avoidance or out of season.

Acknowledgments

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6. General discussion

6.1 Allergic rhinitis: a local disease

The findings in this thesis indicate that allergic rhinitis is a local allergic disease and that all cell types necessary for supporting and maintaining the disease are present in the shock organ, the nose. Local maturation and acquisition of IgE (specific or otherwise) by mast cells is the final step in the whole immune cascade of sensitisation of the allergic nose. However, the cascade of the allergic immune response is ongoing.

6.2 Mast cells and basophils contribute to nasal symptoms in allergic rhinitis

Mast cells and basophils are the sources of mediators (histamine, tachykinins, prostaglandine and leukotriene). The physiological role of mast cells in airway mucosa by healthy controls is unknown. In pathophysiological conditions like allergic rhinitis and asthma, mast cells play a key role in the induction and start of an allergic reaction.

The mast cell mediators are the inducers of allergic upper airway symptoms and inflammation (1). Histamine plays an important role in allergic symptomatology and inflammation (fig. 1) of the upper airways. In allergic rhinitis patients, elevated mast-cell mediator levels in nasal lavage fluid following allergen provocation indicate mast cell degranulation during the early-phase nasal response.

In contrast to our observations, previous studies have suggested that basophils contribute mainly to the late-phase nasal response. This had been concluded from the pattern of mediators released in the late phase (i.e. histamine, leukotriene and others, but not PGD2) (2, 3) and from studies of nasal brushings and lavage fluids that have only shown an increase of basophils, identified as metachromatic cells in the nasal lumen in the late phase (4-6). In this thesis, we have clearly shown an influx of basophils in the epithelium as early as 1 hour after allergen provocation (7). The findings in previous studies cannot be explained only by differences between basophil and mast cell activation. They must also be explained by a different mechanism of mast cell mediator release in the early phase (caused by allergen/IgE cross-linking) and the late phase (primarily neuropeptide-induced). The theory of late-phase mediator release induced by neuropeptides is supported by a number of *in vitro* and *in vivo* observations. *In vitro* experiments show that neuropeptide-induced mast cell mediator release results in high levels of histamine but relatively low levels of PGD2 (8-10). *In vivo* mast cells are often in close proximity to neurogenic fibers in the nasal mucosa (11-13) and, in the late-phase nasal response, elevated levels of neuropeptides are found (8-10). Moreover, *in vivo* experiments in which unilateral challenge results

in bilateral symptomatology (14-16) and Pavlovian conditioning experiments which have shown the induction of symptoms and mast cell degranulation as measured by histamine and tryptase release in the nose of allergic rhinitis patients, without any allergenic stimulation (17, 18), also indicate that neuropeptides play a role in mast cell mediator release.

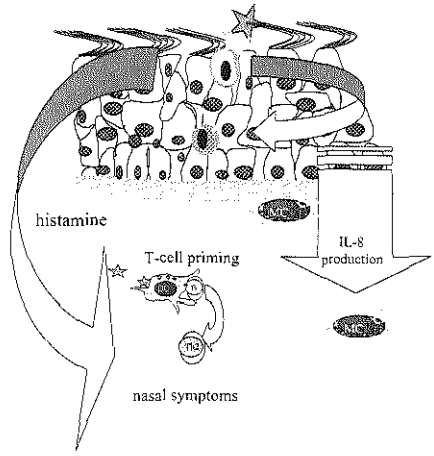

Figure 1. Histamine has immunomodulatory effects. Epithelial IL-8 upregulation (IL-8 has chemotactic properties on mast cells) (by CXCR1 and 2 on mast cells); Increased epithelial permeability by reduction in tight junctions; DCs skew naive T-cells towards Th2 phenotype. Steroid therapy prevents epithelial mast-cell accumulation.

6.3 Microenvironment in the nasal mucosa

In the nasal mucosa, two areas can be distinguished: the epithelium and the lamina propria. The lamina propria can be subdivided into the cell-rich subepithelium and the deeper layers. The available data about the epithelium *in vivo* is limited. Quantification of the epithelial side, which is approximately 1/20 of the total biopsy area, shows only a few cytokine-positive cells as measured by ISH or immunohistochemistry and this has not been evaluated many times because of the low number of expressing cells (19, 20). Data obtained from the lamina propria suggest an upregulation of Th2 cytokines or an influx of Th2 producing cells as a sign of inflammation in allergic rhinitis patients.

Structural cells like epithelial cells, fibroblasts and endothelial cells contribute to the production of several cytokines. Higher levels of RANTES and SCF are observed in the nasal epithelial cells from allergic rhinitis patients than in controls (21-25). No nasal epithelial differences are observed in IL-8 between allergic rhinitis patients and

controls (26). The above-mentioned cytokines play a critical role in the recruitment of eosinophils, basophils and mast cells. Pawankar et al. suggested that the in situ level of SCF in the microenvironment regulates the mast cell phenotype (27, 28).

6.4 Basophil / mast cell maturation and proliferation

Until now it has not been possible to identify a mast cell precursor in blood *in vivo*. *In vitro* mast cells and basophils can be derived from CD34 positive hematopoietic progenitors obtained from blood, cord blood and bone marrow (29-31). Mast cells differentiate due to SCF and basophils develop under the influence of IL-3 (32). CD203c is a recent developed marker that specifically identifies both human basophils and human mast cells (lung, skin) and their CD34(+) progenitors (33, 34).
Differences between basophils and mast cells are based on morphology and immunological markers. In general, basophils are more primitive and have a narrower spectrum of possibilities than mast cells (32). It has been suggested that basophilopoiesis (from bone marrow cells to basophils and mast cells) is a default differentiation pathway in favour of basophils (35). Some blood basophils in allergic patients have been shown to be positive for mast cell proteases (36, 37) and this corresponds to our observation in the nasal mucosa and blood of allergic rhinitis patients (7, 38). Data obtained from skin mast-cell cultures indicated that these mast cells obtained from skin can only proliferate in the absence of serum. This observation strongly suggests that components in serum prevent mast cell proliferation (39, 40). In the light of findings during the past year, we postulate that the difference between basophil and mast-cell maturation is only microenvironment-dependent and the basophil stage may be the immature mast cell stage (38).
The presence of CD34+ (progenitor marker) and CD203c+ (marker for cells from the basophil and mast cell lineage) cells only in the lamina propria of the nasal mucosa of allergic rhinitis patients proves that the maturation site of mast cells is the lamina propria of the nasal mucosa. Basophils can be positive for tryptase, chymase and C-kit in the lamina propria, suggesting that at least some of the basophils have mast-cell characteristics (38).
Arock et al. mentioned that the mast cell line HMC-1 displays the basophilic marker Bsp-1. This is in line with our observation that this cell line is also positive for BB1, another basophil-specific marker (unpublished data). The present mast cell and basophil data provide further support for the existence of hybrid mast cell/basophil progenitors (38).

6.5 Mast cell phenotype plasticity

The microenvironment is an important aspect of the mast cell phenotype, which is not fixed. In the lower airways, the majority of mast cells are single tryptase positive,

while in the upper airways and the skin the majority are tryptase and chymase positive (41-43).

Mast cells in the nasal mucosa of allergic rhinitis patients mostly have a C-Kit+ IgE+ tryptase- chymase- CD203c- phenotype in the epithelium and a C-Kit+ IgE+ tryptase+ chymase+ CD203c+ phenotype in the lamina propria at baseline, indicating a difference in mast cell phenotype between the epithelium and the lamina propria (fig. 2).

The number of mast cells in the lamina propria seems to be relative stable, as indicated by mast cell C-Kit-positive cell numbers, which do not fluctuate during allergen provocation (38). However, the mast cell phenotype changed from a high-amount-tryptase-positive chymase-positive ($MC_T^{HIGH}{}_C$) phenotype into possibly three phenotypes: low-amount-tryptase-positive chymase-positive ($MC_T^{LOW}{}_C$), low-amount-tryptase-positive only (MC_T^{LOW}) or chymase-positive only (MC_C) during repeated allergen provocation. During allergen provocation, the microenvironment changes, as indicated by the influx of inflammatory cells such as basophils, eosinophils and dendritic cells (44, 45) and also by Th2 cytokines produced by T-cells and other inflammatory cells.

The presence of the MC_{TC} and MC_T phenotype is generally accepted (32). However, the MC_C has been introduced recently due to the use of acetone fixation instead of Carnoy's fluid (43). The MC_C phenotype can be induced in culture experiments (46) from normal human progenitors. A possible explanation for the induction of MC_C phenotype could be found in IL-10. Allergen provocation induces IL-10 mRNA in the nasal mucosa of allergic rhinitis patients (20) and in murine studies. IL-10 has been shown to be a critical cytokine, necessary for both the induction and constitutive expression of mouse mast-cell protease 2 (chymase) mRNA and protein (47-49). Cytokine-dependent mast-cell protein expression and mast-cell maturation observations support the idea that the mast cell dynamic state is regulated by the cytokine network to which mast cells are exposed in their different

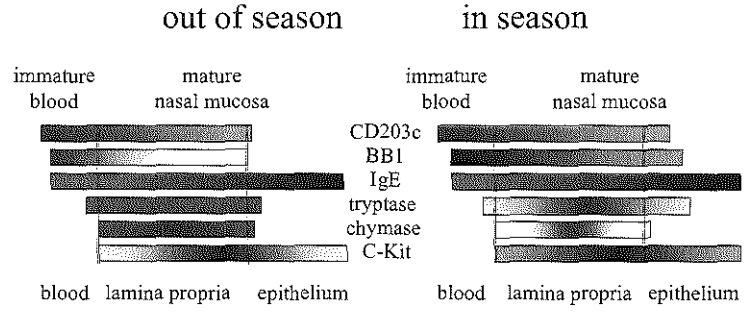

Figure 2. Blood and tissue marker expression of basophilic/mast cells in allergic patients

microenvironments (fig.3) (50). There is a large difference in cytokine mRNA levels between the epithelium and the lamina propria (20), which could explain the different mast cell phenotypes in these compartments.

In our studies, we found no support for the idea of mast cell movement between the lamina propria and epithelium as previously suggested in studies using toluidine blue, IgE and/or tryptase (5, 51). An explanation of the apparent toluidine blue/tryptase-positive cell movement towards the epithelium could be a combination of mast-cell degranulation in the lamina propria, resulting in a reduction of toluidine blue/tryptase-positive cells in the lamina propria and an epithelial basophil influx, resulting in a increase in toluidine blue/tryptase positive cell numbers (20).

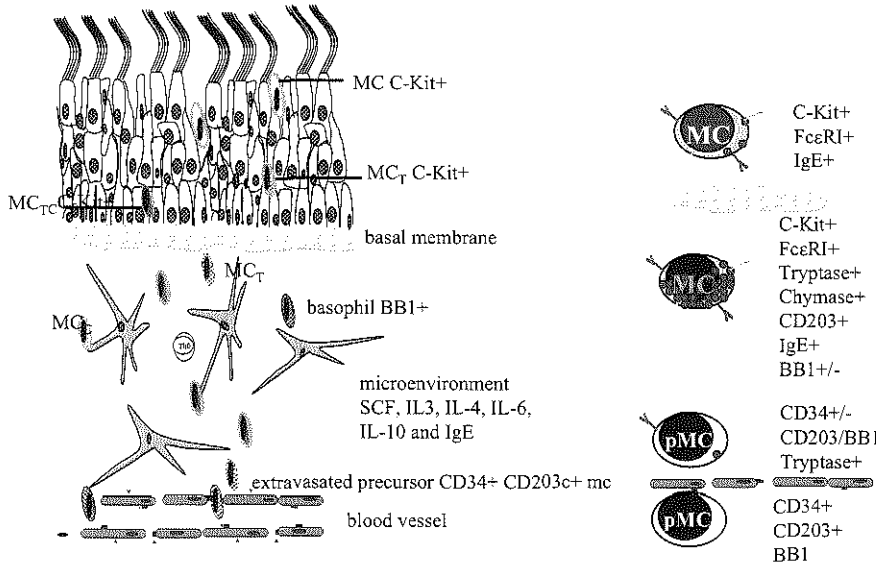

Figure 3. Mast cell maturation in the nasal mucosa of allergic rhinitis patients

6.6 Th2 cytokine and chemokine production in the nasal mucosa of allergic rhinitis patients

Allergic mucosa inflammation is regulated by the local production and release of several Th2 cytokines, such as IL-3, IL-4, IL-5, IL-10 and IL-13 (20, 23, 24, 52-60). Allergen provocation has no effect on Th1 cytokines in the nasal mucosa of allergic rhinitis patients (52, 56, 61-64). IL-4 and IL-13 can support the B-cell switch to IgE production and stimulate the production of immunoglobulins (65, 66). In addition to the well-known Th2 cytokines, chemokines (IL-8 and RANTES) also play a role in eosinophil, basophil and mast cell chemotaxis and recruitment and are well correlated with allergic nasal symptoms in allergic rhinitis patients (20, 67-71). The presence of

functional chemokine receptors on mast cells is a potentially interesting therapeutic target.

6.7 IgE and mast cells

6.7.1 *IgE production in the airways*

Several publications have reported that the production of specific IgE takes place locally in the nasal mucosa (72-77). The nasal mucosa itself contains all the cell types (dendritic cells, T-cells and B-cells (78)) and cytokines (interleukin (IL)-4 and IL-13 (26, 52, 58)) necessary for an IgE immuno-response. The nasal mucosa is a well-organised site in which DCs and other antigen-presenting cells (79) can take up an antigen and stimulate T-cells to produce cytokines and chemokines in order to attract other inflammatory cells (20, 26, 52, 58). Moreover, stimulated T-cells become activated (56, 80) and can proliferate and differentiate in a memory T-cell (81-83). The type of cytokines produced by the T-cell has an influence on other cells. If a B-cell acting as an antigen-presenting cell presents a peptide through the MHC class II molecule, the T-cell which recognises the peptide will stimulate the B-cell to produce immunoglobulins. Moreover, costimulatory molecules are involved as a result of T-B-cell interactions - such as CD154-CD40; CD28-CD80/86; TCR complex-MHC Class II - which are observed in the nasal mucosa of allergic rhinitis patients (84). During this cognate interaction, in the presence of an additional cytokine signal of IL-4 or IL-13, naive B-cells with a surface (s)IgM and sIgD phenotype undergo isotype switching from IgM via IgD through IgG4 and finally to IgE germline transcript (66). This process involves the rearrangement of germline DNA in the immunoglobulin heavy-chain locus. A critical role is postulated for stat-6 in the IL-4-mediated activation of germline-epsilon gene transcript and switch recombination (85).

Zurcher et al (86) demonstrated that functional B-cells, isolated from the nose and cultured in a CD40-stimulating system, could synthesise IgE. Isotype switching of B-cells can also take place in the nasal mucosa (87, 88). Durham et al. reported that local allergen provocation induces ε-germline transcripts in nasal B-cells. In addition, mast cells seem to play critical roles in amplifying IgE production independently of T-cells (65) in allergic rhinitis patients (89). However, the respective contributions of mast cells and the respective contributions of T-cells to the IgE immune response are unclear. Once produced, IgE will sensitise FcεRI-expressing cells (mast cells, basophils and DCs) in the nasal mucosa (44, 76).

The availability of specific IgE-positive plasma cells (84) in peripheral tissue and the knowledge that a plasma cell is an end-stage cell of the B-cell lineage (90, 91) indicate that this cell could contribute to the production of specific intranasal IgE. Before and after local allergen provocation or in and out of season, no changes were

observed in the number of specific IgE-positive cells in the nasal mucosa of seasonal allergic rhinitis patients (38) (unpublished data). Our data is supported by the observation of persistent IgE synthesis ex vivo in an explant culturing system of nasal mucosa obtained from allergic rhinitis patients (77).

Durham and Cameron strongly suggest that the nasal mucosa itself is the site of isotype switching. After allergen provocation, elevated levels of IgE germline transcripts were observed (87, 92). However, the data for IgE germline isotype switching does not exclude the possibility of the influx of B-cells expressing germline transcripts for IgE, supporting the idea that allergy is a systemic disease with local expression, or shock organ-specific homing of inflammatory cells (fig. 4).

It is unclear whether the nasal mucosa is the main site of IgE production because lymphoid tissues may also be involved in the production of immunoglobulin E (93, 94). It has been suggested that IgE is produced in the bone marrow or regional lymph nodes and that IgE reaches mucosa surfaces by diffusion or through migrating cells (95-97).

Local production of IgE has been observed outside the nose in the bronchial mucosa of asthmatics (98, 99). Transplantation case reports describe non-asthmatic recipients of asthmatic lungs who develop asthma after transplantation; however, asthmatic recipients of normal lungs do not develop asthma for up to 3 years after transplantation. This supports the local concept of lung disease in asthma (100).

There is no data on the gastrointestinal mucosa which indicates that the mucosa itself is a source of IgE. There are a few IgE-positive plasma cells in the intestinal mucosa. However, they are not disease-related (101).

No B-cells or even plasma cells have been observed in the skin, indicating that the process in the skin is totally different. The source of IgE production in atopic dermatitis seems to be located more in draining lymph nodes and not in the skin itself.

6.7.2 *IgE loading of mast cells*

The local nasal concentration of specific IgE is high enough for mast cells to acquire specific IgE in the nasal mucosa of allergic rhinitis patients (76). Moreover, the concentration of IgE seems to be higher than in the blood. Basophils acquire IgE in the nasal mucosa of allergic rhinitis patients. Nasal allergen challenge leads to changes in the population of cells bearing IgE (specific and otherwise).

With respect to IgE positivity in basophils, we observed an influx of IgE-positive and IgE-negative basophils after allergen provocation. This basophil influx led to a reduction in the percentage of IgE-positive basophils followed by a slight increase in the percentage of IgE-positive basophils, suggesting that basophils acquire IgE in the nasal mucosa of allergic rhinitis patients after allergen provocation (38). The increase

in the percentage of IgE-positive C-Kit-positive cells illustrates the intranasal IgE acquisition by mast cells of allergic rhinitis patients after allergen provocation (38). The local IgE concentration in the nasal mucosa is the most important reason for IgE loading of mast cells and basophils in the nasal mucosa (fig. 4).

The finding of epithelial grass-pollen-specific IgE-bearing mast cells (5, 26, 53, 56, 76) out of the grass pollen season explains why allergic rhinitis patients immediately show symptoms after a long period of allergen avoidance (4, 5, 45, 56, 102). When the nasal mucosa of seasonal allergic rhinitis patients is exposed for the first time in a season to a low allergen concentration, mast cells release their mediators.

In atopic dermatitis, it is unclear what the IgE source is or which organ is responsible for IgE production. Skin mast cells accumulated IgE in accordance with the bath theory. No plasma cells or B-cells are present in the skin for high local concentration of IgE. In intestinal mucosa, IgE-loaded mast cells are relatively prominent (92%) in the guts of patients with food-related diarrhea who are thought to be atopic on the basis of a positive skin prick test. However, half of normal controls were found to have IgE-positive intestinal mast cells also, suggesting that there is no disease-related IgE mast cell load (103, 104).

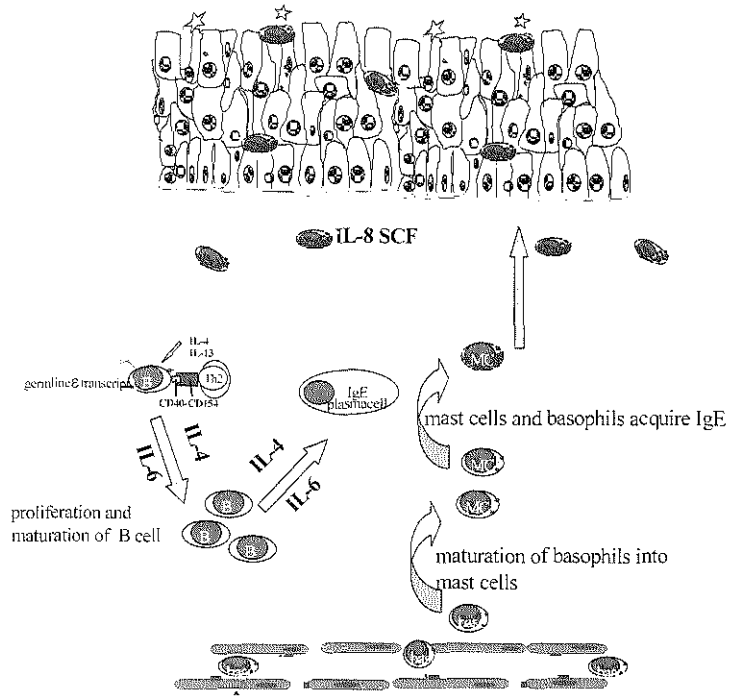

Figure 4. The local IgE immunoresponse in the nasal mucosa of allergic rhinitis patients

The airways and gastrointestinal tract are sources of immunoglobulin. This is in contrast to the skin, which does not contain any immunoglobulin-producing cells. It is well known that the ratio of specific IgE to total IgE is highest in seasonal allergic rhinitis, suggesting that the total amount of IgE produced targets a few allergens. The ratio of specific to total IgE in atopic dermatitis is low because the total IgE level in these patients is usually very high and nothing is known about the most of the targets of IgE in these patients. These observations suggest that the immune system has a general skewing towards a pronounced Th2 response to several antigens. There is a more general atopic constitution in these patients' bodies as a whole.

6.8 Consequences for allergic disease therapy (treatment with intranasal steroids)

In patients with seasonal and perennial allergic rhinitis, the number of nasal mucosa inflammatory cells is higher compared to controls. Intranasal corticosteroid treatment has been shown to reduce these numbers (56, 58, 102, 105-108). Moreover, topical corticosteroid treatment of the nasal mucosa decreases tissue mast cell numbers by down-regulating tissue SCF production required for the survival of local mast cells (109).

Recent findings by our group have demonstrated that intranasal steroid treatment has beneficial effects, reducing the number of mast cells after allergen provocation. Mast cells disappear from the epithelium after active treatment and remain absent despite allergen provocation, indicating high sensitivity in mast cells to steroid treatment, irrespective of mucosa condition (102, 110). Cytokine levels (IL-3, IL-5, IL- 6, IL-13, IFN-γ, RANTES and TNF-α) were also reduced, suggesting a change in microenvironment after steroid treatment and allergen provocation (56, 58, 105, 111-117).

Observations by Durham and Cameron show that germline epsilon transcription is reduced during intranasal FP treatment (87, 118). Moreover, Naclerio observed a significantly lower increase during intranasal FP treatment in serum-specific IgE (119). All these topical steroid studies support the concept that allergic rhinitis patients must be treated topically to reduce nasal symptoms and reduce nasal allergic inflammation and IgE production.

6.9 Mast cell intervention

6.9.1 *Therapeutic intervention in mast cell migration*

Elevated mast cell numbers and elevated mast cell chemoattraction factors, as well as other factors promoting the growth, differentiation and activation of mast cells, have been observed in the nasal mucosa (24, 26, 57, 59, 120-123), especially on the epithelial side (5, 26, 124-127) in allergic rhinitis patients when compared to non-

allergic controls. Nasal allergen provocation also induces elevated levels of these mast cell chemoattraction factors and an increase in mast cell numbers and basophil numbers in the nasal mucosa of allergic rhinitis patients (5, 7, 20, 24, 52, 60, 125, 128).

Murine and *in vitro* experiments show that mast cells migrate toward the agent. Antigens themselves may be responsible for mast cell recruitment (129, 130). Human blood-derived mast cells expressed functional IL-8 receptors (CXCR1 and CXCR2 receptors) on the cell surface (131, 132). These chemokine molecules are interesting targets for recently-developed antagonists and could have a preventive effect on the epithelial accumulation of mast cells.

6.9.2 Therapeutic intervention on mast cells directly

Recently-developed chimeric proteins combining a targeting moiety and a human pro-apoptotic protein such as the killing moiety (Fc-Bak) seem to be successful in the targeted elimination of mast cells and basophils as a new approach for allergy treatment (133). Moreover, there are studies in progress of blocking and inhibiting drugs that influence the signal transduction downstream of FcεRI, preventing migration of the mast cell and basophils (134).

6.9.3 *Therapeutic intervention on mast cell mediators*

Other treatments like anti-histamines, anti-leukotrienes and anti-prostaglandines all have effects on basophils mast cells and their mediators (135-137). However, the mode of action of these therapies is not only focused on the aforementioned cells and mediators because other cells also produce the arachidonic acid metabolites (138) and histamine (139).

6.10 Anti-IgE therapy

Anti-IgE therapy is based on a humanised antibody which targets free IgE at the FcεRI domain of the IgE molecule, thereby preventing its interaction with the FcεRI receptors on mast cells, basophils and dendritic cells (140-143). Although, theoretically, anti-IgE should be very effective, the first clinical studies in allergic rhinitis show that the effect on symptomatology is moderate (144, 145).

However it reduces tissue eosinophilia (146). The finding in this thesis suggests very high local concentrations of specific IgE generated by the local presence of specific IgE-producing plasma cells. In addition, mast cell maturation also takes place in the nasal mucosa and this may help to clarify why anti-IgE therapy seems less effective in the treatment of allergic rhinitis. The observations of anti-IgE therapy suggest that it is not the therapy of first choice in allergic rhinitis patients.

6.10 Concluding remarks

This thesis investigated the role of basophils and mast cells and local IgE production in the nasal mucosa of allergic rhinitis patients. Distinct differences were observed in their nasal mucosa after allergen provocation.

- An influx of basophils is observed in the epithelium of the nasal mucosa as early as one hour after allergen provocation.
- There is mast cell phenotype change after allergen provocation.
- Basophils are positive for specific mast-cell proteins in blood and in the nasal mucosa of allergic rhinitis patients.
- Elevated numbers of Th2 cytokine (IL-10 and IL-13), basophil and mast cell-attracting chemokines (IL-8 and RANTES) were observed after allergen provocation.
- Basophils and mast cells in the nasal mucosa carry specific IgE in allergic rhinitis patients.
- Specific IgE-producing cells are present locally in the nasal mucosa of allergic rhinitis patients.
- Basophils and mast cells acquire IgE on their surface in the nasal mucosa of allergic rhinitis patients.

The IgE-acquired mast cell plays a predominant role in disease manifestation and persistence. A consequence of the present thesis is that new therapy must focus on the prevention of basophil and mast cell presence and activation on the epithelial side and the prevention of specific IgE acquisition on the mast cell surface in the shock organ.

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7. Summary

7.1 The nasal mucosa of allergic rhinitis patients

The present thesis focuses on the role of the nasal mucosa in allergic inflammatory response, with special emphasis on specific IgE production and the IgE acquisition by mast cells of allergic rhinitis patients. The nasal mucosa of allergic rhinitis patients itself contains all the associated cell types (dendritic cells, T-cells and B-cells) and has an IgE - Th2 cytokine supporting microenvironment of interleukin-4 (IL-4) and IL-13. The symptoms of nasal allergic rhinitis were mainly induced by mast cell mediators. Stimulation and regulated secretion of histamine and other mediators by the mast cells and basophils takes place by crosslinking of FcεRI on these cells. This takes place when a multivalent allergen is bound to specific IgE attached to FcεRI in the membrane of nasal mucosa mast cells.

Local production of (specific) IgE and local maturation of mast cells in the nasal mucosa suggest that a mast cell in the nasal mucosa may became positive for (specific) IgE. Allergen provocation induced mast cell phenotype change and basophilic cell influx. Clear interpretation of mast cell dynamic is very difficult because mast cells can flow in and out of the mucosa. Moreover, basophils can change into a mast cell phenotype and both cell types may be obtained from the same progenitor cells. Present results suggest intranasal maturation of basophils into mast cells and the acquisition of IgE by these cells in the nasal mucosa of allergic rhinitis patients.

7.2 Mast cells and basophils

7.2.1 *Mast cell phenotype plasticity*

Both microenvironment and location are important aspects for the basophilic cell and mast cell phenotype, which is not fixed. In the lower airways the majority of mast cells are single tryptase positive, while in the upper airways the majority are tryptase and chymase positive. Observations, we described in chapter 3.1, have indicated that Carnoy's fluid fixation, the formerly fixation method for mast cells, in contrast to acetone fixation, can lead to an underestimation of the number of immunohistochemically stained chymase-positive mast cells. Due to Carnoy's fluid fixation others failed to observe the tryptase negative chymase positive mast cell population. Mast cells can degranulate, after allergen provocation, as indicated by a reduction in number of serine protease positive cells (chapter 3.2 and 3.3). After degranulation mast cell can regranulation and return to their initial numbers.

The mast cell phenotype also displays considerable plasticity in vivo and appears to be controlled by the microenvironment. A clear example illustrating the mast cell

plasticity is the side dependent phenotype. The phenotype of mast cells is different between the epithelium and the lamina propria (chapter 3.3). These observations supported the idea that the local microenvironment is very important not only for maturation and differentiation but also for constitutive expression of proteins responsible for a certain mast cell phenotype.

7.2.2 *Basophil dynamics*

With recently develop basophilic cell markers and allergen provocations, we observed epithelial basophilic cell influx in allergic rhinitis patients only during the early phase (within 1 hour) response and not during the late phase response after allergen provocation (chapter 3.2).Epithelial basophils are elevated only in the early phase after allergen provocation suggesting basophils movement from the lamina propria or blood through the epithelium in the lumen, only during that time period. In the lamina propria the basophilic inflammation starts in the early phase and holds on for at least one week after allergen provocation.

Although the influx of epithelial metachromatically staining cells has been described by several authors as a mast cells redistribution, this influx can only be characterized as an influx of basophilic cell in the epithelium as in detail described in chapter 3.2 and 3.3. The phenotype of tissue basophils in the early phase after allergen provocation is roughly equal to the phenotype of blood basophils (chapter 3.3). Moreover the phenotype of basophils changed, with basophils becoming positive for chymase and C-Kit, to indicate the maturation of basophils into mast cells intranasally (chapter 3.3). In vitro mast cells and basophils can be derived from CD34 positive hematopoietic progenitors obtained from blood, cord blood and bone marrow. CD203c is a recent developed marker that specifically detects human basophils and mast cell (lung, skin) and their CD34(+) progenitors. The presence of CD34 positive CD203c positve cells only in the lamina propria of nasal mucosa of allergic rhinitis imply that the maturation site of mast cells is the lamina propria of the nasal mucosa.

7.3 Th2 cytokines and chemokines predominate the microenvironment of the nasal mucosa in allergic rhinitis patients

Allergic mucosa inflammation is regulated by the local production and release of several Th2 cytokines, such as IL-3, IL-4, IL-5, IL-10 and IL-13. In addition to the well-known Th2 cytokines, chemokines (IL-8 and RANTES) also play a role in eosinophilic cell and mast cell chemotaxis and are well correlated with allergic nasal symptoms in allergic rhinitis patients. Allergen provocation has no effect on Th1 cytokines. However, allergen provocation induced tissue eosinophilia and a increase in IL-8, IL-13 and RANTES mRNA–positive cells. Elevated numbers of eosinophils,

IL-10 and IL-13 mRNA–positive cells compared with baseline numbers, can still be observed 1 week after a single allergen provocation.
Nasal allergen provocation induces elevated levels of IL-8 and RANTES, which are mast cell chemo-attracting factors in allergic rhinitis patients. Interestingly and in line with our (chapter 4) in vivo data are the in vitro observations of mast cell chemotaxis induced by the RANTES and IL-8. The receptors on mast cell, for these chemokines, are an interesting therapeutic target.

7.4 Intranasal production of specific IgE

Several publications have reported that the production of specific IgE take place locally in the nasal mucosa. In chapter 5.1 we described the method to detect cell-bound-specific-IgE in the nasal mucosa of allergic rhinitis patients.
Based on availability of specific IgE positive plasma cells (chapter 5.2) in a peripheral tissue and the knowledge that a plasma cell is an end-stage cell of the B-cell lineage indicates that this cell could contribute to the production of specific IgE intranasal. No alterations were observed in the number of specific IgE positive cells in the nasal mucosa of seasonal allergic rhinitis patients after local allergen provocation in, or out of season (chapter 5.3). Our data is supported by observation of persistent IgE synthesis ex vivo in an explant culturing system of nasal mucosa obtained from allergic rhinitis patients.

7.5 Mast cells and basophils acquire IgE intranasal

Nasal allergen challenge leads to alteration in the population of cells bearing (specific) IgE. Regarding basophils IgE positivity, we observed an influx of IgE positive and IgE negative basophils after allergen provocation. Basophils tended to acquire IgE in the nasal mucosa, suggesting that the local concentration of IgE in the nasal mucosa is higher than in the circulation (chapter 5.3). An increase in the percentage of IgE positive C-Kit positive cells illustrated the intranasal IgE acquisition by mast cells of allergic rhinitis patients after allergen provocation. (chapter 5.3).

7.6 Conclusion

The IgE acquired mast cell plays a predominant role in disease manifestation and persistence. The observation that intranasal tissue basophils are immature mast cells gives new insights in the pathophysiology of allergic rhinitis. A consequence of the present thesis is that new therapy must be focus on prevention of mast cell inflammation on the epithelial side and prevention of specific IgE acquisition on the mast cell surface in the shock organ.

8. Samenvatting

Allergie is een ziekte die zich kan manifesteren in de ademhalingswegen (neus en longen), de huid en het spijsverteringsstelsel (vanaf de mond tot en met de darmen). Allergie wordt gedefinieerd als overgevoeligheid voor stoffen waartegen normaal gesproken geen immunologische reactie optreedt. Al in de jaren '60 is ontdekt dat immunoglobuline E (IgE) een sleutelrol speelt in de overgevoeligheidsreactie.

Wanneer een patiënt verdacht wordt van een allergie, moet onderzocht worden waarvoor hij allergisch is. Het meten van specifiek IgE, ook wel het meten van sensibilisatie (overgevoeligheid) genoemd, kan met behulp van bloedonderzoek of een huidtest plaatsvinden. Het komt regelmatig voor dat de allergietest positief is terwijl de patiënt nooit klachten heeft ervaren voor het desbetreffende allergeen, of omgekeerd: de patiënt heeft klachten die veroorzaakt worden door een bepaald allergeen, maar de allergietest voor dat allergeen valt negatief uit. Tot nu toe is algemeen aangenomen dat allergie een systemische ziekte is die zich lokaal uit. Het zou ook kunnen dat allergie een lokale ziekte is die tevens systemisch gemeten kan worden.

In dit proefschrift worden het neusslijmvlies en het lokale karakter van allergische rhinitis bestudeerd. Speciale aandacht is er voor de belangrijkste cellen en factoren in een allergische ontsteking zoals basofielen, mestcellen en IgE.

Dynamiek van basofielen en mestcellen

Nasale allergeenprovocaties veroorzaken, naast klinische symptomen, ook het binnenstromen van basofielen en eosinofielen in het neusslijmvlies van allergische rhinitis patiënten. Eén uur na allergeenprovocatie is het aantal basofielen in het epitheel het hoogst. Tegelijkertijd neemt in de lamina propria het aantal mestcellen dat gekarakteriseerd is met specifieke markers voor tryptase en/of chymase af. Dit fenomeen is in de jaren '80, op basis van metachromatische kleuringen, beschreven als redistrubitie (herverdeling) en verplaatsing van mestcellen richting het epitheel. Door gebruik te maken van moderne immunohistochemische technieken blijkt dat er geen sprake is van redistributie maar van een afname van metachromatisch kleurende cellen (mestcellen) in de lamina propria en een toename van metachromatisch kleurende cellen (basofielen) in het epitheel.

Ongestimuleerde mestcellen hebben als belangrijkste fenotype (verschijningsvorm) dat ze tryptase-positief en chymase-positief (MC_{TC}) zijn. Mestcellen kunnen na allergeen-provocaties veranderen in grofweg drie mestcel fenotypes: tryptase-positieve mestcel (MC_T), MC_{TC} en chymase-positieve mestcel (MC_C). Zowel de bloedbasofielen als de weefselbasofielen in het neusslijmvlies kunnen mestcelspecifieke eiwitten bevatten. Deze bevinding suggereert dat het verschil tussen basofielen en mestcellen veel geringer is dan tot nu toe werd aangenomen. Wanneer

fenotypes van mestcellen in verschillende delen van het neusslijmvlies met elkaar worden vergeleken, blijken er grote verschillen te zitten tussen de mestcellen in het epitheel en die in de lamina propria. Het fenotype van de mestcellen in de lamina propria is voornamelijk C-Kit-positief IgE-positief tryptase-positief chymase-positief terwijl het fenotype van de mestcellen in het epitheel C-Kit-positief IgE-positief tryptase-negatief chymase-negatief is (hoofdstuk 3).

Cytokines in het neusslijmvlies

Uit analyse van een breed scala van cytokines met behulp van *in situ* hybridisatie blijkt dat, na allergeenprovocatie in het neusslijmvlies, alleen het mRNA voor de Th2 cytokines IL-10 en IL-13 en de chemokines IL-8 en RANTES verhoogd tot expressie wordt gebracht. Opvallend is dat een week na allergeenprovocatie nog steeds een verhoogd aantal eosinofielen, evenals een verhoogd aantal IL-10 en IL-13 mRNA positieve cellen wordt gevonden (hoofdstuk 4).

Specifiek IgE producerende cellen bevinden zich in het neusslijmvlies van allergische rhinitis patiënten

In dit proefschrift wordt een nieuwe methode beschreven om specifiek IgE in het neusslijmvlies op histologisch niveau aan te tonen. Hierbij is gebruik gemaakt van allergenen die gelabeld zijn met biotine. Deze allergenen binden aan cel-gebonden IgE. Het biotine van het gebiotinyleerde allergeen kan microscopisch zichtbaar gemaakt worden. Uit de resultaten van blokkerings- en competitie-experimenten blijkt dat deze methode geschikt is om specifiek IgE aan te tonen. De combinatie van de hiervoor beschreven nieuwe methode en de klassieke immunohistochemie biedt de mogelijkheid om allergeenspecifieke binding aan cellen aan te tonen. Meer dan 90% van de gevonden specifiek IgE positieve cellen waren mestcellen. Bovendien worden allergeenspecifiek IgE producerende plasmacellen gevonden in het neusslijmvlies van allergische rhinitis patiënten. Allergeenprovocaties hebben geen invloed op het aantal (specifiek) IgE producerende cellen in het neusslijmvlies. De mate van IgE belading van mestcellen en basofielen lijkt toe te nemen na allergeenprovocatie. Het merendeel van de mestcellen is beladen met specifiek IgE.

Conclusie

Basofielen en mestcellen lijken twee fenotypes van dezelfde cel te zijn. Zowel het rijpingsproces als de belading met IgE van basofielen en mestcellen vindt plaats in het neusslijmvlies van allergische rhinitis patiënten. Het neusslijmvlies zelf speelt, door de productie van specifiek IgE, een cruciale rol in het onderhouden van de overgevoeligheid voor allergenen.

List of abbreviations	
AEC	3-amino-9-ethylcarbazole
APC	antigen presenting cell
BU	biological unit
CAST	cellular antigen stimulation test
CGRP	calcitonin gene related protein
DC	dendritic cell
ECP	eosinophilic cationic protein
EPO	eosinphil peroxidase
FITC	fluorescein isothiocyanate
GM-CSF	granulocyt monocyt cell stimulating factor
GP	grass pollen
HDM	house dust mite
HEP	histamine equivalent in prick test
HLA-DR	human leukocyte antigen -DR
HRT	histamine release test
ICAM	Intercellular adhesion molecule
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
LRT	leukotriene release test
LT	leukotriene
MBP	major basic protein
MC	mast cell
MC_C	tryptase negative chymase positive mast cell
MCP	monocyte chemotactic protein
MC_T	tryptase positive chymase negative mast cell
MC_{TC}	tryptase positive chymase positive mast cell
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NK	natural killer
NPT	nasal provocation test
PBS	phosphate buffered saline
PG	prostaglandin
RAST	radio allergo sorbent test
SCF	stem cell factor
TGF	transforming growht factor
TNF	tumor necrosis factor
VIP	vasoactive intestinal peptide

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Het proefschrift is klaar. De laatste letters zijn aan het papier toevertrouwd, maar het onderzoek gaat door. Wetenschap is eigenlijk nooit af.

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Alex

Curriculum Vitae

Alex KleinJan is geboren op 18 januari 1966 te Daarle in de gemeente Hellendoorn. Het MAVO-diploma is behaald in Den Ham (O) 1982. Van 1982 tot 1986 heeft hij achtereenvolgens het MLO (klinische chemie) en de propedeuse HLO gehaald aan de HTS/SVL te Hengelo (O). De HLO met als studierichting biologie is in 1990 afgerond aan de Hogeschool Utrecht. Als analist is hij een jaar lang werkzaam geweest op het Instituut Moleculair Biologie van de Universiteit van Utrecht onder supervisie van prof. dr. B.M. Spruijt en prof. dr. W.H. Gispen. Van januari 1991 tot augustus 1994 is hij werkzaam geweest op de afdeling immunologie van de Erasmus Universiteit Rotterdam, alwaar hij onderzoek heeft gedaan aan het slijmvlies van allergische rhinitis patiënten onder supervisie van prof. dr. R. Benner en drs. T. Godthelp. Vanaf augustus 1994 tot juni 2002 heeft hij op de afdeling KNO-heelkunde zijn werkzaamheden voortgezet, hetgeen geresulteerd heeft in dit proefschrift onder begeleiding van prof. dr. W.J. Fokkens. Vanaf juli 2002 is hij werkzaam op de afdeling longziekten onder supervisie van prof. dr. H.C. Hoogsteden en dr. B.N.M. Lambrecht.

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Jazeker, ik beschrijf met deze hand
de tomeloze chaos der natuur,
ik leg de teugels aan van maat en duur,
ik noem de namen, ik leg het verband.

Ik trek de lijnen der geschiedenis,
de lasso's teken ik van zon en maan.
O wereld waar ik in zal onder gaan,
raadsel van orde, die de mijne is.

En toch, en toch, er is een ogenblik
dat mij uit al mijn zekerheid bevrijdt.
In regen van ontferming schrijf ik neer,
in zonlicht van verrukking: hier ben ik,
en daar zijt Gij, en alles wat ons scheidt,
verbindt ons pas: Gij zijt mijn God en Heer.

J.W. Schulte Nordholt
Uit: *Het weefsel Gods* (1965)